**รายงานวิจัยฉบับสมบูรณ์**

**ชื่อชุดโครงการ การคัดเลือกสายพันธุ์ *Lactobacillus* ในช่องปากเพื่อใช้เป็นโพรไบโอติกในช่องปาก**

**Selection of oral *Lactobacillus* for use as probiotic in the oral cavity**

**คณะนักวิจัย**

1 ศาสตราจารย์ ดร.รวี เถียรไพศาล
ภาควิชาโอษฐวิทยา และสถานวิจัยโรคที่พบบ่อยในช่องปากและวิทยาการระบาด
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่

2. ดร. สุพัชรินทร์ พิวัฒน์
ภาควิชาทันตกรรมป้องกัน และสถานวิจัยโรคที่พบบ่อยในช่องปากและวิทยาการระบาด
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่

3. นาย พีระพัฒ หวานนุ่น (นักศึกษาปริญญาเอก)

4. นางสาว อักษรทอง ชูรักษ์ (นักศึกษาปริญญาเอก)

**โครงการนี้ได้รับทุนสนับสนุนจาก งบประมาณแผ่นดิน มหาวิทยาลัยสงขลานครินทร์**
**ประจำปีงบประมาณ 2555 และ 2556**
**รหัสโครงการ DEN550005M**

**1. ชื่อชุดโครงการวิจัย**

การคัดเลือกสายพันธุ์ *Lactobacillus* ในช่องปากเพื่อใช้เป็นโพรไบโอติกในช่องปาก

Selection of oral *Lactobacillus* for use as probiotic in the oral cavity

**2. ชื่อโครงการย่อย**

**โครงการย่อยที่ 1**

(ภาษาไทย) คุณลักษณะทางโมเลกุลของสารโปรตีนต้านจุลชีพของเชื้อสายพันธุ์ *Lactobacillus paracasei L. casei L. rhamnosus* และ *L. salivarius* จากช่องปาก.....

(ภาษาอังกฤษ) Molecular characterization of antimicrobial protein of oral *Lactobacillus paracasei, L. casei, L. rhamnosus* and *L. salivarius*

**โครงการย่อยที่ 2**

(ภาษาไทย) ...คุณลักษณะทางโมเลกุลของสารโปรตีนต้านจุลชีพของเชื้อสายพันธุ์ *Lactobacillus fermentum, L.mucosae* และ *L. plantarum* จากช่องปาก.....

(ภาษาอังกฤษ) ..Molecular characterization of antimicrobial protein of oral *Lactobacillus fermentum L. mucosae* and *L. plantarum*

**โครงการย่อยที่ 3**

(ภาษาไทย) ...ความสามารถในการเกาะติดเยื่อบุช่องปากของเชื้อแลกโตแบซิลลัสจากช่องปาก..

(ภาษาอังกฤษ) ..Adhesion ability of oral *Lactobacillus* on oral epithelial cells.....

**โครงการย่อยที่ 4**

(ภาษาไทย) ความสามารถในการกำจัดอนุมูลอิสระของเชื้อแลกโตแบซิลลัสจากช่องปาก

(ภาษาอังกฤษ) Antioxidative ability of oral *Lactobacillus*

**3. คณะนักวิจัยและคณะ/หน่วยงานต้นสังกัด**

**ผู้อำนวยการแผนงาน**

ศาสตราจารย์ ดร.รวี เถียรไพศาล
สถานวิจัยโรคที่พบบ่อยในช่องปากและวิทยาการระบาด และภาควิชาโอษฐวิทยา
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่
จังหวัดสงขลา โทรศัพท์ 0-7442-9878 โทรสาร 0-7421-2922

**คณะผู้วิจัย**

1. ศาสตราจารย์ ดร.รวี เถียรไพศาล
สถานวิจัยโรคที่พบบ่อยในช่องปากและวิทยาการระบาด และภาควิชาโอษฐวิทยา
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ จังหวัดสงขลา
โทรศัพท์ 0-7442-9878 โทรสาร 0-7421-2922 E-mail:rawee.t@psu.ac.th
2. ผู้ช่วยศาสตราจารย์ ทพญ. ดร. สุพัชรินทร์ พิวัฒน์
สถานวิจัยโรคที่พบบ่อยในช่องปากและวิทยาการระบาด และภาควิชาทันตกรรมป้องกัน
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ จังหวัดสงขลา
โทรศัพท์ 0-7428-7605 โทรสาร 0-7421-2922 E-mail: supacharin.p@psu.ac.th
3. นายอาคมนายอาคม หวานนุ่น (นักศึกษาปริญญาเอก)
สถานวิจัยโรคที่พบบ่อยในช่องปากและวิทยาการระบาด และภาควิชาโอษฐวิทยา
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ จังหวัดสงขลา
โทรศัพท์ 0-7442-9878 โทรสาร 0-7421-2922 E-mail: s5310830003@psu.ac.th
4. นางสาว อักษรทอง ชูรักษ์ (นักศึกษาปริญญาเอก)
สถานวิจัยโรคที่พบบ่อยในช่องปากและวิทยาการระบาด และภาควิชาโอษฐวิทยา
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ จังหวัดสงขลา
โทรศัพท์ 0-7442-9878 โทรสาร 0-7421-2922 E-mail: aksorntong99@gmail.com

**4. บทสรุปผู้บริหาร (Executive Summary)** ประกอบด้วย

**4.1 บทนำ**

โพรไบโอติก คือ กลุ่มจุลินทรีย์ที่มีชีวิต เมื่อบริโภคในจำนวนที่มากพอ จะทำหน้าที่ในการรักษาสมดุลให้กับโฮสต์ที่อาศัยอยู่จึงมีประโยชน์ต่อสุขภาพของโฮสต์นั้น ๆ โพรไบโอติกมีการนำมาใช้มานานโดยเฉพาะในผลิตภัณฑ์อาหารหมัก นักวิทยาศาสตร์คนแรกที่ศึกษาเกี่ยวกับโพรไบโอติก คือ Metchnikof ในปี ค.ศ. 1907 โดยพบว่าชาวบัลกาเรียนมีอายุยืนเนื่องจากการบริโภคนมหมักที่มีเชื้อแลกติก (lactic acid bacteria, LAB) สายพันธุ์ *Lactobacillus bulgaricus* จึงมีข้อสรุปว่าสุขภาพดีมีความสัมพันธ์กับจุลินทรีย์ที่มีชีวิตที่รับประทานเข้าไป

คำว่า "โพรไบโอติก" มีการนิยามขึ้นครั้งแรกโดย Lilly และ Stillwell ในปี ค.ศ. 1965 มีความหมายว่า "สารที่จุลินทรีย์ชนิดหนึ่งสร้างขึ้นและสามารถกระตุ้นการเจริญของจุลินทรีย์อีกชนิดหนึ่ง" Parker (1974) ได้ให้นิยามโพรไบโอติกใหม่ว่า "จุลินทรีย์รวมถึงสารใดๆ ที่ช่วยรักษาสมดุลของจุลินทรีย์ในลำไส้" ต่อมา Fuller (1989) ให้นิยามใหม่ว่า "จุลินทรีย์ที่มีชีวิตที่นำมาใช้ในลักษณะสารเสริมอาหาร ซึ่งมีประโยชน์ต่อสุขภาพในการรักษาสมดุลจุลินทรีย์ในลำไส้" และมีการแก้ไขนิยามอีกหลายครั้งในช่วง ค.ศ. 1992-2001 ล่าสุดในปี ค.ศ. 2002 องค์กร FAO/WHO ได้ให้นิยามใหม่ว่า "จุลินทรีย์ที่มีชีวิตเมื่อรับประทานเข้าไปในปริมาณที่เพียงพอ จะส่งผลต่อสุขภาพของสิ่งมีชีวิตที่ได้รับจุลินทรีย์เหล่านั้นเข้าไป"

โพรไบโอติกที่นิยมนำมาใช้ประโยชน์มากที่สุด คือ แบคทีเรียกลุ่ม *Lactobacillus* และ *Bifidobacterium* โพรไบโอติกที่นำมาใช้ในคนส่วนใหญ่คัดเลือกได้จากระบบทางเดินอาหารของคน ได้แก่ *Lactobacillus plantarum, L. rhamnosus, L. salivarius, L. casei, L. fermentum, L. acidophilus, L. oris, L. paracasei, L. brevis, L. buchneri, L. delbrueckii, L. jensenii, L. gasseri* และ *L. bulgaricus*

หลักเกณฑ์การคัดเลือกโพรไบโอติกโดยองค์การ FAO/WHO (2002) แนะนำคือ

- ต้องไม่เป็นจุลินทรีย์ก่อโรค
- สามารถยับยั้งการเจริญเติบโตเชื้อก่อโรค โดยการผลิตสารต้านจุลินทรีย์ก่อโรค หรือมีความสามารถในการแข่งขันการเจริญเติบโตกับจุลินทรีย์ก่อโรคได้
- สามารถยึดเกาะกับเซลล์เยื่อบุในอวัยวะเป้าหมายได้ดี
- กระตุ้นการสร้างระบบภูมิคุ้มกันของร่างกาย
- มีคุณสมบัติในแง่การแปรรูปที่ดี เช่น อายุการเก็บรักษา ความคงตัว
- ได้รับการยืนยันทางคลินิกในเรื่องความปลอดภัยและผลต่อสุขภาพ

จากการทบทวนทางวรรณกรรมมีรายงานว่าการกินโยเกิร์ตหรือนมที่มี *L. rhamnosus* GG หรือ *L. reuteri* ซึ่งเป็นสายพันธุ์ที่คัดเลือกมาจากทางเดินอาหาร มีผลให้เชื้อก่อโรคฟันผุ *Streptococcus mutans* ในช่องปากลดลง จากผลดังกล่าวทำให้กล่าวว่าโพรไบโอติกนั้นอาจนำมาใช้ในการป้องกันฟันผุได้

อย่างไรก็ตามในเวลาต่อมาได้มีผู้รายงานว่า *L. rhamnosus* GG สามารถอยู่ในร่างกายได้เพียงชั่วคราวเท่านั้น จึงมีข้อคิดว่าการที่เชื้อนี้ไม่สามารถดำรงอยู่ได้นาน อาจเนื่องจากไม่ใช่สายพันธุ์ที่เหมาะในการอยู่ในช่องปาก ดังนั้นจึงควรหาโพรไบโอติก สายพันธุ์ใหม่ที่เหมาะจะอยู่ในช่องปากได้ด้วย

การคัดเลือกสายพันธุ์โพรไบโอติกที่ผ่านมา เป็นการคัดเลือกชนิดสายพันธุ์จุลินทรีย์เพื่อใช้เป็นโพรไบโอติก เพื่อนำไปใช้ประโยชน์เสริมสุขภาพในช่องท้อง จึงมุ่งเน้นในการหาสายพันธุ์ที่มีความสามารถในการยับยั้งการเจริญเติบโตเชื้อก่อโรคในช่องท้อง เช่น *Samonella* spp., *Shigella* spp, *Clostridium defficile*, *Campylobacter pylori* เป็นต้น

นอกจากนี้ศึกษาในการค้นหาสารต้านจุลินทรีย์ก่อโรคของโพรไบโอติกเหล่านั้น เป็นการหาสารซึ่งมักเป็นโปรตีนที่มีความสามารถในการยับยั้งการเจริญเติบโตของเชื้อก่อโรคในช่องท้องเช่นเดียวกัน

## 4.2 วัตถุประสงค์

งานวิจัยนี้จึงต้องการศึกษาคุณสมบัติในด้านต่าง ๆ ของ *Lactobacillus* ที่แยกได้จากช่องปาก เพื่อมุ่งหา probiotic ที่เหมาะสมต่อการประยุกต์ใช้ในช่องปาก

คุณสมบัติที่ต้องการศึกษาได้แก่ 1) ความสามารถในการสร้างสารโปรตีนยับยั้งการเจริญเติบโตของเชื้อก่อโรคในช่องปาก 2) ความสามารถในการเกาะติดเยื่อบุในช่องปาก และ 3) ความสามารถในการสร้างสาร antioxidants

## 4.3 สรุป

โครงการนี้เป็นการต่อยอดจากการศึกษาที่ตีพิมพ์เรื่อง Inhibitory effect of oral *Lactobacillus* against oral pathogens. Lett Appl Microbiol 53:452-459 โดยนำเชื้อ *Lactobacillus* ชนิดสายพันธุ์ต่าง ๆ มาศึกษาคุณสมบัติของโพรไบโอติกในด้านต่าง ๆ ผลการศึกษาโดยสรุปดังนี้

1) พบสารโปรตีนต้านเชื้อก่อโรคในช่องปากจาก *Lactobacillus* สายพันธุ์จำเพาะได้แก่ *L. paracasei* SD1, *L. fermentum* SD11 และ *L. casei* SD2 ได้ศึกษาหากรรมวิธีในการทำบริสุทธิ์โปรตีนจากเชื้อสายพันธุ์ดังกล่าวและศึกษาคุณลักษณะของโปรตีนบริสุทธิ์ ปัจจุบันสามารถทำบริสุทธิ์โปรตีนจากเชื้อ *L. paracasei* SD1 ได้สำเร็จ ตีพิมพ์ 1 เรื่อง (รายละเอียดใน 4.4) และกำลังดำเนินการในส่วนของ *L. fermentum* SD11 และ *L. casei* SD2 คาดว่าจะสามารถตีพิมพ์ได้อีกอย่างน้อย 1 เรื่อง
2) ดำเนินการจดอนุสิทธิบัตร 1 เรื่อง (รายละเอียดใน 4.4)
3) ได้ขยายผลโดย เชื้อ *L. paracasei* SD1 ได้ถูกนำไปศึกษาเป็นโพรไบโอติกในคน ได้ตีพิมพ์ 1 เรื่อง (รายละเอียดใน 4.4)

4) ได้ขยายผลโดย เชื้อ *L. paracasei* SD1 ได้ถูกนำไปศึกษาเป็นโพรไบโอติกในคนและศึกษาผลที่มีต่อภูมิคุ้มกันของอาสาสมัครที่ได้รับโพรไบโอติก ได้ตีพิมพ์ 1 เรื่อง (รายละเอียดใน 4.4)
5) ได้ขยายผลโดยทำการประเมิน เชื้อ *L. paracasei* SD1 ที่ได้ถูกนำไปเตรียมเป็นผลิตภัณฑ์โพรไบโอติกผสมในน้ำผลไม้ ได้ตีพิมพ์ 1 เรื่อง (รายละเอียดใน 4.4)
6) ได้ข้อมูลความสามารถในการเกาะติดของเชื้อ *Lactobacillus* จากช่องปาก สามารถตีพิมพ์ 1 เรื่อง (รายละเอียดใน 4.4)
7) ได้ข้อมูลความสามารถในการผลิตสารต้านอนุมูลอิสระของเชื้อ *Lactobacillus* จากช่องปาก สามารถตีพิมพ์ 1 เรื่อง (รายละเอียดใน 4.4)

## 4.4 เอกสารอ้างอิง

**อนุสิทธิบัตร**

อนุสิทธิบัตร 1 ฉบับ เรื่องกรรมวิธีการเตรียมสารสกัดโปรตีนจากเชื้อ *Lactobacillus fermentum* SD11 และ *Lactobacillus casei* SD2 และการใช้สารสกัดโปรตีนที่ได้จากกรรมวิธีดังกล่าว ในผลิตภัณฑ์อาหารและเวชภัณฑ์ คำขอเลขที่ 1303000002 วันที่ 2 มกราคม 2556 (รายละเอียดดังเอกสารที่แนบ document 1)

**ตีพิมพ์และรอพิจารณาผล**

เรื่องที่ 1 Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. Anaerobe 2014; 27: 17-21. ISI impact factor 2.32 (รายละเอียดในภาคผนวก document 2)

เรื่องที่ 2 Effect of long-term consumption of *Lactobacillus paracasei* SD1 on reducing mutans streptococci and caries risk: a randomized placebo-controlled trial. Dentistry Journal (1st revision) (รายละเอียดในภาคผนวก document 3)

เรื่องที่ 3 Enhancement of salivary human neutrophil peptide 1–3 levels by probiotic supplementation. BMC Oral Health (2015) 15:19: 1-11. DOI 10.1186/s12903-015-0003-0 (รายละเอียดในภาคผนวก document 4)

เรื่องที่ 4 Survival of free and microencapsulated human-derived oral probiotic *Lactobacillus paracasei* SD1 in orange and aloe vera juices. Songkla J Sci Tech (1st revision) (รายละเอียดในภาคผนวก document 5)

เรื่องที่ 5 An assessment of adhesion, aggregation, and surface charges in *Lactobacillus* strains derived from the human oral cavity. Lett Appl Microbiol ($2^{nd}$ revision) (รายละเอียดในภาคผนวก document 6)

เรื่องที่ 6 Antioxidants activity of *Lactobacillus* isolated from oral cavity (manuscript) (รายละเอียดในภาคผนวก document 7)

## คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ การประดิษฐ์

☐ การออกแบบผลิตภัณฑ์

☑ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ.2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ.2535 และพระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ.2542

| สำหรับเจ้าหน้าที่ | |
|---|---|
| วันรับคำขอ 2 มี.ค. 2559 | เลขที่คำขอ 1303000002 |
| วันยื่นคำขอ ๒ พ.ค ๒๕๕๙ | |
| สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ | |
| ใช้กับแบบผลิตภัณฑ์<br>ประเภทผลิตภัณฑ์ | |
| วันประกาศโฆษณา | เลขที่ประกาศโฆษณา |
| วันออกสิทธิบัตร/อนุสิทธิบัตร | เลขที่สิทธิบัตร/อนุสิทธิบัตร |
| ลายมือชื่อเจ้าหน้าที่ | |

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
กรรมวิธีการเตรียมสารสกัดโปรตีนจากเชื้อ *Lactobacillus fermentum* SD11 และ *Lactobacillus casei* SD2 และการใช้สารสกัดโปรตีนที่ได้จากกรรมวิธีดังกล่าว ในผลิตภัณฑ์อาหารและเวชภัณฑ์

2. คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)
มหาวิทยาลัยสงขลานครินทร์
15 ถนนกาญจนวณิชย์
อำเภอหาดใหญ่ จังหวัดสงขลา 90110

3.1 สัญชาติ ไทย
3.2 โทรศัพท์ 0-7428-9322
3.3 โทรสาร 0-7428-9339
3.4 อีเมล์ jittiyut.y@psu.ac.th

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5. ตัวแทน (ถ้ามี) ที่อยู่ (เลขที่ ถนน จังหวัด ประเทศ รหัสไปรษณีย์)
นายจิตติยุทธ เยี่ยมยกกุล
ศูนย์ทรัพย์สินทางปัญญา อุทยานวิทยาศาสตร์
มหาวิทยาลัยสงขลานครินทร์ 15 ถนนกาญจนวณิชย์ อำเภอหาดใหญ่
จังหวัดสงขลา 90110

5.1 ตัวแทนเลขที่ 2266
5.2 โทรศัพท์ 074-289322
5.3 โทรสาร 074-289339
5.4 อีเมล์ jittiyut.y@psu.ac.th

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)
ศ.ดร. รวี เถียรไพศาล ภาควิชาโอษฐวิทยา คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ในวันเดียวกับคำขอรับสิทธิบัตรเลขที่
วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้ โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

8. การยื่นคำขอนอกราชอาณาจักร

| วันยื่นคำขอ | เลขที่คำขอ | ประเทศ | สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ | สถานะคำขอ |
|---|---|---|---|---|
| 8.1 | | | | |
| 8.2 | | | | |
| 8.3 | | | | |

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย

☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9. การแสดงการประดิษฐ์ หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด

วันแสดง วันที่ 2-4 พฤษภาคม 2555 วันเปิดงานแสดง วันที่ 2 พฤษภาคม 2555 ผู้จัด มหาวิทยาลัยสงขลานครินทร์

10. การประดิษฐ์เกี่ยวกับจุลชีพ

| 10.1 เลขทะเบียนฝากเก็บ | 10.2 วันที่ฝากเก็บ | 10.3 สถาบันฝากเก็บ/ประเทศ |
|---|---|---|
| | | |

11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา

☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่น ๆ

12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ เดือน พ.ศ.

☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย

ก. แบบพิมพ์คำขอ 2 หน้า

ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์ 6 หน้า

ค. ข้อถือสิทธิ 1 หน้า

ง. รูปเขียน 2 รูป 2 หน้า

จ. ภาพแสดงแบบผลิตภัณฑ์

☐ รูปเขียน รูป หน้า

☐ ภาพถ่าย รูป หน้า

ฉ. บทสรุปการประดิษฐ์ 1 หน้า

14. เอกสารประกอบคำขอ

☑ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์

☐ หนังสือมอบอำนาจ

☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ

☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย

☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ

☐ เอกสารอื่น ๆ

15. ข้าพเจ้าขอรับรองว่า

☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน

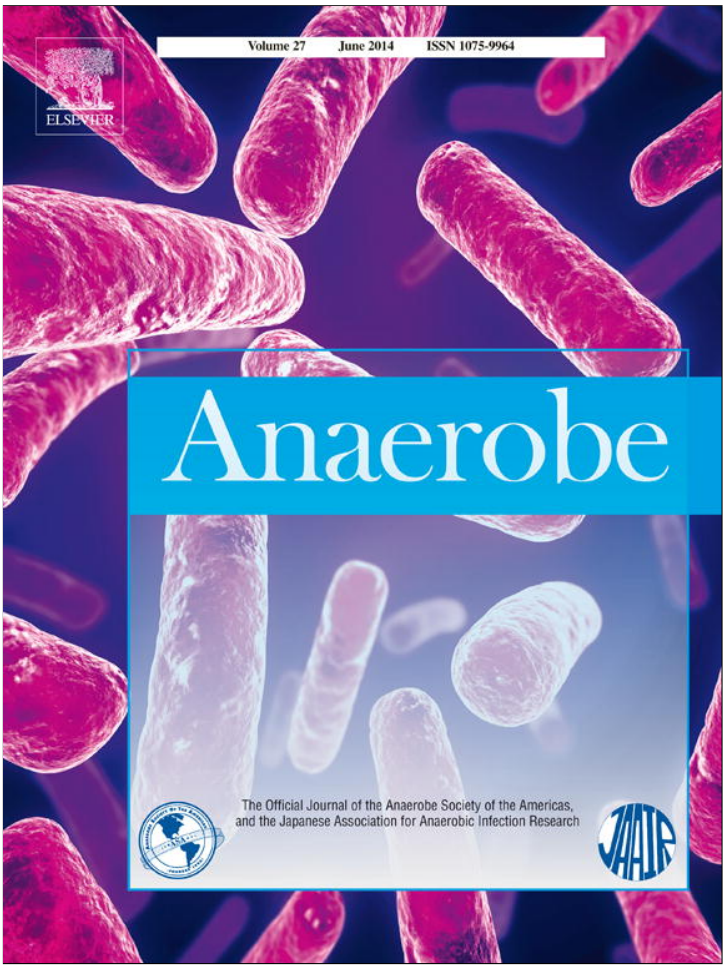
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก.....

16. ลายมือชื่อ ( ☐ ผู้ขอรับสิทธิบัตร / อนุสิทธิบัตร; ☑ ตัวแทน)

(นายจิตติยุทธ เยี่ยมยกกุล)

ตัวแทนผู้รับมอบอำนาจ

**หมายเหตุ** บุคคลใดยื่นคำขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ



Anaerobe 27 (2014) 17–21

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

# Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1

P. Wannun [a], S. Piwat [b], R. Teanpaisan [a,*]

[a] Common Oral Diseases and Epidemiology Research Center and the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai 90112, Thailand
[b] Common Oral Diseases and Epidemiology Research Center and the Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University Hat-Yai 90112, Thailand

ARTICLE INFO

*Article history:*
Received 3 December 2013
Received in revised form
18 February 2014
Accepted 6 March 2014
Available online 15 March 2014

*Keywords:*
*Lactobacillus paracasei*
Paracasin SD1
Bacteriocin
Purification
Characterization
Oral diseases

ABSTRACT

The present study aimed to purify and characterize the antimicrobial protein from *Lactobacillus paracasei* SD1, which is a strain from the human oral cavity. Antimicrobial activity was obtained from purifying the culture supernatant of *L. paracasei* SD1. Purification of the active compound was achieved with ammonium sulfate precipitation followed by chloroform and gel filtration chromatography. As revealed by SDS-PAGE, the active fraction was homogeneous, showing a protein with an approximate molecular weight of 25,000 Da. It was confirmed as having a molecular mass of 24,028.2 Da by mass spectrometry. The antimicrobial compound, named "paracasin SD1", exhibited a broad spectrum against oral pathogens. Paracasin SD1 was stable in a pH range between 3.0 and 8.0 at 100 °C for 5 min, and showed resistance to α-amylase, catalase, lysozyme and whole saliva. However, its activity was lost after proteinase K and trypsin treatment. The results obtained suggest the possibility of using paracasin SD1 for application in prevention/treatment of oral diseases.



## 1. Introduction

There has been an increased interest in the role of probiotic bacteria for promoting and maintaining human health, including oral health [1,2]. The genus *Lactobacillus* includes gram-positive rod-shaped bacteria, which are facultative or microaerophilic anaerobes. They are extensively used as probiotics in the food industry and certain *Lactobacillus* species are also of importance in general health, providing a beneficial microflora in the intestinal tract [2], vagina [3] and oral cavity [4]. They have been proposed to promote oral health due to their ability to inhibit the growth against oral pathogens e.g. cariogenic *Streptococcus* [5–7], and periodontal pathogens [5,7]. The production of antimicrobials against certain pathogens is often considered as an important trait in terms of probiotic efficacy, thus it has been an important criterion in the selection of a probiotic strain. A variety of antimicrobial compounds usually include organic acids, short-chain fatty acids, hydrogen peroxide, and bacteriocin.

Several bacteriocins of *Lactobacillus paracasei* of different origins have been characterized; for example, various bacteriocins were found to be produced by *L. paracasei* subsp. *paracasei* BMK2005 isolated from feces, *L. paracasei* BGBUK2-16 isolated from traditional homemade cheese, and *L. paracasei* HD1.7 isolated from traditional Chinese fermented vegetable food, which contained small peptides sized 2.4, 7.0 and 11.0 kDa, respectively [8–10]. A larger size of bacteriocin (56 kDa) was produced by *L. paracasei* HL32 isolated from an intestinal tract [11]. However, the knowledge of antimicrobial proteins or bacteriocins produced by *L. paracasei* strains having an oral origin is still limited.

*L. paracasei* SD1 is a human oral strain possessing potent antimicrobial activity against cariogenic and periodontal pathogens [7]. In addition, it has been proven to give a benefit for clinical trials in reducing the pathogenic agents in the oral cavity of volunteers [12]. However, the antimicrobial protein substance has not been studied as yet. Thus, the present study aimed to purify and characterize the antimicrobial protein of *L. paracasei* SD1.

## 2. Materials and methods

### 2.1. Bacterial strains and strain selection

A total of 21 *L. paracasei* strains, 20 clinical isolates from the oral cavity and a *L. paracasei* CCUG 32212 reference strain, were

* Corresponding author. Tel.: +66 74 429878; fax: +66 74 212922.
*E-mail address:* rawee.t@psu.ac.th (R. Teanpaisan).

http://dx.doi.org/10.1016/j.anaerobe.2014.03.001
1075-9964/

obtained from the previous study of Piwat et al. [13], and the culture collection was kept at −80 °C in the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Thailand. All strains were identified using restriction fragment length polymorphism (RFLP) analysis of a polymerase chain reaction (PCR) [14]. Sequencing of the 16S-rRNA was performed for strains with uncertain identifications.

Initially, the cell free supernatant and supernatant of sonicated cells of all tested strains were screened for inhibitory activity, and *Streptococcus mutans* ATCC 25175 was used as an indicator strain at this step. After recovery from storage, each tested strain was inoculated into a De Man Rogosa Sharpe (MRS) broth at 37 °C for 24 h. Bacterial cells were separated from the broth culture by centrifugation at 8000 g for 10 min. The supernatant was adjusted to pH 6.5 with 1 M NaOH and then treated with proteinase K, catalase and lysozyme at a final enzyme concentration of 1 mg $mL^{-1}$. The treated supernatants were tested for antimicrobial activity using an agar well diffusion assay.

The supernatant was concentrated 10-fold in a Speed-Vac concentrator, and then the pH was adjusted to 6.5 with 1 M NaOH. One part of the concentrated supernatant was extracted with equal volume of either chloroform or ethyl acetate separately. After thoroughly mixing, the organic phase evaporated and the sediment was resuspended in phosphate buffer saline (PBS, pH 7.0) to the original volume.

The bacterial cells were washed three times with PBS, and the sediment was than resuspended in PBS to the original volume. The washed cells suspension was sonicated with a cell disrupter to lyse the bacterial cell wall for 5 min in an ice bath. Cell debris was removed by centrifugation at 8000 g for 10 min, and the supernatant was used as the sonicated cell free extract.

All samples were examined for inhibitory activity against *S. mutans* ATCC 25175 using a microdilution assay. The inhibitory activity was defined as >80% inhibition growth of an indicator strain by the tested sample compared to the control (indicator strains with medium only).

From our screening study, the results indicated that the strongest inhibitory activity was observed in the aqueous phase of the supernatant of *L. paracasei* SD1. Thus, the strain was subjected to a further bacteriocin purification study.

### 2.2. Purification of the bacteriocin

One liter of the supernatant of *L. paracasei* SD1 was precipitated with 40% ammonium sulfate overnight at 4 °C with stirring. The precipitated proteins were collected by centrifugation of 20,000 g at 4 °C for 20 min and then resuspended in 10 mL of 0.1 M PBS. The precipitated proteins were dialyzed twice against 2 L of 0.05 M PBS for 24 h using a dialysis bag with a molecular weight cutoff of 3.5 kDa. The dialyzed proteins were mixed thoroughly with an equal volume of chloroform and then centrifuged with 6000 g at 4 °C for 10 min. The organic phase was evaporated and the sediment was resuspended in PBS.

The sample (0.5 mL) was loaded on a Superdex 200 HR 10/30 column (LKB-Pharmacia, Uppsala, Sweden); a fast protein liquid chromatography (FPLC) system. A buffer was applied in 0.5 mL $min^{-1}$ with a 0.05 M sodium phosphate buffer (pH 6.5) with the addition of 0.15 M NaCl for elution, with a time interval of 20 min after the samples were injected. The elution was monitored simultaneously at 280 nm and controlled in 0.5 mL $min^{-1}$. Fractions of 3 mL each were collected and then dialyzed two times against 2 L of distilled water for 24 h using a dialysis bag with a molecular weight cutoff of 3.5 kDa. The flow-through and the dialysates were concentrated by lyophilization before testing for bacteriocin activity.

### 2.3. Determination of protein concentration

The protein content was estimated using Bradford's assay [15], a reagent kit of Bio-Rad Laboratories, USA. A sample (20 μL) was added to 1 mL of the Bradford reagent, and then mixed for 1 min at room temperature. The mixture was measured for the protein concentrations at 595 nm by comparing with bovine serum albumin (Sigma–Aldrich, USA) as the standard.

### 2.4. Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (tricine SDS-PAGE)

Tricine-SDS PAGE analysis of purified bacteriocin was performed according to Schagger et al. [16], with 12% acrylamide gel. Following electrophoresis, the gel was cut into two parts; one was stained with Coomassie brilliant blue R-250 (Bio-Rad, Canada), whilst the other was fixed in 20% (v/v) isopropanol and 10% (v/v) acetic acid for 1.5 h, and then rinsed in sterile distilled water for 16 h. After the final rinse step, the gel was overlaid with soft agar medium inoculated with the tested indicator strain.

### 2.5. Mass spectrometry

The active fraction was dissolved at the concentration of 1 mg $mL^{-1}$ in 50% acetonitrile in water, and 10 μL of sample was applied in liquid chromatography mass spectrometry (LC-MS, model V BIO-Q triple-quadrupole; Biotech, Altrincham, UK).

The sample was pumped at a flow rate of 5 μL $min^{-1}$, delivered by a 140-A solvent delivery system (Applied Biosystems, Foster City, Cailf.). The capillary tip was set at a voltage of 3.2 kV, and the sample cone voltage was set at 30 V. The mass spectrometer was set to scan the mass range of 20,000–30,000 Da. The mass spectrometer was calibrated by preliminary analysis of horse heart myoglobin (Sigma–Aldrich, USA).

### 2.6. Sensitivity to heat, pH, and enzymes

The thermal sensitivity of purified bacteriocin was evaluated by heating at 60, 80 and 100 °C and autoclaved at 120 °C for 20 min in a water bath. After cooling, the inhibitory activity was tested and compared with the nontreated control. A residual inhibitory activity was measured using the microdilution assay. The stability of the lyophilized bacteriocin during a long-term storage was tested at 25, 4 and −20 °C over 6 months. Bacteriocin aliquots were taken from storage every month and were measured for antimicrobial activity.

The effect of pH on purified bacteriocin activity was tested at various pH values from 3.0 to 9.0. The bacteriocin powder was dissolved with the buffer, incubated for 4 h at 37 °C, and then the pH was adjusted to 6.5 with either 1 M NaOH or 1 M HCl. Then the samples were assayed as described above.

Sensitivity to enzymatic proteolysis was tested by treatment of bacteriocin aliquots in 0.05 M PBS pH 7.0 with proteolytic enzymes (trypsin and proteinase K), α-amylase, catalase and lysozyme at a final enzyme concentration of 1 mg $mL^{-1}$. Sensitivity to whole saliva was also tested. After 3 h of incubation at 37 °C, the reaction was stopped by boiling for 3 min and then the samples were subsequently assayed for antimicrobial activity. The control was prepared in parallel by incubating without the enzyme. In all the sensitivity tests, *S. mutans* ATCC 25175 was used as the indicator strain.

### 2.7. Inhibitory spectrum of the bacteriocin

The bacteriocin was tested for its inhibitory activity using the broth microdilution assay. Several indicator strains (see Table 3)

**Table 1**
Purification stage and antimicrobial activity of *L. paracasei* SD1.

| Purification stage | Volume (mL) | Total protein (μg) | Specific activity (AU $\mu g^{-1}$) |
|---|---|---|---|
| Culture supernatant | 1000 | 63,000 | 53.8 |
| Ammonium sulfate precipitation | 50 | 700 | 204.1 |
| Chloroform extraction | 50 | 135 | 1048.2 |
| Gel filtration chromatography | 1 | 0.8 | 46,875.0 |

Note. AU (arbitrary units) was expressed as the reciprocal highest dilution inhibiting the growth of the indicator strain.

were chosen on the basis of their importance in and relatedness to the oral ecosystem. All test strains were grown overnight in MRS or brain heart infusion broth. The bacteriocin sensitivity of various indicators was presented using MIC. Antimicrobial protein titers were reported in arbitrary units (AU) per mL or μg, and one AU was defined as the reciprocal of the highest dilution showing inhibitory action towards the sensitive strains.

## 3. Results

### 3.1. Screening antimicrobial protein production

A total of 20 clinical *L. paracasei* strains and a reference strain, *L. paracasei* CCUG 32212, were screened for inhibitory activity against *S. mutans* ATCC 25175. It was demonstrated that a 10 times-concentrated supernatant of all tested strains exhibited a stronger activity compared to the supernatant from the sonicated cell extract. Extract with chloroform and ethyl acetate did not result in any removal of the substance from the aqueous phase. Of those, *L. paracasei* SD1 exhibited the strongest inhibitory growth on *S. mutans* ATCC 25175. The inhibitory activity from the supernatant of *L. paracasei* SD1 was partially lost after treatment with catalase and lysozyme, and it was completely absent after treatment with proteinase K (Fig. 1).

### 3.2. Purification of bacteriocin from L. paracasei SD1

Initially, the antimicrobial protein was precipitated from a cell free supernatant of *L. paracasei* SD1 with 40, 60 or 80% ammonium sulfate. It was shown that the strongest antimicrobial activity against *S. mutans* ATCC 25175 was recovered in the protein pellet, which was saturated with 40% ammonium sulfate. Increased specific activity of the precipitated protein (from 204.1 to 1048.2 AU $\mu g^{-1}$) was found after treatment with chloroform (1:1 v/v) (Table 1).

The 40% ammonium sulfate precipitated protein was further purified using gel filtration chromatography (Superdex 200 HR 10/30 column) with the FPLC system. It was revealed that the specific antimicrobial activity increased from 53.8 AU $\mu g^{-1}$ (in the supernatant) to 46,875 AU $\mu g^{-1}$ (in the active fraction). Also an inhibition zone of the specific antimicrobial activity against *S. mutans* ATCC 25175 was detectable, and the tricine SDS-PAGE electrophoresis estimated the protein molecular mass at approximate 25,000 Da (Fig. 2a). The LC-MS analysis demonstrated a specific protein molecular mass of 24,028.2 Da (Fig. 2b).

### 3.3. Characterization of bacteriocin activity

#### 3.3.1. Enzyme sensitivity

The bacteriocin activity was completely inactivated by trypsin and proteinase K, and a relative loss of activity was observed after treatment with lysozyme. The bacteriocin activity was found to be resistant to α-amylase, catalase and whole saliva (Table 2).

#### 3.3.2. pH and heat sensitivity

The antimicrobial activity seemed to be active across a broad pH range between pH 3.0–8.0, and the most active activity was observed with acidic pH between 5.0 and 6.0. The antimicrobial activity was completely lost at pH value 9.0.

The antimicrobial activity did not show any detectable loss of activity when heated at 60 °C, and there was a slight decrease of activity at 80 °C. There was no detectable activity observed when it was heated at 120 °C (Table 2). It was found that storage of paracasin SD1 for 6 months at 25, 4 and −20 °C did not affect its activity.

#### 3.3.3. Spectrum of activity

The antibacterial activity of the purified antimicrobial protein produced by *L. paracasei* SD1 was tested against selected oral pathogens using the microdilution assay. A total of 12 strains belonging to 6 species of Gram-positive, Gram-negative bacteria and *Candida albicans* were examined. The results demonstrated that all tested strains were sensitive to the purified protein except *Fusobacterium nucleatum*. It was noted that Gram-positive bacteria was more sensitive than Gram-negative bacteria (Table 3).

## 4. Discussion

This work describes the purification and characterization of an antimicrobial protein produced by a strain of *L. paracasei* SD1 isolated from the human oral cavity. An earlier report showed that *L. paracasei* SD1 had strong antimicrobial activity against a wide range of oral pathogens [7]; however, the specific antimicrobial compound has not yet been identified. In this study, it was demonstrated that the antimicrobial compounds of *L. paracasei* SD1 were found in the cell-free supernatant, which may include bacteriocin, hydrogen peroxide and antioxidative activity. The finding of partial loss activity after the catalase treatment indicated that there was hydrogen peroxide in the supernatant. The active compound also had a proteinaceous nature because its activity was lost after treatment with proteinase K. The organic solvent could

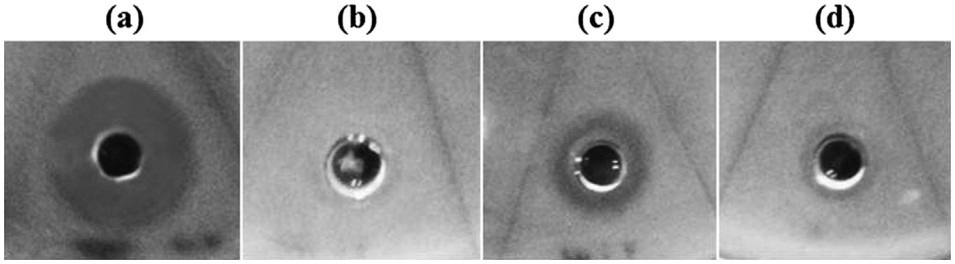



**Fig. 1.** The inhibitory activity of the culture supernatant of *L. paracasei* SD1 against *S. mutans* ATCC 25175 treated with various enzymes. (a) untreated supernatant, (b) treated with proteinase K, (c) treated with catalase, and (d) treated with lysozyme.

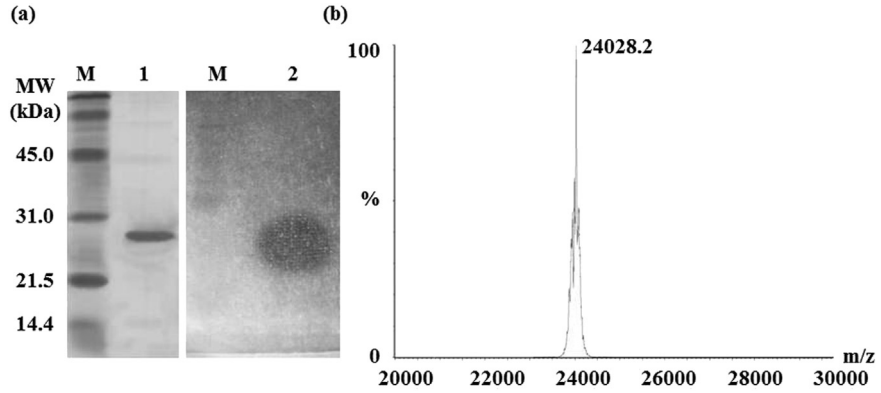



**Fig. 2.** (a) Tricine-SDS PAGE analysis of purified bacteriocin from *L. paracasei* SD1: (Lane 1) Coomassie brilliant blue stained gel, (Lane 2) gel overlaid with soft agar containing *S. mutans* ATCC 25175, and (Lane M) molecular weight standard, low range (Bio-Rad), and (b) Liquid chromatography mass spectrometry of paracasin SD1; only one peak was obtained corresponding to a molecular mass of 24,028.2 Da.

not remove any antimicrobial protein from the aqueous phase of the culture supernatant, which indicated that the bacteriocin produced from *L. paracasei* SD1 has a hydrophilic character.

In this study, we report on purification and characterization of the bacteriocin named "paracasin SD1", produced by *L. paracasei* SD1. It was demonstrated that paracasin SD1 was purified from cell-free supernatant by precipitation with ammonium sulfate, chloroform extraction and gel filtration chromatography. The chloroform extraction of the ammonium precipitated protein increased the antimicrobial activity, suggesting a high degree of purification of paracasin SD1. The antimicrobial activity of ammonium sulfate precipitated proteins increased after mixing with chloroform. It may explain by that some bacteriocins appear in their native state as aggregates with other molecules e.g. lipid materials. These aggregates may mask the antimicrobial activity partially. Such complexities can be eliminated by extraction with chloroform [17]. This was supported by a study of Contreras et al. [18], who demonstrated that a simple one-step methanol-chloroform extraction could remove most fatty acid contamination from the ammonium sulfate precipitated bateriocin, which resulted in a pure bacteriocin [18].

The molecular mass of the purified protein was determined by LC-MS, and it was found to be 24,028.2 Da. To our knowledge, paracasin SD1 might be a novel bacteriocin produced by *L. paracasei* SD1 since its molecular mass differs from previous bacteriocins reported by others [8–10,19]. Paracasin SD1 has a rather high molecular weight compared with those of peptides (2–4 kDa) produced by non-oral origin *L. paracasei* strains.

Accumulating evidence suggests that probiotics exert various biological roles through several mechanisms, one of the most considered being bacteriocin production. The results in the present study agree with previous studies indicating that bacteriocin production is a specific feature of certain strains. Among the *L. paracasei* strains screened for their bacteriocin in this study, an antimicrobial protein band was found only in *L. paracasei* SD1 (data not shown). For this reason, bacteriocin production has been an important criterion in the selection of certain probiotic strains. Moreover few studies have demonstrated the impact of bacteriocin production on the ability of a strain to complete within the GI tract and/or positively influence the health of the host [20]. Apart from directly inhibiting competing strains or pathogens of bacteriocins, they may function as colonizing peptides, facilitating the introduction and/or dominance of a producer into an already occupied niche [21]. In addition, bacteriocins may function as signaling peptides, either signaling other bacteria through quorum sensing and bacterial cross talk within microbial communities or signaling cells of the host immune system [22–26]. For these reasons, ability of strains to produce bacteriocin has always received attention.

**Table 2**
Effects of enzyme, pH and heat treatment on purified paracasin SD1 produced from *L. paracasei* SD1.

| Treatment | Antimicrobial activity | Treatment | Antimicrobial activity |
|---|---|---|---|
| Enzyme | | Heat °C/min | |
| α-amylase | + + + | 60/5 | + + + |
| Catalase | + + + | 80/5 | + + + |
| Lysozyme | + | 100/5 | + |
| Proteinase K | – | 120/5 | – |
| Trypsin | – | 60/10 | + + + |
| Whole saliva | + + + | 80/10 | + + |
| pH | | 100/10 | – |
| 3.0 | + | 120/10 | – |
| 4.0 | + + | 60/20 | + + + |
| 5.0 | + + + | 80/20 | + |
| 6.0 | + + + | 100/20 | – |
| 7.0 | + + | 120/20 | – |
| 8.0 | + | | |
| 9.0 | – | | |

Note. Antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + +, antimicrobial activity ≥37,500 AU $mL^{-1}$; + +, antimicrobial activity ≥25,000 AU $mL^{-1}$; +, antimicrobial activity ≥12,500 AU $mL^{-1}$; -, non-detected antimicrobial activity. All tests were performed in triplicate.

**Table 3**
Bacterial strains used and antimicrobial activity of purified paracasin SD1.

| Indicator strains | Antimicrobial activity |
|---|---|
| **Gram positive bacteria:** | |
| *Streptococcus mutans* ATCC 25175 | + + + |
| *Streptococcus sobrinus* ATCC 33478 | + + + |
| *Lactobacillus casei* ATCC 393 | + + + |
| *Lactobacillus fermentum* ATCC 14931 | + + + |
| *Lactobacillus paracasei* CCUG 32212 | + + |
| *Lactobacillus plantarum* ATCC 14917 | + + + |
| *Lactobacillus rhamnosus* ATCC 7469 | + + |
| *Lactobacillus salivarius* ATCC 11741 | + + |
| **Gram negative bacteria:** | |
| *Aggregatibacter actinomycetemcomitans* ATCC 33384 | + |
| *Fusobacterium nucleatum* ATCC 25586 | – |
| *Porphyromonas gingivalis* ATCC 33277 | + |
| **Yeast:** | |
| *Candida albicans* ATCC 90028 | + + |

Note. Antimicrobial activity was determined by the microdilution assay against an oral pathogenic. + + +, antimicrobial activity ≥37,500 AU $mL^{-1}$; + +, antimicrobial activity ≥25,000 AU $mL^{-1}$; +, antimicrobial activity ≥12,500 AU $mL^{-1}$; -, non-detected antimicrobial activity. All tests were performed in triplicate.

Regarding the characterization of paracasin SD1, a broad spectrum of antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and even anti-*Candida* was found. It was noted that Gram-positive bacteria was more sensitive to paracasin SD1 than Gram-negative bacteria. This may be explained by the observation that bacteriocins usually exhibit antimicrobial activity against closely related strains of bacteria to the producer [27]. However, why *F. nucleatum* was not sensitive to paracasin SD1, is difficult to explain.

The antimicrobial activity may be influenced by the action of the pH, temperature and proteolytic enzymes. This study has demonstrated that paracasin SD1 could be active in a broad pH range from 3.0 to 8.0, although maximum activity was detected within a narrow pH range of 5.0–6.0. The antimicrobial activity appeared to be relative heat-stable, since inhibitory activity was maintained upon heating at 100 °C for 5 min. Therefore, paracasin SD1 could be used in pasteurized products. In addition, paracasin SD1 was stable and unchanged antimicrobial activity over long periods of storage.

Concerning sensitivity tests to various enzymes, it was found that paracasin SD1 was inactivated by trypsin, a proteolytic enzyme, which usually presents in the small intestine. However, the antimicrobial activity of paracasin SD1 was not sensitive to $\alpha$-amylase, catalase, lysozyme and whole saliva, indicating that paracasin SD1 may be practical to use as a topical application in the oral cavity.

## 5. Conclusion

Paracasin SD1 displayed an attractive antimicrobial activity, being active against the following oral pathogens: *S. mutans, Streptococcus sobrinus, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans.* This and other interesting features make paracasin SD1 an attractive candidate for oral applications, especially for prevention and/or treatment of oral diseases.

## Acknowledgments

This work was supported by the National Research Council of Thailand (DEN550005M), the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (AGR 540555M), and the National Science and Technology Development Agency (NSTDA) of Thailand.

## References

[1] Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, de Vos WM, et al. Demonstration of safety of probiotics a review. Int J Food Microbiol 1998;44:93–106.
[2] Parvez S, Malik KA, Ah Kang S, Kim HY. Probiotics and their fermented food products are beneficial for health. J Appl Microbiol 2006;100:1171–85.
[3] Reid G. Probiotic and prebiotic applications for vaginal health. J AOAC Int 2012;95:31–4.
[4] Meurman JH, Stamatova I. Probiotics: contributions to oral health. Oral Dis 2007;13:443–51.
[5] Koll-Klais P, Mandar R, Leibur E, Marcotte H, Hammarstrom L, Mikelsaar M. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. Oral Microbiol Immunol 2005;20:354–61.
[6] Simark-Mattsson C, Emilson CG, Hakansson EG, Jacobsson C, Roos K, Holm S. *Lactobacillus*-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. Eur J Oral Sci 2007;115:308–14.
[7] Teanpaisan R, Piwat S, Dahlen G. Inhibitory effect of oral *Lactobacillus* against oral pathogens. Lett Appl Microbiol 2011;53:452–9.
[8] Lozo J, Vukasinovic M, Strahinic I, Topisirovic L. Characterization and antimicrobial activity of bacteriocin 217 produced by natural isolate *Lactobacillus paracasei* subsp. *paracasei* BGBUK2-16. J Food Prot 2004;67:2727–34.
[9] Ge J, Ping W, Song G, Du C, Ling H, Sun X, et al. Paracin 1.7, a bacteriocin produced by *Lactobacillus paracasei* HD1.7 isolated from Chinese cabbage sauerkraut, a traditional Chinese fermented vegetable food. Wei Sheng Wu Xue Bao 2009;49:609–16.
[10] Bendjeddou K, Fons M, Strocker P, Sadoun D. Characterization and purification of a bacteriocin from *Lactobacillus paracasei* subsp. *paracasei* BMK2005, an intestinal isolate active against multidrug-resistant pathogens. World J Microbiol Biotechnol 2012;28:1543–52.
[11] Pangsomboon K, Kaewnopparat S, Pitakpornpreecha T, Srichana T. Antibacterial activity of a bacteriocin from *Lactobacillus paracasei* HL32 against *Porphyromonas gingivalis*. Arch Oral Biol 2006;51:784–93.
[12] Teanpaisan R, Piwat S. *Lactobacillus paracasei* SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. Clin Oral Investig; 2013, in press, doi:10.1007/s00784-013-1057-5.
[13] Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlen G. *Lactobacillus* species and genotypes associated with dental caries in Thai preschool children. Mol Oral Microbiol 2010;25:157–64.
[14] Teanpaisan R, Dahlen G. Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral *Lactobacillus* species. Oral Microbiol Immunol 2006;21:79–83.
[15] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[16] Schagger H, Aquila H, Von Jagow G. Coomassie blue-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for direct visualization of polypeptides during electrophoresis. Anal Biochem 1988;173:201–5.
[17] Parada LJ, Caron CR, Medeiros AB, Soccol CR. Bacteriocins from lactic acid bacteria: purification,Properties and use as biopreservatives. Braz Arch Biol Technol 2007;50:521–42.
[18] Contreras BG, De Vuyst L, Devreese B, Busanyova K, Raymaeckers J, Bosman F, et al. Isolation, purification, and amino acid sequence of lactobin A, one of the two bacteriocins produced by *Lactobacillus amylovorus* LMG P-13139. Appl Environ Microbiol 1997;63:13–20.
[19] Pangsomboon K, Bansal S, Martin GP, Suntinanalert P, Kaewnopparat S, Srichana T. Further characterization of a bacteriocin produced by *Lactobacillus paracasei* HL32. J Appl Microbiol 2009;106:1928–40.
[20] Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc Natl Acad Sci U S A 2007;104:7617–21.
[21] Riley MA, Wertz JE. Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie 2002;84:357–64.
[22] Czaran TL, Hoekstra RF, Pagie L. Chemical warfare between microbes promotes biodiversity. Proc Natl Acad Sci U S A 2002;99:786–90.
[23] Di Cagno R, De Angelis M, Limitone A, Minervini F, Simonetti MC, Buchin S, et al. Cell-cell communication in sourdough lactic acid bacteria: a proteomic study in *Lactobacillus sanfranciscensis* CB1. Proteomics 2007;7:2430–46.
[24] Gobbetti M, De Angelis M, Di Cagno R, Minervini F, Limitone A. Cell-cell communication in food related bacteria. Int J Food Microbiol 2007;120:34–45.
[25] Meijerink M, van Hemert S, Taverne N, Wels M, de Vos P, Bron PA, et al. Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. PLoS One 2010;5:e10632.
[26] Majeed H, Gillor O, Kerr B, Riley MA. Competitive interactions in *Escherichia coli* populations: the role of bacteriocins. ISME J 2011;5:71–81.
[27] Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek 1996;70:113–28.

*Dent. J.* **2014**, *2*, 1-x; doi:10.3390/dj20x000x

OPEN ACCESS

*dentistry journal*

**ISSN 2304-6767**

www.mdpi.com/journal/dentistry

*Article*

# Effect of long-term consumption of *Lactobacillus paracasei* SD1 on reducing mutans streptococci and caries risk: a randomized placebo-controlled trial

**Rawee Teanpaisan[1,*], Supatcharin Piwat[2], Sukanya Tianviwat[2], Benchamat Sophatha[1] and Thanyanan Kampoo[1]**

[1] Common Oral Diseases and Epidemiology Research Center and the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, 90112, Thailand; E-Mails: benchamat02@gmail.com (B.S.); kanokporn.k@psu.ac.th (T.K.)

[2] Common Oral Diseases and Epidemiology Research Center and the Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, 90112, Thailand; E-Mails: supacharin.p@psu.ac.th (S.P.); sukanya.ti@psu.ac.th (S.T.)

***** Rawee Teanpaisan; E-Mail: rawee.t@psu.ac.th; Tel.: +66-89-6555836; Fax: +66 74 212922

External Editor:

*Received: / Accepted: / Published:*

---

Abstract: **Background:** A previous study revealed *Lactobacillus paracasei* SD1, a probiotic strain, could reduce mutans streptococci (MS). The aim of this study was to evaluate the long-term effects of *L. paracasei* SD1 on the colonization of MS, and whether caries lesions developed. **Methods:** After informed consent, 122 children were recruited and randomly assigned to the probiotic or control groups. The probiotic group received milk-powder containing *L. paracasei* SD1 and the control group received standard milk-powder once daily for 6 months. Salivary MS and lactobacilli were enumerated using differential culture at baseline and at 3-month intervals for 12 months. The persistence of *L. paracasei* SD1 was investigated using AP-PCR for DNA-fingerprinting. Oral health was examined at baseline and at the end of the study according to WHO criteria. **Results:** The long-term consumption could prolong colonization of *L. paracasei* SD1. Significantly reduced MS counts and increased lactobacilli levels were found among children in the probiotic group. There was less caries in the probiotic group at the end of the study. A significant reduction of the development of new caries lesions (4.5 times) was observed in the high caries risk group but not in the low caries risk group. **Conclusions:** Results

demonstrate that the long-term daily ingestion of the human-derived probiotic *L. paracasei* SD1 significantly reduces the number of MS and caries risk.

**Keywords:** *Lactobacillus paracasei* SD1; mutans streptococci; probiotics; dental caries

## 1. Introduction

Dental caries still remains one of the most common diseases worldwide, especially in developing countries. A great effort has been made searching for the means to reduce cariogenic microflora, however, this seems to have been unsuccessful in completely eradicating caries-associated microorganisms. A number of studies have reported the use of probiotic strains for the prevention of oral diseases, including dental caries [1, 2].

*Lactobacillus* species are the major organisms that have been previously evaluated as potential probiotics for the prevention of dental caries. This is mainly due to their purported inhibitory activity against cariogenic mutans streptococci (MS) [3-5], and the fact that they are generally considered safe for oral administration in humans [6]. A number of *in vivo* clinical control trials have been performed and most of those demonstrated positive effects of probiotics on reducing MS [2]. Probiotic strains previously used for oral care are those mainly derived from non-oral origins e.g. the gastrointestinal tract. They might not be ideal strains for the oral environment since this quite differs from the gastrointestinal habitat. This has been supported by the findings that such strains usually colonize in the oral cavity for only a short time [7-9]. However, most of the information received has been from short-term consumption of probiotics. A long term study is needed to clarify whether how long the probiotics would persist and whether a cariogenic microbial shift would be induced.

Another point of concern is that most studies observed the number of MS as the outcome, while reports of outcomes of caries lesion development are still lacking. This is impossible to do so unless a long-term study is carried out.

Our previous studies demonstrated that *Lactobacillus paracasei* SD1, an oral human-derived strain, is a good candidate to be a probiotic for oral health. The randomized double blinded studies showed that a short-term, 4-week consumption of milk powder containing *L. paracasei* SD1 could reduce salivary MS in volunteers. The strain could still be found up to 4 weeks following cessation of dosing [10, 11].

The aims of the present study were to investigate the long-term effect of *L. paracasei* SD1 on the colonization of MS, and the persistence of *L. paracasei* SD1 *in vivo* was also evaluated. Moreover, caries lesion development was monitored.

## 2. Results and Discussion

In recent years, bacteriotherapy in the form of probiotic bacteria with an inhibiting effect on oral pathogens has been a promising concept, thus, probiotic applications for prevention and/or treatment of oral diseases have received increased attention. Short- and long-term intervention in clinical trials have been performed, and most have shown that receiving probiotic strains could decrease the MS levels in

either saliva or dental plaque. However, some different results have also been reported. This inconsistency has been explained by the different strains used or differences in study design and/or study population [12-18].

Most studies on probiotics and dental caries have measured changes in MS counts, but only a few studies have used dental caries as the endpoint measurement. This study was designed as a randomized double-blinded clinical long-term trial on the effects of *L. paracasei* SD1, a human oral-origin strain, on both MS level and dental caries. The number of children investigated in this study is given in the flow chart of Figure 1. The baseline characteristics of the children in both groups who participated in the study are presented in Table 1. There were no statistically significant differences between the groups in age, pH of saliva and caries status (DMFT) at the baseline ($P > 0.05$). The sex in both groups was a significant difference ($P < 0.05$), there were more females in the probiotic group than in the control group. The dropout rate from the baseline was low, and compliance with the study protocol during the intervention was satisfactory based on the information from the logbook.

**Figure 1.** Flow-chart showing the progress of children participating at each time period of the 12 months study

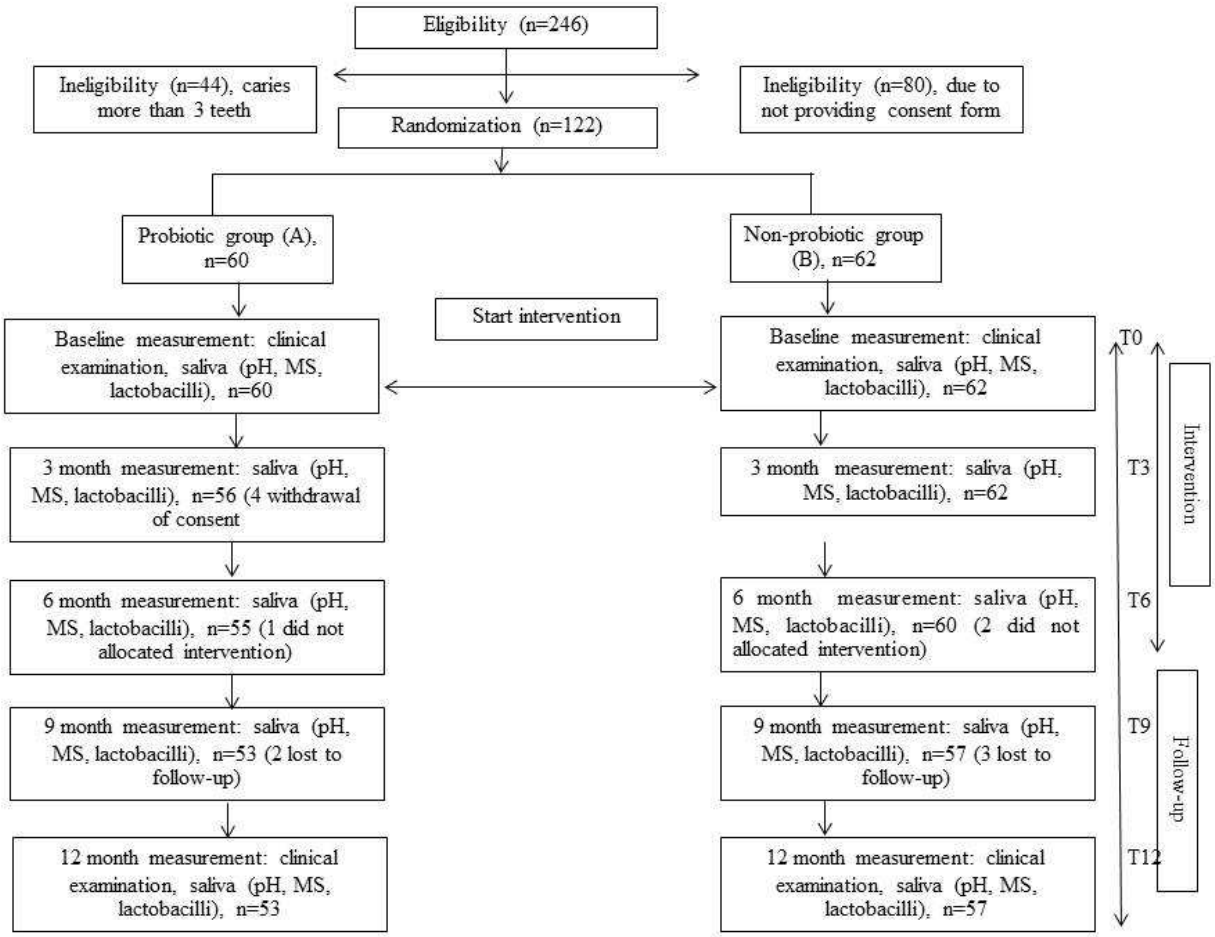


**Table 1.** General characteristics of children

| Characteristics | Control | Probiotic |
|---|---|---|
| Age (years) | 13.13±0.71 | 13.25±0.73 |
| Sex: | | |
| male | 41 | 23 |
| female | 22 | 36 |
| DMFT at baseline | 0.91 ± 1.40 | 0.86 ± 1.12 |
| pH of saliva: | | |
| T0 | 7.47±0.29 | 7.50±0.30 |
| T3 | 7.18±0.42 | 7.38±0.49 |
| T6 | 7.60±0.60 | 7.78±0.36 |
| T9 | 7.70±0.41 | 7.57±0.49 |
| T12 | 7.32±0.47 | 7.30±0.56 |

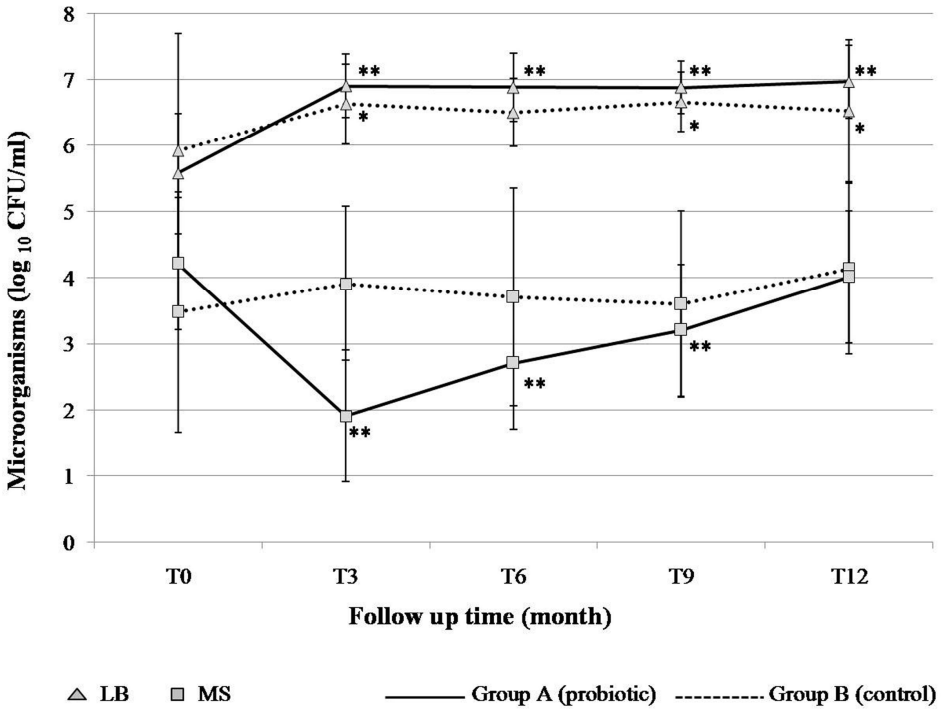
In previous studies, the follow-up samplings were generally conducted at the termination of probiotic intervention, and any information on the post intervention re-growth of the suppressed target bacteria afterwards was minimal. This study monitored the MS levels more frequently during the intervention and follow-up time. It was hypothesized that long-term exposure to probiotics could prevent or delay MS colonizing, and accordingly prevent dental caries. Thus, evaluation of the study was based on MS colonization and the increment of dental caries between the children receiving probiotic milk and control milk. The levels of salivary MS and lactobacilli at baseline and at the end of the study are demonstrated in Figure 2. At baseline, the mean counts ($\log_{10}$ CFU/ml ± SD) of MS in the probiotic and control groups were 4.21±1.01 and 3.48±1.82, respectively, which was not a statistically significant difference ($P > 0.05$). Significant differences of MS levels between the two groups were found at T3 ($P < 0.001$) and T6 ($P = 0.001$). The MS levels did not change significantly among children taking the control milk throughout the study, except that it significantly increased during T9-T12 ($P = 0.011$). The number of MS in the saliva of children in the probiotic group was found to have significantly decreased during and 3 months after receiving the probiotic milk (T3-T9) compared to the baseline ($P < 0.001$). At the end of the study (T12), the MS level was not significantly different compared to the baseline. It was assumed that the presence of *L. paracasei* SD1 resulted in an inhibiting effect on MS. However, it should be noted that probiotic action involves several mechanisms including production of antimicrobial substances, competition with pathogens by preventing cellular adhesion and invasion, and modulation of local and systemic immune functions [19]. We have also found a significant increase of salivary innate immune among children receiving the probiotic milk at T3 (published separately). So far, there is no clear explanation for the observation why there was a rapid decrease of MS at T3, it might be the result of several mechanisms of probiotic action in combination. This requires the further elucidation.

At baseline, the mean counts of lactobacilli in the probiotic and control groups were 5.57±0.91 and 5.92±1.77, respectively, being significantly lower in the probiotic group than in the control group ($P < 0.001$). The significant differences of lactobacilli counts between both groups were found at T3 ($P = 0.027$), T6 ($P = 0.003$), T9 ($P = 0.043$) and T12 ($P < = 0.027$). During the follow-up period, the level of lactobacilli increased significantly in both groups compared to the baseline; however, it increased more in the probiotic group than in the control group. An oral colonization with *L. paracasei* SD1 showed good compliance due to the presence of *L. paracasei* SD1 in the saliva. At baseline, *L.*

*paracasei* SD1 was not detectable in either the control or test group. During the study, the finding of *L. paracasei* SD1 could be detected as 85%, 80%, 60% and 0% at T3, T6, T9 and T12, respectively, among the children who were receiving probiotic milk, but it was not found in the control group.

The finding above raised the question of whether the probiotic bacteria can permanently colonize in the mouth and whether or not there is any residual effect after discontinuation. Some studies showed that different strains of probiotic strains act as a transient colonizer in the oral cavity, and studies on plaque and saliva have shown that ingested probiotic bacteria were recoverable up to 1-2 weeks after termination of intake [7-9]. However, the prolonged time for colonization of *L. paracasei* SD1, of at least 3 months (T6-T9) after cessation of dosing, in the mouth of the children taking the probiotic milk was noted in the present study. The colonization of *L. paracasei* SD1 seemed to last longer than the other studies [7-9]. This may relate to its origin from the human mouth, where the environment is more suitable for the strain to survive. Another possibility is that prolonged exposure to the probiotic strains may be needed. Our previous study evaluated the short-term (4-weeks) consumption of milk containing *L. paracasei* SD1, and demonstrated that the strain could be detected for a further 4 weeks afterwards. A short contact time of an extrinsically administered probiotic to the oral cavity may be a limiting factor, since the already established microbial ecology may prevent any introduced probiotics from colonizing and becoming a part of the commensal oral biofilm.

**Figure 2.** The number of salivary mutans streptococci (MS) and lactobacilli (LB) at the baseline (T0) and during the 12 months of study (T3-T12). The parameters were evaluated by the Wilcoxon signed-rank test: * Significance ($P < 0.05$), ** Significance ($P << 0.001$) difference versus baseline.




Any residual effect after a long-term taking of products containing probiotics has always been an issue of interest. The establishment of probiotic strains in the oral cavity could promote caries development due to their acidogenic and aciduric properties. However, *L. paracasei* strains have been

shown being less acid-producers compared to strong acid producers e.g. *Lactobacillus salivarius* [20]. In this study, lactobacilli level significantly increased in both groups, but the increase was found much more in the probiotic than in the control group. All participants were advised not to take food or drink containing probiotic (lactobacilli) during the study. However, increase of lactobacilli level in the control group might be derived from an unexpected source e.g. pickling vegetables and fruits, which is commonly ingested among Thai people. However, it was noted that there were no negative side effects following the probiotic intervention. Although fluctuations in saliva pH were observed, they were within the neutral range. Throughout the study, the salivary pH was found closely the same from the baseline to the end of the study in both groups (Table 1).

There have been only a few reports of randomized controlled clinical trials using caries as the outcome, especially regarding, clinical evaluation of probiotic bacteria that originated from the mouth. Most strains that have been isolated and developed for gastrointestinal health have been adopted for use in dental research. The 6 months intervention followed by 6 months observation in this study may still be too short for monitoring caries progression. Fluoride is commonly used in Thailand, which may be another factor delaying caries progression. However, our results revealed that *L. paracasei* SD1 showed a beneficial effect on dental caries; there was less dental caries among the children in the probiotic group at the end of study (Table 2). The mean of decay teeth (DT) in the probiotic group was lower than in the control group, although there was no statistically significant difference (Table 2). When the caries increment (∆ DT) and caries risk were taken for analysis, a significant increase in the caries increment was observed among children in the control group with high caries risk compared to the probiotic group (Table 2). In addition, it revealed that the long-term intake of milk containing *L. paracasei* SD1 reduced the risk of caries significantly (OR = 4.55, $P = 0.019$) in children with high caries risk (Table 3). Among children with low caries risk, it seemed that the probiotic group had more caries increase (≥ 1) than the control group, however, it was not statistically significant (OR = 0.45, $P = 0.342$) (Table 3). The definition of caries risk here is based on either dental caries alone or combined with microbiological examination. This is in agreement with another study of caries trial reporting the effect of probiotics in reduction of caries risk and caries incidence [12]. Theoretically, a reduction of MS over time would reduce caries risk, at least of new lesions. The clinical significance of our findings supported that a long-term exposure to milk containing *L. paracasei* SD1 could prevent or delay MS colonization resulting in reduction of caries risk.

**Table 2.** Mean decay teeth (DT) ± SD at T0 and mean caries increments (∆ DT) ± SD at T12 in the control and probiotic groups

| | **Control group** | **Probiotic group** |
|---|---|---|
| Total: (n=110) | | |
| DT at T0 | 0.61 ± 1.10 | 0.68 ± 0.98 |
| ∆ DT at T12 | 0.57 ± 0.95 | 0.30 ± 0.57 |
| Low caries risk: (n=56) | | |
| DT at T0 | 0.0 | 0.0 |
| ∆ DT at T12 | 0.30±0.79 | 0.35±0.56 |
| High caries risk: (n=54) | | |

| | | |
|---|---|---|
| DT at T0 | 1.43±1.31 | 1.26±1.03 |
| △ DT at T12* | 0.91±1.04 | 0.26±0.57 |

* Significance (p = 0.007), the parameters were evaluated by the Mann-Whitney U test

**Table 3.** Number of children with caries increment of the control and probiotic groups with low and high caries risk

| | **Number of children (%) with** | | | |
|---|---|---|---|---|
| | Low caries risk (n=56) | | High caries risk (n=54)* | |
| | Control | Probiotic | Control | Probiotic |
| No caries increase | 25 (83.3) | 18 (69.2) | 11 (47.8) | 25(80.6) |
| Caries increase ≥ 1 | 5 (16.7) | 8 (30.8) | 12 (52.2) | 6 (19.4) |
| Total | 30 (100) | 26 (100) | 23 (100) | 31 (100) |
| OR | 0.45 | | 4.55 | |

* Significance (p = 0.019), the parameters were evaluated by the Chi-Square test

It has been accepted that the effects of probiotic strains are strain specific, not all the *Lactobacillus* are probiotics that possess the ability to confer health benefits for the host [21]. Thus exploring the desirable properties of certain strains is needed. Our previous study revealed that *L. paracasei* SD1 could produce paracasin SD1, a specific antimicrobial protein, against various oral pathogens including cariogenic bacteria [22]. It may support the advantages of this strain in respect to its potential use as a bacterial replacement, which is a means of combatting infections by the administration of non-pathogenic bacteria to displace pathogenic microorganisms.

## 3. Experimental Section

*Subjects*

To be considered for invitation, subjects had to have good oral health with caries ≤ 3 teeth, absence of untreated active carious lesions, absence of either gingivitis or periodontal disease, be a non-smoker, and have daily tooth-brushing habits using fluoride-containing toothpaste. The exclusion criteria were (i) habitual consumption of probiotics or xylitol, (ii) systemic antibiotic medication taken within 6 weeks, (iii) allergy to cows' milk, lactose intolerance, severe food allergy, and (iv) systemic or severe chronic diseases.

The sample size calculation was based on our previous study [11]; it was calculated that there would be an estimated 80% power at the 0.05 level of significance, using two-sided testing, to detect a mean difference on the logarithm scale of 0.55 for MS count assuming a standard deviation of 0.63. Twenty participants per group were needed. After adjustment to evaluate the result of the caries increment and for 20% dropouts, a total sample size of 120 was judged as necessary for this study.

The project was thoroughly explained to the children and their parents in a meeting organized at the school. From a total of 246 children, 202 were eligible but only 122 volunteered after informed consent was given by their parents. The study group comprised 122 children (58 females, 64 males), 12–14 years of age (mean: 13.19 ± 0.72 years). A flow chart of the study is outlined in Figure 1. All subjects were asked to immediately report any adverse side effects and to fill in the questionnaire form after 6 months of milk consumption.

*Study Design*

The prospective investigation was a follow-up of a double-blinded, randomized placebo-controlled trial in two parallel groups, with a study period of 12 months. The study was approved by the Faculty of Dentistry Ethics Committee at the Prince of Songkla University, Thailand. Children were randomized to the study or control group by means of drawing lots, and they were coded as A or B. The code was kept by an independent monitor. This code was not unveiled until all data had been analyzed. Neither the researchers nor clinicians and health care personnel knew whether the children received control or intervention milk during the course of the study. The project was registered at the http://www.clinicaltrials.in.th/, one of the primary of WHO Registry Network, Clinical Trials identifier TCTR20130904001.

*Intervention*

Children in the probiotic group drank 5 g of reconstituted milk powder with probiotic *L. paracasei* SD1 in 50 ml water once daily, whereas children in the control group drank 5 g of reconstituted milk powder without probiotic bacteria in 50 ml water once daily for 6 months. The probiotic milk powder contained *L. paracasei* SD1 $10^7$ CFU/mg and was prepared according to Teanpaisan *et al*. [23]. The test and the control milks were delivered in plastic bags marked ‘‘A’’ or ‘‘B’’, and all children were asked to drink under observation everyday by the health care staff. On the holidays or weekends, children were asked to drink their milk at home and to return the plastic bags.

The compliance was checked by the health care staff who filled in a logbook everyday with information on attendance of children and whether or not the children had been drinking the milk.

*Oral Examination*

Oral examinations were performed at baseline and at the end of the study after 12 months by 3 dentists (PS, TS and TK). Before the start of the study, the 3 examination teams were calibrated against each other and inter-examiner reliability tests were carried out before the baseline and before the re-examination.

Dental caries status was recorded according to WHO criteria [24]. The caries increment (Δ DT) was calculated for each participating individual as the difference between the baseline score and the 12-month score.

Caries risk was determined on the basis of combined clinical and microbiological results according to the modification of Nase et al. [12], which classified the children into ‘high risk’ and ‘low risk’. ‘High risk’ was recorded if the child had either a DT score > 0 and/or MS count ≥ $10^5$ cfu/ml. If there was no caries (DT score = 0) and the MS count < $10^5$ cfu/ml, the child was recorded as ‘low risk’.

*Microbial evaluation*

Unstimulated saliva samples were collected at baseline (T0), 3 (T3), 6 (T6), 9 (T9) and 12 (T12) months. Each sample was made in a 10-fold serial dilution and cultured on a Mitis Salivarius Bacitracin agar or a de Man, Rogosa and Sharpe (MRS) agar being the selective media for MS and

lactobacilli, respectively. After 48 h of incubation in anaerobic condition at 37°C, the number of MS and lactobacilli were counted as colony forming units (CFU)/ml.

Five to ten colonies of lactobacilli on MRS plates were collected, purified and kept at -80°C for tracing of *L. paracasei* SD1 using AP-PCR fingerprints [10].

*Analysis of data*

All numerical data were presented as means and standard deviations. The general characteristics of the children (gender, age, pH of saliva and caries status-DMFT) between control and probiotic groups were analyzed using the chi-square test for categorized/dichotomized variables and the Mann-Whitney U test for interval variables. The number of colony counts of MS and lactobacilli were presented as $\log_{10}$ CFU/ml. The changes in bacterial counts and salivary pH from baseline to the intervention period were analyzed using Wilcoxon Signed rank test. The number of colony counts of MS, and lactobacilli between the two groups were analyzed using the Mann-Whitney U test. The difference of caries increment between the two groups was analyzed by the Mann-Whitney U test. The chi-square test was used to compare the percentages of children with caries increment between the study groups. The software package used was the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), and the differences were considered significant when $P < 0.05$.

## 4. Conclusions

In conclusion, the long-term daily consumption of milk powder containing *L. paracasei* SD1 has resulted in the reduction of both numbers of MS in saliva and caries risk. The long-term exposure seems to be an essential factor for prolonged colonization of the probiotic strain. Therefore, prolonged administration or the repeated application of the probiotics may be needed to maintain the desired probiotic effect for benefits on oral health.

## Acknowledgments

This work was supported by the National Research Council of Thailand (DEN550005M), the National Science and Technology Development Agency (NSTDA) of Thailand, and the Thailand Research Fund (TRF) through the Royal Golden Jubilee PhD program (PhD/0217/2553/AN.2) for B.S.

## Author Contributions

R.T. conceived and designed the experiments; R.T. and B.S. performed the microbiological study; S.P., S.T. and T.K. performed the clinical study; S.P. and R.T. analyzed the data; R.T. wrote the paper.

## Conflicts of Interest

"The authors declare no conflict of interest".

## References

1. Meurman, J.H.; Stamatova, I. Probiotics: contributions to oral health. *Oral Dis* **2007**, *13*, 443-451.

2. Cagetti, M.G.; Mastroberardino, S.; Milia, E.; Cocco, F.; Lingström, P.; Campus, G. The use of probiotic strains in caries prevention: a systematic review. *Nutrients* **2013**, *5*, 2530-2550.
3. Simark-Mattsson, C.; Emilson, C.G.; Hakansson, E.G.; Jacobsson, C.; Roos, K.; Holm, S. *Lactobacillus*-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. *Eur J Oral Sci* **2007,** *115*, 308-314.
4. Strahinic, I.; Busarcevic, M.; Pavlica, D.; Milasin, J.; Golic, N.; Topisirovic, L. Molecular and biochemical characterizations of human oral lactobacilli as putative probiotic candidates. *Oral Microbiol Immunol* **2007**, *22*, 111-117.
5. Teanpaisan, R.; Piwat, S.; Dahlen, G. Inhibitory effect of oral *Lactobacillus* against oral pathogens. *Lett Appl Microbiol* **2011**, *53*, 452-459.
6. Adams, M.R.; Martean, P. On the safety of lactic acid bacteria from food. *Int J Food Micro* **1995,** *27*, 263-264.
7. Yli-Knuuttila, H.; Snall, J.; Kari, K.; Meurman, J.H. Colonization of *Lactobacillus rhamnosus* GG in the oral cavity. *Oral Microbiol Immunol* **2006,** *21*, 129-131.
8. Caglar, E.; Topcuoglu, N.; Cildir, S.K.; Sandalli, N.; Kulekci, G. Oral colonization by *Lactobacillus reuteri* ATCC 55730 after exposure to probiotics. *Int J Paediatr Dent* **2009,** *19*, 377-381.
9. Ravn, I.; Dige, I.; Meyer, R.L.; Nyvad, B. Colonization of the oral cavity by probiotic bacteria. *Caries Res* **2012,** *46*, 107-112.
10. Teanpaisan, R.; Piwat, S. *Lactobacillus paracasei* SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. *Clin Oral Investig* **2014**, *18*, 857-862.
11. Ritthago,l W.; Saetang, C.; Teanpaisan, R. Effect of probiotics containing *Lactobacillus paracasei* SD1 on salivary mutans streptococci and lactobacilli in orthodontic cleft patients: A double-blinded, randomized, placebo-controlled study. *Cleft Palate Craniofac J* **2014**, *51*, 257-263.
12. Nase, L.; Hatakka, K.; Savilahti, E.; Saxelin, M.; Ponka, A.; Poussa, T.; Korpela, R.; Meurman, J.H. Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. *Caries Res* **2001**, *35*, 412-420.
13. Cildir, S.K.; Germec, D.; Sandalli, N.; Ozdemir, F.I.; Arun, T.; Twetman, S.; Caglar, E. Reduction of salivary mutans streptococci in orthodontic patients during daily consumption of yoghurt containing probiotic bacteria. *Eur J Orthod* **2009,** *31*, 407-411.
14. Cildir, S.K.; Sandalli, N.; Nazli, S.; Alp, F.; Caglar, E. A novel delivery system of probiotic drop and its effect on dental caries risk factors in cleft lip/palate children. *Cleft Palate Craniofac J* **2012,** *49*, 369-372.
15. Caglar, E.; Cildir, S.K.; Sandallin, N.; Ergeneli, S.; Twetman, S. Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. *Acta Odontol Scand* **2006**, *64*, 314-318.
16. Caglar, E.; Kuscu, O.O.; Cildir, S.K.; Kuvvetli, S.S.; Sandallin, N. A probiotic lozenge administered medical device and its effect on salivary mutans streptococci and lactobacilli. *Int J Paediatr Dent* **2008**, *18*, 35-39.

17. Aminabadi, N.A.; Erfanparast, L; Ebrahimi, A.; Oskouei, S.G. Effect of chlorhexidine pretreatment on the stability of salivary lactobacilli probiotic in six- to twelve-year-old children: a randomized controlled trial. *Caries Res* **2011,** *45,* 148-154.
18. Jindal, G.; Pandey, R.K.; Agarwal, J.; Singh, M. A comparative evaluation of probiotics on salivary mutans streptococci counts in Indian children. *Eur Arch Paediatr Dent* **2011,** *12,* 211-215.
19. Parvez, S.; Malik, K.A.; Ah Kang, S.; Kim, H.Y. Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* **2006,** *100,* 1171-1185.
20. Piwat, S.; Teanpaisan, R.; Dahlén, G.; Thitasomakul S.; Douglas C.W. Acid production and growth by oral *Lactobacillus* species *in vitro. J Investig Clin Dent* **2012,** *3,* 56-61.
21. Joint FAO/WHO Working Group: Guidelines for the evaluation of probiotics in food. **2002.**
22. Wannun, P.; Piwat, S.; Teanpaisan, R. Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. *Anaerobe* **2014**, *15*, 17-21.
23. Teanpaisan, R.; Chooruk, A.; Wannun, A.; Wichienchot, S.; Piwat, S. Survival rates of human-derived probiotic *Lactobacillus paracasei* SD1 in milk powder using spray drying. *Songklanakarin J Sci Technol* **2012**, *34*, 241-245.
24. World Health Organization: Oral Health Surveys, Basic Methods, ed. 3. Geneva, *World Health Organization*, **1987**.

Wattanarat et al. BMC Oral Health (2015) 15:19
DOI 10.1186/s12903-015-0003-0

BMC Oral Health

RESEARCH ARTICLE Open Access

# Enhancement of salivary human neutrophil peptide 1–3 levels by probiotic supplementation

Onnida Wattanarat[1], Anupong Makeudom[2], Thanapat Sastraruji[3], Supatcharin Piwat[4,6], Sukanya Tianviwat[4,5,6], Rawee Teanpaisan[4,7] and Suttichai Krisanaprakornkit[3*]

## Abstract

**Background:** Probiotic supplementation can reduce mutans streptococci (MS) numbers. One of its proposed mechanisms is immunomodulation. Salivary human neutrophil peptide 1–3 (HNP1-3) levels have previously been demonstrated to be higher in caries-free than in caries-susceptible children, suggesting their preventive role against caries. We aimed to compare salivary HNP1-3 levels between an intervention group with probiotics and a control group.

**Methods:** A randomized double-blinded clinical trial was conducted. Sixty schoolchildren were equally allocated to either an intervention or control group. The use of a probiotic strain, *Lactobacillus paracasei* SD1, has shown to reduce MS numbers in volunteers. In unstimulated whole saliva, HNP1-3 levels were assayed by ELISA, and MS and lactobacilli counts were assayed by colony counting at baseline (T0) and at 3 (T3), 6 (T6), and 12 months (T12). The International Caries Detection and Assessment system was used to assess caries status.

**Results:** In the intervention group, salivary HNP1-3 levels were significantly greater than those in the control group at T3 and T6 ($p < 0.001$), whereas MS counts were significantly decreased ($p < 0.01$). In the intervention group, positive and negative correlations were found between HNP1-3 levels and lactobacilli counts and between MS and lactobacilli counts, respectively. However, there was no significant correlation between enhanced HNP1-3 levels and decreased MS numbers. The caries increment for the pit and fissure surface, but not for the smooth surfaces, was significantly decreased in the intervention group compared with the control group ($p = 0.01$).

**Conclusions:** Probiotics can temporarily enhance salivary HNP1-3 levels; however, their action to reduce new pit and fissure caries probably involves microbial interactions.

**Trial registration:** TCTR20130904001 (registration date: September 04, 2013).

**Keywords:** Alpha-defensins, Dental caries, Mutans streptococci, Probiotics, Saliva

## Background

Dental caries is one of the most prevalent diseases in both children and adults worldwide [1]. However, the success of all caries preventive programs has been impeded by its multifactorial nature. The disease is a result of demineralization, caused by the interactions of cariogenic bacteria, a diet rich in fermentable carbohydrates, and host components, such as tooth and saliva properties [2]. Although many bacterial species can play a role in the caries process [3], mutans streptococci (MS) have been considered major pathogens associated with early caries development [4]. The main virulence properties of MS are acidogenicity, acid tolerance, biofilm formation and tooth adhesion [5]. Strong association between MS quantities and the pathogenesis of dental caries is demonstrated by several previous studies, reviewed in [6].

Probiotic administration is considered a potential strategy for improving or maintaining oral health. According to the World Health Organization (WHO), probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [7]. Several mechanisms have been proposed for the probiotic action, including production of antimicrobial substances,

* Correspondence: suttichai.k@cmu.ac.th
[3]Center of Excellence in Oral and Maxillofacial Biology, Department of Oral Biology and Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand
Full list of author information is available at the end of the article

competition with pathogens by preventing cellular adhesion and invasion, and modulation of local and systemic immune functions [8-11]. In a recent systematic review [12], several clinical trials have shown the capacity of probiotic supplementation to reduce MS counts, but the decreasing effect is variable and short-lasting. Moreover, only very few studies have so far investigated the quantities of lactobacilli (LB) and the prevention of new caries occurrence by probiotic supplementation.

Although LB, commonly used as probiotics, have been associated with caries progression [13], a recent study has revealed that only a certain species, *i.e.*, *Lactobacillus salivarius*, is more related to caries development by its ability to produce high levels of acids [14]. In contrast to these cariogenic bacteria, *Lactobacillus paracasei* isolated from caries-free subjects possesses an ability to suppress MS growth [15,16]. In this study, *Lactobacillus paracasei* SD1 was introduced as a probiotic strain and used in the oral cavity because of its several previously-demonstrated properties, including inhibition of MS growth, less acid production than other LB, and good adherence to oral epithelial cells [17].

Human neutrophil peptides 1–3 (HNP1-3) are small cationic antimicrobial peptides that provide the first line of host defense against a broad spectrum of microorganisms [18]. HNP1-3 are expressed in ductal epithelial cells of submandibular salivary glands and secreted into saliva [19]. They are also produced by neutrophils and released into gingival crevicular fluid [20]. The preventive role of HNP1-3 against dental caries has been suggested by the significantly higher salivary HNP1-3 levels in caries-free children than in those experiencing caries demonstrated by Tao et al. [19]. Since one of the probiotic mechanisms has been proposed to be involved with host immune regulation, we, therefore, hypothesized that probiotic supplementation might help prevent dental caries by augmentation of local host immunity via enhanced production of salivary HNP1-3. The aims of this study were to examine the effects of probiotic intervention on salivary HNP1-3 levels, MS counts, and LB counts, and to determine the correlations between these host and microbial parameters. Furthermore, new occurrences of carious lesions for the second permanent molars during a 12-month clinical trial were assessed and compared between the control and intervention groups.

## Methods

### Overview of study design

This trial was designed as a randomized, double-blinded and placebo-controlled method with two parallel groups. The study protocol was approved by the Human Experimentation Committees of the Faculties of Dentistry, Chiang Mai University and Prince of Songkla University, Thailand. Informed consent was obtained from the parents or guardians of each participant before the commencement of this study. Sixty eligible and healthy participants out of 246 children were allocated equally to either of the two groups, the control and probiotic groups, using a simple randomization procedure by means of drawing lots (Figure 1). The inclusion and exclusion criteria are identified in Participants and in Figure 1. For the double-blinded method, the code was kept by an independent monitor. This code was not unveiled until all data had been analyzed. None of the researchers, the clinicians, the participants, the teachers, or the statistician knew whether the children received control or intervention milk throughout the entire course of this study. The trial was registered at the http://www.clinicaltrials.in.th/, one of the primary WHO Registry Networks, Clinical Trials identifier TCTR20130904001. The whole experimental period lasted for 12 months, and the outcome measurement of this study consisted of four parameters: salivary HNP1-3, MS and LB levels at four different time points: T0 (baseline), T3 (three months of intervention), T6 (six months of intervention) and T12 (six months after cessation of the intervention), and the presence of carious or demineralized lesions at T0 and T12 (Figure 1). The *priori* sample size calculation was performed with a focus on mean differences of MS counts between two independent groups using G Power software [21], with the effect size equal to 0.8 at 5% statistical significance level and 90% power of test. The calculation yielded no fewer than 28 participants in each group.

### Preparation of milk powder supplemented with probiotics

The probiotic strain, *Lactobacillus paracasei* SD1, was isolated from caries-free volunteers and has been previously shown to exert the maximal inhibitory effect on *Streptococcus mutans in vitro* [17]. The bacterial strain was identified by PCR-RFLP of the 16S ribosomal RNA gene and sodium dodecyl-polyacrylamide gel electrophoresis [22]. The probiotic intervention was manufactured in a form of milk powder by the spray drying technique. The culture conditions and the spray drying technique were performed as previously described [23]. Briefly, a culture of *Lactobacillus paracasei* SD1 was inoculated in a mixture of 1% probiotics and a 3-liter quantity of heat-treated (50°C for 30 min) and 20% reconstituted skimmed milk, and then spray dried with a spray dryer (model B191 Buchi mini spray dryer; Flawil, Switzerland). The final product was yellowish white powder with moisture contents of 3.44 ± 0.85% and viable counts of $7.5 \pm 0.20 \times 10^8$ cfu/g, which was stored at 4°C. The viability of *Lactobacillus paracasei* SD1 in the inoculated milk powder was previously assessed in a six-month study by examining its growth in triplicate Man Rogosa and Sharpe (MRS) pour plates under anaerobic

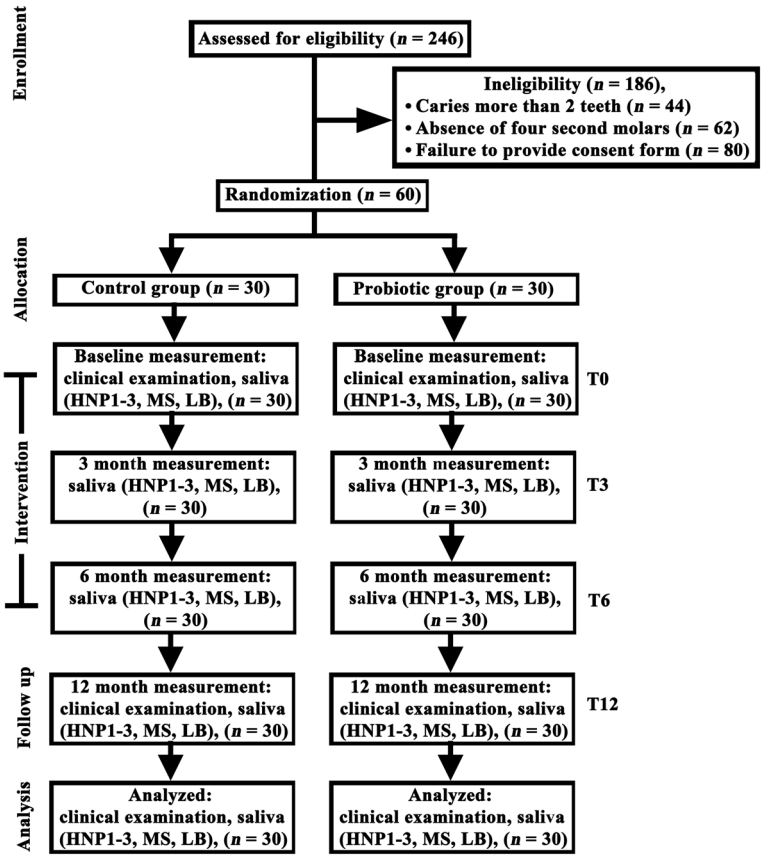



**Figure 1 A consort flow chart showing the number of participants in the control and probiotic groups at the beginning and completion of the trial.** Out of 246 children enrolled in this trial, 60 participants remained eligible due to the inclusion and exclusion criteria. Sixty participants were equally allocated to either the control or the probiotic group by simple randomization described in the Methods. Participants received milk powder with or without probiotics during the first six-month intervention, and saliva samples were collected for analyses of HNP1-3 levels, MS and LB counts at four different time points: T0, T3, T6 and T12. Oral examination for evaluation of ICDAS scores was performed at T0 and T12.

incubation for three days at 37°C [23]. It was demonstrated that the maximum survival rate of *Lactobacillus paracasei* SD1 in the skimmed milk powder was 99% if the milk powder was stored at 4°C for six months [23]. However, the survival rate declined sharply if the milk powder was stored at 25°C [23]. The milk powder in general appearance for both probiotic and control groups looked identical, except for the absence of the probiotic strain in the control group, and was packaged in the same clear plastic bags marked with a name of each participant. The packaging and delivering steps were performed by a research assistant who was blinded from every step of sampling and analyzing.

### Participants

The inclusion criteria were: (i) good oral health with caries in two or fewer teeth (ii) presence of four second molars in the mouth (iii) absence of untreated active deep carious lesions (iv) absence of periodontitis (v) non-smoking and (vi) daily tooth brushing habit using fluoride toothpaste. The exclusion criteria were teenagers with systemic diseases, receiving systemic antibiotics within six weeks, routine consumption of probiotics or xylitol, allergy to cows' milk, lactose intolerance and severe food allergy. Prior to the commencement of the clinical trial, all participants were informed not to consume any products containing probiotics, such as yoghurt, drinking yoghurt, cheese, etc., throughout the entire duration of this trial. The participants in both groups were instructed to mix 5 g of milk powder in 50 ml water and drink once daily, except on the days of saliva collection, for six months under the supervision of instructors. On the holidays or the weekends, all children were informed to drink their milk at home and to return the empty plastic bags. The compliance was checked by their teachers who filled in a logbook every day with information on school attendance of children and on whether or not the children had been drinking the milk. It was apparent that no children participating in this study were absent from their classes due to sick leave during the first six-month intervention. All participants were asked to immediately report any side effects.

### Analyses of salivary HNP1-3 levels

A 2-ml quantity of unstimulated whole saliva was collected and equally divided in two aliquots, one for analysis of HNP1-3 levels and the other for analysis of microbiological levels. The first aliquot was added with Nonidet P-40 (Sigma-Aldrich, St. Louis, MO) to the final concentration of 0.1% (v/v), centrifuged at 15,000 rpm at 4°C for 10 min [19], and the cleared supernatant was collected and stored frozen for further analysis of HNP1-3 levels by an HNP1-3 ELISA kit (HyCult Biotechnology, Uden, the Netherlands) according to the manufacturer's instruction. In brief, diluted saliva samples (1:200) were applied to a pre-coated plate in triplicate with the specific primary antibody to HNP1-3, and incubated for 1 h at room temperature. For a negative control, dilution buffer without addition of the saliva samples was added to the pre-coated plate. After washing four times with washing buffer, each well was incubated with the biotinylated tracer antibody for 1 h at room temperature. Then, the streptavidin-peroxidase conjugate was added and incubated for 1 h. After that, 3,3′,5,5′-tetramethylbenzidine, a chromogenic substrate, was added for 20 min, and the reaction was then stopped by the addition of 2% oxalic acid. The concentrations of HNP1-3 in saliva samples were calculated from a standard curve established by various known concentrations of an HNP1-3 standard, used as the ELISA system control. The concentrations of salivary HNP1-3 were then normalized by their total protein content using a BCA protein assay (Pierce Inc., Rockford, IL) according to the manufacturer's instruction.

### Microbiological assays

The quantities of salivary MS and LB were evaluated by a typical colony counting method at T0, T3, T6 and T12. The second aliquot of saliva samples was ten-fold diluted from 1:10 to 1:10,000. Each dilution (10 μl) was dropped onto the selective agar plates, Mitis Salivarius Bacitracin (MSB) agar for MS (Difco Laboratories, Detroit, MI) and MRS agar for LB (Difco Laboratories). The conditions for incubation were anaerobic, 10% $H_2$, 10% $CO_2$ and 80% $N_2$ at 37°C for 48 h. The colony counting was performed under a microscope in duplicate.

### International Caries Detection and Assessment System (ICDAS)

At the beginning and the end of this study (T0 and T12), all participants were examined for oral health and dental caries status by two experienced dentists (S.P. and S.T.) with the Cohen's kappa values 0.85 and 0.82 for the intra- and inter-examiner calibrations, respectively. The examination was performed by using a mouth mirror and an air syringe under an operating light. The caries data for each tooth surface, including occlusal (pit and fissure) and smooth surface (buccal, lingual, mesial and distal) caries, were recorded according to the criteria of the International Caries Detection and Assessment System (ICDAS) [24]. The ICDAS codes consist of: 0 = sound, 1 = first visual change in enamel, 2 = distinct visual change in enamel, 3 = localized enamel breakdown, 4 = underlying dentin shadow, 5 = distinct cavity with visible dentin, and 6 = extensive cavity within visible dentin. Caries risk assessment in this study was determined by the modified criteria previously described by Nase and co-workers [25], which include both clinical caries status and MS quantities as follows. The "high risk" participants were defined as having both an ICDAS score >0 and MS levels $\geq 10^5$ cfu/ml, whereas the "moderate risk" ones had either an ICDAS score >0 or MS levels $\geq 10^5$ cfu/ml and the "low risk" ones had an ICDAS score =0 and MS levels $<10^5$ cfu/ml.

### Statistical analyses

The Mann–Whitney *U* test was used to analyze intergroup differences in salivary HNP1-3 levels. Differences in salivary HNP1-3 levels among four different periods in each group were analyzed by the Kruskal-Wallis test, followed by the Mann–Whitney *U* test. The number of colony counts for MS and that for LB were presented as log cfu/ml and analyzed by the independent sample *t*-test. Correlations between each pair of the data from HNP1-3 levels, MS and LB counts in each group were tested by the Pearson Correlation test. Regression analysis was performed for the significant correlation data. An odds ratio (OR) for caries increment was calculated by Crosstabulation. The software package used was the Statistical Package for Social Sciences (SPSS version 17.0 Inc., Chicago, IL), and the differences were considered significant when *p*-values were less than 0.05. All of these statistical methods were performed by a biostatistician (T.S.).

## Results

### Demographic data

A total of 60 participants (age ranges 13–15 years; $n = 26$ for males and 34 for females) completed this 12-month clinical trial (Figure 1). No adverse side effects from probiotics or milk powder intake in this cohort were reported. The compliance for daily consumption of milk powder in both groups was carefully monitored under the supervision of schoolteachers and well controlled throughout the first six-month intervention period. The assessment for caries risk revealed that the percentages of participants classified in the "high risk", the "moderate risk" and the "low risk" groups were 50, 31.7 and 18.3, respectively. Although the randomized procedure was performed to allocate all 60 eligible participants to the

control or probiotic group, it was coincident that the percentages of high, moderate and low caries risk in the control versus the probiotic group were almost equal as follows: 50.0 versus 50.0, 33.3 versus 30.0, and 16.7 versus 20.0, respectively.

### Raised salivary HNP1-3 levels in the probiotic group

The median salivary HNP1-3 levels, expressed in the unit of μg/mg of total proteins, in the probiotic group were significantly greater than those in the control group at T3 (1.0250 versus 0.5145 μg/mg; $p < 0.001$) and T6 (1.1470 versus 0.5415 μg/mg; $p < 0.001$), but not at T0 or T12 (Figure 2). With respect to the kinetics of salivary HNP1-3 levels in the probiotic group during the 12-month period of this clinical trial, it was demonstrated that the HNP1-3 levels were temporarily elevated during the six-month intervention period (Figure 2). In other words, these levels were significantly increased at T3 and T6 ($p < 0.001$) and gradually declined at T12. However, no significant changes in salivary HNP1-3 levels in the control group at four time points (T0, T3, T6 and T12) were observed (Figure 2).

### Decreased MS counts as opposed to increased LB counts by probiotic intervention

The mean counts and standard errors for salivary MS and LB (log cfu/ml) at T0, T3, T6 and T12 in the control and probiotic groups are demonstrated in a linear graph (Figure 3). At baseline (T0), it was demonstrated that the mean salivary LB count in the probiotic group (a solid line) was not significantly different from that in the control group (a dotted line), while the mean salivary MS count in the probiotic group was significantly higher than that in the control group ($p < 0.05$; Figure 3). However, at T3 and T6, a significant reduction in MS counts in the probiotic group was clearly evident when compared with those in the control group (T3 = $p < 0.001$ and T6 = $p < 0.01$), whereas a significant increase in LB counts was instead observed ($p < 0.05$; Figure 3). In contrast to the probiotic group, no significant changes in the mean counts for salivary MS and LB were found in the control group (dotted lines in Figure 3). At T12, the mean count for salivary MS in the probiotic group was increased and returned almost to the baseline, while the mean count for salivary LB in the probiotic group was still significantly higher than that in the control group ($p < 0.01$; Figure 3), suggesting the ability of LB to be retained in the oral cavity despite cessation of the probiotic intervention for six months.

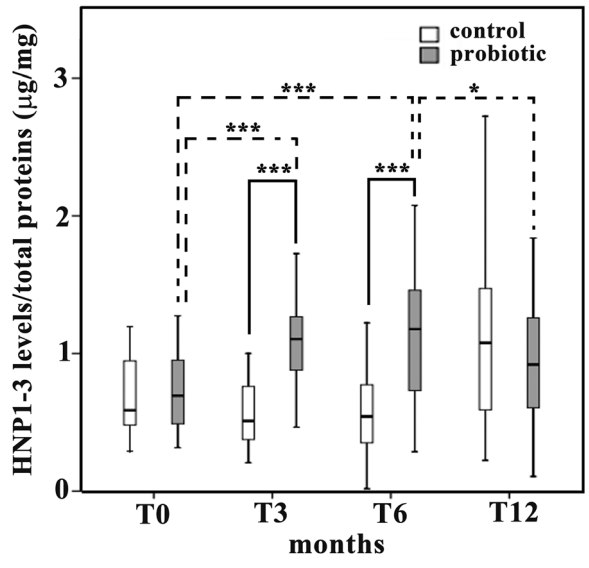



**Figure 2 Significant but transient increase in salivary human neutrophil peptide (HNP) 1–3 levels by probiotic supplementation.** The *y*-axis of box plot graph demonstrates salivary HNP1-3 levels normalized by total protein concentration in the unit of μg/mg for the control (empty boxes) and the probiotic (gray boxes) groups from four different periods of saliva collection: T0, T3, T6 and T12. * = $p < 0.05$; *** = $p < 0.001$. The solid lines show the significant differences between two groups, while the dotted lines represent the significant differences within the probiotic group.

### Significant reduction in the caries increment for the pit and fissure surface in the probiotic group

The percentages of sound (ICDAS score =0) and unsound surfaces (ICDAS scores =1–6) from all five tooth surfaces, including pit and fissure, mesial, distal, buccal, and lingual, at T0 and T12 in both control and probiotic groups are illustrated as stacked bars to a total of 100% (Figure 4A). It was apparent that the percentages of carious lesions in the second permanent molars in both control and probiotic groups increased in almost all tooth surfaces during this 12-month study and that the pit and fissure caries in both groups was more prevalent in our cohort than was caries in the other four smooth surfaces, including mesial, distal, buccal and lingual (Figure 4A). Sums of the percentages of caries increment for all five tooth surfaces, including the pit and fissure surface and four smooth surfaces, and for four smooth surfaces in the probiotic group versus the control group were 25.5 versus 44.2 and 15.2 versus 21.4, respectively (Figure 4B). The percentages of caries increment only in the pit and fissure surface were 10.3 versus 22.8 (Figure 4B). Interestingly, the percentage of new caries occurrence for all five tooth surfaces (OR = 1.605; 95% CI 1.007–2.557; $p = 0.045$) and for the pit and fissure surface (OR = 2.582; 95% CI 1.235–5.400; $p = 0.01$) was significantly lower in the probiotic group than that in the control group, whereas there was no significant difference in new caries occurrence between two groups for the four smooth surfaces (OR = 1.506; 95% CI 0.726–3.126; $p = 0.269$).

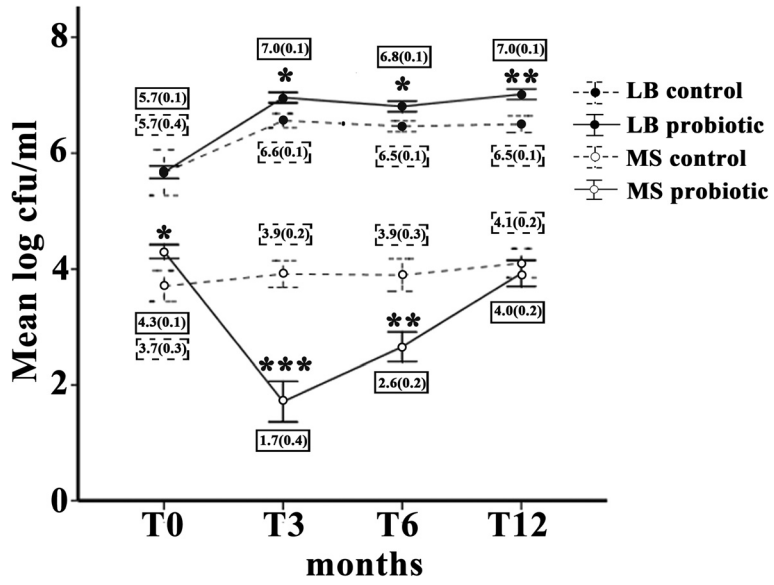



**Figure 3 Significant but transient reduction in salivary mutans streptococci (MS) counts by probiotic intervention.** The linear graph illustrates mean log counts of MS (open circles) and those of lactobacilli (LB; black circles) in the unit of cfu/ml (*y*-axis) in both probiotic (solid lines) and control (dotted lines) groups from four different periods of saliva collection (*x*-axis): T0, T3, T6 and T12. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Error bars represent standard errors (SE). The values of mean log MS and LB counts and (SE) are shown in the solid boxes for the probiotic group and in the dotted boxes for the control group in each period of saliva collection.

### Lack of correlation between increased levels of HNP1-3 and decreased numbers of MS

Correlations between each pair of salivary HNP1-3 levels in μg/mg of total protein, MS counts and LB counts in log cfu/ml in both control and probiotic groups were determined by the Pearson Correlation test (see Additional file 1 for the data of each parameter), and it was revealed that significant correlations between these parameters were only found in the probiotic group, but not in the control group (Figure 5). Particularly, salivary HNP1-3 levels were positively correlated with LB counts ($r = 0.376$; $p < 0.001$; Figure 5B) with the equation from the regression analysis: $y = 0.653x + 5.929$, where $y$ is the log LB count and $x$ is the HNP1-3 level, whereas LB counts were inversely associated with MS counts ($r = -0.282$; $p = 0.002$; Figure 5F). Nevertheless, no significant correlation was noted between salivary HNP1-3 levels and MS counts in the probiotic group (Figure 5D), suggesting that enhanced salivary HNP1-3 levels by the probiotic intervention are not associated with decreased MS quantities.

## Discussion

The main findings from this study include (a) a significant but temporary increase in salivary HNP1-3 levels at T3 and T6 in the probiotic group; (b) a significant increase in LB counts as opposed to a transient decrease in MS counts during probiotic intervention; (c) positive and negative correlations found between HNP1-3 levels and LB counts and between LB counts and MS counts, respectively, in the probiotic group; and (d) a significant reduction in caries increment for the pit and fissure surface, not for the smooth surfaces. Among these four major findings, the significant increase in salivary HNP1-3 levels by probiotic supplementation during the six-month intervention period is of great interest since it is probable that *Lactobacillus paracasei* SD1 may exert an immunostimulatory effect by enhanced production of innate immune effectors, especially HNP1-3, from ductal epithelial cells of submandibular salivary glands [19], which are then secreted into the saliva. The possible immunostimulatory effect of *Lactobacillus paracasei* SD1 warrants further investigations. In addition, the innate immune activation by *Lactobacillus paracasei* SD1 is corroborated with the significant correlation between elevated salivary HNP1-3 levels and increased LB counts seen in Figure 5B. Activation of host immunity, particularly increased production of secretory immunoglobulin A (sIgA), has been previously shown to be one of the principal mechanisms for probiotics in the gastrointestinal tracts [26-28] and in the oral cavity [29-31]. In this study, the rationale to investigate only the salivary HNP1-3 levels during probiotic intervention was based on the observation from a single previous study that demonstrated significantly higher HNP1-3 levels in the saliva samples from caries-free children than those from caries-susceptible children [19]. However, it is necessary to further determine the salivary levels of other

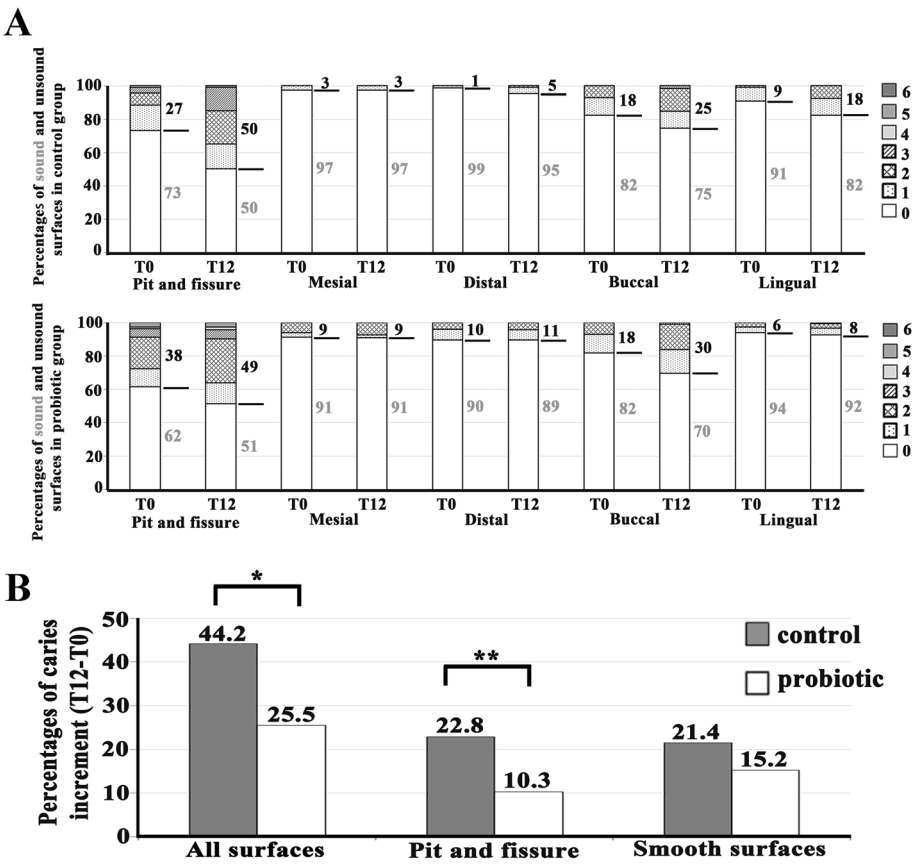



**Figure 4 Significant decrease in the percentage of caries increment for the pit and fissure surface, but not for the smooth surfaces, by probiotic intervention. (A)** The 100% stacked bars demonstrate the percentages of sound (white area; ICDAS score =0) and unsound (filled areas; ICDAS scores =1–6) surfaces from all five tooth surfaces, including pit and fissure, mesial, distal, buccal, and lingual, in the control (upper panel) and the probiotic (lower panel) groups. Note higher percentages of unsound surfaces in the pit and fissure surface than those in the other four surfaces. **(B)** The bar graph reveals significant reduction in the percentages of caries increment in the probiotic group for a combination of all five tooth surfaces and for the pit and fissure surface alone, but not for the combined four smooth surfaces, including mesial, distal, buccal and lingual surfaces. * = $p < 0.05$; ** = $p = 0.01$.

immune-related effector molecules, especially other antimicrobial peptides besides HNP1-3, in a future study. It is noted that the levels of salivary HNP1-3 detected in our cohort were comparable to those measured by the same ELISA method performed by Tao and co-workers [19]. Interestingly, the HNP1-3 levels were temporarily elevated during the six-month intervention period and gradually declined to the baseline levels after cessation of probiotic intake. The temporary induction of HNP1-3 levels was in line with the transient induction of sIgA levels in saliva by probiotic intake [30], indicating that daily consumption of probiotics seems to be required for a sustainable increase in host immune responses.

Nevertheless, the raised salivary HNP1-3 levels did not significantly correlate with the decreased MS counts, but the LB counts were instead correlated inversely with the MS counts, suggesting that the mechanism for probiotic action in the oral cavity may probably involve the competition and/or the interaction between two distinct microorganisms as previously shown by Teanpaisan and Piwat [22] rather than enhanced production of HNP1-3. It is worthwhile to note that the inverse correlation between MS and LB counts was weak, so this suggests that other factors that were not tested in this study, such as different MS strains recovered from different individuals [32], may play a role in the regulation of MS numbers by LB. In contrast to no significant change in MS counts in the control group, it was evident that the MS counts were transiently decreased in the probiotic group at T3 and T6, consistent with the finding from a previous study [33]. This, again, confirms the short-lasting effect of probiotics against MS numbers, similar to the transient increasing effect on HNP1-3 levels. It is interesting to note a prolonged increase in LB counts irrespective of the cessation of probiotic intervention, which may be explained by the ability of *Lactobacillus paracasei* SD1 to be retained in the oral cavity, as previously reported [22] and by the counting method for total LB on MRS agar

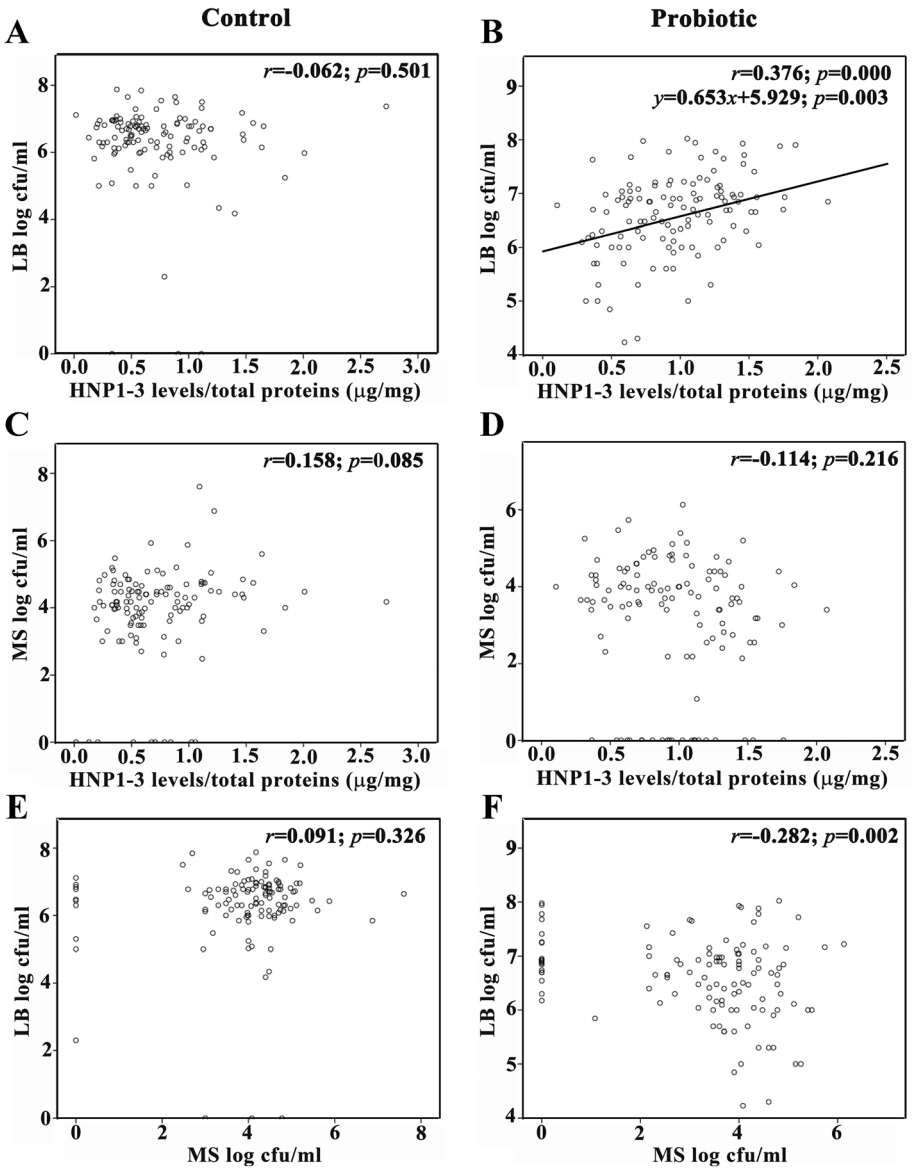



**Figure 5 Positive and negative correlations between salivary human neutrophil peptide (HNP) 1–3 levels and log counts of lactobacilli (LB) and between log counts of mutans streptococci (MS) and those of LB, respectively, in the probiotic group.** The correlation coefficients (*r*) and the significance levels (*p*-values) between each pair of three parameters, including HNP1-3 levels in μg/mg, LB counts in log cfu/ml and MS counts in log cfu/ml were determined and illustrated for both control **(A, C and E)** and probiotic **(B, D and F)** groups. The regression analysis could be determined only for the significant correlation between increased HNP1-3 levels and increased LB counts in B, and a regression line was drawn with the equation: $y = 0.653x + 5.929$; $p = 0.003$.

in this study, rather than the specific quantification of *Lactobacillus paracasei* SD1. Consequently, it is possible that the increased MS counts back to the baseline levels at 12 months were attributable to the absence of *Lactobacillus paracasei* SD1, although the total LB counts were still high. In this study, we did not examine the morphology of LB recovered from saliva samples in great detail with Gram stain, although some colonies of LB on MRS plates were randomly selected and checked for the presence of *Lactobacillus paracasei* SD1 by a qualitative assay like Arbitrarily Primed-Polymerase Chain Reaction fingerprints [22]. Therefore, the inability to confirm the morphology of LB and to quantitatively compare the levels of *Lactobacillus paracasei* SD1 between the control and probiotic groups at each time point is a limitation of this study and this inability may preclude the possibility of *Lactobacillus paracasei* SD1 retention in the oral cavity.

In addition to the laboratory results regarding HNP1-3 levels and microbial counts in saliva, we extended our

investigation into the clinical findings relating to caries increment in our cohort. The significant reduction by approximately 2.6 fold for caries increment within 12 months was observed for the pit and fissure caries in the probiotic group when compared with the control group (Figure 4B), suggesting that probiotic intervention in milk powder helps reduce the occurrence of new carious lesions for the pit and fissure surface. In addition to considering the pit and fissure surface alone, the sum of the percentages for caries increment in all five tooth surfaces was also significantly reduced in the probiotic group (Figure 4B). These results differ from the findings from two previous studies, which demonstrated a low odds ratio for caries prevention on occlusal surfaces from milk containing *Lactobacillus rhamnosus* GG in preschool children [25] and lack of significant difference in new caries occurrence between the probiotic and control groups [34]. A possible explanation for this discrepancy is that the participants in each of those studies were categorized as low caries risk, whereas half of the participants in our study were categorized as high caries risk, according to the modified criteria for caries assessment mentioned in the Methods. Therefore, as suggested by the results of those studies and ours, probiotic intervention may be beneficial to reduce the new occurrence of pit and fissure caries in children with high caries risk.

In this study, it was demonstrated that the probiotic intervention did not reduce new caries occurrence on the smooth surfaces. This may be explained by the fact that pit and fissure caries is more associated with MS quantities than is smooth surface caries, consistent with the critical role of MS in pit and fissure caries development [35]. Moreover, it was apparent that the effect of probiotics on smooth surface caries varied among different smooth surfaces (Figure 4A). Particularly, the probiotics appeared to have a caries-reducing effect on the distal and lingual surfaces, but not on the mesial and buccal surfaces. Nevertheless, such mixed effects did not reach a statistically significant level when sum of the percentages for caries increment in four smooth surfaces was determined (Figure 4B). Some factors that can influence distinct outcomes between occlusal and smooth surface caries and then preclude statistical verification include the location of second permanent molars (whether in maxillae or in mandibles), the sample size, and the 12-month duration of this study. Therefore, it is suggested that a larger study with a longer follow-up duration and with special attention to the tooth location be further conducted to more precisely verify all of the statistically significant and non-significant findings and to define the extent and mechanism behind any observable beneficial effects from probiotic intervention.

Utilizing non-sweetened milk powder as a suitable vehicle to introduce the probiotics in this study provided several health benefits for the participants, including provision of more nutrients, good compliance, and additional caries prevention from some constituents in milk powder, such as casein, calcium and phosphorous [36]. Moreover, among several different LB species that have been reported as probiotics, it has been previously shown that *Lactobacillus paracasei* exerted a maximum inhibitory effect on MS [15,16], possibly by its ability to produce paracasin SD1 that functions against MS in the oral cavity [37]. Furthermore, compared with various probiotic strains used in other studies, predominantly isolated from the gastrointestinal tract [33,34,38,39], *Lactobacillus paracasei* SD1 used in our study was isolated from the oral cavity of caries-free volunteers. However, this probiotic strain could only exert a temporary effect on MS reduction and HNP1-3 induction despite a prolonged increase in LB counts in the saliva samples. This may be because the colony counting method on MRS agar cannot differentiate our probiotic strain from other LB strains that already reside in the participants' mouths. Otherwise, it is probable that our probiotic strain can possibly increase the number of other LB strains, a possibility that warrants further investigations.

Although cariogenic progression varies between different individuals and sometimes lasts longer than a year, the 12-month randomized and double-blinded clinical trial was designed in order to control different inherent factors between the control and probiotic groups. Additionally, the ICDAS was chosen as a tool to assess carious lesions in this short period of study because it is sensitive enough to detect an early carious lesion [40]. Further studies into the underlying mechanisms of increased HNP1-3 production by our probiotics in different oral cell types are currently being investigated, and additional studies into the activation of other oral immune effectors classified in acquired and innate immunity are essential to verify the real potential benefits and applications of probiotics for good oral health, especially for caries prevention. Several confounding factors that were beyond our control in each individual, including variations in the baseline levels of salivary HNP1-3, in the different quantities and strains of MS, in the quantities of fermentable carbohydrate consumption, and in the quality of oral hygiene care, are noted and may affect the outcomes of this study.

## Conclusions

Probiotic supplementation with *Lactobacillus paracasei* SD1 can temporarily enhance salivary HNP1-3 levels and decrease the numbers of MS. The significant correlation, found between increased LB counts and decreased MS counts, suggests that probiotic action may involve

the competition or interaction between two distinct microorganisms. In this study, the increment of pit and fissure caries, but not of smooth surface caries, was diminished by probiotic supplementation in the form of milk powder.

### Available supporting data

The data of mutans streptococci (MS) and lactobacilli (LB) counts and human neutrophil peptide (HNP) 1–3 levels at T0, T3, T6 and T12 are provided in the Additional file 1.xls on BMC Oral Health website.

## Additional file

**Additional file 1: Data of mutans streptococci (MS) and lactobacilli (LB) counts and human neutrophil peptide (HNP) 1-3 levels at T0, T3, T6 and T12.**

#### Abbreviations

BCA: Bicinchoninic acid assay; cfu: Colony forming unit; ELISA: Enzyme-linked immunosorbent assay; HNP: Human neutrophil peptide; ICDAS: International caries detection and assessment system; LB: Lactobacilli; MRS: Man Rogosa and Sharpe; MS: Mutans streptococci; MSB: Mitis Salivarius Bacitracin; OR: Odds ratio; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; RNA: Ribonucleic acid.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

OW: Measurement of salivary HNP1-3 levels, data analyses and interpretation, and manuscript preparation. AM: Measurement of salivary HNP1-3 levels and manuscript preparation. TS: Statistical analyses and manuscript preparation. SP: Oral examination and ICDAS scores, saliva collection. ST: Oral examination and ICDAS scores, saliva collection. RS: Preparation of probiotics in the milk powder, microbiological assays, study design. SK: Data analyses and interpretation, manuscript preparation, and corresponding author. All authors have read and approved this manuscript in its final version.

#### Acknowledgements

This study was supported by the National Research Council of Thailand (DEN550005M); the National Science and Technology Development Agency (NSTDA) of Thailand to R.T.; the Royal Golden Jubilee-Thailand Research Fund (PHD/0051/2556) to A.M.; the Intramural Endowment Fund, Chiang Mai University to O.W.; and the Thailand Research Fund (BRG5680001) to S.K. We thank Dr. M. Kevin O Carroll, Professor Emeritus of the University of Mississippi School of Dentistry, USA, and Faculty Consultant at Chiang Mai University Faculty of Dentistry, Thailand, for his critical reading of this manuscript.

#### Author details

[1]Department of Pediatric Dentistry and Orthodontics, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand. [2]Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand. [3]Center of Excellence in Oral and Maxillofacial Biology, Department of Oral Biology and Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand. [4]Common Oral Diseases and Epidemiology Research Center, Faculty of Dentistry, Prince of Songkla University, Songkhla 90112, Thailand. [5]Rural Oral Health Center of Southern Thailand, Faculty of Dentistry, Prince of Songkla University, Songkhla 90112, Thailand. [6]Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Songkhla 90112, Thailand. [7]Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Songkhla 90112, Thailand.

Received: 5 November 2014 Accepted: 29 January 2015
Published online: 10 February 2015

#### References

1. Selwitz RH, Ismail AI, Pitts NB. Dental caries. Lancet. 2007;369:51–9.
2. Fejerskov O. Concepts of dental caries and their consequences for understanding the disease. Community Dent Oral Epidemiol. 1997;25:5–12.
3. Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol. 2005;33:248–55.
4. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 1986;50:353–80.
5. Banas JA. Virulence properties of *Streptococcus mutans*. Front Biosci. 2004;9:1267–77.
6. Tanzer JM, Livingston J, Thompson AM. The microbiology of primary dental caries in humans. J Dent Educ. 2001;65:1028–37.
7. World Health Organization. Guidelines for the Evaluation of Probiotics in Food [http://who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf]
8. Fooks LJ, Gibson GR. *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiol Ecol. 2002;39:67–75.
9. Meurman JH, Antila H, Korhonen A, Salminen S. Effect of Lactobacillus rhamnosus strain GG (ATCC 53103) on the growth of Streptococcus sobrinus in vitro. Eur J Oral Sci. 1995;103:253–8.
10. Doron S, Gorbach SL. Probiotics: their role in the treatment and prevention of disease. Expert Rev Anti Infect Ther. 2006;4:261–75.
11. Perdigon G, Maldonado Galdeano C, Valdez JC, Medici M. Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr. 2002;56 Suppl 4:21–6.
12. Cagetti MG, Mastroberardino S, Milia E, Cocco F, Lingstrom P, Campus G. The use of probiotic strains in caries prevention: a systematic review. Nutrients. 2013;5:2530–50.
13. van Houte J. Role of micro-organisms in caries etiology. J Dent Res. 1994;73:672–81.
14. Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlen G. Lactobacillus species and genotypes associated with dental caries in Thai preschool children. Mol Oral Microbiol. 2010;25:157–64.
15. Simark-Mattsson C, Emilson CG, Hakansson EG, Jacobsson C, Roos K, Holm S. Lactobacillus-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. Eur J Oral Sci. 2007;115:308–14.
16. Sookkhee S, Chulasiri M, Prachyabrued W. Lactic acid bacteria from healthy oral cavity of Thai volunteers: inhibition of oral pathogens. J Appl Microbiol. 2001;90:172–9.
17. Teanpaisan R, Piwat S, Dahlen G. Inhibitory effect of oral Lactobacillus against oral pathogens. Lett Appl Microbiol. 2011;53:452–9.
18. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 2003;3:710–20.
19. Tao R, Jurevic RJ, Coulton KK, Tsutsui MT, Roberts MC, Kimball JR. Salivary antimicrobial peptide expression and dental caries experience in children. Antimicrob Agents Chemother. 2005;49:3883–8.
20. McKay MS, Olson E, Hesla MA, Panyutich A, Ganz T, Perkins S, et al. Immunomagnetic recovery of human neutrophil defensins from the human gingival crevice. Oral Microbiol Immunol. 1999;14:190–3.
21. Faul F, Erdfelder E, Buchner A, Lang AG. Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses. Behav Res Methods. 2009;41:1149–60.
22. Teanpaisan R, Piwat S. *Lactobacillus paracasei* SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. Clin Oral Investig. 2014;18:857–62.
23. Teanpaisan R, Chooruk A, Wannun A, Wichienchot S, Piwat S. Survival rates of human-derived probiotic *Lactobacillus paracasei* SD1 in milk powder using spray drying. Songklanakarin J Sci Technol. 2012;34:241–5.
24. Ismail AI, Sohn W, Tellez M, Amaya A, Sen A, Hasson H, et al. The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. Community Dent Oral Epidemiol. 2007;35:170–8.
25. Nase L, Hatakka K, Savilahti E, Saxelin M, Ponka A, Poussa T, et al. Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. Caries Res. 2001;35:412–20.
26. Fukushima Y, Kawata Y, Hara H, Terada A, Mitsuoka T. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. Int J Food Microbiol. 1998;42:39–44.
27. Kaila M, Isolauri E, Saxelin M, Arvilommi H, Vesikari T. Viable versus inactivated lactobacillus strain GG in acute rotavirus diarrhoea. Arch Dis Child. 1995;72:51–3.

28. Link-Amster H, Rochat F, Saudan KY, Mignot O, Aeschlimann JM. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol. 1994;10:55–63.
29. Surono IS, Koestomo FP, Novitasari N, Zakaria FR, Yulianasari, Koesnandar. Novel probiotic *Enterococcus faecium* IS-27526 supplementation increased total salivary sIgA level and bodyweight of pre-school children: a pilot study. Anaerobe. 2011;17:496–500.
30. Ericson D, Hamberg K, Bratthall G, Sinkiewicz-Enggren G, Ljunggren L. Salivary IgA response to probiotic bacteria and mutans streptococci after the use of chewing gum containing *Lactobacillus reuteri*. Pathog Dis. 2013;68:82–7.
31. Stensson M, Koch G, Coric S, Abrahamsson TR, Jenmalm MC, Birkhed D, et al. Oral administration of *Lactobacillus reuteri* during the first year of life reduces caries prevalence in the primary dentition at 9 years of age. Caries Res. 2014;48:111–7.
32. Phattarataratip E, Olson B, Broffitt B, Qian F, Brogden KA, Drake DR, et al. *Streptococcus mutans* strains recovered from caries-active or caries-free individuals differ in sensitivity to host antimicrobial peptides. Mol Oral Microbiol. 2011;26:187–99.
33. Yli-Knuuttila H, Snall J, Kari K, Meurman JH. Colonization of *Lactobacillus rhamnosus* GG in the oral cavity. Oral Microbiol Immunol. 2006;21:129–31.
34. Taipale T, Pienihakkinen K, Alanen P, Jokela J, Soderling E. Administration of *Bifidobacterium animalis* subsp. *lactis* BB-12 in early childhood: a post-trial effect on caries occurrence at four years of age. Caries Res. 2013;47:364–72.
35. Marsh PD. Microbiology of dental plaque biofilms and their role in oral health and caries. Dent Clin North Am. 2010;54:441–54.
36. Twetman S, Stecksen-Blicks C. Probiotics and oral health effects in children. Int J Paediatr Dent. 2008;18:3–10.
37. Wannun P, Piwat S, Teanpaisan R. Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. Anaerobe. 2014;17:21–7.
38. Caglar E, Kuscu OO, Cildir SK, Kuvvetli SS, Sandalli N. A probiotic lozenge administered medical device and its effect on salivary mutans streptococci and lactobacilli. Int J Paediatr Dent. 2008;18:35–9.
39. Haukioja A, Yli-Knuuttila H, Loimaranta V, Kari K, Ouwehand AC, Meurman JH, et al. Oral adhesion and survival of probiotic and other lactobacilli and bifidobacteria *in vitro*. Oral Microbiol Immunol. 2006;21:326–32.
40. Jablonski-Momeni A, Stachniss V, Ricketts DN, Heinzel-Gutenbrunner M, Pieper K. Reproducibility and accuracy of the ICDAS-II for detection of occlusal caries *in vitro*. Caries Res. 2008;42:79–87.

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY

# Survival of free and microencapsulated human-derived oral probiotic Lactobacillus paracasei SD1 in orange and aloe vera juices



| | |
|---|---|
| Journal: | *Songklanakarin Journal of Science and Technology* |
| Manuscript ID: | SJST-2014-0198.R1 |
| Manuscript Type: | Original Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Teanpaisan, Rawee; Faculty of Dentistry, Prince of Songkla University, Stomatology<br>Chooruk, Aksorntong; Faculty of Dentistry, Prince of Songkla University, Stomatology<br>Kampoo, Thanyanan; Faculty of Dentistry, Prince of Songkla University, Stomatology |
| Keyword: | Biotechnology and Agro-Industry |
| | |

# Survival of free and microencapsulated human-derived oral probiotic *Lactobacillus paracasei* SD1 in orange and aloe vera juices

Rawee Teanpaisan, Aksorntong Chooruk, Thanyanan Kampoo

Common Oral Diseases and Epidemiology Research Center, and the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, 90112, Thailand

**Correspondence author:**

Rawee Teanpaisan,

Common Oral Diseases and Epidemiology Research Center, and the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University,

Hat-Yai, 90112, Thailand

Tel: +66 74 429878; Fax: +66 74 212922

**Abstract**

Microencapsulation was evaluated as a means of preserving *Lactobacillus paracasei* SD1, a human-derived strain with probiotic potential, in orange and aloe vera juices. The microencapsulation parameters included alginate concentration, calcium chloride concentration and hardening-time, and the efficacy of microencapsulation to preserve the survival of microencapsulated bacteria compared to free cells during exposure in fruit juices were determined. The results revealed that the viable count of free-cell form markedly decreased compared to microencapsulated form. The microencapsulation of 2% alginate (w/v) and 0.05 M $CaCl_2$ gave the best result to preserve the probiotic. It was found that viability of microencapsulated probiotic bacteria was found significantly higher than free-cell in fruit juices during 8 weeks of storage time at the refrigerator. The potential probiotic trait related to inhibitory effect was not affected after microencapsulation process. In summary, the microencapsulation method may be an alternative way in preserving the viability of probiotic *L. paracasei* SD1.

**Key words**: Microencapsulation, Probiotic, *Lactobacillus paracasei* SD1, Fruit juices

## 1. Introduction

There has been an increased interest in the role of probiotic bacteria for promoting and maintaining human health including oral health (Salminen *et al.*, 1998; Parvez *et al.,* 2006), and *Lactobacillus* has been proposed recently to promote oral health. Most studies have reported an inhibitory activity of oral *Lactobacillus* against cariogenic *Streptococcus* (Simark-Mattsson *et al.*, 2007; Teanpaisan *et al.*, 2011), and some also have demonstrated growth suppression of periodontal pathogens (Koll-Klais *et al.,* 2005; Teanpaisan *et al.,* 2011). Therefore, probiotic strains have been increased an attention to incorporate into a variety of food products including yoghurts, cheese, drinks and dietary products. Of those, fruit juices may be alternative products for the incorporation of probiotic. Nowadays, most people consume fruit juices as daily soft drink due to their nutritional relevance e.g. organic acids, minerals, fiber and a good source of antioxidant compound (vitamins A, C and E). It has recommended that the probiotic bacteria should be alive and be present at amount of at least $10^6$-$10^7$ CFU/ml or g in the products (Rybka and Kailasapathy, 1995). However, it was reported that the free-cell probiotic bacteria may not survive in sufficient numbers when incorporated into food products. The viability of probiotic strains could be influenced by several factors of soft drinks e.g. acidic pH, oxygen level, antibacterial components and packaging conditions (Vinderola *et al.,* 2011).

Among the available techniques for immobilizing living cells, entrapment in calcium alginate beads has been proven to be useful for the immobilization of various probiotic strains including *Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus plantarum, L. acidophilus, L. paracasei, Bifidobacterium bifidum, Bifidobacterium infantis* and *Bifidobacterium lactis* (Khalil and Mansour, 1998; Chandramouli *et al*., 2004; Ding and Shah, 2008). Calcium alginate beads act as a physical barrier to protect probiotic living cells from adverse environment e.g. acidic pH. In addition, alginate has the benefits of being non-

toxic to the cells being immobilized, and it is an accepted as food additive (Prevost and Divies, 1992). However, there are different conditions for the use of calcium alginate as a matrix for bacterial cells microencapsulation. The reported concentrations of sodium alginate vary widely from 0.5% to 4% (w/v). Also, the concentration of calcium chloride and hardening time of capsules in the calcium chloride solution for stabilization of the beads vary greatly from 0.05 to 0.2 M and 5 min to 2 h, respectively (Sheu and Marshall, 1993; Jankowski at al., 1997; Khalil and Mansour, 1998; Truelstrup Hansen et al., 2002). Therefore, the appropriate condition of calcium alginate bead for individual product needs to be optimized.

We have previously reported the use of spray drying to preserve the probiotic *Lactobacillus paracasei* SD1 in milk power, and such produce has been proven to give a benefit for clinical trials in reducing the pathogenic agents in the oral cavity (Teanpaisan and Piwat, 2013; Ritthagol *et al.*, 2013). Thus, we now extend the use of calcium alginate as an alternative way to preserve human-derived *Lactobacillus paracasei* SD1. It hypothesized that calcium alginate could preserve the probiotic bacterial cells, therefore, the survival time of the probiotic bacteria in the microencapsulated form would longer than the free cell form in fruit juices. The aims of the present study were to optimize the microencapsulation parameters including alginate concentration, calcium chloride concentration and hardening time, and to evaluate the efficacy of microencapsulation to preserve the survival of microencapsulated probiotic bacteria compared to free cells during exposure to fruit juices.

## 2. Materials and methods

### 2.1 Bacterial strains and culture conditions

Probiotic strain *Lactobacillus paracasei* SD1 was isolated previously from the human oral cavity, and was identified as *L. paracasei* according to 16S-rRNA gene profiles by

restriction fragment length polymorphism analysis (PCR-RFLP) and protein profiles by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Teanpaisan and Dahlén, 2006). The strain was stored at -80°C until used.

The probiotic strain was activated by growing twice on MRS agar at 37ºC in anaerobic condition (80% $N_2$, 10% $H_2$, and 10% $CO_2$) for 24 h, and the strain was then propagated in 500 ml MRS broth in the same condition for 24 h. Cells were harvested by centrifugation at 3,000 × g for 10 min, and washed twice with 0.85% (w/v) normal saline. Cell count was determined by anaerobic on MRS agar after 48 h at 37ºC in anaerobic condition. The cell suspension was subsequently used either directly (free cells) in assays or subjected to microencapsulation as described below.

**2.2 Preparation of microencapsulation of probiotic**

Initially, the microencapsulation parameters including alginate concentration (1.5%, 2% and 3% (w/v), calcium chloride concentration (0.01, 0.05 and 0.1 M) and hardening time (5 min, 30 min, and 1 h) were optimized for viability of microencapsulated bacteria.

The alginate and bacteria suspension with initial cell load approximately $10^9$ CFU/ml was slowly dispensed using a pipette into a beaker containing a solution of calcium chloride with a magnetic stirrer at 200 rpm. After certain hardening time, the calcium alginate beads were removed and washed thoroughly with sterile distilled water. The calcium alginate beads were then kept at 4ºC in 0.85% saline solution until further use.

**2.3 Evaluation of bacterial viability in free-cell form and microencapsulated form**

To determine the viable counts of *L. paracasei* SD1 in free-cell form and microencapsulated form, the initial cell loads of free-cell form (3 × $10^6$ CFU/ml) and microencapsulated form (1-5 × $10^6$ CFU/ml) were monitored monthly over a period of 6 months of preserving at 4ºC. For the enumeration of the microencapsulated cells,

microcapsules were disrupted and counted using MRS agar and incubation at 37°C for 48 h in anaerobic condition. Also, free-cells were counted as the same procedure.

**2.4 Evaluation of bacterial viability in fruit juices**

Commercially sterilized (according to the manufacturing system) orange and aloe vera (TIPCO F&B Co., Ltd., Thailand) were used for all experiments. Fruit juices with no added preservatives and a long shelf life were obtained.

Probiotic free-cell form or microencapsulated form was added in 100 ml of each fruit juice at final $10^9$ CFU/ml, and thoroughly mixed under sterilized process in a laminar flow cabinet. Fruit juices contained probiotic free-cell form or microencapsulated form were separated to a small portion in sterile bottles, and kept in room temperature (25°C) or refrigerator (4°C). For monitoring of probiotic viability, an enumeration of the probiotic cells was performed weekly over a period of 6 weeks and at 8 and at 12 weeks, using colony counting on MRS agar with incubation at 37ºC for 48 h under anaerobic condition. The pH of juices contained free- or microencapsulated bacteria were also monitored using pH meter. All experiments were performed in triplicate to determine an average mean and standard deviation.

**2.5 Inhibitory effect of *L. paracasei* SD1 before and after microencapsulation**

Before and after microencapsulation, the inhibitory effect of *L. paracasei* SD1 against *Streptococcus mutans* was assessed by an agar overlay method (Teanpaisan *et al.,* 2011). In brief, *L. paracasei* SD1 was inoculated on the surface of the brain heart infusion agar and incubated anaerobically (80% $N_2$, 10% $H_2$, and 10% $CO_2$) for 24-48 h at 37°C to develop visible macro-colonies.

*Streptococcus mutans* ATCC 25175 was precultivated in the brain heart infusion broth (BHI), and then the suspension of cells was adjusted to an optical density (OD) 0.25 at 600 nm. Thereafter, 5 ml of BHI soft agar (7 g/l agar) were seeded with 100 µl of an

overnight culture of *S. mutans* ATCC 25175 strain and immediately poured over the macro-colonies of *L. paracasei* SD1. The plate was incubated anaerobically at 37°C for 24 h. An inhibitory zone, resulting in the releasing of inhibitory substance of *L. paracasei* SD1 against the growth of *S. mutans*, was observed.

**2.6 Statistical analysis**

All the results were expressed as mean ± SD from three individual experiments. Differences among treatments were evaluated by one-way ANOVA and paired *t*-test. Significant differences between means within and among the treatment were determined using Tukey's HSD test. Results were considered statistically significant when $P < 0.05$. All statistical analysis was carried out using SPSS (Chicago, Illinois, USA).

## 3. Results and Discussion

### 3.1 Effect of alginate concentration and $CaCl_2$ concentration on viability of *L. paracasei* SD1

In the present study, we investigated the use of microencapsulation to preserve viable cells of the oral human-derived *L. paracasei* SD1 strain.

The initial microencapsulated parameters were investigated to determine the conditions which were optimum for probiotic viability. There was 2- to 3-log CFU decrease in bacterial cells count (from $10^9$ CFU/ml) after microencapsulation. The data obtained in these trials indicated that the viability count of *L. paracasei* SD1 significantly decreased ($P < 0.05$, one way ANOVA) when the $CaCl_2$ concentration was further increased to 0.10 M (Figure 1). An increased survival of probiotic was found in the microcapsules that had been hardened for 30 min compared with that in the microcapsules which had been hardened for only 5 min (data not shown).

### 3.2 Survival of free cells and microencapsulated *L. paracasei* SD1 during storage at refrigerator

There is no uniformity in the reported microencapsulation procedure for certain use, thus, the microencapsulation parameters, including alginate concentration, calcium chloride concentration and hardening time of capsules in calcium chloride, were quantitatively determined and optimized in this study. The survival time of probiotic bacteria in the microencapsulated form compared to the free-cell form was performed. The detail of result is given in Figure 2. The viable count of free-cell decreased markedly, and no viable cells were recovered after 3 months of refrigerated storage. It demonstrated that the survival time of probiotic bacteria in the microencapsulated form was longer than in free cell form. Generally, viability of microencapsulated *L. paracasei* SD1 in various concentrations of alginate and $CaCl_2$, gradually decreased from a 2-log to 4-log CFU of probiotic counts over 6 months of refrigerated storage. Results were similar to the previous reports (Khalil and Mansour, 1998; Chandramouli *et al*., 2004; Ding and Shah, 2008), which revealed that calcium alginate could preserve the bacterial cell viability. This is due to its ability of calcium alginate act as a physical barrier to protect probiotic cells from adverse effect.

There were probiotic count differences (1-log to 2-log CFU) among the various concentrations of calcium alginate. However, the microencapsulation of 2% alginate (w/v) and 0.05 M $CaCl_2$ gave the maximum viable cells count of probiotic and uniform spherical microcapsule formation. Thus, it was selected for further use in the experiment of fruit juices.

### 3.3 Survival of free-cell form and microencapsulated form of *L. paracasei* SD1 in fruit juices during storage at either refrigerator or room temperature

The results of survival of probiotic bacteria in free-cell form and microencapsulated form in orange and aloe vera juices at a refrigerator and room temperature are shown in Figure 3. It revealed that viability of microencapsulated probiotic bacteria was significantly

($P < 0.05$, paired *t*-test) higher than that of free probiotic bacteria in both orange and aloe vera juices during 12 weeks of storage time at either a refrigerator (Fig. 3A) or room temperature (Fig. 3B).

After 8 weeks, only the microencapsulated form could preserve probiotic bacteria at the level of $10^6$ CFU/ml, which means number of $1.02 \pm 0.43 \times 10^6$ and $2.51 \pm 0.28 \times 10^6$ CFU/ml for orange and aloe vera juices at a refrigerator, respectively (Fig. 3A). As it has recommended that the probiotic bacteria should be alive and be present at amount of at least $10^6$-$10^7$ CFU/ml or g in the products (Rybka and Kailasapathy, 1995). Therefore, results in this study have proven that microencapsulated probiotic in a refrigerated condition was able to maintain viability in the fruit juices in satisfied number ($10^6$ CFU/ml) for at least 8 weeks.

The loss of viability of probiotic bacteria seemed to be greater in the orange juice than in the aloe vera juice when it was compared in the same form (either encapsulated form or free cells form). However, it was not statistically significant ($P > 0.05$, paired *t*-test). Some reports also showed that acid environment influenced the survival rate of probiotic bacteria (Kailasapathy and Rybka, 1997; Ding and Shah, 2008).

It is noted that the storage time in this study could be more prolonged than the others, in which storage time was only 2 week (Adhikari *et al.*, 2000; Saarela *et al.*, 2006). Microcapsules may provide a favorable environment for the probiotic strain, as well as a physical barrier from the harsh, e.g. acidic condition of the fruit juices.

**3.4 pH changes in fruit juices containing free cells and microencapsulated probiotic bacteria during a storage at either a refrigerator or room temperature**

The pH changes in the orange juice and the aloe vera juice containing free- and microencapsulated probiotic bacteria during a storage period of 12 weeks at either a refrigerator (Fig. 4A) or room temperature (Fig. 4B) were monitored. It was found that the pH of orange juice ($3.3 \pm 0$) and aloe vara juice ($4.2 \pm 0$) containing microencapsulated

probiotic cells did not change over the whole period of 6 weeks of storage at either a refrigerator or room temperature. The pH of juices with free-cell form started to drop after 3 and 5 weeks at room temperature and a refrigerator, respectively. Free probiotic bacteria may have utilized sucrose in juices and produced small amounts of organic acids, lowering the pH of the product during storage. The results indicated that microencapsulation could maintain the pH of juices containing probiotic cells.

### 3.5 Inhibitory effect of *L. paracasei* SD1 before and after microencapsulation

*L. paracasei* SD has been reported as a novel probiotic strain that could reduce cariogenic bacteria in volunteers receiving milk powder containing *L. paracasei* SD1 (Teanpaisan and Piwat, 2013). It has been shown previously that *L. paracasei* SD1 produces a broad-spectrum antimicrobial activity that exhibits activity against oral pathogens such as *Streptococcus mutans, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Tanerella forsythia* (Teanpaisan *et al*., 2011). Antimicrobial activity is a desirable trait for probiotic cultures (Collins *et al*., 1998) and may be used to competitively exclude undesirable microorganisms in the oral cavity, thereby playing a role in probiotic persistence in the host. In this study, it was shown that the potential probiotic trait related to inhibitory activity was not affected after microencapsulation process compared to before microencapsulation process (Figure 5).

## 4. Conclusions

Our findings highlight the need to take into consideration the technological properties of probiotic strains, and emphasize the importance of strain selection with regard to processing, as well as health-promoting properties. In this study, it was shown that 2% alginate and 0.05 M $CaCl_2$ could provide a survival of *L. paracasei* SD1 up to 8 weeks. The fruit juices contained microencapsulated *L. paracasei* SD1 had high levels of viable probiotic ($\geq 10^6$ CFU/g) in the refrigerated storage. In addition, inhibitory effect of *L. paracasei* SD1

was not affected after microencapsulation process. Thus, a supplemented fruit juice by incorporating microencapsulated *L. paracasei* SD1 in calcium alginate beads might be an alternative source for providing a live beneficial organism to consumers. However, 8 weeks survival of *L. paracasei* SD1 in microencapsulated form may be too short for the manufacturing products, it will need to improve for a longer time.

**Acknowledgements**

This work was supported by the National Research Council of Thailand (DEN550005M), the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (AGR 540555M), and the National Science and Technology Development Agency (NSTDA) of Thailand.

**References**

Adhikari, K., Mustapha, A., Grum, I.U. and Fernando, L. 2000. Viability of microencapsulated bifidobacteria in set yoghurt during refrigerated storage. Journal of Dairy Science. 83, 1946-1951.

Chandramouli, V., Kailaspathy, K., Peiris, P. and Jones, M., 2004. An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. Journal of Microbiological Methods. 56, 27–35.

Collins, J.K., Thornton, G. and Sullivan, G.O. 1998. Selection of probiotic strains for human applications. International Dairy Journal. 8, 487-490.

Ding, W.K. and Shah, N.P. 2008. Survival of free and microencapsulated probiotic bacteria in orange and apple juices. International Food Research Journal. 15, 219- 232.

Jankowski, T., Zielinska, M. and Wysakowska, A. 1997. Encapsulation of lactic acid bacteria with alginate/starch capsules. Biotechnology Techniques. 10, 31-34.

Kailasapathy, K. and Rybka, S. 1997. *Lactobacillus acidophilus* and *Bifidobacterium* spp. Their therapeutic potential and survival in yogurt. Australian Journal of Dairy Technology. 52, 28–35.

Khalil, ALI H. and Mansour, ESAM H. 1998. Alginate Encapsulated Bifidobacteria Survival in Mayonnaise. Journal of Food Science. 63, 702-705.

Koll-Klais, P., Mandar, R., Leibur, E., Marcotte, H., Hammarstrom, L. and Mikelsaar, M. 2005. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. Oral Microbiology and Immunology. 20, 354-361.

Parvez, S., Malik, K.A., Ah Kang, S. and Kim, H.Y. 2006. Probiotics and their fermented food products are beneficial for health. Journal of Applied Microbiology. 100, 1171-1185.

Prevost, H. and Divies, C. 1992. Cream fermentation by a mixed culture of Lactobacilli entrapped in two-layer calcium alginate beads. Biotechnology Letters. 14, 583-588.

Ritthagol, W., Saetang, C. and Teanpaisan, R. 2013. Effect of probiotics containing *Lactobacillus paracasei* SD1 on salivary mutans streptococci and Lactobacilli in orthodontic cleft patients: A double-blinded, randomized, placebo-controlled study. Cleft Palate-Craniofacial Journal. 51, 257-263.

Rybka, S. and Kailasapathy, K. 1995. The survival of culture bacteria in fresh and freeze-dried AB yoghurts. Australian Journal of Dairy Technology. 50, 51–57.

Saarela, M., Virkajarvi, I., Alakomi, H.L., Mattila, P. and Matto, J. 2006. Stability and functionality of freeze-dried probiotic *Bifidobacterium* cells during storage in juice and milk. International Dairy Journal. 16, 1477-1482.

Salminen, S., Ouwehand, A.C. and Isolauri, E. 1998. Clinical applications of probiotic bacteria. International Dairy Journal. 8, 563-572.

Sheu, T.Y. and Marshall, R.T. 1993. Micro-encapsulation of Lactobacilli in calcium alginate gels. Journal of Food Science. 54, 557-61.

Simark-Mattsson, C., Emilson, C.G., Hakansson, E.G., Jacobsson, C., Roos, K. and Holm, S. 2007. *Lactobacillus*-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. European Journal of Oral Sciences. 115, 308-314.

Teanpaisan, R. and Dahlen, G. 2006. Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral *Lactobacillus* species. Oral Microbiology and Immunology. 21, 79-83.

Teanpaisan, R., Piwat, S. and Dahlen, G. 2011. Inhibitory effect of oral *Lactobacillus* against oral pathogens. Letters in Applied Microbiology. 53, 452-59.

Teanpaisan, R. and Piwat, S. 2013. *Lactobacillus paracasei* SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. Clinical Oral Investigations. 18, 857-862.

Truelstrup Hansen, L., Allan-Wojtas, P. M., Jin, Y. L. and Paulson, A. T. 2002. Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. in milk and simulated conditions. Food Microbiology. 19, 35-45.

Vinderola, G., Binetti, A., Burns, P. and Reinheimer, J. 2011. Cell viability and functionality of probiotic bacteria in dairy products. Frontiers in Microbiology. 2, 1-5.

**Figure captions:**

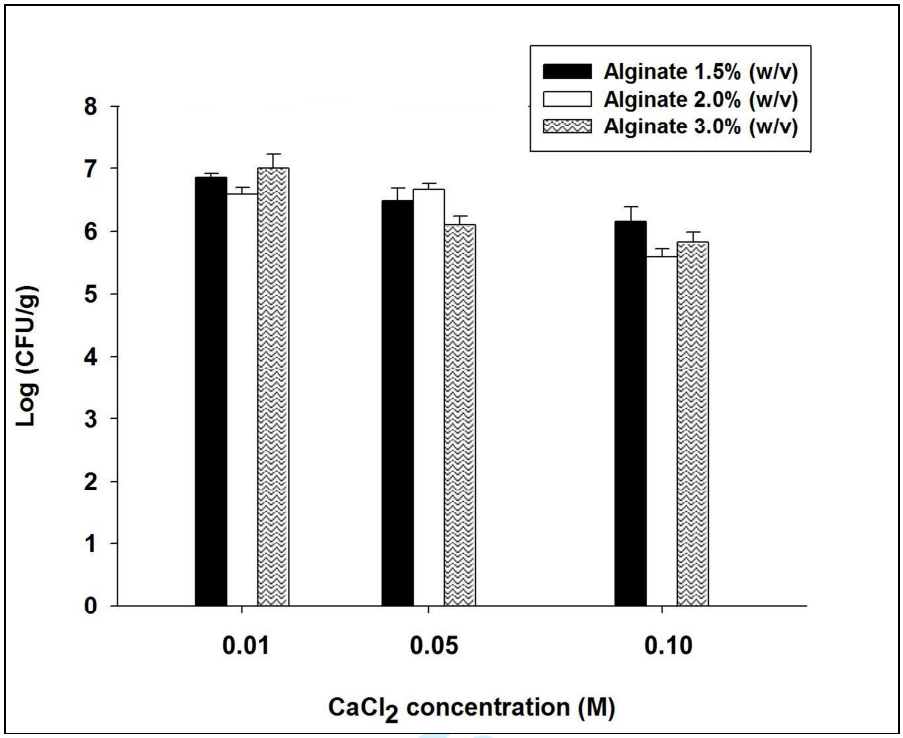



**Figure 1**. Effect of alginate concentration and $CaCl_2$ concentration on viability of *L. paracasei* SD1

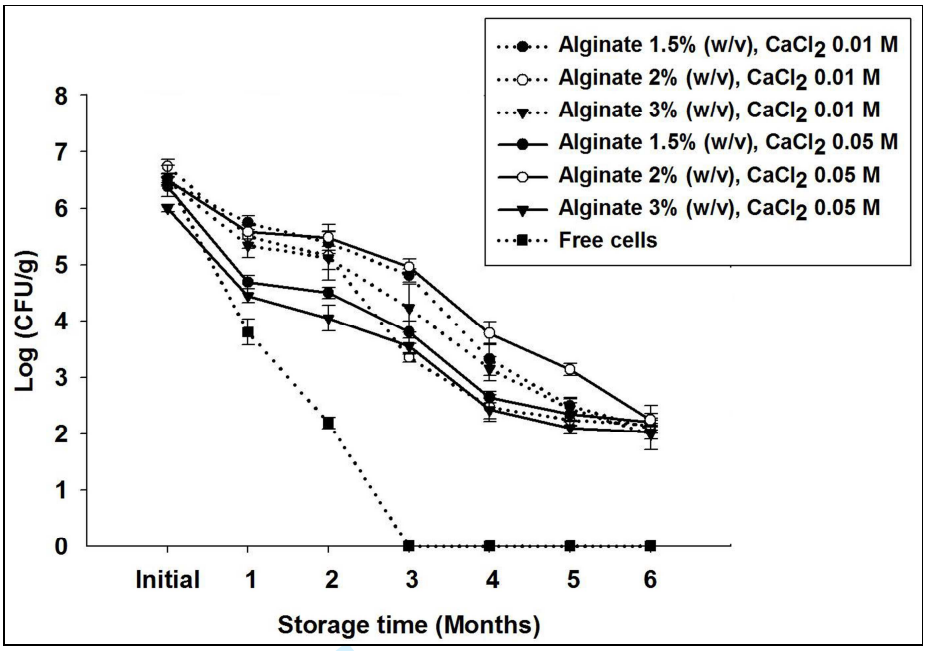



**Figure 2.** Survival of free-cell and microencapsulated *L. paracasei* SD1during storage in a refrigerator

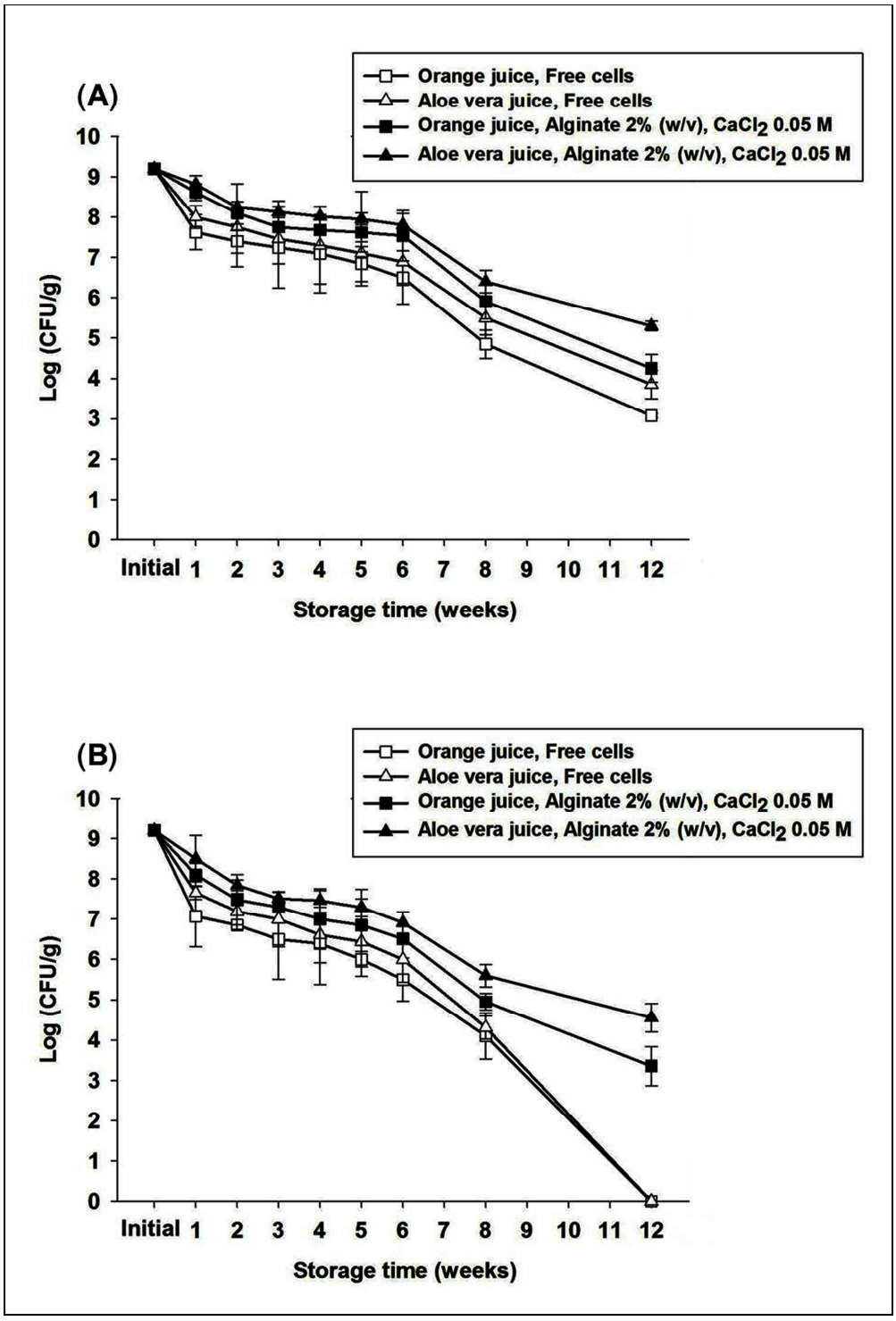



**Figure 3.** Survival of free-cell and microencapsulated *L. paracasei* SD1 in juices during storage at a refrigerator (A) and room temperature (B)

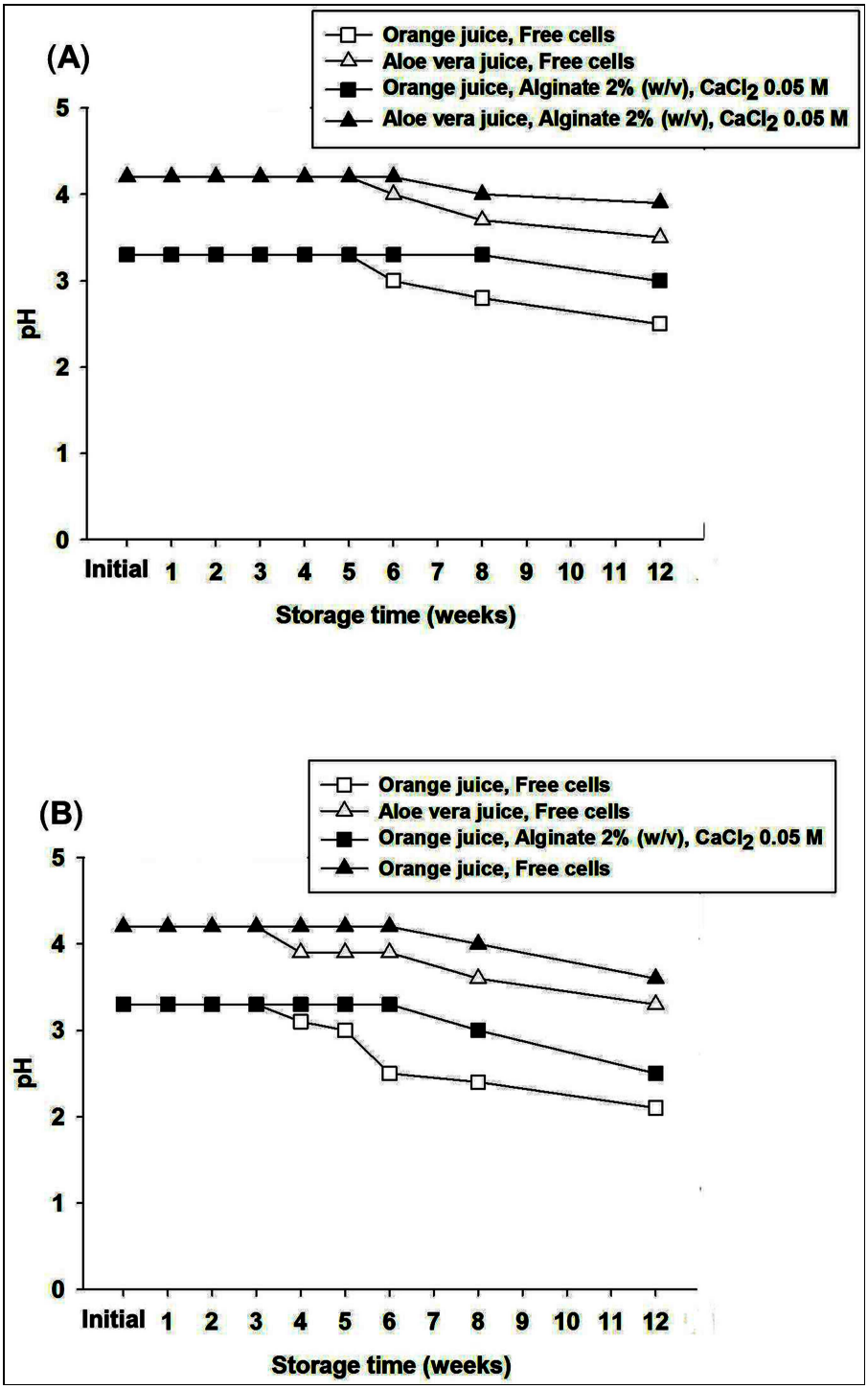



**Figure 4.** pH changes in fruit juices containing free-cell and microencapsulated probiotic bacteria during a storage at a refrigerator (A) and room temperature (B)

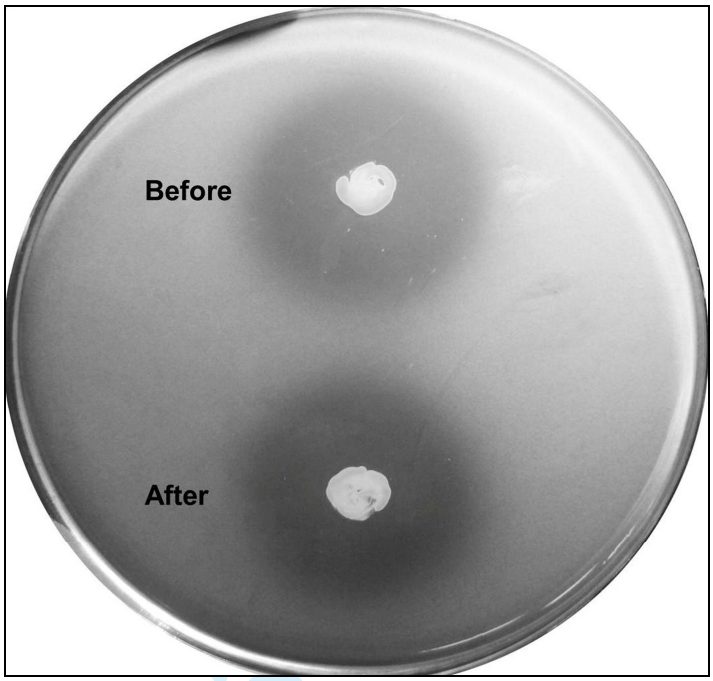



**Figure 5.** Inhibitory effect of *L. paracasei* SD1 against *S. mutans* ATCC 25175 tested before and after microencapsulation process.

# An assessment of adhesion, aggregation, and surface charges in Lactobacillus strains derived from the human oral cavity

| | |
|---|---|
| Journal: | *Applied Microbiology* |
| Manuscript ID: | LAM-2014-1799.R2 |
| Journal Name: | Letters in Applied Microbiology |
| Manuscript Type: | LAM - Original Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Piwat, Supatcharin; Prince of Songkla University, Common Oral Diseases and Epidemiology Research Center, Department of Preventive Dentistry, Faculty of Dentistry<br>Sophatha, Benchamat; Prince of Songkla University, Common Oral Diseases and Epidemiology Research Center, Stomatology<br>Teanpaisan, Rawee; Prince of Songkla University, Stomatology, Faculty of Dentistry; Common Oral Diseases and Epidemiology Research Center, |
| Key Words: | Lactobacillus, Probiotics, Lactic acid bacteria |
| | |

# An assessment of adhesion, aggregation, and surface charges in *Lactobacillus* strains derived from the human oral cavity

Supatcharin Piwat[1, 2], Benchamat Sophatha[1, 3] and Rawee Teanpaisan[1, 3]

[1] Common Oral Diseases and Epidemiology Research Center, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Thailand

[2] Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Thailand

[3] Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Thailand

Correspondence author:

Rawee Teanpaisan,

Common Oral Diseases and Epidemiology Research Center and the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, 90112, Thailand

Tel: +66 74 429878; - Fax: +66 74 212922

E-mail address: rawee.t@psu.ac.th

Running title: The adhesion and aggregation of *Lactobacillus*

Keywords: adhesion, aggregation, physicochemical properties, oral keratinocyte H357 cell, *Lactobacillus* strains

## Abstract

There is limited information concerning the adhesion and aggregation of human oral lactobacilli. In this study, the adhesion of 10 *Lactobacillus* species was investigated using H357 oral keratinocyte cells as an *in vitro* model for oral mucosa. Coaggregation with the representative oral pathogen, *Streptococcus mutans* ATCC 25175, and the physicochemical cell properties was also evaluated. The results demonstrated significant variations in adhesion (42-96%) and aggregation (autoaggregation, 14-95%; coaggregation, 19-65%). All strains showed a high affinity for chloroform, and most strains had a moderate-to-high hydrophobicity. All strains, except *L. casei* and *L. gasseri,* showed a moderate affinity for ethyl acetate. There was a strong association of autoaggregation with coaggregation ($r_s$=0.883, $P$<0.001). The highest mean for autoaggregation (74%) and coaggregation (47%) belonged to the *L. gasseri* strains. Correlations between the adhesion and surface characteristics and aggregation were observed among the *L. fermentum* and *L. paracasei* strains; however, there was a variation in the strains properties within and between species. This study indicated that the *L. gasseri, L. fermentum,* and *L. paracasei* strains might be potential probiotics for the human oral cavity given their desirable properties. It should also be emphasised that a selective process for probiotic strains is required.


## Significance and Impact of the Study

Adhesion to host tissues and bacterial aggregation (auto- and coaggregation) are high important criteria for selecting strains with probiotic potential. These abilities are commonly involved with surface-charged characteristics. This is the first study to investigate the oral *Lactobacillus* species using an oral keratinocyte cell line. Significant results were found for the correlations between the adhesion and surface charge characteristics and for aggregation among certain strains of *L. gasseri, L. fermentum,* and *L. paracasei*. This observation could

be useful when collecting background information for the selection of probiotic strains for use in oral health.

## Introduction

The genus *Lactobacillus* spp. contains over 160 species that are gram-positive, rod-shaped, non-spore-forming and catalase-negative bacteria. They are found in diverse environments such as food, vegetables and sewage, and they live in close contact with humans in the gastrointestinal tract, vagina and oral cavity. Their ability to inhibit the growth of various pathogens has gained attention, and they have been used as probiotics in the gut for decades (Parvez *et al.* 2006; Andrews and Tan 2012).

In the oral cavity, *Lactobacillus* usually comprises less than 1% of the total microbiota in the normal oral microflora (London 1976); however, some strains have previously been reported in relation to the progression of dental caries (van Houte 1994; Teanpaisan *et al.* 2007). It has been suggested that there were differences among various strains with respect to their ability to produce acid (Piwat *et al.* 2012). These findings implied that not all *Lactobacillus* spp. strains had a caries-inducing effect (Piwat *et al.* 2010; Piwat *et al.* 2012). Some strains have been demonstrated to be potential probiotics for oral health (Näse *et al.* 2001; Nikawa *et al.* 2004; Teanpaisan and Piwat 2014), although the mechanisms of their interactions are not entirely understood.

Adhesion to host tissues is the first step in bacterial colonisation and also influences the subsequent phases leading to commensalism or infectious diseases. The ability to adhere to mucosal host surfaces has always been considered to be an important property among the bacterial strains used as probiotics (FAO/WHO 2002). The bacterial cell surface charge and hydrophobicity have been shown to influence the strength of bacterial adhesion (Harty and Knox 1991; Piette and Idziak 1992). In addition, some studies have reported that aggregation ability was found to be related to bacterial adherence property (Collado *et al.* 2008; Xu *et al.* 2009; Tuo *et al.* 2013).

Bacterial aggregation between microorganisms of the same strain (autoaggregation) or between different species and strains (coaggregation) is of considerable importance in several niches, such as the human gut and oral cavity, where probiotics are active (Collado *et al*. 2008; Keller *et al*. 2011). Probiotics can inhibit the adherence of pathogenic bacteria to mucosa either by forming a barrier via autoaggregation or by direct coaggregation with the pathogens. Therefore, the adhesion and aggregation abilities are prerequisite factors in developing selection criteria for new probiotic strains. Most studies have been performed with the non-oral *Lactobacillus* strains, and information on the adhesion of *Lactobacillus* strains derived from the human oral cavity is still limited.

The objectives of the present study were to assess the adhesion and aggregation abilities and the surface charges of various *Lactobacillus* species derived from the human oral cavity, and to determine whether there was any correlation among the adhesion, surface charges, and aggregation.

## Results and Discussion

Bacterial adhesion to host cells is a complex process involving contact between the bacterial cell membrane and interacting surfaces. The ability to adhere to epithelial cells and mucosal surfaces has been considered to be one of the selection criteria for probiotic strains. Because it is difficult to investigate bacterial adherence *in vivo*, adhesion has been studied using cell lines of human origin in culture as *in vitro* models (Xu *et al.* 2009). To our knowledge, this study may be the first to examine the adhesion ability of various oral *Lactobacillus* strains to the oral mucosa in an *in vitro* model using an oral keratinocyte H357 cell culture. An adhesion assay used in this study provided the advantages of internalisation and externalization data for various *Lactobacillus* strains towards oral keratinocyte H357 cell culture. The results for the total adhesion, externalization abilities, and internalisation abilities between and within 10 *Lactobacillus* species are shown in Fig. 1. There was great variation in the total adhesion, externalization abilities, and internalisation abilities among individual strains at the inter- and intra-species levels, which ranged from 42-96%, 2-83% and 0-84%, respectively. *L. rhamnosus* and *L. salivarius* had the highest mean value for total adhesion (79%) followed by *L. mucosae* (74%) and *L. vaginalis* (71%). However, the internalisation of *L. vaginalis* (50%) and *L. mucosae* (47%) was much higher than the externalisation (21% and 26%, respectively). *L. gasseri* showed high externalisation (40%), whereas its internalisation was low (22%). It was noted that the strains within the *L. fermentum*, *L. paracasei* and *L. gasseri* species showed a wide range of externalisation and internalisation.

Concerning the aggregation ability, all strains tested in this study performed autoaggregation and coaggregation with *S. mutans* ATCC 25175. Aggregation among the oral *Lactobacillus* strains depended on the time of incubation. The autoaggregation and coaggregation of all strains were different (Fig. 2). The highest means for autoaggregation

(74%) and coaggregation (47%) belonged to the *L. gasseri* strains. The lowest mean autoaggregation (31% and 38%) and coaggregation (28% and 26%) values were found among the *L. oris* and *L. mucosae* strains, respectively. There was a strong correlation between autoaggregation and coaggregation ($r_s = 0.883$) with a significance of $P < 0.001$, as shown in Fig. 3.

Many studies have demonstrated that bacterial surface charges are involved in the adhesion to the host mucosa and in attachments within and between the bacterial species (Wadström *et al*., 1987; Pelletier *et al.* 1997; Del Re *et al.* 2000; Collado *et al*. 2007; Xu *et al*., 2009). The bacterial surface charges are also involved in aggregation (Kos *et al.,* 2003; Del Re *et al.* 2000; Collado *et al*. 2007; Xu *et al*. 2009). There were positive correlations between the adhesion ability and aggregation to hydrophobicity, as demonstrated by the high adherence or high aggregation to xylene, an apolar solvent (Harty and Knox 1991; Collado *et al*. 2007; Xu *et al*. 2009; Darilmaz *et al*. 2012). In this study, the oral *Lactobacillus* strains were derived from saliva, and the results were generally consistent with the study of lactobacilli that were isolated from various locations in the mouth (Colloca *et al.* 2000). Most oral *Lactobacillus* strains were revealed to have moderate-to-high hydrophobicity and hydrophilic surface charges; however, *L. gasseri* had a low hydrophobicity and affinity for ethyl acetate (Fig. 4). All strains, except *L. casei* and *L. gasseri,* showed moderate affinity for ethyl acetate, a basic solvent. All *Lactobacillus* strains displayed a higher affinity for chloroform, an acidic solvent, than for the ethyl acetate solvent, which was similar to the results of a previous study (Pelletier *et al.* 1997). This finding indicates that *Lactobacillus* strains are strong electron donors. This is expected because most bacterial surfaces possess an overall electronegative charge (Beveridge and Graham 1991).

The correlation between the total adhesion ability and the internalisation of all *Lactobacillus* strains and surface charges is shown in Table 1; however, this information was

clearly observed in only the *L. fermentum* and *L. paracasei* strains (Table-2-SuppInfo). The individual strain with high surface charges was significant in internalisation and in aggregation, especially when the data from the 15 selected *L. fermentum* strains was analysed, given that the coefficient correlation was much higher (Figure-5-SuppInfo). A similar result was also observed for the coaggregation of all strains, in which a significant correlation with all solvents was found (Table 1). However, this correlation was clearly revealed in only the *L. fermentum* and *L. paracasei* strains (Table-2-SuppInfo, Figure-5-SuppInfo and Figure-6-SuppInfo).

The results implied that the internalisation of certain oral *Lactobacillus* strains, for example *L. fermentum* and *L. paracasei*, may play an important role in adhesion within the human oral cavity. However, it was noted that *L. gasseri* strains showed high externalisation and aggregation (auto- and coaggregation) abilities and that they had low hydrophobicity and low affinity to ethyl acetate. This finding indicates that *L. gasseri* strains might perform their adhesion and aggregation via a different mechanism. This may be supported by the findings of a specific protein, an aggregation-promoting factor, of *L. gasseri* (Boris *et al*. 1998; Ventura *et al*. 2002). Adhesion is a complex process involving non-specific (hydrophobicity and hydrophilic) and specific ligand-receptor mechanisms. In this study, the mechanisms of adhesion were not studied. Thus, it was not possible to define which strains were bound because of the specific interaction mediated by adhesin(s) and in strains bound by non-specific interactions. Future work is needed to study the adhesion mechanisms that involve specific interaction as mediated by adhesin(s).

A relation between the aggregation and adhesion ability has been reported for some bifidobacterial species and *Lactobacillus* strains derived from the gut origin (Perez *et al.* 1998; Del Re *et al.* 2000; Kos *et al*. 2003; Rickard *et al*. 2003). In this study, a positive

correlation between aggregation and internalisation was found among *L. paracasei* and 15 selected *L. fermentum* strains (Figure-7-SuppInfo and Figure-8-SuppInfo).

The findings in this study indicated that *L. paracasei* and *L. fermentum* strains may be potential probiotics for the human oral cavity according to the desirable properties for selecting probiotic strains. It should also be emphasised that a selective process for probiotic strains is required.

In addition, the ability of microorganisms to adhere to oral mucosal surface and to aggregate (auto- and coaggregation) may have ecological significance in the oral cavity. In this study, most oral *Lactobacillus* strains revealed high adhesion (either through externalisation or internalisation) ability to oral keratinocyte H357 cells, which may have resulted from the findings of moderate-to-high hydrophobicity and the surface charges of those strains. The ability to adhere to the host cells reportedly correlated to surface charges, which would aid in the initial attachment.

A certain strain with high auto- and coaggregation might result in a low level of detection in the oral cavity because aggregation followed by swallowing is generally regarded to be an important factor in the removal of organisms from the mouth. In addition, many authors have reported that the aggregation ability is a desirable property for selecting probiotics strains (Collado *et al*. 2007; Keller *et al*. 2011; Darilmaz *et al*. 2012; Tuo *et al*. 2013). Organisms with the ability to coaggregate with other bacteria such as pathogens may have an advantage over non-coaggregating organisms, by aggregating and removing the pathogens from the environment. This finding may support the evidence showing that *L. gasseri* was found to be less prevalent in the saliva of high-caries children (2.6%) than in that of low-caries children (15%) (Piwat *et al*. 2010). In addition, another study (Koll-Klais *et al.* 2005) reported that *L. gasseri* was significantly less prevalent in chronic periodontitis patients

(8%) than in healthy subjects (64%). In this study, *L. gasseri* was found to have a high coaggregation ability with a cariogenic pathogen (*S. mutans*), which may explain the phenomenon above. It should be stated that this is a preliminary study on the aggregation of oral *Lactobacillus* strains, and only *S. mutans* has been used as a representative oral pathogen. Therefore, it is advisable to include more oral strains for investigation to identify whether the aggregation of *Lactobacillus* strains could influence the oral microbiome.

In conclusion, the results demonstrated that various *Lactobacillus* spp. derived from the oral cavity showed different abilities in terms of adhesion and aggregation. Correlations between the adhesion and surface characteristics and aggregation were observed among the *L. fermentum* and *L. paracasei* strains; however, there was a variation in the strain properties within and between species. The *L. grasseri* strains exhibited higher auto- and coaggregation with *S. mutans* than the other strains. To our knowledge, this is the first study to investigate oral *Lactobacillus* species with oral keratinocyte cell line, which could be useful in the collection of background information for selecting probiotic strains for use in oral health. It should also be emphasised that a case-by-case assessment is needed to select strains that provide the most suitable bacteriotherapy in the oral cavity.

## Materials and Methods

### Bacterial strains and culture conditions

A total of 197 oral *Lactobacillus* strains from 10 species were obtained from the previous study by Piwat *et al.* (2010), and they had already been identified using a restriction fragment length polymorphism (RFLP) analysis of a polymerase chain reaction (PCR) (Teanpaisan and Dahlen 2006) and 16S rRNA PCR-Denaturing gradient gel electrophoresis (Piwat and Teanpaisan 2013). The 16S-rRNA sequencing was performed for strains with uncertain identifications. The culture collection was kept at -80°C in the Department of Stomatology at the Faculty of Dentistry, Prince of Songkla University, Thailand. The strains that were used in this study included 10 strains of *Lactobacillus casei*, 61 strains of *Lactobacillus fermentum*, 17 strains of *Lactobacillus gasseri*, 12 strains of *Lactobacillus mucosae*, 9 strains of *Lactobacillus oris*, 22 strains of *Lactobacillus paracasei*, 12 strains of *Lactobacillus plantarum*, 18 strains of *Lactobacillus rhamnosus*, 27 strains of *Lactobacillus salivarius,* and 9 strains of *Lactobacillus vaginalis*.

The *Lactobacillus* strains were initially cultured for 18-24 h on MRS agar, and a distinct colony of each bacterium was then transferred to MRS broth for an additional 18-24 h incubation. *Streptococcus mutans* ATCC 25175, the strain used in the aggregation assay, was cultured on a blood agar plate for 18-24 h, and a colony was transferred to brain heart infusion broth for an additional 18-24-h incubation. All strains were incubated under anaerobic conditions (10% $H_2$, 10% $CO_2$ and 80% $N_2$) at 37°C.

### Study of adhesion ability

The H357 keratinocyte, the oral squamous carcinoma cell line used in this study, was a kind gift from Professor Paul Speight of the University of Sheffield, UK. The cells were

grown and maintained in keratinocyte growth medium containing 3 parts Dulbecco's modified Eagle's medium (DMEM) plus 1 part Ham's F-12 nutrient mixture supplemented with 10% foetal calf serum, 10 ng $ml^{-1}$ of epidermal growth factor, 5 g $ml^{-1}$ of insulin, 0.5 g $ml^{-1}$ of hydrocortisone, 100 IU $ml^{-1}$ of penicillin, 100 g $ml^{-1}$ of streptomycin and 2.5 g $ml^{-1}$ of amphotericin B $ml^{-1}$. To harvest the cells for experiments, the cells were lifted from a culture plate by trypsinisation with 0.25% trypsin and 0.05% EDTA at 37°C for 10 to 15 min and collected by centrifugation. The keratinocytes were subcultured in 24-well plates at approximately $10^5$-cells $well^{-1}$ and were grown at 37°C in 5% $CO_2$ for 2 days to reach confluence.

A total adhesion (externalisation and internalisation) assay was performed as described by Kintarak *et al.* (2004). Each tested *Lactobacillus* strain was grown anaerobically overnight in 10 ml of MRS broth at 37°C. The bacterial cells were harvested and washed twice with phosphate-buffered saline (PBS). A bacterial inoculum containing approximately $10^8$ CFU $ml^{-1}$ suspended in DMEM was added to each cell culture well, and the plate was incubated at 37°C in 5% $CO_2$ for 90 min. Non-adherent bacteria were washed off, and then the adherent bacteria plus intracellular bacteria were quantified as the total adhesion.

For internalisation, the procedure was the same as the one mentioned above. After washing off the non-adherent bacteria, 1 ml of a solution containing 100 g $ml^{-1}$ of gentamicin in DMEM was added to each well to kill extracellular bacteria. The plates were incubated with gentamicin for 2 h at 37°C in 5% $CO_2$ and then washed twice with PBS.

To determine the number of bacteria, the keratinocytes were trypsinised with trypsin-EDTA and lysed with 0.1% Triton X-100, and serial dilutions were plated onto MRS agar to determine the viable bacterial counts.

Total adhesion or internalisation was reported as percentages from duplicates according to the formula for total adhesion or internalization as follows: $(\%) = (A_0/A) \times 100$,

where A and $A_0$ were the $\log_{10}$ number of bacterial cells (CFU $ml^{-1}$) before and after adhesion or internalisation. Adhesion (externalisation) was calculated as total adhesion minus internalization.

**Microbial adhesion to solvents**

The microbial adhesion to solvents (MATS) test was performed according to the method of Rosenberg *et al.* (1980) with some modifications. Bacterial cells were washed twice with PBS and resuspended in the same buffer to an absorbance ($A_{600nm}$) of approximately 0.2 ± 0.05 to standardise the number of bacteria to approximately $10^8$ CFU $ml^{-1}$. Three millilitres of bacterial suspension was added with 1 ml of each of the following solvents: xylene (nonpolar neutral solvent), chloroform (monopolar acidic solvent), and ethyl acetate (monopolar basic solvent). After a 10-min pre-incubation at room temperature, the two-phase system was mixed by vortexing for 2-min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance was measured at 600 nm. The results were reported as percentages from triplicates according to the formula MATS (%) = $(A\text{-}A_0/A) \times 100$, where A and $A_0$ were the absorbance before and after mixing solvents, respectively. The strains were classified as having low (0-35%), moderate (36-70%), or high (71-100%) hydrophobicity or charged surfaces.

**Study of aggregation (autoaggregation and coaggregation) ability**

The autoaggregation of each *Lactobacillus* strain was performed using a spectrophotometric assay according to the method of Kos *et al*. (2003) with some modifications. Bacterial cells were prepared as above. Cell suspensions (4 ml) were mixed by vortexing for 10 s, and autoaggregation was determined after 1, 2, and 24 h of incubation at 37°C. The autoaggregation percentage was expressed as $(1\text{-}\ A_{time}/A_{initial}) \times 100$.

The coaggregation of each *Lactobacillus* strain was examined, and *Streptococcus mutans* ATCC 25175 was chosen to represent a cariogenic bacterium in this experiment. Bacterial suspensions were prepared as described for autoaggregation analysis, and *S. mutans* was cultured in a brain heart infusion broth. Equal volumes of bacterial cells (2 ml) from the tested *Lactobacillus* strains and *S. mutans* were mixed and incubated at 37°C for 1, 2, and 24 h. The absorbance (600 nm) was determined for the mixture and for the bacterial suspensions alone. The percentage of coaggregation was determined as $[(A_{Lactobacillus} + A_{S.\ mutans})/2-(A_{mix})/(A_{Lactobacillus} + A_{S.\ mutans})/2 \times 100]$.

**Statistical analysis**

All experiments were independently conducted two or three times, and each assay was performed in duplicate. The results were expressed as the means ± standard deviation. The software package used for the analyses was the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA). Correlation coefficients were performed using Spearman's rho test, and the differences were considered significant when $P < 0.05$.

## Acknowledgements

-

## Funding

This study was supported by the National Research Council of Thailand (DEN550005), the Thailand Research Fund (TRF) through the Royal Golden Jubilee PhD program (PhD/0217/2553/AN.2), and the National Science and Technology Development Agency (NSTDA) of Thailand.

## Conflict of interest

None declared.

## Ethical approval

Not required.

## References


Andrews, J.M. and Tan, M. (2012) Probiotics in luminal gastroenterology: the current state of play. *Intern Med J* **42**, 1287-1291.

Beveridge, T.J. and Graham, L.L. (1991) Surface layers of bacteria. *Microbiol Rev* **55**, 684-705.

Boris, S., Suárez, J.E., Vázquez, F. and Barbés, C. (1998) Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infect Immun* **66,** 1985-1989.

Collado, M.C., Meriluoto, J. and Salminen, S. (2008) Adhesion and aggregation properties of probiotic and pathogen strains. *Eur Food Res Technol* **226,** 1065-1073.

Collado, M.C., Surono, I., Meriluoto, J. and Salminen, S. (2007) Indigenous Dadih lactic acid bacteria: Cell-surface properties and interactions with pathogens. *J Food Sci* **72,** M89-M93.

Colloca, M.E., Ahumada, M.C., Lopez, M.E. and Nader-Macias, M.E. (2000) Surface properties of lactobacilli isolated from healthy subjects. *Oral Dis* **6,** 227-233.

Darilmaz, D.O., Beyatli, Y. and Yuksekdag, Z.N. (2012) Aggregation and hydrophobicity properties of 6 dairy Propionibacteria strains isolated from homemade Turkish chesses. *J Food Sci* **71**, M20-M24.

Del Re, B., Sgorbati, B., Miglioli, M. and Palenzona, D. (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett Appl Microbiol* **31,** 438-442.

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. *Food and Agricultural Organization of the United Nations and World Health Organization Working Group report.*

Harty, D.W.S. and Knox, K.W. (1991) An *in vitro* study of adhesion of various *Lactobacillus*. *Microb Ecol Health Dis* **4,** 19-28.

Keller, M.R., Hasslof, P., Stecksen-Blicks, C. and Twetman, S. (2011) Co-aggregation and growth inhibition of probiotic lactobacilli and clinical isolates of mutans streptococci: An *in vitro* study. *Acta Odontol Scand* **69,** 263-268.

Kintarak, S., Whawell, S.A., Speight, P.M., Packer, S. and Nair, S.P. (2004) Internalization of *Staphylococcus aureus* by human keratinocytes. *Infect Immun* **72,** 5668-5675.

Koll-Klais, P., Mandar, R., Leibur, E., Marcotte, H., Hammarstrom, L. and Mikelsaar, M. (2005) Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. *Oral Microbiol Immunol* **20**, 354-361.

Kos, B., Susković, J., Vuković, S., Simpraga, M., Frece, J. and Matosić, S. (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus M92*. *J Appl Microbiol* **94,** 981-987.

London, J. (1976) The ecology and taxonomic status of lactobacilli. *A Rev Microbiol* **30,** 279-301.

Näse, L., Hatakka, K., Savilahti, E., Saxelin, M., Pönkä, A., Poussa, T., Korpela, R. and Meurman, J.H. (2001) Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. *Caries Res* **35,** 412-420.

Nikawa, H., Makihira, S., Fukushima, H., Nishimura, H., Ozaki, Y. and Ishida K. (2004) *Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci. *Int J Food Microbiol* **95**, 219-223.

Parvez, S., Malik, K.A., Ah Kang, S. and Kim, H.Y. (2006) Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* **100**, 1171-1185.

Pelletier, C., Bouley, C., Cayuela, C., Bouttier, S., Bourlioux, P. and Bellon-Fontaine, M.N. (1997) Cell surface characteristics of *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, and *Lactobacillus rhamnosus* strains. *Appl Environ Microbiol* **63,** 1725-1731.

Perez, P.F., Minnaard, Y., Disalvo, E.A. and De Antoni, G.L. (1998) Surface properties of bifidobacterial strains of human origin. *Appl Environ Microbiol* **64**, 21-26.

Piette, J.P.G. and Idziak, E.S. (1992) A model study of factors involved in adhesion of *Pseudomonas fluorescens*. *Appl Envirom Microbiol* **58**, 2783-2791.

Piwat, S. and Teanpaisan, R. (2013) 16S rRNA PCR-Denaturing gradient gel electrophoresis of oral *Lactobacillus casei* group and their phenotypic appearances. *ISRN Microbiol* 1-6.

Piwat, S., Teanpaisan, R., Dahlen, G., Thitasomakul, S. and Douglas, C.W.I. (2012). Acid production and growth by oral *Lactobacillus* species in vitro. *J Investig Clin Dent* **3**, 56-61.

Piwat, S., Teanpaisan, R., Thitasomakul, S., Thearmontree, A. and Dahlén, G. (2010) *Lactobacillus* species and genotypes associated with dental caries in Thai preschool children. *Mol Oral Microbiol* **25,** 157-164.

Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E. and Handley, P.S. (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* **11,** 94-100.

Rosenberg, M. and Gutnick and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* **9,** 29-33.

Teanpaisan, R. and Dahlen, G. (2006) Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral *Lactobacillus* species. *Oral Microbiol Immun* **21,** 79-83.

Teanpaisan, R. and Piwat, S. (2014) *Lactobacillus paracasei* SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. *Clin Oral Investig* **18**: 857-862.

Teanpaisan, R., Thitasomakul, S., Piwat, S., Thearmontree, A., Pithpornchaiyakul, W. and Chankanka, O. (2007) Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3–24 month old Thai children. *Int Dent J* **57**, 445-451.

Tuo, Y., Yu, H., Ai, L., Wu, Z., Guo, B. and Chen, W. (2013) Aggregation and adhesion properties of 22 *Lactobacillus* strains. *J Dairy Sci* **96**, 4252-4257.

van Houte, J. (1994) Role of micro-organisms in caries etiology. *J Dent Res* **73**, 672–681.

Ventura, M., Jankovic, I., Walker, D.C., Pridmore, R.D. and Zink, R. (2002) Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri. Appl Environ Microbiol* **68,** 6172-6181.

Wadström, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S. and Gullmar, B. (1987) Surface properties of lactobacilli isolated from the small intestine of pigs. *J Appl Bacteriol* **62,** 513-520.

Xu, H., Jeong, H.S., Lee, H.Y., and Ahn, J. (2009) Assessment of cell surface properties and adhesion potential of selected probiotic strains. *Lett Appl Microbiol* **49,** 434-442.

**Figure legends**

**Figure 1** The total adhesion **(a),** externalisation **(b)** and internalisation **(c)** of different oral *Lactobacillus* spp. were demonstrated. The boxplot shows the median, percentile (first and third percentile), and minimum-maximum distribution of value. Different letters are significantly different at $P < 0.05$.

**Figure 2** The autoaggregation (□) and coaggregation (■) of different oral *Lactobacillus* species. Different letters (compared within the same parameter) are significantly different at $P < 0.05$.

**Figure 3** The 24 h autoaggregation vs 24 h coaggregation (○) correlation of different oral *Lactobacillus* species with $r_s = 0.883$, $P < 0.001$.

**Figure 4** The characterisation of bacterial adhesion to different solvents using xylene (□), chloroform (▨) and ethyl acetate (■) of different oral *Lactobacillus* species. Different letters (compared within the same parameter) are significantly different at $P < 0.05$.

**Figure-5-SuppInfo** The correlation of different solvents vs internalisation (●) and different solvents vs coaggregation (○): **(a)** xylene, of 61 total *L. fermentum* strains with $r_s = 0.257$, $P = 0.045$ and $r_s = 0.558$, $P < 0.001$, respectively; **(b)** chloroform, of 15 selected *L. fermentum* strains with $r_s = 0.795$, $P < 0.001$ and $r_s = 0.821$, $P < 0.001$, respectively; **(c)** ethyl acetate, of 15 selected *L. fermentum* strains with $r_s = 0.624$, $P = 0.013$ and $r_s = 0.664$, $P = 0.007$, respectively; and **(d)** xylene, of 15 selected *L. fermentum* strains with $r_s = 0.763$, $P = 0.001$ and $r_s = 0.821$, $P < 0.001$, respectively.

**Figure-6-SuppInfo** The correlation of different solvents vs internalisation (●) and different solvents vs coaggregation (○) of 22 *L. paracasei*; **(a)** chloroform, with $r_s = 0.561$, $P = 0.007$ and $r_s = 0.525$, $P < 0.012$, respectively; **(b)** ethyl acetate, with $r_s = 0.195$, $P = 0.385$ and $r_s =$

418 0.496, $P = 0.019$, respectively; and **(c)** xylene, with $r_s = 0.453$, $P = 0.034$ and $r_s = 0.715$, $P <$
419 0.001, respectively.

420 **Figure-7-SuppInfo** The correlation of internalisation vs autoaggregation (▲) and
421 internalisation vs coaggregation (△) of 22 *L. paracasei* strains with $r_s = 0.443$, $P = 0.039$ and
422 $r_s = 0.607$, $P = 0.003$, respectively.

423 **Figure-8-SuppInfo** The correlation of internalisation vs autoaggregation (▲) and
424 internalisation vs coaggregation (△) of 15 selected *L. fermentum* strains with $r_s = 0.806$, $P <$
425 0.001, and $r_s = 0.788$, $P < 0.001$, respectively.

426

**Table 1** Correlation coefficient of Spearman ($r_s$) between surface properties and adhesion or aggregation

| Assay | Bacterial adhesion to solvent | | |
|---|---|---|---|
| | Xylene | Chloroform | Ethyl acetate |
| Total adhesion | 0.341 (0.000) | 0.093 (0.191) | 0.192 (0.007) |
| Externalization | 0.008 (0.912) | 0.122 (0.088) | 0.043 (0.550) |
| Internalization | 0.269 (0.000) | 0.222 (0.002) | 0.188 (0.008) |
| Aggregation: | | | |
| Autoaggregation | 0.223 (0.002) | 0.308 (0.000) | 0.235 (0.001) |
| Coaggregation | 0.223 (0.002) | 0.341 (0.000) | 0.183 (0.010) |

( ) indicates *P*-value

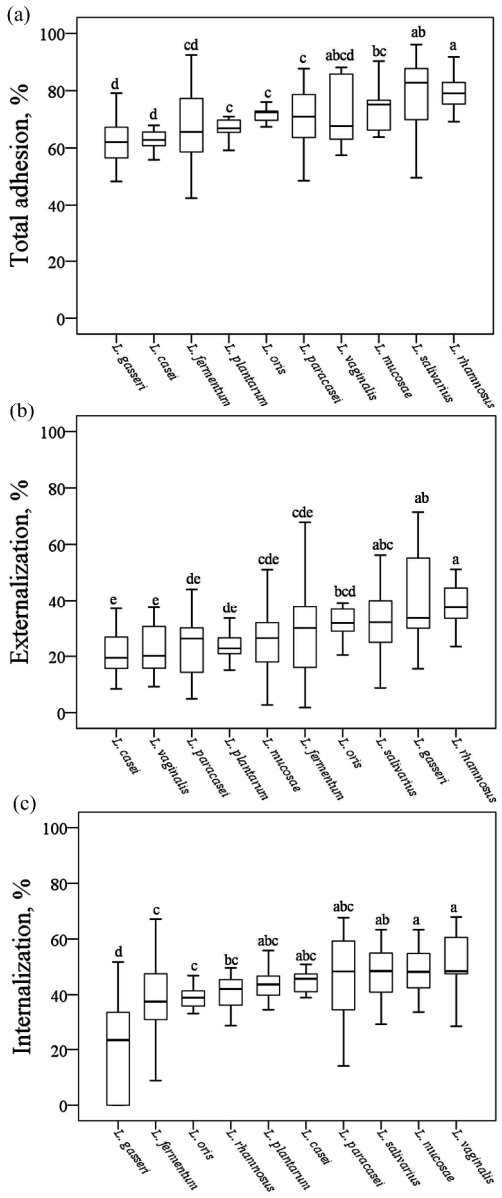



Figure 1 The total adhesion (a), externalisation (b) and internalisation (c) of different oral Lactobacillus spp. were demonstrated. The boxplot shows the median, percentile (first and third percentile), and minimum-maximum distribution of value. Different letters are significantly different at $P < 0.05$.
199x476mm (300 x 300 DPI)

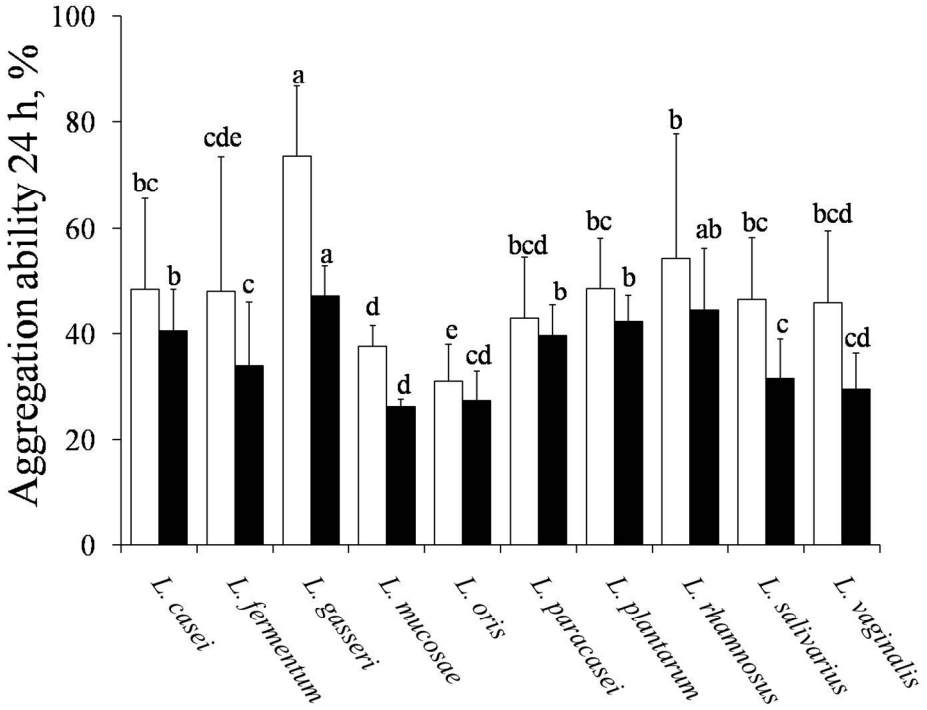



Figure 2 The autoaggregation (≤) and coaggregation (′) of different oral Lactobacillus species. Different letters (compared within the same parameter) are significantly different at $P < 0.05$.
254x190mm (300 x 300 DPI)

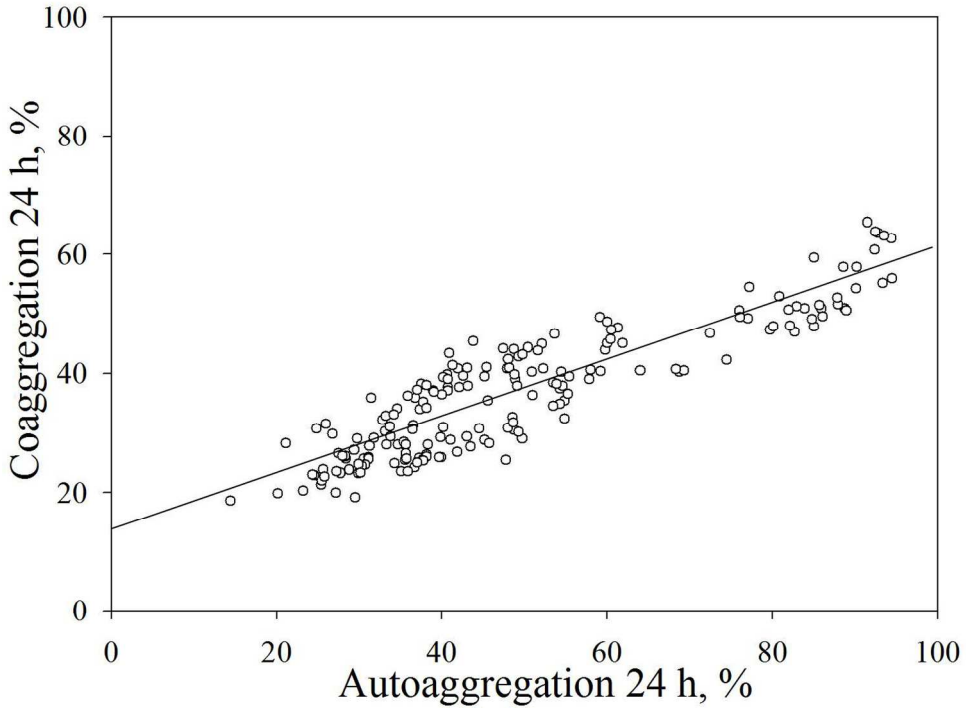



Figure 3 The 24 h autoaggregation vs 24 h coaggregation (□) correlation of different oral Lactobacillus species with rs = 0.883, P < 0.001.
254x192mm (300 x 300 DPI)

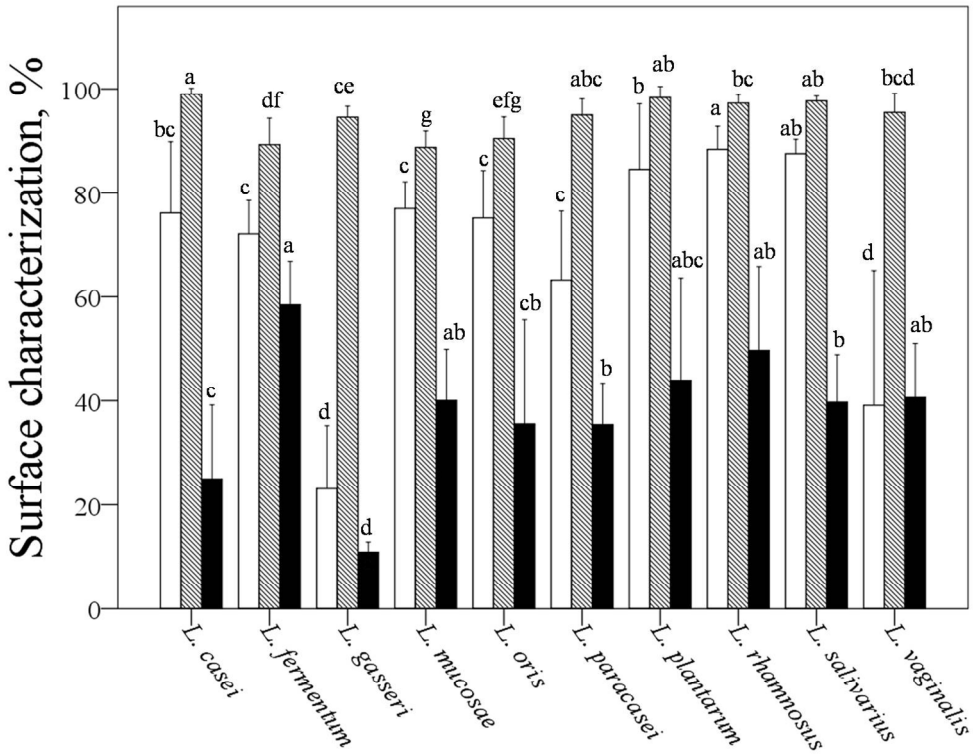



Figure 4 The characterisation of bacterial adhesion to different solvents using xylene (≤), chloroform ( ) ) and ethyl acetate (′) of different oral Lactobacillus species. Different letters (compared within the same parameter) are significantly different at P < 0.05.
274x214mm (300 x 300 DPI)

**Antioxidant activity of *Lactobacillus* isolated from oral cavity**

Aksorntong Chooruk[1, 2], Supatcharin Piwat[1, 3], Rawee Teanpaisan[1, 2]

1 Common Oral Diseases and Epidemiology Research Center, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Thailand

2 Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Thailand

3 Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Thailand

Correspondence author:

Rawee Teanpaisan,

Common Oral Diseases and Epidemiology Research Center and Department of Stomatology, Faculty of Dentistry, Prince of Songkla University,

Hat-Yai, 90112, Thailand

Tel: +66 74 429878; Fax: +66 74 212922

e-mail address: rawee.t@psu.ac.th

**Abstract**

This study aims to evaluate the antioxidant activity of oral *Lactobacillus* strains in vitro. Two-hundred and one strains of oral *Lactobacillus* were investigated antioxidant properties through scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, inhibition of lipid peroxidation, and resistance to reactive oxygen species (ROS). In addition, the levels of enzymatic antioxidant as superoxide dismutase (SOD) activity and non-enzymatic antioxidant as glutathione (GSH) content of intracellular cell-free extracts were assessed, and was also evaluated the relationship between antioxidant parameters of *Lactobacillus* and caries status. The results showed that all *Lactobacillus* strains exhibited wide dispersion of free radical scavenging, inhibition of lipid peroxidation, SOD activity and GSH content within/between species, indicating that antioxidant properties may be strain dependent. Among ten species of *Lactobacillus, L. paracasei*, *L. fermentum* and *L. plantarum* showed among the highest free radical scavenging, inhibition of lipid peroxidation, SOD activity and GSH contents. Moreover, *L. paracasei*, *L. fermentum* and *L. rhamnosus* showed high viability in 1 mM hydrogen peroxide and 1 mM hydroxyl radicals. Inhibition of lipid peroxidation and GSH were found to be the factor significantly negative associated with caries status ($p \leq 0.05$). The odds ratios of developing caries status decrease when having inhibition of lipid peroxidation and GSH activity were 0.951 and 0.844 respectively. It can be concluded that the antioxidant efficacy of *Lactobacillus* may be involve in defence mechanism against oxidative stress and could also be used to alternative dental therapies.

**Key words:** Antioxidant activity, Oxidative stress, *Lactobacillus*.

## 1. Introduction

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS), or free radicals and antioxidant defenses leading to the disturbance of the human normal health. It is believed that oxidative stress plays critical roles in various diseases such as cancer, diabetes, neurodegenerative diseases, emphysema, cirrhosis, atherosclerosis, myocardial infarction and arthritis. It has been claimed that antioxidant is the first line to defense against ROS [1]. In oral cavity, antioxidant defense system has been found to implicate with oral diseases that the level of antioxidant could be altered in response to an infection or diseases. For examples, antioxidant capacity of saliva was sometime found significantly increased in children with active and/or severe caries [2, 3]. In addition, some studies reported that total antioxidant capacity of serum in periodontitis was lower than in health [4]. It indicated that the balance of oxidant-antioxidant system may also involve in oral diseases.

The formation of ROS can be prevented by antioxidants which found in natural sources such as vegetables, fruits and microbial sources [5]. Thus, consumption of antioxidant supplements has been recommended to prevent human bodies from oxidative damage. Several studies have reported that consumption of living or lyophilized cultures of probiotic bacteria can reduce free radicals thereby reducing oxidative stress, due to their ability to produce antioxidants [6, 7]. Antioxidant activities including superoxide dismutase (SOD), glutathione reductase (GR) and glutathione (GSH) have been reported for some *Lactobacillus* species [2, 8-11]. Most reports have studied the antioxidative potential in probiotic strains *Lactobacillus* spp. and *Bifidobacterium* spp. derived from the gut and fermented foods [8, 10, 12, 13]. There is limited information on antioxidant activities of *Lactobacillus* strains isolated from oral cavity. Whether those antioxidant activities of *Lactobacillus* strains have an influence on oral diseases is unknown. The aims of this study

were to investigate the activities and types of antioxidants of oral *Lactobacillus*, and to determine whether there was any association between antioxidants and caries status.

## 2. Materials and methods

### 2.1 Bacterial strains and culture condition

A total 201 of oral *Lactobacillus* strains represent 10 different species were obtained from the previous study [14] , and the culture collection was kept at -80°C in the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Thailand. The details including the number of strains, type of species and caries status of the individuals are shown in Table 1. All strains were identified using the restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) and the denaturing gradient gel electrophoresis (DGGE) [15, 16]. Sequencing of the 16S-rRNA was performed for strains with uncertain identifications.

### 2.2 Preparation of intact cells and intracellular cell-free extracts

The strains were recovered from the -80°C storage and cultured on a Man Rogosa and Sharpe (MRS) agar plate at 37ºC for 24-48 h under anaerobic condition (80% $N_2$, 10% $H_2$, and 10% $CO_2$). After recovery, each strain was inoculated into 50 mL MRS broth in anaerobic condition at 37ºC for overnight. Bacterial cells were then harvested by centrifugation at 5,000 rpm for 10 min, and washed three times with phosphate buffered saline (PBS, pH 7.4). The washed cells were resuspended in PBS, and adjusted to approximately $10^9$ CFU/mL. The cells suspension was used as intact cells.

The washed cells suspension was sonicated with a cell disrupter for 5 min in an ice bath. The cell debris was removed by centrifugation at 10000 rpm for 10 min, and the supernatant was used as the intracellular cell-free extracts.

### 2.3 Determination of antioxidant activity

#### 2.3.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The DPPH radical-scavenging activity of various *Lactobacillus* species was evaluated using the modified method of Lin and Chang [10]. In brief, 100 µL of intact cells or intracellular cell-free extracts and 100 µL of freshly prepared DPPH solution (0.2 mM in

ethanol) were mixed and allowed to react for 30 min at room temperature. Deionized water and DPPH solution without bacteria were used as controls. The ability of scavenged DPPH was monitored by the decrease in absorbance at 517 nm. The scavenging ability was defined as:

$$\text{Scavenging activity (\%)} = [1 - (A_{sample}/A_{control})] \times 100$$

### 2.3.2 Inhibition of lipid peroxidation

The antioxidant activity of *Lactobacillus* strains was determined using the TBA method of Lin and Chang [10], based on the monitoring inhibition of linoleic acid peroxidation. Linoleic acid was chosen as the source for unsaturated fatty acid and, a $Fe/H_2O_2$ system was used for the catalysis of oxidation. The linoleic acid emulsion was made up of 0.1 mL of linoleic acid, 0.2 mL of Tween 20 and 19.7 mL of deionized water. The total volume of reaction mixture (2.3) mL contained 0.5 mL PBS (0.02 M, pH 7.4), 1.0 mL linoleic acid emulsion, 0.2 mL $FeSO_4$ 0.01% (w/v), 0.2 mL 0.02% $H_2O_2$ (v/v), and 0.4 mL samples were mixed and incubated at 37°C for 24 h. Deionized water was substituted for samples as the control. After 24 h of incubation, 2 mL of the reaction solution were mixed with 0.2 mL of trichloroacetic acid 4% (w/v), 2 mL of thiobarbituric acid 0.8% (w/v) and 0.2 mL of butylated hydroxytoluene 0.4% (w/v). The mixture was incubated at 100°C for 30 min and allowed to cool. Chloroform was then added for extraction. The extract was obtained and the absorbance was measured at 532 nm. The ability of inhibition by the samples was defined as:

$$\text{Inhibition activity (\%)} = [1- (A_{sample}/A_{control})] \text{ x}100$$

## 2.4 Determination the types of antioxidants

### 2.4.1 Superoxide dismutase (SOD) activity of intracellular cell-free extracts

The SOD activity was the ability of tested samples to inhibit the forming of red formazan nitroblue tetrazolium produced by xanthine and xanthine oxidase reaction [17]. The SOD activity in this study was performed using the method of Ukeda et al.[18]. In brief, the

reaction mixture contained 2.4 mL of 50 mM sodium phosphate buffer (pH 7.8), 0.1 mL of 3 mM xanthine, 0.1 mL of 3 mM ethylenediaminetetraacetic acid, 0.1 mL of 0.75 mM nitroblue tetrazolium (NBT), 0.1 mL of 15% bovine serum albumin and either 0.1 mL of intracellular cell-free extracts or of PBS as blank. After the reaction mixture were incubated at 37°C for 5 min, followed adding 0.1 mL of 0.01 U xanthine oxidase was incubated at 37°C for 20 min. SOD activity was measured at 560 nm that was determined by referring to a standard curve. Inhibition of NBT reduction to 50% of maximum was defined as 1 U of SOD activity, and SOD activity was expressed in unit per milliliter (Unit/mL).

#### 2.4.2 Glutathione (GSH) content of intracellular cell-free extracts

GSH of in intracellular extracts of *Lactobacillus* strains was performed using Ellman's reagent [19] 5,5-dithiobis-2-nitrobenzoic acid (DTNB). The reaction mixture contained 0.2 mL of intracellular cell-free extracts, 2.3 mL of 0.2 M phosphate buffer (pH 7.5) and 0.5 mL of 1.0 mM DTNB and incubated at 25°C for 10 min in the dark. The sulphydryl groups present in GSH forms a colored complex with DTNB, which was measured by colorimeter at 412 nm. An increase in absorbance indicated an increase in GSH levels. The amount of GSH was determined using its molar extinction coefficient (ε) of 13600 M/cm/ and expressed in terms of nanomole per milliliter (nmol/mL). The GSH was calculated as the following equation.

$$GSH = (A - A_0) \text{ x dilution factor of sample} / \varepsilon$$

A and $A_0$ are the absorbances of in the presence and the absence of sample, respectively.

### 2.5 Study the resistance of lactobacilli to reactive oxygen species (ROS)

The effect of different ROS namely hydrogen peroxide and hydroxyl radicals on the viability of different *Lactobacillus* strains was determined using the method of Kullisaar et al. [9]. *L. fermentum* 10 strains, *L. paracasei* 9 strains, *L. rhamnosus* 9 strains, *L. oris* 5 strains, *L. gasseri* 5 strains and *L. salivarius* 5 strains were selected from caries free and caries individual.

### 2.5.1 Resistance to hydrogen peroxide and hydroxyl radical

Bacterial strains were harvested by centrifugation at 5000 rpm for 10 min after overnight incubation at 37°C in anaerobic condition, and washed twice in 0.85% NaCl. The cell suspensions contained $10^9$ CFU/mL, and incubated with 1.0 mM $H_2O_2$ at 37°C. At 2 h intervals, the removed aliquots were plated onto MRS agar, and incubated at 37°C in anaerobic condition for 48 h. The number of bacteria survival was counted.

In addition, the cell suspensions were exposed to hydroxyl radicals that were generated via the Fenton reaction by which [20] contains 10 mM terephthalic acid (1,4-benzenedicarboxylic acid, Sigma) in phosphate buffer, *Lactobacillus* cultures at the level of $10^9$ CFU/mL, and $CuSO_4.5H_2O$. The reaction was initiated by the addition of 1.0 mM $H_2O_2$. Aliquots were drawn at 15 min time intervals, and the number of bacteria survival was performed as described above.

## 2.6 Data analysis

All values were expressed as mean±standard deviation (X±SD). A Kolmogorov-Smirnov test was applied to examine the normality of data distribution. Differences in antioxidant activity and type of antioxidant among the *Lactobacillus* species were evaluated using Kruskal-Wallis test followed by Mann-Whitney U test, and were considered to be significant at $p<0.05$. Logistic regression analysis was used to evaluate the odds ratios of these factors for carries status. Significance was considered at $p < 0.05$. All statistical analysis was carried out using SPSS (Chicago, Illinois, USA).

## 3. Results

### 3.1 Antioxidant activity of *Lactobacillus* species

DPPH free radical scavenging activity and inhibition of lipid peroxidation within 10 *Lactobacillus* species are shown in Figure (1 A,B). There was a wide variation in the antioxidant activities of different *Lactobacillus* strains. The free radical scavenging activity of each strain ranged from 11.3 to 53.2% and inhibition of lipid peroxidation ranged from 10.30 to 77.8% respectively. The antioxidant activity of *Lactobacillus* strains was expressed in both intact cells and intracellular cell-free extracts. There was a great diversity in their antioxidant activity. The antioxidant activity in terms of inhibition of lipid peroxidation of some *Lactobacillus* species was higher than for free radical scavenging activity. *L. paracasei* had the highest scavenging activity mean of 34.3% followed by *L. mucosae, L. rhamnosus, L. casei, L. fermentum* and *L. salivarius.* The least scavenging activity was found for *L. plantarum, L. vaginalis, L. oris* and *L. gasseri* that were significantly lower than the rest. (Fig. 1 A).

In the inhibition of lipid peroxidation tests, the results revealed that *L. plantarum, L. rhamnosus* and *L. mucosae* had strong an inhibition activity mean of 64.8%, 59.1% and 55.1%, while *L. salivarius, L. fermentum, L. vaginalis, L. casei* and *L. paracasei* exhibited moderate inhibition activity, and *L. oris* and *L. gasseri* had the significantly lowest inhibition activity ($P<0.05$) (Fig. 1 B).

### 3.2 Antioxidants types of *Lactobacillus*

The SOD activity of all strains showed a ranged from 0 to 0.69 Unit/mL (Fig. 2 A). *L. fermentum* strains showed a high variation and significantly higher SOD activity (mean 0.42 Unit/mL) than other *Lactobacillus* spp. Some strains of *L. paracasei* also showed high activity while the lowest activity was found for *L. oris* and *L. gasseri*

The level of GSH content of the *Lactobacillus* strains was ranged from 0 to 22.41 nmol/mL (Fig. 2 B). The strains exhibited the highest content were *L. paracasei* (mean 13.28

nmol/mL) followed by *L. casei* and *L. fermentum* (mean 11.93, 11.95 nmol/mL respectively). *L. gasseri* and *L. oris* (4.96, 3.97 nmol/mL) showed the lowest activity.

**3.3 Resistance to hydrogen peroxide and hydroxyl radicals**

The effect of hydrogen peroxide on the viability of *Lactobacillus* strains are shown in Figure 3. We found that *L. fermentum, L. paracasei* and *L. rhamnosus* showed the most resistant against 1.0 mM hydrogen peroxide after 8 h, while *L. oris, L. gasseri* and *L. salivarius* displayed the most sensitive to hydrogen peroxide (Fig. 3A). Moreover, *Lactobacillus* strains in caries free showed higher viable than caries. However, it was not statistically significant ($P > 0.05$), except *L. fermentum* and *L. paracasei* in caries free can resistance to 1.0 mM hydrogen peroxide after 8 h higher than caries ($P < 0.05$) (Fig. 3 B).

In the presence of 1.0 mM hydroxyl radicals, *L. fermentum*, *L. paracasei* and *L. rhamnosus* survived up to 60 min, while *L. oris, L. gasseri* and *L. salivarius* survived for 15 and 45 min respectively (data not shown).

**3.4 Association between all of the antioxidant parameters and caries status**

The binary logistic regression revealed that inhibition of lipid peroxidation and GSH were found to be the factor significantly negative associated with caries status (Table 2). The odds ratios of developing caries status decrease when having inhibition of lipid peroxidation and GSH were 0.951 and 0.844 respectively.

## 4. Discussion

Recently, natural antioxidants have received much attention from scientists and general public due to their role in the maintenance of human health and prevention and treatment of diseases. *Lactobacillus* strains are considered to be beneficial microorganisms and it has been increasing interest worldwide in the potential probiotic. Several studies have reported that some *Lactobacillus* strains were isolated from fermented food and intestinal tract can improve the antioxidant status in vitro and in vivo [8, 12, 21]. However, there have been a few published studies investigating the role of antioxidant activity of *Lactobacillus* species isolated from oral cavity. Also, the antioxidant of *Lactobacillus* strains on caries status is relatively little unknown. Therefore, the field of research has focused on the antioxidant activities of *Lactobacillus* strains, and correlations between antioxidant parameters of *Lactobacillus* strains and caries status were also determined.

Since the antioxidant mechanisms for in vitro assay methods were diverse, the antioxidant activity should be determined by different ways. Two-hundred and one strains of oral *Lactobacillus* examined derived from caries free and caries individuals. In this study, our results showed that *Lactobacillus* strains possessed antioxidant activities of both intact cells and intracellular cell-free extracts, which displayed through two major mechanisms. It is DPPH free radical scavenging (direct method) and inhibition of lipid peroxidation (an indirect method) [22]. *L. paracasei*, *L. mucosae* and *L. fermentum* showed high scavenging activity, and *L. plantarum*, *L. rhamnosus* and *L. mucosae* showed high inhibition of lipid peroxidation while *L. oris* and *L. gasseri* showed low activities in both mechanisms. It was noted that the strains within each species of *Lactobacillus* showed a wide range of antioxidant activities. Different *Lactobacillus* strains possess different level of antioxidant activity. Results were similar to the previous reports Achuthan et al., 2012 and Amaretti et al., 2013 [8, 23] which revealed that the antioxidant activity of *Lactobacillus* are varies among strains,

and antioxidant activity have also been found variety of mechanisms. Our results indicate that *Lactobacillus* exhibited antioxidant activity dependent on species/strain.

Furthermore, intracellular cell-free extracts exhibited stronger antioxidant activity than intact cells. This was probably because many substances in *Lactobacillus* could be the source of the antioxidant activity. Previous studies have shown that SOD and GSH in *Lactobacillus* are the major compound antioxidant which plays an important role in protecting organisms against toxicity and detoxifying superoxide anion, hydrogen peroxide and hydroxyl radical. These are regarded as a major source of oxidative stress [24, 25]. Therefore, we have investigated type of antioxidant in the intracellular cell-free extracts of *Lactobacillus* strains. The present of this study found that almost all *Lactobacillus* strains possessed both SOD and GSH. We also observed that *L. fermentum* and *L. paracasei* showed high SOD activity, GSH content and antioxidant activities, while *L. oris* and *L. gasseri* showed low SOD activity, GSH content and antioxidant activities. This result can be supported by previous research which revealed that SOD and GSH may be the main function of antioxidant activities of *Lactobacillus* strains [9, 24, 25].

In addition, the high antioxidant activity of *Lactobacillus* strains is one of the reasons for their able to decrease the risk of accumulation of ROS. All the three species *(L. fermentum, L. paracasei* and *L. rhamnosus*) displayed tolerance in presence of 1.0 mM hydrogen peroxide (weakly reactive, but it is highly diffusive and has long lifetime) up to 8 h significantly longer than other species. In the presence of highly damaging hydroxyl radicals (highly reactive), *L. fermentum, L. paracasei* and *L. rhamnosus* remained viable after 60 min, while *L. oris, L. gasseri* and *L. salivarius* survived for 15 and 45 min (data not shown). It demonstrated that the viability of *Lactobacillus* strains may depend on antioxidant activity, types of antioxidants and upon dose of hydrogen peroxide and hydroxyl radicals [13, 21]. Besides, the survival time of *Lactobacillus* strains from caries free seemed to be greater in those isolated from individuals with caries. However, it was not statistically significant

(P>0.05), except *L. fermentum* and *L. paracasei* in caries free can resistance to 1.0 mM hydrogen peroxide after 8 h higher than caries (P < 0.05). *L. fermentum* and *L. paracasei* are predominant species in the oral cavity due to type of antioxidants could be influence on survival of these species [24, 25]. It is possible to exert antioxidant mechanisms to protect themselves against oxidative stress. This is agreement with some reports showing that almost all bacteria are well protected against ROS by antioxidant activity [21].

In the oral cavity, dental caries and periodontal disease are the most common infectious diseases worldwide. Recently it has been claimed that oxidative stress may play an important role in the onset and the development of several inflammatory oral pathologies and dental caries may also be included [2, 26]. Most studies have reported that the antioxidant activity of salivary associated with dental caries and also periodontal [2, 4], while only a few studies have examined the relationship between antioxidant activity of *Lactobacillus* strains and caries status. Thus, in this study verify their relationship status. Our results found that antioxidant activity in term inhibition of lipid peroxidation and GSH to be the factor significant negative associated with caries status (P<0.05). Previous studies have reported that lipid peroxidation is used to indicate oxidative stress while GSH may play a major role in reduced the deleterious effect of lipid peroxidation [27, 28]. To the best of our knowledge, our data provides the first evidence of a logistic regression relationship between antioxidant parameters of *Lactobacillus* and caries status. Our data suggest that a high GSH level from *Lactobacillus* could be help decrease caries. Amaretti et al., 2013 reported that *Lactobacillus* strains may enhance antioxidant defenses in the host by producing and releasing GSH which are absorbed and distributed in the organism [23]. Moreover, Ahmad et al., 2013 reported that antioxidant activity was higher in caries active group[2]. Similarly, Han et al., 2013 showed that who had DMFT showed a higher concentration of GSH [29]. Therefore, our results suggested that *Lactobacillus* have to build up antioxidant mechanisms to protect themselves against oxidative stress, and also can protect the host from attack free radicals when

*Lactobacillus* colonized and propagated in the oral cavity [30]. Thus, we indicated the antioxidant activity was higher in salivary caries due to there are a large number of lactobacilli which can produce antioxidant.

Additionally, antioxidant from *Lactobacillus* has an indirect function to prevention or treatment of oral diseases [30]. For example, Peran et al., 2006 reported that GSH was released from *L. fermentum* can prevents colonic inflammation in the TNBS model of rat colitis [31], and also consistent with Mendi et al., 2014 found that antioxidant activity of Lactobacilli could protective effect on gingival fibroblasts [21]. In this study, we also observed that *L. fermentum L. paracasei, L. casei, L. plantarum* and *L. rhamnosus* showed high inhibition of lipid peroxidation and GSH activity. Interestingly, these species plays an important role in maintaining good oral health, and also used as probiotic [30]. The antioxidant activity appeared to be relative viability of *Lactobacillus.* Therefore, those strains may be considered for further study in clinical trials. In the present study, suggested that antioxidant activity derived from *Lactobacillus* strains could be a good natural therapy agent for counteract ROS and reduce the risk of oral disease.

## 5. Conclusion

The result of this study believed that *Lactobacillus* species exhibited antioxidant activities in all major ways, and antioxidant properties of *Lactobacillus* strain are variation within/between species. To our knowledge this is the first study to investigate antioxidant activity of oral *Lactobacillus* species, and also used as base on information of antioxidant activity. We suggested that the antioxidant activity from oral *Lactobacillus* could be a good natural therapy agent for oral diseases.

**Acknowledgements:** This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (AGR 540555M) and the National Research Council of Thailand.

**7. References**

[1] Toyokuni S. Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 1999;49:91-102.

[2] Ahmadi-Motamayel F, Goodarzi MT, Hendi SS, Kasraei S, Moghimbeigi A. Total antioxidant capacity of saliva and dental caries. Med Oral Patol Oral Cir Bucal 2013;18:e553-6.

[3] Kumar D, Pandey RK, Agrawal D. An estimation and evaluation of total antioxidant capacity of saliva in children with severe early childhood caries. Int J Paediatr Dent 2011;21:459-64.

[4] Diab-Ladki R, Pellat B, Chahine R. Decrease in the total antioxidant activity of saliva in patients with periodontal diseases. Clin Oral Investig 2003;7:103-7.

[5] Duda-Chodak A, T. Tarko and M. Statek, . The effect of antioxidants on Lactobacillus casei cultures. Acta Sci Pol,Technol Aliment 2008;7(4) 39-51

[6] Martarelli D, Verdenelli MC, Scuri S, Cocchioni M, Silvi S, Cecchini C, et al. Effect of a probiotic intake on oxidant and antioxidant parameters in plasma of athletes during intense exercise training. Curr Microbiol 2011;62:1689-96.

[7] Chin FL., MP. T. In Vitro Effects of Lactic Acid Bacteria on Cancer Cell Viability and Antioxidant Activity. Journal of Food and Drug Analysis 2010;18:77-86.

[8] Achuthan AA, Duary RK, Madathil A, Panwar H, Kumar H, Batish VK, et al. Antioxidative potential of lactobacilli isolated from the gut of Indian people. Mol Biol Rep 2012;39:7887-97.

[9] Kullisaar T, Zilmer M, Mikelsaar M, Vihalemm T, Annuk H, Kairane C, et al. Two antioxidative lactobacilli strains as promising probiotics. Int J Food Microbiol 2002;72:215-24.

[10] Lin MY, Chang FJ. Antioxidative effect of intestinal bacteria Bifidobacterium longum ATCC 15708 and Lactobacillus acidophilus ATCC 4356. Dig Dis Sci 2000;45:1617-22.

[11] Jansch A, Korakli M, Vogel RF, Ganzle MG. Glutathione reductase from Lactobacillus sanfranciscensis DSM20451T: contribution to oxygen tolerance and thiol exchange reactions in wheat sourdoughs. Appl Environ Microbiol 2007;73:4469-76.

[12] Li S, Zhao Y, Zhang L, Zhang X, Huang L, Li D, et al. Antioxidant activity of Lactobacillus plantarum strains isolated from traditional Chinese fermented foods. Food Chem 2012;135:1914-9.

[13] Das D, Goyal A. Antioxidant activity and ฮณ-aminobutyric acid (GABA) producing ability of probiotic Lactobacillus plantarum DM5 isolated from Marcha of Sikkim. LWT - Food Science and Technology 2015;61:263-8.

[14] Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlen G. Lactobacillus species and genotypes associated with dental caries in Thai preschool children. Mol Oral Microbiol 2009;25:157-64.

[15] Matsumiya Y, Kato N, Watanabe K, Kato H. Molecular epidemiological study of vertical transmission of vaginal Lactobacillus species from mothers to newborn infants in Japanese, by arbitrarily primed polymerase chain reaction. J Infect Chemother 2002;8:43-9.

[16] Piwat S, Teanpaisan R. 16S rRNA PCR-Denaturing Gradient Gel Electrophoresis of Oral Lactobacillus casei Group and Their Phenotypic Appearances. ISRN Microbiol 2013;2013:342082.

[17] Nishikimi M. Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system. Biochem Biophys Res Commun 1975;63:463-8.

[18] Ukeda H, Maeda S, Ishii T, Sawamura M. Spectrophotometric assay for superoxide dismutase based on tetrazolium salt 3'--1--(phenylamino)-carbonyl--3, 4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate reduction by xanthine-xanthine oxidase. Anal Biochem 1997;251:206-9.

[19] Ellman G. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-5

[20] Barreto JC, Smith GS, Strobel NH, McQuillin PA, Miller TA. Terephthalic acid: a dosimeter for the detection of hydroxyl radicals in vitro. Life Sci 1995;56:PL89-96.

[21] Mendi A, Aslim B. Antioxidant lactobacilli could protect gingival fibroblasts against hydrogen peroxide: a preliminary in vitro study. Probiotics Antimicrob Proteins 2014;6:157-64.

[22] Buenger J, Ackermann H, Jentzsch A, Mehling A, Pfitzner I, Reiffen KA, et al. An interlaboratory comparison of methods used to assess antioxidant potentials. Int J Cosmet Sci 2006;28:135-46.

[23] Amaretti A, di Nunzio M, Pompei A, Raimondi S, Rossi M, Bordoni A. Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities. Appl Microbiol Biotechnol 2012.

[24] Lee J, HWANG KT, CHUNG MY, CHO DH, Park CS. Resistance of Lactobacillus casei KCTC 3260 to Reactive Oxygen Species (ROS): Role for a Metal Ion Chelating Effect. JOURNAL OF FOOD SCIENCE 2005;8:388-91.

[25] Kullisaar T, Songisepp E, Aunapuu M, Kilk K, Arend A, Mikelsaar M, et al. Complete glutathione system in probiotic Lactobacillus fermentum ME-3. Prikl Biokhim Mikrobiol 2010;46:527-31.

[26] Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000 2007;43:160-232.

[27] C. C. Tsai, H. S. Chen, S. L. Chen, Y. P. Ho, K. Y. Ho YMW, Hung CC. Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. JOURNAL OF PERIODONTAL RESEARCH 2005;40:378–84.

[28] Metgud R, Bajaj S. Evaluation of salivary and serum lipid peroxidation, and glutathione in oral leukoplakia and oral squamous cell carcinoma. J Oral Sci 2014;56:135-42.

[29] Han DH, Kim MJ, Jun EJ, Kim JB. The role of glutathione metabolism in cariogenic bacterial growth and caries in Korean children. Arch Oral Biol 2013;58:493-9.

[30] Pranay J., S. P. Probiotics and their efficacy in improving oral health: a review. Journal of Applied Pharmaceutical Science 2012;2: 151-63.

[31] Peran L, Camuesco D, Comalada M, Nieto A, Concha A, Adrio JL, et al. Lactobacillus fermentum, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis. Int J Colorectal Dis 2006;21:737-46.

**8. Legend**

Table 1 Bacterial strains in the oral cavity used in this study

| Species | Caries free<br>0 dmft<br>[subjects/strains] | Caries<br>≥ 1 dmft<br>[subjects/strains] | Total |
|---|---|---|---|
| *L. fermentum* | 5/9 | 28/42 | 33/51 |
| *L. salivarius* | - | 15/41 | 15/41 |
| *L. plantarum* | - | 4/11 | 4/11 |
| *L. mucosae* | - | 6/12 | 6/12 |
| *L. casei* | - | 6/10 | 6/10 |
| *L. paracasei* | 2/4 | 5/18 | 7/22 |
| *L. rhamnosus* | 1/4 | 5/10 | 6/14 |
| *L. vaginalis* | 1/8 | 1/2 | 2/10 |
| *L. oris* | - | 5/12 | 5/12 |
| *L. gasseri* | - | 4/18 | 4/18 |
| Total | 9/25 | 79/176 | 88/201 |

Decayed, missing and filled teeth (DMFT) index

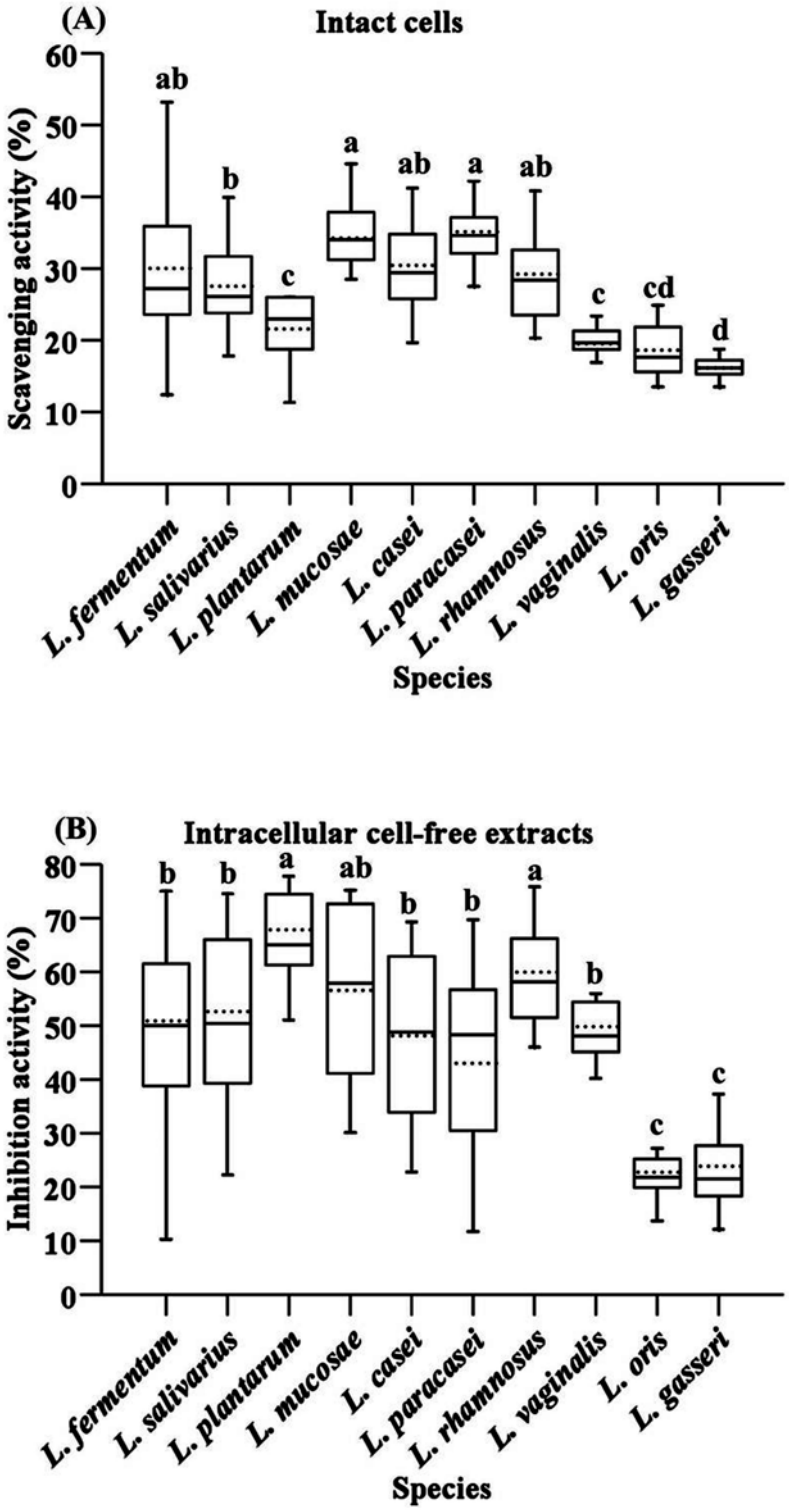



Fig. 1. The antioxidant activity of intact cells and intracellular cell-free extracts of *Lactobacillus* species isolated from oral cavity. Boxplotes indicate the mean, median, 25th and 75th percentiles, and the minimum-maximum values. Different letters means statistically significant difference ($P < 0.05$)

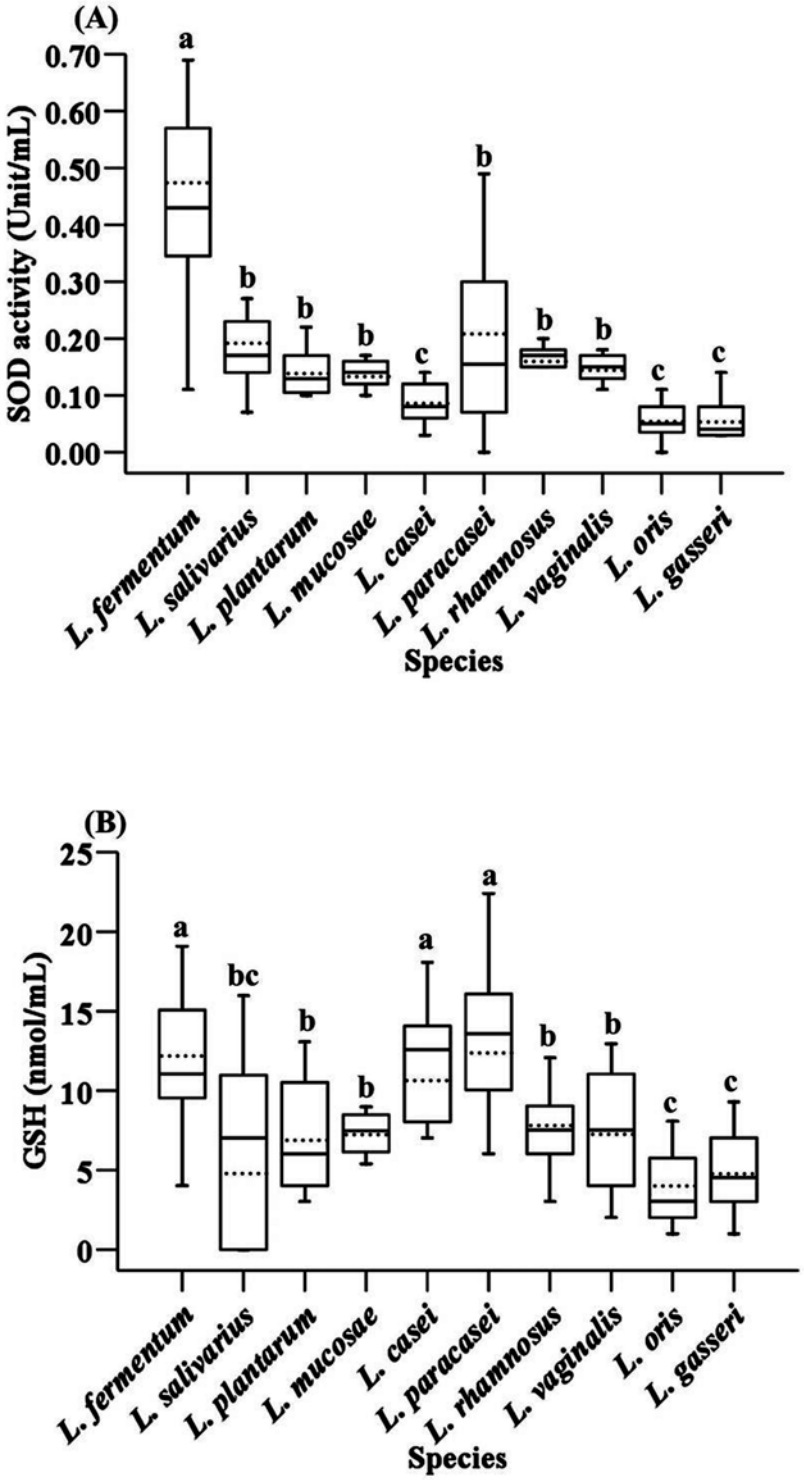



Fig. 2. SOD activity and GSH content of intracellular cell-free extracts of *Lactobacillus* strains isolated from oral cavity. Boxplotes indicate the mean, median, 25th and 75th percentiles, and the minimum-maximum values. Different letters means statistically significant difference ($P < 0.05$)

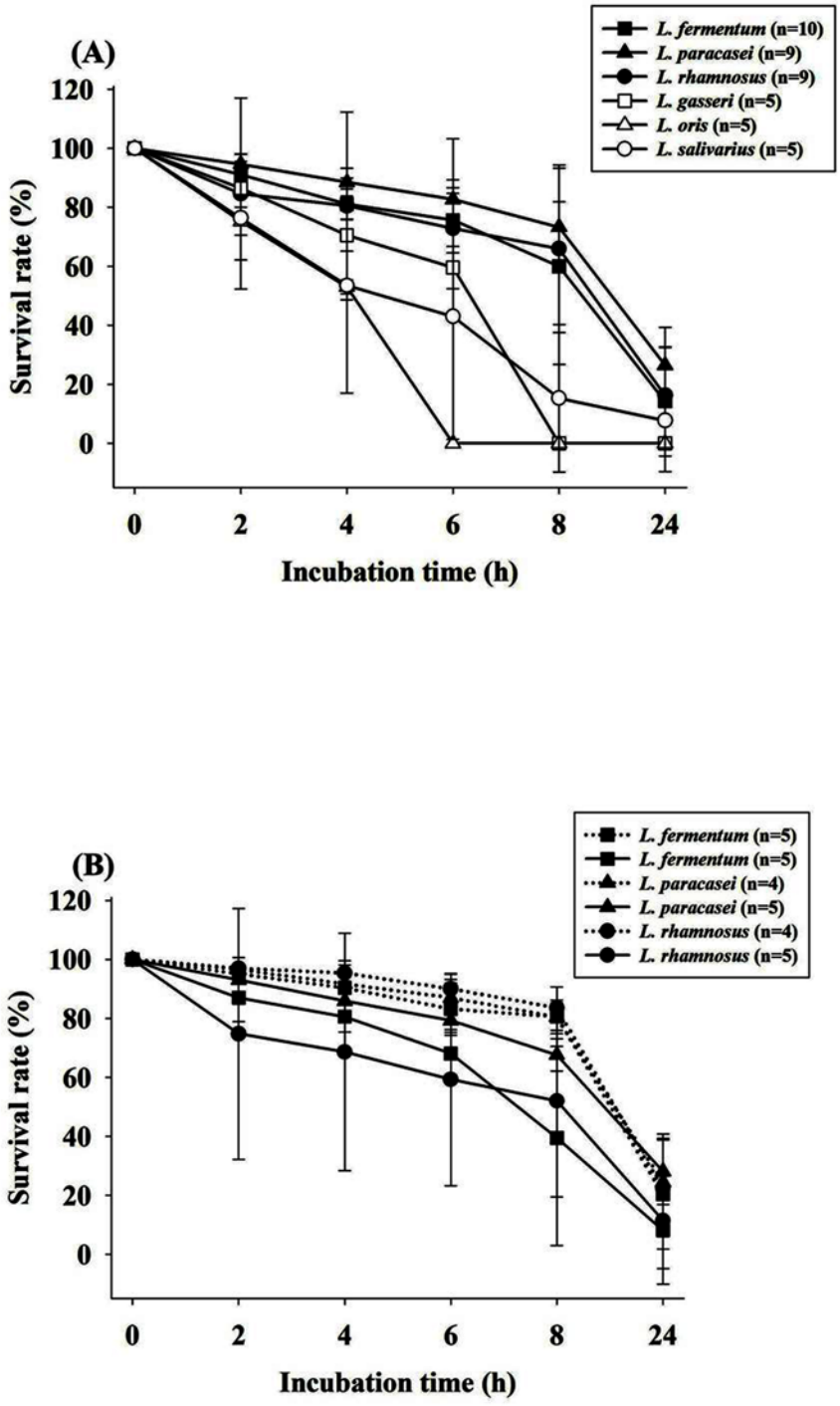



Fig. 3. (A,B) The survival of *Lactobacillus* strains in the presence of 1.0 mM hydrogen peroxide. (B); Dotted lines and solid lines indicate caries free and caries respectively. * $p < 0.05$; (Mann-Whitney *U*-test), statistically-significantly different between caries free and caries.

Table 2 Association between caries status* and antioxidant activities and types of antioxidants

| Variables | No. of strains | β | Odds ratios | 95%CI | P-value |
|---|---|---|---|---|---|
| **Antioxidant activity** | | | | | |
| Scavenging activity (%) | 201 | -0.003 | 0.997 | 0.943-1.054 | 0.920 |
| Inhibition activity (%) | 201 | -0.039 | 0.962 | 0.932-0.993 | 0.016 |
| **Types of antioxidants** | | | | | |
| SOD (Unit/mL) | 201 | -2.038 | 0.130 | 0.010-1.645 | 0.115 |
| GSH (nmol/mL) | 201 | -0.166 | 0. 847 | 0.763-0.941 | 0.002 |

* Caries free; dmft=0, Caries; dmft ≥ 1