Purification and Characterization of Antimicrobial Protein Produced by Oral Lactobacilli

Phirawat Wannun

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Oral Health Sciences)

Prince of Songkla University

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ผู้เขียน นายพีระวัฒ หวานนุ่น
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บทคัดย่อ

เชื้อแลคโตบาซิลลัสได้รับการยอมรับในการใช้เป็นเชื้อโปรไบโอติกซึ่งช่วยในการส่งเสริมการมีสุขภาพที่ดีของมนุษย์ทั้งในระบบทางเดินอาหาร ระบบทางเดินปัสสาวะ และในช่องปาก เชื้อแลคโตบาซิลลัสบางสายพันธุ์สามารถผลิตแบคเทอริโอซิน ที่ถูกสังเคราะห์ขึ้นจากไรโบโซม ซึ่งเป็นเปปไทด์หรือโปรตีนต้านจุลชีพ โปรตีนชนิดนี้ออกฤทธิ์ในการยับยั้งการเจริญเติบโตหรือฆ่าเชื้อแบคทีเรียที่มีพันธุกรรมที่คล้ายคลึงหรือมีความใกล้ชิดกัน ซึ่งมีแบคเทอริโอซินหลายชนิดที่ผลิตจากเชื้อแลคโตบาซิลลัสจากต้นกำเนิดที่แตกต่างกัน ยกตัวอย่างเช่น ระบบทางเดินปัสสาวะ ลำไส้ อุจจาระและอาหาร ซึ่งจากการศึกษาได้เผยให้เห็นถึงบทบาทของแบคเทอริโอซินที่ใช้ในการเก็บรักษาอาหารและการป้องกันการติดเชื้อในลำไส้และช่องคลอด แต่อย่างไรก็ตามองค์ความรู้ของแบคเทอริโอซิน หรือโปรตีนต้านจุลชีพที่ผลิตโดยเชื้อแลคโตบาซิลลัส สายพันธุ์ที่แยกได้จากช่องปากของมนุษย์ และความสามารถในการป้องกันการติดเชื้อในช่องปากยังมีอยู่อย่างจำกัด โดยจุดมุ่งหมายของวิทยานิพนธ์ในครั้งนี้ประกอบด้วย: 1) เพื่อค้นหาเชื้อแลคโตบาซิลลัสสายพันธุ์ที่แยกได้จากช่องปากที่มีความสามารถในการผลิตโปรตีนต้านจุลชีพ 2) เพื่อทำให้โปรตีนที่มีฤทธิ์ในการต้านจุลชีพ ที่ผลิตได้จากเชื้อแลคโตบาซิลลัสที่แยกได้จากช่องปากมีความบริสุทธิ์ 3) เพื่อประเมินผลของเอนไซม์ ความเป็นกรด-ด่าง อุณหภูมิ และระยะเวลาในการบ่ม ต่อการออกฤทธิ์ของโปรตีนต้านจุลชีพของเชื้อแลคโตบาซิลลัสที่แยกได้จากช่องปาก และ 4) เพื่อศึกษาสภาวะที่เหมาะสมสำหรับการเจริญเติบโตของเชื้อและการผลิตโปรตีนต้านจุลชีพของเชื้อแลคโตบาซิลลัสที่แยกได้จากช่องปาก

ในการศึกษาครั้งนี้ใช้เชื้อแลคโตบาซิลลัสจำนวน 90 สายพันธุ์ ซึ่งประกอบไปด้วย *Lactobacillus casei*, *L. fermentum*, *L. gasseri*, *L. mucosae*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* และ *L. salivarius* ซึ่งแยกได้จากเด็กที่มีและไม่มีฟันผุ โดยเชื้อแลคโตบาซิลลัสทุกสายพันธุ์จะถูกนำมาทดสอบฤทธิ์การต้านเชื้อ *Streptococcus mutans* ATCC 25175 ซึ่งจากการศึกษา

พบว่า เชื้อ *L. paracasei* SD1 และ *L. fermentum* SD11 ซึ่งแยกได้จากคนที่ไม่มีฟันผุ มีฤทธิ์การต้านเชื้อสูงที่สุด การทำให้สารที่มีฤทธิ์ในการต้านเชื้อมีความบริสุทธิ์ประกอบด้วย การตกตะกอนด้วยแอมโมเนียมซัลเฟต ต่อด้วยการสกัดด้วยคลอโรฟอร์ม การแยกด้วยคอลัมป์โครมาโทกราฟฟีชนิดแยกตามขนาดโมเลกุล การแยกด้วยคอลัมป์โครมาโทกราฟฟีชนิดผันกลับได้ หลังจากผ่านขั้นตอนการทำให้บริสุทธิ์ การทดสอบด้วยวิธี SDS-PAGE พบว่าโปรตีนที่มีฤทธิ์ในการต้านเชื้อที่ผลิตได้จากเชื้อ *L. paracasei* SD1 และ *L. fermentum* SD11 มีความบริสุทธิ์สูง และมีน้ำหนักโมเลกุลประมาณ 25 และ 33 กิโลดาลตัน ตามลำดับ และยืนยันน้ำหนักโมเลกุลอีกครั้งด้วยวิธี LC-MS ซึ่งพบว่า มีน้ำหนักโมเลกุลเป็น 24.03 และ 33.59 กิโลดาลตัน ตามลำดับ

สารต้านจุลชีพจากเชื้อ *L. paracasei* SD1 และ *L. fermentum* SD11 ถูกตั้งชื่อเป็น “paracasin SD1” และ “fermencin SD11” ตามลำดับ และสารต้านจุลชีพทั้งสองมีฤทธิ์ในการต้านเชื้อก่อโรคในช่องปากได้หลายชนิด ไม่ว่าจะเป็นเชื้อก่อโรคฟันผุ โรคปริทันต์ และยีส (*Candida*) จากการศึกษาพบว่า paracasin SD1 มีความคงตัวที่ดีในช่วงค่าความเป็นกรด-ด่าง ระหว่าง 3.0 ถึง 8.0 มีความคงทนที่อุณหภูมิ 100 องศาเซลเซียส เป็นเวลา 5 นาที ในขณะที่ fermencin SD11 มีความคงตัวได้ดีที่อุณหภูมิระหว่าง 60 ถึง 80 องศาเซลเซียส ในช่วงค่าความเป็นกรด-ด่างที่ 3.0 ถึง 7.0 สารต้านจุลชีพทั้งสองถูกทำลายได้ด้วยเอนไซม์ย่อยโปรตีน (โปรติเนส-เค และทริปซิน) แต่ไม่ได้รับผลกระทบจากเอนไซม์แอลฟาอะไมเลส คะตะเลส ไลโซไซม์ และน้ำลาย ยกเว้น paracasin SD1 ที่มีการสูญเสียกิจกรรมบางส่วนเมื่อถูกบ่มด้วยเอนไซม์ไลโซไซม์ สภาวะที่เหมาะสมสำหรับการเจริญเติบโตและการผลิตแบคเทอริโอซินของเชื้อ *L. paracasei* SD1 และ *L. fermentum* SD11 คือการเพาะเลี้ยงในอาหารที่มีความเป็นกรด-ด่าง อยู่ในช่วงระหว่าง 5.0 ถึง 6.0 ที่อุณหภูมิ 37 หรือ 40 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง ทั้งในสภาวะที่มี หรือไม่มีออกซิเจน

จากการศึกษาครั้งนี้สรุปได้ว่า เชื้อ *L. paracasei* SD1 และ *L. fermentum* SD11 ซึ่งเป็นสายพันธุ์ที่คัดแยกได้จากเด็กที่ไม่มีฟันผุ สามารถผลิตโปรตีนต้านจุลชีพได้ ซึ่งโปรตีนต้านจุลชีพนี้ถูกทำให้บริสุทธิ์ และตั้งชื่อเป็น paracasin SD1 และ fermencin SD11 ตามลำดับ โดยสารต้านจุลชีพทั้งสองสามารถออกฤทธิ์ได้ดีในสภาวะความเป็นกรด-ด่างที่กว้าง และมีความสามารถในการยับยั้งเชื้อก่อโรคในช่องปากได้หลายชนิด ซึ่งสภาวะที่เหมาะสมสำหรับการเจริญเติบโตและการผลิตแบคเทอริโอซินของเชื้อ *L. paracasei* SD1 และ *L. fermentum* SD11 คือการเพาะเลี้ยงในอาหารที่มีความเป็นกรด-ด่าง อยู่ในช่วงระหว่าง 5.0 ถึง 6.0 ที่อุณหภูมิ 37 หรือ 40 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง ทั้งในสภาวะที่มี หรือไม่มีออกซิเจน จากการศึกษานี้มีความเป็นไปได้ที่จะใช้เชื้อ *L. paracasei* SD1 และ *L. fermentum* SD11 รวมทั้งแบคเทริโอซินที่เชื้อผลิตขึ้น เป็นทางเลือกหนึ่งที่ช่วยส่งเสริมการมีสุขภาพช่องปากที่ดี หรือการป้องกันโรคในช่องปาก เช่น โรคฟันผุ และโรคปริทันต์ ซึ่งจำเป็นต้องมีการทดลองทางคลินิกต่อไป

Thesis Title Purification and Characterization of Antimicrobial Protein Produced by Oral Lactobacilli

Author Mr. Phirawat Wannun

Major Program Oral Health Sciences

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ABSTRACT

Lactobacillus has been proposed as probiotics for promoting and maintaining human health. Certain species of *Lactobacillus* produce and secrete bacteriocins, which are ribosomally synthesized antimicrobial peptides. This peptides exhibit a bacteriostatic or bacteriocidal activity against genetically closely related bacteria. A number of bacteriocins of *Lactobacillus* from different origins have been studied; for example, genitourinary tract, intestinal tract, feces and food. The studies have revealed the role of bacteriocin in food preservation, and the protection of intestinal and vaginal infections. However, the knowledge of bacteriocins or antimicrobial proteins produced by human orally *Lactobacillus* on the protective ability on oral infection is still limited. The aims of the present thesis were therefore: 1) to screen antimicrobial protein activity from oral *Lactobacillus*, 2) to purify active protein from oral *Lactobacillus*, 3) to evaluate the enzymes, pH, temperature, and time incubator on antimicrobial protein activity from oral *Lactobacillus*, and 4) to investigate the optimum conditions for bacterial growth and antimicrobial protein production by oral *Lactobacillus*.

A total 90 strains of oral *Lactobacillus* including *L. casei*, *L. fermentum*, *L. gasseri*, *L. mucosae*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. salivarius* from children with caries and caries free. All strains were screened for inhibitory activity against *Streptococcus mutans* ATCC 25175. *L. paracasei* SD1 and *L. fermentum* SD11 isolated from caries free subjects were exhibited the strongest inhibitory activity. Purification of the active compound was achieved with ammonium sulfate precipitation followed by chloroform extraction, gel filtration chromatography and/or reverse-phase high-performance liquid chromatography. As revealed by SDS-PAGE, the active fraction from *L. paracasei* SD1 and *L. fermentum* SD11 were unadulterated, showing a proteins with an approximate molecular weight of 25 and 33 kDa respectively. They were confirmed as having a molecular mass of 24.03 and 33.59 kDa by liquid chromatography-mass spectrometry.

The antimicrobial compound from *L. paracasei* SD1 and *L. fermentum* SD11, named "paracasin SD1" and "fermencin SD11" respectively, exhibited a broad spectrum against oral pathogens including cariogenic and periodontogenic pathogens, and *Candida*. Paracasin SD1 was stable in a pH range between 3.0 and 8.0 at 100 °C for 5 min while fermencin SD11 was stable between 60-80 °C in a pH range of 3.0 to 7.0. They were sensitive to proteolytic enzymes (proteinase K and trypsin), but not affected by α-amylase, catalase, lysozyme and saliva except paracasin SD1, it was relative loss of activity after treatment with lysozyme. The optimum conditions for growth and bacteriocin production of both *L. paracasei* SD1 and *L. fermentum* SD11 were cultured at acidic with pHs of 5.0-6.0 at 37 °C or 40 °C under aerobic or anaerobic conditions for 12 h.

In conclusion, *L. paracasei* SD1 and *L. fermentum* SD11, the strains derived from caries free children, could produce an antimicrobial proteins, from which later the purified proteins, namely paracasin SD1 and fermencin SD11 respcetively. They was active over a pH range with a broad spectrum against various oral pathogens. The optimum conditions for growth and bacteriocin production of *L. paracasei* SD1 and *L. fermentum* SD11 were cultured with acidic pHs of 5.0-6.0 at 37 °C or 40 °C under aerobic or anaerobic conditions for 12 h. It is promising that *L. paracasei SD1* and *L. fermentum* SD11 and there bacteriocins may be an alternative approach for promoting oral health or prevention of oral diseases e.g. dental caries and periodontitis, that would require further clinical trials.

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Phirawat Wannun

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LIST OF ABBERVIATIONS AND SYMBOLS

ATCC	American Type Culture Collection
AU	Arbitrary units
°C	degree Celcius
CFU	colony forming unit
CCUG	Culture Collection, University of Göteborg
Da	Dalton
g	gram
h	hour
i.e.	id est
kDa	kiloDalton
l	liter
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimole/liter
M	mole/liter
PBS	phosphate-buffered saline
rpm	revolutions per minute
SD	standard deviation
v/v	volume by volume
w/v	weigh by volume
µg	microgram
µl	microliter
%	percent
α	alpha
β	beta

LIST OF PAPERS AND PROCEEDING

International Journal Papers

1. Phirawat Wannun, Supatcharin Piwat and Rawee Teanpaisan. Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. ***Anaerobe*** 2014; 27: 17-21.
2. Phirawat Wannun, Supatcharin Piwat and Rawee Teanpaisan. Purification, characterization and optimum conditions of fermencin SD11, a bacteriocin produced by human orally *Lactobacillus fermentum* SD11. ***Appl Biochem Biotechnol*** 2016; 179: 572-582.

Conference Proceeding

1. Wannun A. and Teanpaisan R. Antimicrobial protein produced by oral *Lactobacillus* against dental caries pathogen. The 2nd Current Drug Development International Conference; 2012 May 2-4; Phuket Graceland Resort & Spa Hotel, Phuket, Thailand. Quintessence Publishing; 2012. p. 179-180.

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With best regards,

Teerapol Srichana
Chair of CDD 2012

1. INTRODUCTION

Background and Rationale

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, facultative anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria. They are widely used as probiotics in the food industry. Many studies have shown that certain *Lactobacillus* species are also of importance in human health, providing a beneficial microflora in the vagina,[3] intestinal tract,[2] 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. The main mechanisms of *Lactobacillus* strains to inhibit growth of pathogens due to their produce antimicrobial substances, for example organic acids, hydrogen peroxide and bacteriocins.[6, 7]

Bacteriocins are small bacterial proteins or peptides synthesized by ribosomes, and they exhibit a bacteriostatic or bacteriocidal activity against genetically closely related bacteria.[8] Bacteriocin producing bacteria as well as bacteriocins are increasing interests because they can be used as biological controls in the manufacture of beverages and ferment products.[9, 10] In addition, it may be used as a pharmaceutical product for local applications e.g. in the vagina.[11, 12] In application for commercial production, a number of studies have reported that physicochemical factors, e.g. pH, temperature and aeration, have a dramatic effect on the production of bacteriocins.[13-16] Thus, the optimization for biomass production to improve the yield of bacteriocins is necessary.

Several bacteriocins of *Lactobacillus* spp. from different origins have been characterized; for example, various bacteriocins were found to be produced by *L. fermentum* L23 isolated from vaginal, *L. paracasei* HD1-7 isolated from Chinese cabbage sauerkraut, *L. paracasei* BGBUK2-16 isolated from homemade white-pickled cheese, *L. casei* CRL 705 isolated from dry fermented sausage, *L. acidophilus* LF221 isolated from infant faeces, *L. plantarum* LE5 and LE27

isolated from ensiled corn, *L. animalis* TSU4 isolated from fish and *L. rhamnosus* 160 isolated from vaginal.[17-23] The studies have revealed the role of bacteriocin in food preservation, and the protection of intestinal and vaginal infections. However, the knowledge of bacteriocins or antimicrobial proteins produced by human orally *Lactobacillus* on the protective ability on oral infection is still limited.

Previous studies of Piwat *et al*. have shown that certain *Lactobacillus* species were associated with the presence and progression of dental caries in Thai children and adults.[24] Nevertheless, the study and others have shown that some *Lactobacillus* species have been suggested as potential probiotics for caries prevention, mainly because of their inhibitory activities against cariogenic *Streptococcus* spp.[5, 7, 25-27] The presence of certain oral lactobacilli is correlated with good oral health, this may be due to their ability to inhibit the growth of oral pathogen s*in vitro*.[7, 27] In addition, the prominent species, i.e. *L. fermentum*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. salivarius,* have been shown to reduce the growth of oral pathogens.[5, 7, 28] However, the antimicrobial substances have not been studied as yet. Thus, the present study was to screen antimicrobial protein activity, purified, characterized and optimized in conditions for bacterial growth and bacteriocin production of human oral *Lactobacillus*.

Literatures review

1. *Lactobacillus*

Lactobacillus species are Gram positive bacilli, catalase negative. They are considered to be important organisms due to their fermentative ability as well as their health and nutritional benefits.[29] During recent decades lactobacilli have gained importance as probiotics, 'live microorganisms which when administered confer a health benefit on the host'.[30] They are member of lactic acid bacteria (LAB), since it has similarity in production of lactic acid as a major end-product of carbohydrate catabolism.[31] Generally, LAB are generally recognized as safe (GRS) status except *Leuconostocs* and *L. rhamnosus* which are prohibited in European countries.[32] Initially lactobacilli are the magnificent interest for food fermentation and food preservation because they are able to inhibit the growth of undesirable microorganisms (e.g. *Clostridium botulinum*, *Bacillus* spp., *Enterococcus feacalis*, *Listeria monocytogenes* and *Staphylococcus aureus*) that cause food spoilage

and food-borne diseases, resulting in less use of chemical preservatives. Subsequently, there has been an increase in potential heath purposes as a live microorganism ingestion for promoting or supporting a balance of indigenous microflora as a probiotic, such as probiotic yogurts or yogurt-like products as well as providing a potential source of antimicrobial agents, such as bacteriocins, for the prevention of certain infectious diseases.[31] Recently, probiotic has been used for greater than ever: reducing carcinogenesis in preventative therapy for intestinal tumors; restoring the imbalance in the gut microflora, reducing blood level of urine toxin in treatment of chronic kidney failure, degradation of oxalate to prevent the subsequent evolution of kidney stones, reducing blood levels of cholesterol, and enhancing immunity by stimulating the activity of splenic NK cells and increasing gut IgA.[33] However, few studies available on the role and effects of probiotic for oral health.[34]

2. Role of *Lactobacillus* in oral cavity

The oral cavity shelters a very numerous and various microbial flora. More than 700 bacterial species have been detected in the oral cavity, of which over 50% have not been cultivated.[35] More than 300 individual microbial species can be cultivated have been identified from a human mouth.[36] Lactobacilli appear in the oral cavity during the first years of a child's life.[37]

Lactobacillus species are ubiquitous microorganisms colonizing the gastrointestinal tract, mucosal surfaces of the mouth and genitourinary tract. This bacterium has been reported infrequently as a cause of lethal infections in either immune competent or immune compromised hosts. *L. casei, L. fermentum, L. gasseri, L. paracasei, L. plantarum, L. rhamnosus* and *L. salivarius* have been reported the human oral cavity.[5, 27] The presence of certain oral *lactobacillus* are correlated with good oral health, this may be due to their ability to inhibit the growth of oral pathogens *in vitro*.[7, 27] In addition, the dominant species, i.e. *L. fermentum, L. paracasei, L. plantarum, L. rhamnosus* and *L. salivarius* have been shown to inhibit the growth of oral pathogens.[5, 7, 28]

The studied of Ahumada *et al*.[38] have reported that, lactobacilli isolated from the tongue were exhibited able to inhibit the growth of *S. mutans*. The inhibitive substances production were found in the homofermentative group more the heterofermentative group. In addition, this result same as other report, lactobacilli isolated from patients with caries active could produce inhibitory substances more than streptococci species (*mutans, sanguis, anginosus, salivarius*).

The studied of Koll-Klais *et al*.[5] have shown that, 88 % of oral lactobacilli could inhibited *Actinobacillus actinomycetemcomitans,* 82 % *Porphyromonas gingivalis,* 65 % *Prevotella intermedia* and 69 % *S. mutans.* The highest antimicrobial activity was associated to *L. plantarum, L. salivarius L. paracasei* and *L. rhamnosus*. The lowest antimicrobial activity against *S. mutans* was founded in the strains isolated from periodontically sound patients than the strains isolated from patients with chronic periodontitis.

The studied of Sookhee *et al*.[27] have shown that, *L. paracasei* and *L. rhamnosus* were isolated in the oral cavities of sound subjects could produce antimicrobial substances against *Actinomyces viscosus, Porphyromonas gingivalis, Staphylococcus aureus, S. mutans*, *S. salivarius, S. sanguis*, and *Candida*.

The studied of Ishikawa *et al*.[39] have reported that, *L. salivarius* could inhibited, *Prevotella nigrescens, Prevotella intemedia* and *Porphyromonas gingivalis* within 24 h. In addition, a clinical study have shown that daily intake of tablets containing *L. salivarius* could decreased the number of anaerobic rods bacteria in saliva after 4 weeks of administration.

Many studies have evaluated the role of *Lactobacillus* as probiotic in oral health. According to Nase *et al*.[25] showed that consumption milk containing *L. rhamnosus* GG for 7 month could reduce caries risk in preschool children. The studied of Petti *et al*.[40] have shown that, consumption of *L. bulgaricus* in yoghurt could decreased lactobacilli and *S. mutans* in saliva; but, as *L. bulgaricus* contained in this yoghurt do not colonize the oral cavity. One week consumption yoghurt containing *L. acidophilus* and *L. casei* caused a removal of other lactobacilli in saliva and dental plaque.[41]

The studied of Ahola *et al*.[26] have shown that, a shot-term consumption of cheese containing with *L. rhamnosus* GG and *L. rhamnosus* LC 705 reduces caries-related bacteria from saliva in young adults. This cheese was also reduce the risk of a high number of yeast, and reduce a high number of *S. mutans*, cause to reduce the carious risk.[25, 26, 40, 41] Also, our group has previously demonstrated that some oral *Lactobacillus* species have been proposed as probiotics in oral cavity, mainly because of their inhibitory activities against oral pathogens.[6, 25, 42]

3. Types of antimicrobial substances of *Lactobacillus*

The antimicrobial properties of LAB are derived during fermentation and the production of one or more antimicrobial active metabolites, including organic like lactic and acetic acid, hydrogen hydroxide, ethanol, carbon dioxide and also the compound, such as bacteriocins.[43] The antimicrobial compound produced by LAB act as biopreservatives in food, with founds dating back to approximately 6,000 before Christ.[44, 45] The role of antimicrobial substances are not only for promoting and maintaining human health but rather for one bacterium gaining advantage over another that competes for the same environment and same energy source.[46]

3.1. Organic acid

Acid is a major inhibitory factor produced by LAB. The lowers of pH of environment to the point that the growth of other bacteria could be inhibited.[47] During fermentation, heterofermentative of LAB produce amounts of lactic acid, acetic acid, ethanol and carbon dioxide while homofermentative produce lactic acid alone.[48] The reduction of pH in the fermented food resulting from acid production by the fermentation process that the primary preserving actions of the LAB.[49] The organic acids are believed to disrupt the mechanisms responsible for maintaining the membrane potential, thereby inhibiting active transport.[45, 50]

The productions of lactic acid and other organic acids were caused by sugar fermentation. Acid producing LAB is very important factor for the inhibition of growth of undesired microorganisms, and it also important in helping to maintain the balance of microorganisms in the intestinal tracts.[51, 52] However, the intensity of the antagonistic action may not be directly related to the amount of acid produced. It also has been recognized that LAB are capable of producing other inhibitory substances than organic acids.[53] The reduction of pH makes organic acids lipossoluble, allowing them to destroy the cell membrane and reach the cytoplasm of pathogens.[54] Lactic acid caused prominent permeabilization in Gram negative bacteria *in vitro* and it is responsible for low intracellular pH.[55] For instance, *L. acidophilus* No 4356 produced a large amount of lactic acid that completely inhibited the growth of *H. pylori* in a mixed culture.[56] Urease activity allowed pathogens

such as *Yersinia enterocolitica* or *H. pylori* to develop in the environment with low pH; however the production of acid from lactobacilli were able to inhibit this activity.[56-58]

3.2. Hydrogen peroxide

LAB have the ability to produce hydrogen peroxide when grown in the presence of oxygen through the action of NADH oxidases, super oxide dismutase and flavoprotein-containing oxidases.[46, 59, 60] Because of LAB are catalase-negative, hydrogen peroxide can accumulate to high and act inhibitory to the growth of some microorganisms.[59] However, it is argued that the hydrogen peroxide does not accumulate significant amounts because it could decomposed by flavoproteins, pseudocatalases and peroxidases *in vivo*.[61]

The production of hydrogen peroxide by *L. salivarius* CECT 5713 showed antimicrobial activity against *L. monocytogenes* Ohio and *Klebsiella oxytoca* CECT 860T *in vitro*.[62] The supernatants of *L. johnsonii* NCC533 or *L. gasseri* CRL1421 exhibited antimicrobial activity against growth of pathogen while untreated with catalase, suggesting the implication of H_2O_2.[63, 64] H_2O_2 was also shown to be able to inhibit the formation of *Staphylococcus epidermidis* CSF41498 biofilm *in vitro*, and to reduce the transcription of *icaADBC* operon involved in polysaccharide intercellular adhesion in this species.[65]

3.3. Carbon dioxide

Heterofermentative of LAB could produce carbon dioxide and contributes to an anaerobic environment, and it cause toxic to various aerobic microorganisms that living in the area. Moreover, the carbon dioxide has an antimicrobial ability.[66] The mechanism of this activity is not known, but it is believed that the accumulates of carbon dioxide in the lipid bilayer due to the inhibition of enzymatic decarboxylation,[67] causing dysfunction of membrane permeability.[66] It have been report that low levels of carbon dioxide have been found to enhance the growth of certain microorganisms, whereas high carbon dioxide concentrations led to growth inhibition.[66]

3.4. Diacetyl

Various LAB can produce diacetyl (2,3-butanedione) during heterofermentation, including the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*.[68, 69] Diacetly is more sensitive to Gram-negative bacteria, molds and yeast than Gram positive bacteria. The antimicrobial mechanism of diacetyl was action on the arginine-binding protein of Gram-negative bacteria leading to interference with arginine utilization.[45, 68] Diacetyl is well known for its ability to enhance the flavor of butter. Moreover, there is also imparts to the fermented dairy products, but this property as well as the high concentration necessary to restrict the use of diacetyl in food storage as food preservatives.

3.5. Bacteriocin

Bacteriocins are gene-coded, ribosomally synthesized antimicrobial peptides, and they exhibit a bacteriostatic or bacteriocidal activity against genetically closely related bacteria.[46, 70, 71] Most of bacteria produce only one bacteriocin, but some species can produce several different bacteriocins. [72] The first discovered of bacterocin by Gratia[73] in 1925. He was demonstrated that *Escherichia coli* produced antimicrobial substance, named "colicin", and had the ability to inhibit growth of other *E. coli* strains. Since then, many studies have been identified and characterized of antimicrobial peptides produced from both Gram positive and Gram negative bacteria, and there antimicrobial proteins were collective term "bacteriocins".[74]

4. Bacteriocin

4.1. Bacteriocin definition

Gene-encoded, ribosomally synthesized antimicrobial peptides are being produced as constituents of their innate immune systems in nature by microorganisms, plants, animals and humans, which is a common defense strategy against bacteria.[75, 76] The bacteriocins are often cationic and amphiphilic, and most of them kill bacteria by causing cell-leakage by permeabilizing the target

cell membranes. Antimicrobial peptides can be roughly categorized into those that have high content of a certain amino acids, most often proline, those contain intramolecular disulfide bridges, and those with an amphiphilic region in their molecule if they display an α-helical structure.[77]

Ribosomally synthesized antimicrobial peptides produced by bacteria are generally referred to bacteriocins, which may be either plasmid encoded[78] or chromosomally encoded.[79] The producer cells are also immunized at nominal levels of their own inhibitors.[80] Their net charges, with pIs varying from 8.3 to 10, are common features.[81] Even bacteriocins's structure resembles many of the antimicrobial peptides produced by eukaryotes; they seem to be more potent and much higher specific activity than those produced by animals and plants.[77] Higher potencies and specificity of bacteriocins were due to their high affinity binding to specific receptors or docking sites on the target-cell surface.[82] The bacteriocins produced by Gram- positive bacteria, proteins are typically smaller than 6 kDa in contrast to most of the bacteriocins produced by Gram- negative bacteria often produce bacteriocins are peptides, which are larger. 20 kDa. The bacteriocin productions are not necessary linked to one species or restricted to organisms sharing a specific environment. Among the lactobacilli, there has been much interest in *L. acidophilus* and *L. plantarum*, due to their potential application as starter bacteria in food fermentation product.

4.2. Bacteriocin classification

Bacteriocins produced by Gram-positive LAB are grouped into four distinct class by Klaenhammer's[8] in 1993. These groupings have formed on the basis of their structure and function. Klaenhammer's suggested four classes of bacteriocins as follows:

- Class I bacteriocins, also known as lantibiotics, containing the unusual post translationally modified residues lanthionine, β-methyl lanthionine and other modified residues, such as dehydroalanine and dehydrobutyrine. A small membrane-active peptides (<5 kDa) containing 19 to 38 amino acids. Nisin is the most characterized of the Class I bacteriocins.
- Class II bacteriocins, also known as non-lantibiotics, consist of peptides without modified residues, a small heat-stable peptides (<10 kDa). The class is further divided into three subclasses; subclass IIa, IIb and IIc.

- Subclass IIa are the pediocin-like bacteriocins, which have *Listeria*-active peptides, consensus sequence in the N-terminal of -Tyr-Gly-Asn-Gly-Val-Xaa-Cys-
- Subclass IIb are poration complexes consisting of two difference peptide for activity.
- Subclass IIc, which contains one-peptide bacteriocin, thiol-activated peptides requiring reduced cysteine residues for activity

- Class III are consist of large (>30 kDa), heat-labile proteins, it often with enzymatic activity.
- Class IV are complex proteins, composed of one or more chemical moieties, either lipid or carbohydrate.

There are a number of bacteriocins produced by different *Lactobacillus* spp. (Table 1), and they can be classified according to their biochemical and genetic characteristics.[83]

5. Isolation of bacteriocin

Mostly bacteriocins were isolated from LAB, and from different sources such as food product, plant material and human or animal isolates. The nature of the inhibitory substance produced by organisms in the initial screening by the agar well diffusion technique. [74] According to Moreno *et al*.[84] have isolate 167 strains of *L. lactis* from cheese and milk. The antimicrobial activity was detected through the agar well diffusion assay. The antagonism activity from hydrogen peroxide and organic acid was avoided by added catalase and neutralize by phosphate buffer in the cultured medium. The results found that, 8.4 % of isolated strains were inhibited the growth of indicator strain. *Tahara et al*.[85] have reported that, the screening of antimicrobial activity from *L. acidophilus* JCM 1132 was performed by agar well diffusion assay. The inhibitory activity of neutralized cell-free supernatant was not affected by catalase, indicating that a contribution of hydrogen peroxide was ruled out.

Table 1. Bacteriocins of *Lactobacillus* spp. and their main characteristics

Producing species	Bacteriocin	Spectrum of action	Characteristics
L. acidophilus	Acidocin CH5	Gram-positive bacteria	Class II bacteriocin, forms high molecular weight aggregates
	Lactacin F	*Lactobacillus* *L. fermentum* *Enterococcus faecalis* *L. delbrueckii*	Class II bacteriocin, 6,3 kDa, 57 amino-acids, heatstable at 121 °C for 15 minutes
	Lactacin B	*L. helveticus* *L. debrweckii* *L. helveticus* *L. bulgaricus.* *Lactococcus lactis.*	Class III bacteriocin, 6,3 kDa, heat-stable, detected only in cultures maintained between pH 5.0 to 6.0
L. amylovorus	Lactobin A	*L. acidophilus* *L. delbrueckii*	Class II bacteriocin, 4,8 kDa, 50 amino-acids, narrow spectrum of activity
L. casei	Lactocin 705	*Listeria monocytogenes* *L. plantarum*	Class II two-component bacteriocin (33 amino-acids each component), 3,4 kDa,
L. sake	Lactocin S	*Lactobacillus* *Leuconostoc* *Pediococcus*	Class I bacteriocin, 3,7 kDa, active between pH of 4,5 and 7,5
	Sakacin P	*Listeria monocytogenes*	Class II bacteriocin, 4,4 kDa, heat-stable
L. curvatus	Curvacin A	*Listeria monocytogenes* *Enterococcus faecalis*	Class II bacteriocin, 4,3 kDa
L. helveticus	Helveticin J	*L. bulgaricus* *Lactococcus lactis*	Class III bacteriocin, 37 kDa, narrow spectrum of action, sensitive to proteolytic enzymes, reduction of activity after 100 °C for 30 min

Parada *et al.* [9]

Lakshminarayanan *et al.* [86] have screened of *Lactobacillus* over 70,000 colonies, from faecal samples collected from 266 subjects. The antimicrobial activity of the cultured supernatants of the isolated strains were determined by the agar well diffusion assay. The results shown that 276 colonies exhibiting antimicrobial activity against indicator strain. The predominant antimicrobial-producing species were identified as *L. acidophilus, L. crispatus, L. gasseri, L. salivarius* and *Enterococcus* spp.

Ndlovu *et al.*[87] have screened of 155 LAB strains isolated from red wines. The antimicrobial activity was detected by the agar spot test method. The results shown that 8 isolates were identified to be bacteriocin producers and were identified as *E. faecium*. The peptides were preliminarily identified as enterocin P.

6. Bacteriocin purification

Several techniques have been used to obtain purified or partially purified bacteriocins. The purification scheme may be varied for some applications. Highly purified preparations would be needed for determination of a bacteriocin's amino acid composition and sequence. However, high yields of active bacteriocin will be the focus of a food biopreservative system. Most purifications start with a method that concentrates bacteriocins from culture supernatants, because bacteriocins usually are extracellular products. Ammonium sulfate precipitation is well established as an initial step in the purification process.[88]

Dialysis and ultrafiltration are valuable methods of concentrating and purifying bacteriocins. By using membranes of specific pore size, the researcher can retain proteins above a particular size and allow smaller proteins to pass through.

Several methods of chromatography, such as gel filtration, ion exchange, and/or hydrophobic interaction chromatography, have been recommended for achieving significant purification of bacteriocins. Especially, reverse-phase chromatography has been frequently used as a final step for several bacteriocins, including bacteriocin TSU4[23], curvaticin FS47[89], lactacin F[90], plantaricin A[91], bavaricin A[92], bacteriocin LS1[93] and bacteriocin L23.[18]

Many lactobacilli species have been identified as producers of bacteriocins, and the purification processes of their bacteriocins are depend on their characterization. The different approach for purifying the bacteriocin such as lactacin F, produced from *L. acidophilus* 11088, was

purified by ammonium sulfate precipitation, gel filtration chromatography, and high performance liquid chromatography. The purification methods resulted in a 474 fold increase in specific activity. Purified lactacin F was established to have a molecular mass of 2.5 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). [90]

Bacteriocin SL1, produced from *L. salivarius* BGHO1, was purified by ammonium sulfate precipitation, reverse-phase chromatography on FPLC and HPLC systems. The purified Bacteriocin SL1 was established to have a molecular mass of approximately 10 kDa by tricine SDS-PAGE.[93]

Plantaricin ZJ5, a new Bacteriocin produced from *L. plantarum* ZJ5, was purified by ammonium sulfate precipitation followed by cation exchange, hydrophobic interactions and reverse-phase chromatography. As revealed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), the molecular mass of plantaricin ZJ5 was found to be 25.7 kDa.[94]

Plantaricin ZJ008, produced from *L. plantarum* ZJ008, was purified by macroporous resin column (XAD 2), cation exchange, gel filtration chromatography, and RP-HPLC. After purification, activity unit was increasing from 426 IU/ml to 8,471 IU/ml. Mass spectrometry based on MALDI-TOF indicated that the bacteriocin had a molecular of 1.33 kDa.

Curvaticin FS47, produced by *L. curvatus FS47*, was purified by ammonium sulfate precipitation, solid-phase extraction and reverse-phased HPLC. The purification methods resulted in a 25 fold increase in specific activity and 0.0034 % recovered. Molecular mass of curvaticin FS47 was found to be 4.07 kDa based on mass spectrometry.[89]

Lactacin F, a bacteriocin produced by *L. acidophilus* 11088, was purified and characterized by Muriana and Klaenhammer.[90] Lactacin F was purified by ammonium sulfate precipitation, gel filtration, and reversed-phase HPLC resulted in a 474-fold increase in specific activity.

7. Bacteriocin characterization

The physical parameters such as temperature, pH and enzyme were dramatic effected on bacteriocin activity. Some bacteriocins show that the molecules can be active under a certain range of temperature and pH. Generally, bacteriocins were sensitive to proteolytic enzymes such as proteinase K, pepsin and trypsin.[95] According to Bendjeddou *et al.*[96] reported that, paracaseicin A, a heat-labile protein produced by *L. paracasei* subsp. *paracasei* BMK2005. Paracaseicin A was active only at acidic pH range; inactivated by treatment with papain and proteinase K; stable at 60 and at 80 °C, and no detectable was observed after heating at 120 °C for 10 min.

Bacteriocin L23 produced by *L. fermentum* L23 was active whide pH range (4.0-7.0); inactivated by treatment with proteolytic enzymes (trypsin and protease VI); heat stable (60 min at 100 °C).[18] Cell free culture filtrate of probiotic *L. rhamnosus* 231 exhibited a broad spectrum of activity against Gram-positive and Gram-negative bacteria.[97] Acidocin D20079 produced by *L. acidophilus* DSM 20079 was extremely heat-stable (30 min at 121 °C); inactivated by trypsin, ficin, pepsin, papain, and proteinase K. Acidocin D20079 has a narrow inhibitory spectrum, especially the genus *Lactobacillus.*[98]

8. Bacterocin production

Most studies performed to optimize bacteriocin production have used commercial media to provide a rich supply of growth nutrients. Physicochemical factors such as pH or temperature have a dramatic effect on the production of bacteriocins.[13, 16, 99] For example, Barefoot *et al.*[100] reported that pH was an important factor in the production of lactatib B by *L. acidophilus*. A maximum activity was obtained at pH 6.0. Maximum production of lactacin F by *L. acidophilus* 11088 was obtained at pH 7.0.[90] Production of lactococcin 140 produced by *Lactococcus lactis* 140NWC was pH dependent, with maximum activity of 1.54×10^4 AU/ml was obtained at pH 5.5.[101] The production of acidocin 8912 was depended on temperature. Maximum production of acidocin 8912 was obtained at 37 °C in MRS Broth.[102]

Optimal production of bacteriocin can occur at different growth phases. Some bacteriocins such as lactococcin 140 and nisin are produced during the exponential phase. [101, 103]

During the late exponential and early stationary phase of growth, many bacteriocins, such as helveticin J[104], pediocin AcH[13], propionicin PLG-1[105] and pediocin SJ-1[106] are produced extracellularly. This suggests that these bacteriocins are secondary metabolites.

Objectives

1. To screen antimicrobial protein activity from oral *Lactobacillus*
2. To purify active protein from oral *Lactobacillus*
3. To evaluate the enzymes, pH, temperature, and time incubator on antimicrobial activity from oral *Lactobacillus*
4. To investigate the optimum conditions for bacterial growth and antimicrobial protein production by oral *Lactobacillus*

2. MATERIALS AND METHODS

Bacterial strains and culture conditions

A total 90 strains of oral *Lactobacillus* including *L. casei* (5 strains), *L. fermentum* (40 strains), *L. gasseri* (5 strains), *L. mucosae* (5 strains), *L. paracasei* (20 strains), *L. plantarum* (5 strains), *L. rhamnosus* (5 strains) and *L. salivarius* (5 strains), a clinical collection strains were obtained from the previous study of Piwat *et al*.[24], 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).[107] Sequencing of the 16S-rRNA was performed for strains with uncertain identifications. *Lactobacillus* strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth for 24-48 h, at 37 °C in an anaerobic condition (80 % N_2, 10 %H_2 and 10 % CO_2).

The antimicrobial activity of the antimicrobial protein produced by oral *Lactobacillus* were determined against a broader spectrum of oral bacteria strained will be performed. For details, see Table 2.

Antimicrobial activity assay

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 MRS broth at 37 °C for 24 h. Bacterial cells were separated from the broth culture by centrifugation at 8,000×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. The treated supernatants were tested for antimicrobial activity using an agar well diffusion assay.

Table 2. Microorganisms used as indicator strains to determine the activity spectrum of the bacteriocins produced by oral *Lactobacillus*

Indicator strains	**Medium and growth conditions**
Gram positive bacteria:	
Lactobacillus casei ATCC 393	MRS, 37 °C and anaerobic condition
Lactobacillus fermentum ATCC 14931	MRS, 37 °C and anaerobic condition
Lactobacillus paracasei CCUG 32212	MRS, 37 °C and anaerobic condition
Lactobacillus plantarum ATCC 14917	MRS, 37 °C and anaerobic condition
Lactobacillus rhamnosus ATCC 7469	MRS, 37 °C and anaerobic condition
Lactobacillus salivarius ATCC 11741	MRS, 37 °C and anaerobic condition
Streptococcus mutans ATCC 25175	BHI, 37 °C and anaerobic condition
Streptococcus sobrinus ATCC 33478	BHI, 37 °C and anaerobic condition
Gram negative bacteria:	
Aggregatibacter actinomycetemcomitans ATCC 33384	BHI, 37 °C and anaerobic condition
Fusobacterium nucleatum ATCC 25586	BHI, 37 °C and anaerobic condition
Porphyromonas gingivalis ATCC 33277	BHI with 2% (w/v) Vitamin K (0.03 M), 37 °C and anaerobic condition
Yeast:	
Candida albicans ATCC 90028	SDA, 37 °C and aerobic condition

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 to the original volume.

The bacterial cells were washed three times with phosphate buffer saline (PBS, pH 7.0), then the sediment was 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 8,000×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). Antimicrobial protein titers were reported as 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.

From our screening study, the results indicated that the strongest inhibitory activity was observed in the aqueous phase of the supernatant of *L. paracasei* SD1and *L fementum* SD11. Thus, the strains were subjected to a further bacteriocin purification study.

Purification of the bacteriocin

1. Ammonium sulfate precipitation

One liter of the supernatant from *Lactobacillus* strain was adjusted to pH 6.5 with 1.0 M NaOH, and was precipitated with ammonium sulfate at various saturation levels (40 %, 60 % and 80 %). The mixture was stirred overnight at 4 °C, the protein precipitate was centrifuged (20,000×g, 20 min, 4 °C) and the resulting pellet was dissolved in 50 ml of 0.1 M sodium phosphate buffer pH 7.0. The precipitated protein was dialysed using a dialysis tubing (molecular weight cutoff of 3.5 kDa) against 1 liter 0.1 M sodium phosphate buffer pH 7.0 at 4 °C. The entire dialysis required

four changes of the same buffer over 2 days. At each saturation levels of ammonium sulfate, the resulting suspension was assayed for inhibitory activity. Protein concentration was determined by the method of Bradford's. [108]

From our screening study of ammonium sulfate at various saturation levels, the results indicated that the strongest inhibitory activity of *L. paracasei* SD1and *L fementum* SD11were observed at the 40 % saturation of ammonium sulfate. Thus, the 40 % saturation was subjected to a further bacteriocin purification study.

2. Solvent extraction

The precipitated bacteriocin (50 ml) was mixed with equal volume of solvents; chloroform or ethyl acetate.[109] Phase separation was achieved after the samples standing at room temperature for approximately 30 min. The samples were centrifuged with 6,000×g at 4 °C for 10 min. The water phase was dried using freeze-drying while solvent phase was dried using speed vac concentrator. The pellet was resuspended in 0.1 M sodium phosphate buffer pH 7.0. The antimicrobial activity will be determined by the broth microdilution assay.

From the study of solvent extraction, the results indicated that the strongest inhibitory activity of *L. paracasei* SD1and *L fementum* SD11were observed in the water phase after extracted with chloroform. Thus, the extraction with chloroform was subjected to a further bacteriocin purification study.

3. Column chromatography

3.1. Purification of bacteriocin produced from *L. paracasei* SD1

- Gel filtration chromatography

The sample (0.5 ml) form *L. paracasei* SD1 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 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. Fractions of 0.5 ml each were collected and then dialyzed two times against 0.5 l of distilled water for 24 h using a dialysis bag with a molecular weight cutoff of 3.5 kDa. The individual fraction was lyophilized under pressure (25 mHg) at -110 °C of freeze-drying (CoolSafe 110-4 Pro, ScanLaf, Denmark) for 24 h. Each fraction was then tested for bacteriocin activity using a broth microdilution assay.

3.2. Purification of bacteriocin produced from *L. fermentum* SD11

- Gel filtration chromatography

The sample (0.5 ml) was loaded on a Fractogel® EMD BioSEC (1.5×90 cm) column (MERCK, Germany). The column was equilibrated and eluted by a 50 mM sodium phosphate buffer pH 6.5 with the addition of 0.15 M NaCl at a flow rate of 0.5 ml/min, with a time interval of 20 min after the samples were injected. Fractions of 0.5 ml each were collected and then dialyzed two times against 0.5 l of distilled water for 24 h using a dialysis bag with a molecular weight cutoff of 3.5 kDa. The individual fraction was lyophilized as mentioned above. Each fraction was then tested for bacteriocin activity using a broth microdilution assay.

- Cation exchanger chromatography

The active fraction (0.5 ml) from gel filtration chromatography was loaded on a SP Sepharose® Fast Flow 1.0/15 cm column (Pharmacia-Biotech, Canada). Equilibrate with 20 mM sodium acetate. After sequentially washing the column with 60 ml of 160 and 320 mM sodium chloride, the active fraction will be elute at a flow rate of 1.0 ml/min with 500 mM sodium chloride. Fractions of 1 ml each were collected and then dialyzed two times against 0.5 l of distilled water for 24 h using a dialysis bag with a molecular weight cutoff of 3.5 kDa. The individual fraction was lyophilized as mentioned above. Each fraction was then tested for bacteriocin activity using a broth microdilution assay.

From this study, cation exchanger chromatography could not remove contamination proteins from the bacteriocin. Thus, the further purification step was applied to reverse-phase HPLC system.

- Reverse-phase HPLC

For further purification of bactericon produced by *L fementum* SD11, the active eluted fraction (100 µl) from gel filtration chromatography was applied to RP-HPLC on a C_{18} Sep-Pack column (Millipore Corp., Milford, Mass.) at a flow rate of 0.5 ml/min. Elution was carried out by applying a linear gradient of acetonitrile with deionized water from 15 to 100 % within 30 min, with a time interval of 10 min after the samples were injected. Absorbencies were recorded between 210 and 280 nm using a photodiode array detector (model 996; Waters). All collected fractions were evaporated and then tested for bacteriocin activity. The purity of active fraction was confirmed with second RP-HPLC run in the same conditions.

Determination of protein concentration

The protein content was estimated using Bradford's assay,[108] a reagent kit of Bio-Rad Laboratories, USA, and bovine serum albumin (Sigma-Aldrich, USA) was used as a standard. A sample (20 µl) was added to 1 ml of the Bradford reagent, and then mixed for 1 min at room temperature. The solution was monitored for the protein concentrations at 595 nm by comparing with bovine serum albumin as the standard.

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

Tricine-SDS PAGE analysis of purified bacteriocin was performed according to Schagger *et al.*[110], 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.

Mass spectrometry

The active fraction was dissolved at the concentration of 1 mg/ml 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 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 to 40 kDa. The mass spectrometer was calibrated by preliminary analysis of horse heart myoglobin (Sigma).

Characterization of bacteriocin produced by *L. paracasei* SD1 and *L. fermentum* SD11

1. Sensitivity to enzymes, pHs and heats

Enzyme sensitivity: Aliquots of 1.0 ml of the purified bacteriocin (containing approximately 30 µg) were powdered using freeze-drying and added to 1.0 ml of enzyme solutions. These solutions were prepared at 1.0 mg/ml as follows: trypsin (Sigma-Aldrich), proteinase K, α-amylase (Merck), catalase (Merck), lysozyme (Merck) in 0.05 M PBS (pH 7.0) [111, 112] and saliva. After incubation for 4 h at 37 °C, reactions were stopped by boiling the mixture for 3 min. The antimicrobial activity was determined against *S. mutans* ATCC 25175 by broth microdilution assay.

pH sensitivity: The antimicrobial activity of the bacteriocin was tested at various pH values from 3.0 to 9.0. Aliquots of 1.0 ml of the purified bacteriocin (containing approximately 30 µg) were powdered using freeze-drying and separately mixed with 1.0 ml of 0.05 M buffers as follows: citrate-phosphate (pH 3.0, 4.0, 5.0 and 6.0), sodium-phosphate (pH 7.0), Tris–HCl (pH 8.0)

and glycine NaOH (pH 9.0).[96] The mixtures were incubated for 4 h at37 °C. The antimicrobial activity was determined against *S. mutans* ATCC 25175 by broth microdilution assay.

Heat sensitivity: Aliquots of 1.0 ml of the purified bacteriocin (containing approximately 30 µg) were heated at: 60, 80, 100 and 120 °C for 5, 10 and 20 min in a water bath.[96] Next, the samples were cooled at room temperature. The mixtures were incubated for 4 h at37 °C. The antimicrobial activity was determined against *S. mutans* ATCC 25175 by broth microdilution assay. The stability of the lyophilized bacteriocin during long-term storage was tested at 25 °C, 4 °C and -20 °C over 6 months. Bacteriocin aliquots were taken from storage every month and were measured for antimicrobial activity.

2. Inhibitory spectrum of the bacteriocin

The bacteriocin was tested for its inhibitory activity using the broth microdilution assay. Several indicator strains (see Table 2) were chosen on the basis of their importance in and relatedness to the oral ecosystem. All tested 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 AU per ml.

Determination of optimum growth and bacteriocin production of *L. paracasei* SD1 and *L. fermentum* SD11

The experiment was performed using a biofermenter (BioFlo®/CelliGen115, Germany) where MRS broth (2 l) was inoculated with 100 ml of *L. paracasei* SD1 or *L. fermentum* SD11 culture for 12-18 h at 37 °C to an initial cell density of 10^8 CFU/ml.

Initially, the experiments were set up to explore the optimum growth and bacteriocin production by incubating at 37 °C under aerobic or anaerobic conditions with different pHs of 5.0, 6.0, 7.0 and 8.0. To monitor the bacterial growth, an aliquot of culture was removed at 0, 3, 6, 12, 24 and 48 h time interval and measured at 600 nm absorbance. After concentrating the supernatant with

a Speed-Vac concentrator and adjusting pH to 6.5, bacteriocin activity against *S. mutans* ATCC 25175 was determined using a microdilution assay.

The optimum growth and bacteriocin activity were evaluated by comparing the growth rate and bacteriocin activity between times that were calculated as follows:

Growth rate or bacteriocin activity = $X_2 - X_1 / t_2 - t_1$; where X_1 and X_2 were growth (OD) or bacteriocin activity (AU) at the time point 1 (t_1) and time point 2 (t_2), respectively.

The results indicated that at pHs 5.0 and 6.0 under either aerobic or anaerobic condition provided the optimum growth and bacteriocin production of *L. paracasei* SD1 and *L. fermentum* SD11. However, it was more convenient to conduct the experiment under aerobic condition to explore for the optimum temperature. Thus, the biofermenter was set to pH 6.0 under aerobic condition at different temperatures of 25, 37 and 40 °C, and the bacterial growth rate and bacteriocin production was monitored as mentioned above.

Statistics analysis

The following parameters were used to characterize of bacteriocin: enzyme, pH, temperature and inhibitory spectrum were used as evaluated parameter. The average value of each parameter is presented as mean ± standard deviation (SD) of AU. Normality and homogeneity of variance assumptions: pH, temperature, aeration and incubation time points were assessed by Shapiro-Wilks test. Nonparametric tests were used due to non-normal distributions of the data. Multiple comparisons of growth rate and bacteriocin activity at each incubation time points (0-3, 3-6, 6-12, 12-24 and 24-48 h) with various parameters: pH (5.0, 6.0 or 7.0), temperature (25, 37 or 40 °C) and aeration (aerobe or anaerobe) were analyzed by Kruskal-Wallis test. Mann-Whitney U Test was used to test differences of growth rate and bacteriocin activity at each pair of pH levels, temperature levels and aeration levels. Data were analyzed by the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL), and the significant differences were considered as $p < 0.05$.

3. RESULTS

Screening antimicrobial protein production

A total of 90 clinical *Lactobacillus* strains 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 and *L. fermentum* SD11, the strains isolated from caries free subjects exhibited the strongest inhibitory growth on *S. mutans* ATCC 25175. The inhibitory activity from the supernatant of *L. paracasei* SD1 and *L. fermentum* SD11 (inhibition zone 2.2 $\pm$ 0.1 and 2.0 $\pm$ 0.15 cm respectively) were partially lost after treatment with catalase (inhibition zone 1.6 $\pm$ 0.1 and 1.5 $\pm$ 0.15 cm respectively), and it was completely absent after treatment with proteinase K (Fig. 1 and 2).

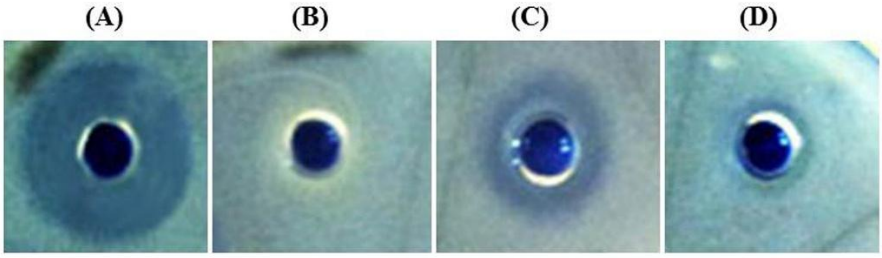

Fig. 1. The inhibition zone of the supernatant from *L. paracasei* SD1 against *S. mutans* ATCC25175. The supernatant was treated with various enzymes. (A) untreated supernatant, (B) treated with proteinase K, (C) treated with catalase, and (D) treated with lysozyme.

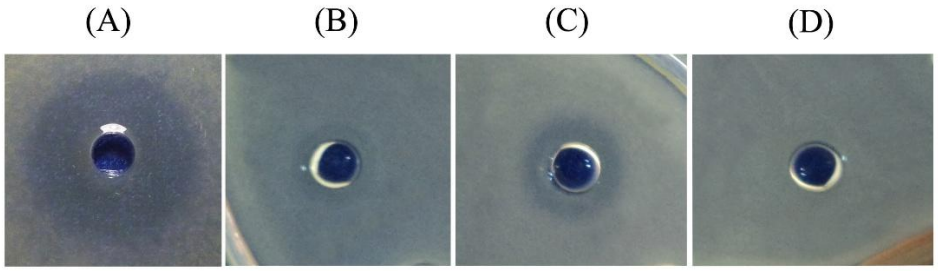

Fig. 2. The inhibition zone of the supernatant from *L. fermentum* SD11 against *S. mutans* ATCC25175. The supernatant was treated with various enzymes. (A) untreated supernatant, (B) treated with proteinase K, (C) treated with catalase, and (D) treated with lysozyme.

Purification of bacteriocin

Initially, the antimicrobial protein was precipitated from cell free supernatant of *L. paracasei* SD1 and *L. fermentum* SD11 with 40 %, 60 % or 80 % ammonium sulfate. It was shown that the strongest antimicrobial activity of *L. paracasei* SD1 and *L. fermentum* SD11against *S. mutans* ATCC 25175 was recovered in the protein pellet which was saturated with 40 % ammonium sulfate (4,123 ± 59 and 3,938 + 25 AU/ml respectively). Increased specific activity of the precipitated protein from 204.1 to 1,048.2 AU/µg (bacericon from *L. paracasei* SD1) and 103.8 to 317.53 AU/µg (bacericon from *L. fermentum* SD11) was found after treatment with chloroform (1:1 v/v) (Table 3).

1. Purification of bacteriocin from *L. paracasei* SD1

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/µg (in the supernatant) to 46,875 AU/µg (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 approximately 25 kDa (Fig. 3a). The LC-MS analysis demonstrated a specific protein molecular mass of 24.03 kDa (Fig. 3b). This antimicrobial protein was named as "paracasin SD1".

Table 3. Purification of antimicrobial proteins from *L. paracasei* SD1 and *L. fermentum* SD11

Sample	Volume (ml)	Total protein (µg)	Total activity (Au)	Specific activity (AU/µg)	Activity recovered (%)
***L. paracasei* SD1**					
Culture supernatant	1,000	63,000	3,386,100	53.8	100
Ammonium sulfate precipitation	50	700	142,855	204.1	4.2
Chloroform extraction	50	135	141,505	1,048.2	4.2
Gel filtration chromatography	1	0.8	37,500	46,875	1.1
***L. fermentum* SD11**					
Culture supernatant	1,000	33,000	606,060.6	18.36	100
Ammonium sulfate precipitation	50	850	88,235.29	103.8	14.5
Chloroform extraction	25	243	77,160.49	317.53	12.7
Gel filtration chromatography	1	2.2	13,636.36	6,198.34	2.24
Reverse-Phase HPLC	1	0.16	125,000	781,250	20.6

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

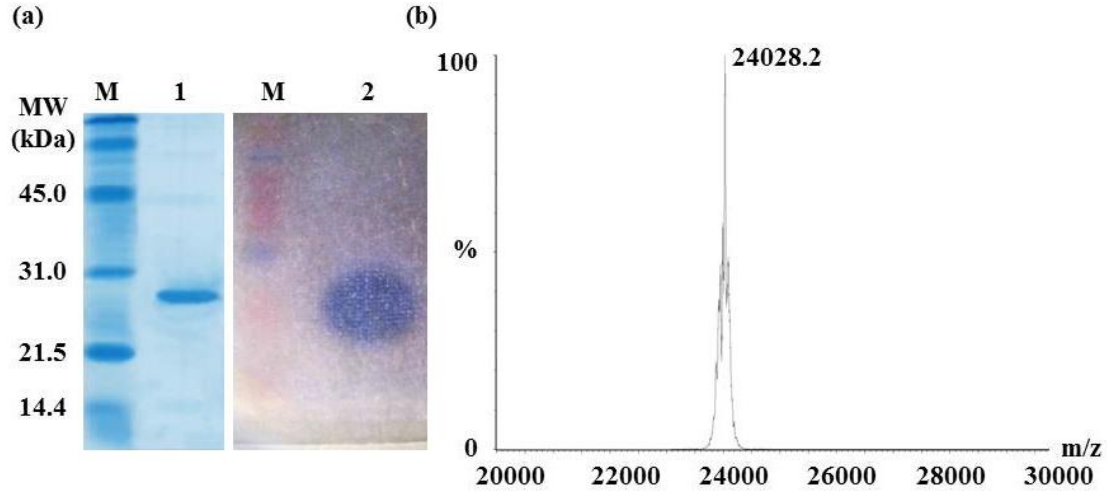

Fig. 3. (a) Tricine-SDS PAGE analysis of purified bacteriocin from *L. paracasei* SD1: (Lane 1) Coomassie brilliant blue stained gel, (Lane 2) gel overlaid with soft agar containing *S. mutans* ATCC 25175, and (Lane M), molecular weight standard, low range (Bio-Rad), and (b) Liquid chromatography mass spectrometry of paracasin SD1; only one peak was obtained corresponding to a molecular mass of 24.03 kDa.

2. Purification of bacteriocin from *L. fermentum* SD11

The bacteriocin was purified using the following steps: 40 % ammonium sulfate precipitation, chloroform extraction, gel filtration chromatography, cation exchange chromatography and PR-HPLC. The results demonstrated that, the first step of purification by gel filtration chromatography could remove most contaminating proteins from the ammonium sulfate precipitated and chloroform extracted bacteriocin. Consequently, the specific antimicrobial activity increased from 18.36 AU/µg (in the supernatant) to 6,198.34AU/µg (in the active fraction) (Table 3). LC-MS analysis of the active faction of gel filtration chromatography was comprised three peaks of proteins with the similar molecular weight (Fig. 4). This active fraction was applied to cation exchanger column. It was revealed that the active fraction of cation exchanger column was also shown three separated peaks (Fig. 5) after LC-MS analysis. Then the purification of bacteriocin was successfully achieved by C_{18} PR-HPLC. This step of purification could remove contaminating proteins. Consequently, the specific activity increased from 18.36 AU/µg (in the supernatant) to 781,250 AU/µg (in the active fraction) (Table 3). The first RP-HPLC run revealed the present of a major peak

eluted at 16-17 min retention time (Fig. 6a). The active fraction was confirmed by a second RP-HPLC run, gave only one active peak eluted at the same retention time (Fig. 6b). The tricine SDS-PAGE and bioautographic analysis revealed one active protein against *S. mutans* ATCC 5175, having an estimated molecular mass of approximately 33 kDa (Fig. 7a), and it was confirmed as having a molecular mass of 33.59 kDa by LC-MS (Fig. 7b). This antimicrobial protein was named as "fermencin SD11".

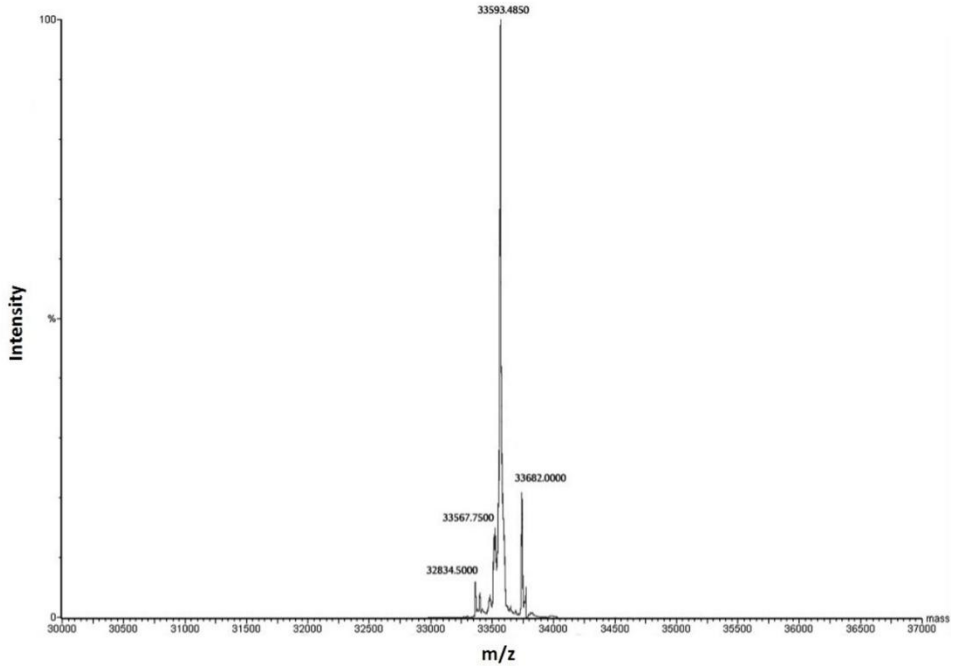

Fig. 4. Liquid chromatography mass spectrometry (LC-MS) of the active fraction produced by *L. fermentum* SD11after purified by gel filtration chromatography. The active faction was comprised three separate peaks of proteins.

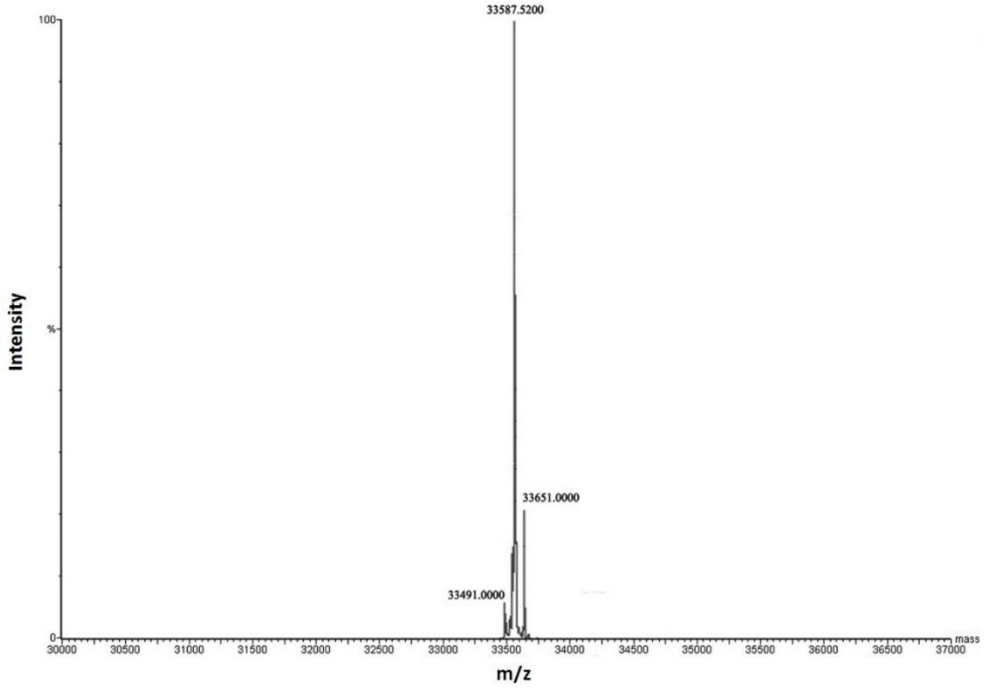

Fig. 5. Liquid chromatography mass spectrometry (LC-MS) of the active fraction produced by *L. fermentum* SD11 after purified by cation exchange chromatography. The active faction was comprised three separate peaks of proteins.

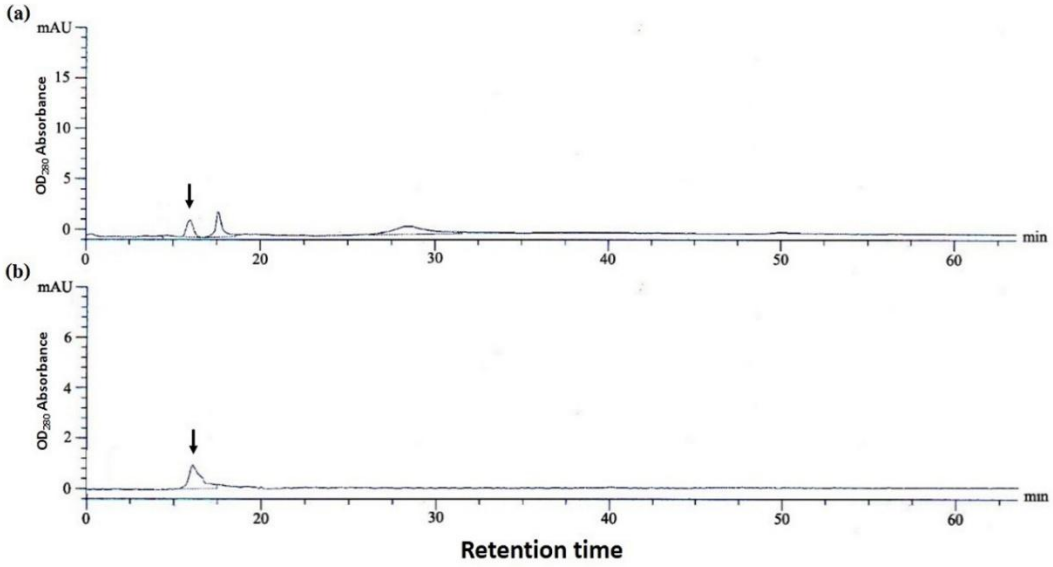

Fig. 6. C_{18} reverse-phase HPLC analysis of bacteriocin produced by *L. fermentum* SD11. Active fraction was obtained after a 16-17 min retention time (a). The purity of the obtained fraction was confirmed with a second RP-HPLC under the same conditions (b).

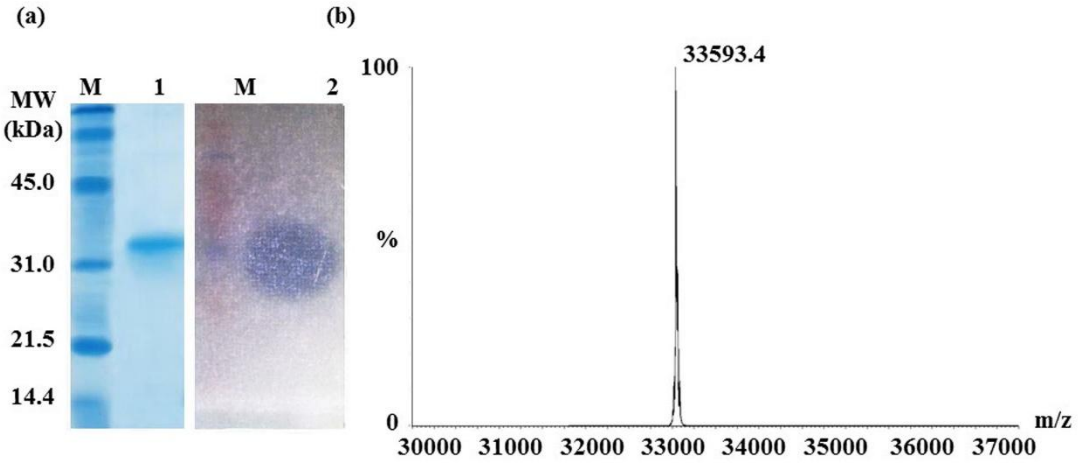

Fig. 7. (a) Tricine-SDS PAGE analysis of purified bacteriocin from *L. fermentum* SD11: Lane 1, Coomassie brilliant blue stained gel; Lane 2, gel overlaid with soft agar containing *S. mutans* ATCC 25175; and Lane M, molecular weight standard (low range of Bio-Rad); and
(b) Liquid chromatography mass spectrometry of fermencin SD11; only one peak was obtained corresponding to a molecular mass of 33.59 kDa.

Characterization of bacteriocin activity

1. Enzyme sensitivity:

Paracasin SD1 and fermencin SD11 were completely inactivated by trypsin and proteinase K. The bacteriocin activity was found to be resistant to α-amylase, catalase, lysozyme and saliva except paracasin SD1, it was relative loss of activity after treatment with lysozyme. (Table 4).

Table 4. Effects of enzyme on paracasin SD1 and fermencin SD11 activity produced by *L. paracasei* SD1 and *L. fermentum* SD11

Effect of enzyme on antimicrobial activity	Antimicrobial activity*	
	paracasin SD1	**fermencin SD11**
α-amylase	+ + +	+ + +
catalase	+ + +	+ + +
lysozyme	+	+ + +
protenase K	-	-
trypsin	-	-
saliva	+ + +	+ + +

Note. **Paracasin SD1**; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + + is antimicrobial activity ≥37,500 ± 0.0 AU/ml, + + is antimicrobial activity ≥25,000 ± 0.0 AU/ml, + is antimicrobial activity ≥12,500 ± 0.0 AU/ml, and - is non-detected antimicrobial activity. All tests were performed in triplicate.

Fermencin SD11; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans.* + + +, antimicrobial activity ≥125,000 ± 0.0 AU/ml; + +, antimicrobial activity ≥13,636.36 ± 0.0 AU/ml; +, antimicrobial activity ≥9,090.9 ± 0.0 AU/ml; -, non-detected antimicrobial activity. All tests were performed in triplicate.

2. pH and heat sensitivity:

The antimicrobial activity of paracasin SD1 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 5.0-6.0. The antimicrobial activity was completely lost at pH value 9.0. Meanwhile, the antimicrobial activity of fermencin SD11 was stable at a wide range of pH levels (3.0-7.0) with optimal activity at pH 5.0 and 6.0. The antimicrobial activity was completely lost at alkaline pH (8.0-9.0) (Table 5).

The antimicrobial activity of paracasin SD1 did not show any detectable loss of activity when heated at 60 °C, and it was a slight decrease of activity at 80 °C. There was stable at

100 °C for 5 min, and no detectable activity observed when it was heated at 120 °C. For fermencin SD11, it was stable between 60-80 °C and retained more than 60 % of its activity after heating at 80 °C for 10 min, and no detectable activity was observed after heating at 100 °C for 10 min (Table 6). It was found that storage of paracasin SD1 and fermencin SD11 for 6 months at 25 °C, 4 °C and -20 °C did not affect its activity.

3. Spectrum of activity:

Paracasin SD1 and fermencin SD11 presented a wide range of inhibitory spectrum against the indicators listed in Table 7. A total of 12 strains belonging to 6 species of Gram-positive, Gram-negative bacteria and *Candida albicans* were examined. The results demonstrated that both of the bacteriocin having the strongest antagonistic activity towards Gram positive bacteria followed by the *Candida* strain. Meanwhile, paracasin SD1 was no effected to *Fusobacterium nucleatum*. (Table 7).

Table 5. Effects of pH on paracasin SD1 and fermencin SD11 activity produced by *L. paracasei* SD1 and *L. fermentum* SD11

Effect of pH on antimicrobial activity	Antimicrobial activity*	
	paracasin SD1	**fermencin SD11**
3.0	+	+
4.0	+ +	+ +
5.0	+ + +	+ + +
6.0	+ + +	+ + +
7.0	+ +	+ +
8.0	+	-
9.0	-	-

Note. **Paracasin SD1**; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + + is antimicrobial activity ≥37,500 ± 0.0 AU/ml, + + is antimicrobial activity ≥25,000 ± 0.0 AU/ml, + is antimicrobial activity ≥12,500 ± 0.0 AU/ml, and - is non-detected antimicrobial activity. All tests were performed in triplicate.

Fermencin SD11; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans.* + + +, antimicrobial activity ≥125,000 ± 0.0 AU/ml; + +, antimicrobial activity ≥13,636.36 ± 0.0 AU/ml; +, antimicrobial activity ≥9,090.9 ± 0.0 AU/ml; -, non-detected antimicrobial activity. All tests were performed in triplicate.

Table 6. Effects of heat treatment on paracasin SD1 and fermencin SD11 activity produced by *L. paracasei* SD1 and *L. fermentum* SD11

Effect of heat treatment on antimicrobial activity	Antimicrobial activity*	
	paracasin SD1	**fermencin SD11**
5 min		
60	+ + +	+ + +
80	+ + +	+ + +
100	+	-
120	-	-
10 min		
60	+ + +	+ + +
80	+ +	+ +
100	-	-
120	-	-
20 min		
60	+ + +	+ + +
80	+	+
100	-	-
120	-	-

Note. **Paracasin SD1**; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + + is antimicrobial activity ≥37,500 ± 0.0 AU/ml, + + is antimicrobial activity ≥25,000 ± 0.0 AU/ml, + is antimicrobial activity ≥12,500 ± 0.0 AU/ml, and - is non-detected antimicrobial activity. All tests were performed in triplicate.

Fermencin SD11; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + +, antimicrobial activity ≥125,000 ± 0.0 AU/ml; + +, antimicrobial activity ≥13,636.36 ± 0.0 AU/ml; +, antimicrobial activity ≥9,090.9 ± 0.0 AU/ml; -, non-detected antimicrobial activity. All tests were performed in triplicate.

Table 7. Bacterial strains used in this study and their sensitivity to paracasin SD1 and fermencin SD11 produced by *L. paracasei* SD1 and *L. fermentum* SD11

Indicator strains	**Antimicrobial activity**	
	paracasin SD1	**fermencin SD11**
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. **Paracasin SD1**; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + + is antimicrobial activity ≥37,500 ± 0.0 AU/ml, + + is antimicrobial activity ≥25,000 ± 0.0 AU/ml, + is antimicrobial activity ≥12,500 ± 0.0 AU/ml, and - is non-detected antimicrobial activity. All tests were performed in triplicate.

Fermencin SD11; antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + +, antimicrobial activity ≥125,000 ± 0.0 AU/ml; + +, antimicrobial activity ≥13,636.36 ± 0.0 AU/ml, and +, antimicrobial activity ≥9,090.9 ± 0.0 AU/ml. All tests were performed in triplicate.

Determination of optimum growth and bacteriocin production by *L. paracasei* SD1 and *L. fermentum* SD11

Initially, the experiments were conducted to explore the optimum growth and bacteriocin production by incubating at 37 °C under aerobic or anaerobic conditions with different pHs of 5.0, 6.0, 7.0 and 8.0.

Results demonstrated that the increase of bacteriocin activity depended on the increase of the growth rate. After considering all the different conditions used, the maximum growth rate (approximately 0.15/h) and bacteriocin production (approximately 496 AU/h) of *L. paracasei* SD1 were achieved between 6-12 h of culturing in MRS broth at 37 °C. Also to *L. fermentum* SD11, the maximum growth rate (approximately 0.11/h) and bacteriocin production (approximately 293 AU/h) were achieved between 6-12 h of culturing in MRS broth at 37 °C. Both pHs, 5.0 and 6.0 of two strains, gave significantly higher growth rate and bacteriocin production of than pH 7.0. There were no significant differences of growth rate and bacteriocin production when culturing under aerobic or anaerobic conditions (Fig. 8-9). No bacterial growth and bacteriocin activity was found under pH 8.0 (data not shown).

After trials for the optimum temperature of both strains at 25, 37 and 40 °C, at 37 °C and 40 °C provided the higher bacterial growth and bacteriocin production compared to 25 °C (Fig. 10-11). The results indicate that the optimum conditions for growth and bacteriocin production of *L. paracasei* SD1 and *L. fermentum* SD11 were cultured with acidic pHs of 5.0-6.0 at 37 °C or 40 °C under aerobic or anaerobic conditions for 12 h.

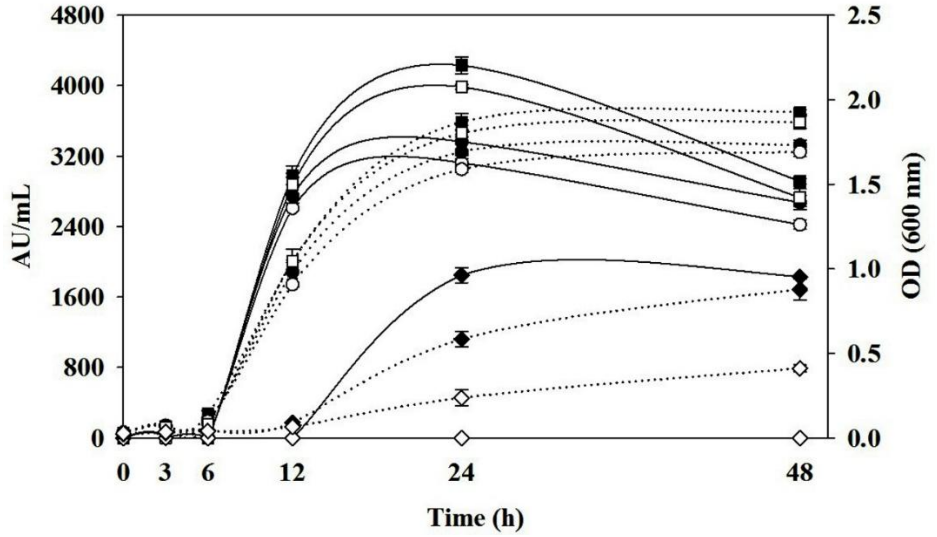

Fig. 8. The bacteriocin activity (solid line) and growth (dotted line) of *L. paracasei* SD1 in MRS broth at 37 °C with different pHs: pH 5.0 (●), pH 6.0 (■) and pH 7.0 (♦) under aerobic condition; pH 5.0 (○), pH 6.0 (□) and pH 7.0 (◊) under anaerobic condition.

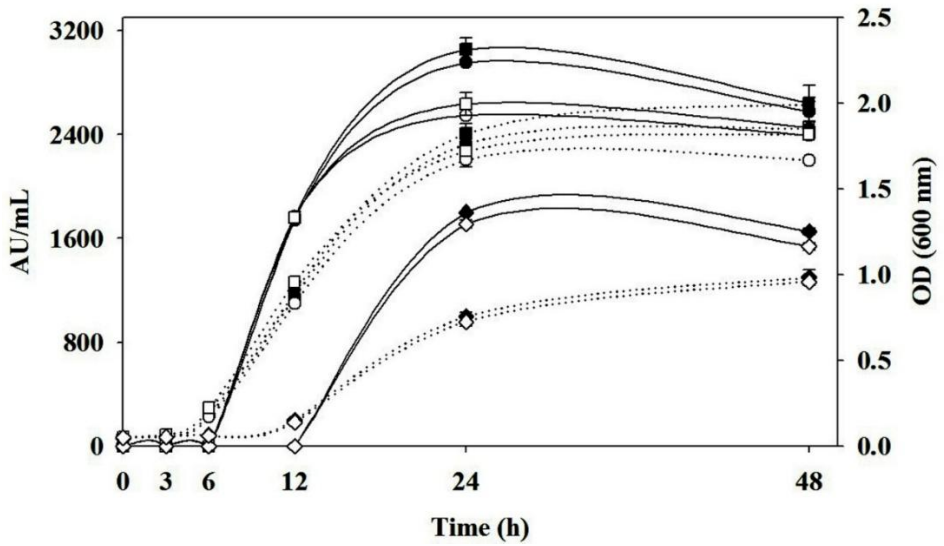

Fig. 9. The bacteriocin activity (solid line) and growth (dotted line) of *L. fermentum* SD11 in MRS broth at 37 °C with different pHs: pH 5.0 (●), pH 6.0 (■) and pH 7.0 (♦) under aerobic condition; pH 5.0 (○), pH 6.0 (□) and pH 7.0 (◊) under anaerobic condition.

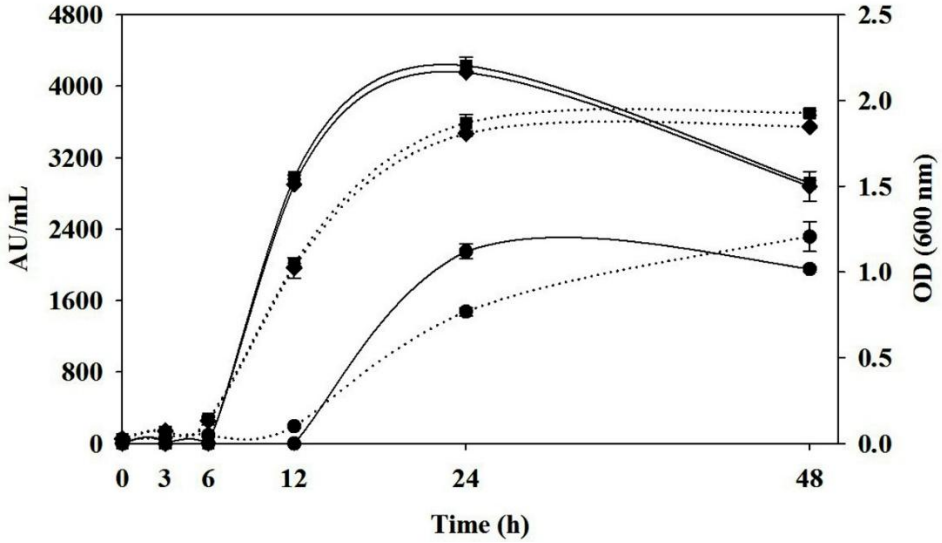

Fig. 10. The bacteriocin activity (solid line) and growth (dotted line) of *L. paracasei* SD1 in MRS broth at pH 6.0 under aerobic condition with different temperatures: 25 °C (●), 37 °C (■) and 40 °C (♦).

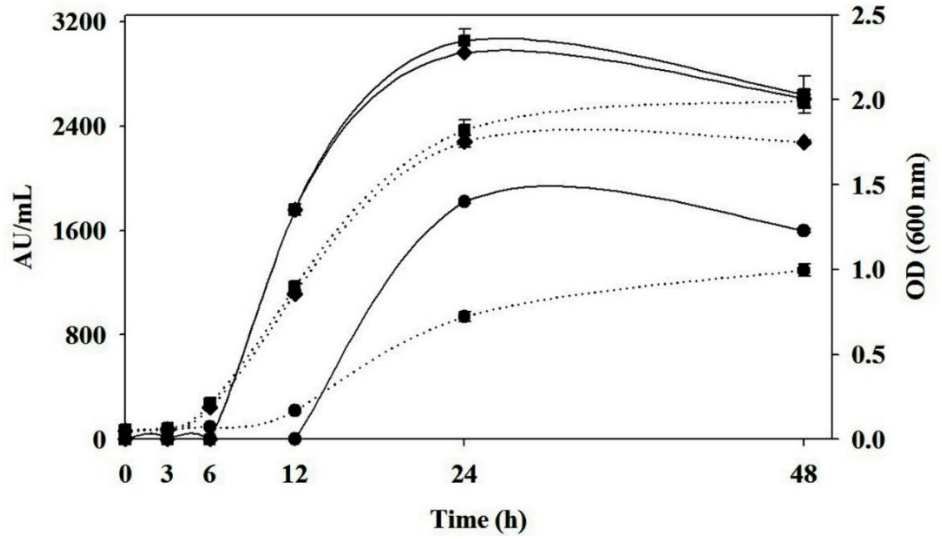

Fig. 11. The bacteriocin activity (solid line) and growth (dotted line) of *L. fermentum* SD11 in MRS broth at pH 6.0 under aerobic condition with different temperatures: 25 °C (●), 37 °C (■) and 40 °C (♦).

4. DISCUSSION

Screening examination for selection of bacterial strains

This work describes the purification, characterization and optimum conditions for bacteriocin production of an antimicrobial protein produced by *Lactobacillus,* a strains isolated from the human oral cavity. A previous 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 been identified.

In this study, a total of 90 strains of *Lactobacillus*, from caries and caries free subjects were screened for antimicrobial production against *S. mutans* ATCC 25175, and a bacteriocin-like antagonism against an indicator strain was observed in the supernatants and sonicated cell free extract of all strains tested. However, after adjusting each culture filtrate to pH 6.5, the supernatants of only two strains; *L. paracasei* SD1 and *L. fermentum* SD11 isolated from caries free subjects, still showed inhibitory activity. This concurs with earlier findings confirmed that the probiotic property is strain specific.[113] Moreover, this study supports the previous reports[6] in which inhibitory activity of certain *Lactobacillus* strains could be found in subjects with good oral health. It was demonstrated that the effective inhibition of *Lactobacillus* against cariogenic *S. mutans* strains was found to relate to caries free subjects. Koll-Klais *et al*.[5] also showed that *Lactobacillus gasseri* from healthy persons had a stronger inhibitory activity against periodontogenic *Aggregatibacter* (*Actinobacillus) actinomycetemcomitans* than did the strains from patients with chronic periodontitis.

L. paracasei SD1 and *L. fermentum* SD11 were exhibited partial loss activity after the catalase treatment indicated that there were hydrogen peroxide in the supernatants. The active compound also had a proteinaceous nature because there activity were lost after treatment with proteinase K. The organic solvent could not remove any antimicrobial protein from the aqueous phase of the culture supernatant, which indicated that the bacteriocins produced from *L. paracasei* SD1 and *L. fermentum* SD11 have a hydrophilic character.

Purification of the bacteriocin

In this study were purified of two bacteriocins named “paracasin SD1”, produced by *L. paracasei* SD1, and “fermencin SD11” produced by *L. fermentum* SD11. It was demonstrated that paracasin SD1 was purified from cell-free supernatant by precipitation with ammonium sulfate, chloroform extraction and gel filtration chromatography. Meanwhile, fermencin SD11 was purified from cell-free supernatant by ammonium sulfate precipitation, chloroform extraction, gel filtration chromatography and reverse-phase HPLC. In this study cation exchanger column was also performed, but it could not purify fermencin SD11 in one peak. LC-MS analysis of the active faction of cation exchanger column showed three peaks of proteins. It may explain by that those proteins had the same cationic character of the molecules.

The chloroform extraction of the ammonium precipitated protein increased the antimicrobial activity, suggesting a high degree of purification of paracasin SD1 and fermencin SD11. 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.[9] This was supported by a study of Contreras *et al*.[112] 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.[112]

The molecular mass of paracasin SD1 and fermencin SD11 were determined by LC-MS, and they were found to be 24.03 and 33.59 kDa respectively. To our knowledge, paracasin SD1 and fermencin SD11 might be a novel bacteriocin produced by *L. paracasei* SD1 and *L. fermentum* SD11 since its molecular mass differs from previous bacteriocins reported by others.[17-19, 96, 114, 115]

Several bacteriocins of *L. paracasei* and *L. fermentum* 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, *L. paracasei* HD1.7 isolated from traditional Chinese fermented vegetable food, and *L. paracasei* HL32 isolated from an intestinal tract that, contained peptides sized 2.4, 7.0, 11.0 and 56 kDa, respectively.[17, 19, 96, 116] Of those, two strains, *L. fermentum* L23 and *L. fermentum* CS57, originated from vagina and one strain, *L. fermentum* Beijerinck CCRC 14018, originated from food that, contained peptides sized < 7, > 30 and 1-3 kDa, respectively.[18, 115, 117] It indicated that

Lactobacillus strains from different sources could produce various sizes of bacteriocin proteins. However, the knowledge of antimicrobial proteins or bacteriocins produced by *L. paracasei* and *L. fermentum* strains having an oral origin is still limited.

Characterization of bacteriocin activity

Regarding the characterization of paracasin SD1 and fermencin SD11, 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 and fermencin SD11 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.[118] However, why *Fusobacterium 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 and fermencin SD11could be active in a broad pH range, although maximum activity was detected within a narrow pH range of 5.0-6.0. This finding was in agreement with previous studies reporting that bacteriocin activity from LAB was most stable when acidic and neutral.[18, 115, 117]

The antimicrobial activity of paracasin SD1 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 the other hand, fermencin SD11 was stable between 60-80 °C and retained more than 60 % of its activity after heating at 80 °C for 10 min. No detectable activity was observed after heating at 100 °C for 10 min, suggesting that the antimicrobial substance was a heat labile protein. According to Klaenhammer's classification, fermencin SD11 should be classified into bacteriocin class III due to its properties of large molecules (> 30 kDa) and sensitivity to heat.[8] In addition, paracasin SD1 and fermencin SD11 were stable and unchanged antimicrobial activity over long periods of storage.

Concerning sensitivity tests to various enzymes, it was found that paracasin SD1 and fermencin SD11 were inactivated by trypsin, a proteolytic enzyme, which usually presents in the small intestine. However, the antimicrobial activity of paracasin SD1 and fermencin SD11 were not

sensitive to α-amylase, catalase, lysozyme and whole saliva, indicating that paracasin SD1 and fermencin SD11 may be practical to use as a topical application in the oral cavity.

Determination of optimum growth and bacteriocin production of *L. paracasei* SD1 and *L. fermentum* SD11

Traditionally, optimization of bacteriocin production has been performed by physiological and metabolic control. A number of studies have reported that physicochemical factors, e.g. pH, temperature and aeration, have a dramatic effect on the production of bacteriocins.[13-16] Thus, the optimization for biomass production to improve the yield of bacteriocins is necessary.

In this study, our finding demonstrated that the increase of bacteriocin activity depended on the increase of the growth rate. After considering all the different conditions used, the maximum growth rate and bacteriocin production of *L. paracasei* SD1 and *L. fermentum* SD11 were achieved between 6-12 h of culturing in MRS broth at 37 °C. Both pHs, 5.0 and 6.0 of two strains, gave significantly higher growth rate and bacteriocin production than pH 7.0, and pH 8.0 not detected bacterial growth and bacteriocin production. There were no significant differences of growth rate and bacteriocin production when culturing under aerobic or anaerobic conditions. It indicated that the maximum paracasin SD1 and fermencin SD11 occurred during the exponential phase when bacterium was actively dividing. This concurs with earlier findings where bacteriocin activity generally increased rapidly during the exponential growth phase.[119-121] Only in a few cases could bacteriocin production occur during the stationary phase. Bacteriocin activity frequently decreased in the late stages of the culture; this may be due to proteolytic degradation, aggregation or adsorption to cells.[122]

Application of oral *Lactobacillus* and their bacteriocins

The application of probiotics to promote for good oral health received an attention from researchers. The results to date have shown that certain probiotic strains are useful in the prevention and treatment of oral infections such as dental caries and periodontitis. *L. rhamnosus* GG is one of the probiotics which has been widely studies in dental field. The studies have shown that *L. rhamnosus* GG has benefits in the prevention of oral diseases.[25, 26] Our previous studies reported that *L. paracasei* SD1, a selected strain had a bacteriocin protein sized 24.03 kDa, which showed an antimicrobial activity against various oral pathogens.[123] The strain has been applied into various products e.g. milk powder, yogurt and fruit juices[124, 125], and products that are contained probiotics have been studied clinical trials. The studies has proven that *L. paracasei* SD1 is a useful probiotic strain for caries prevention due to the significant reduction of cariogenic bacteria (mutans streptococci) and less caries lesions among the volunteers who received the probiotic milk powder compared to the controls. [126-128] *L. fermentum* SD11, another one selected strain in this study, may be a potential probiotic for oral health, because the strain had a bacteriocin protein sized 33.59 kDa which also showed an antimicrobial activity against various oral pathogens. It would require for further clinical trials.

Many studies have been carefully selected and purified bacteriocins for applications in food technology.[71, 129, 130] Thus, bacteriocins may be a potential proteins for replacing antibiotics in the treatment of the pathogens. In this study, paracasin SD1 and fermencin SD11 exhibit broad spectrum of antibacterial activity against oral photogenes. Hence, the application of both bacteriocins as products for use in the oral cavity may be an effective approach to control oral pathogens that would require for further trials.

5. CONCLUSION

L. paracasei SD1 and *L. fermentum* SD11 were isolated from caries free subjects. The two strains could produce an antimicrobial proteins, from which later the purified proteins, namely paracasin SD1 and fermencin SD11 respectively. Paracasin SD1 was purified from cell-free supernatant by precipitation with ammonium sulfate, chloroform extraction and gel filtration chromatography. Meanwhile, fermencin SD11 was purified from cell-free supernatant by ammonium sulfate precipitation, chloroform extraction, gel filtration chromatography and reverse-phase HPLC. The chloroform extraction of the ammonium precipitated protein increased the antimicrobial activity of both bacteriocins. The molecular mass of paracasin SD1 and fermencin SD11 were determined by LC-MS, and they were found to be 24.03 and 33.59 kDa respectively.

Regarding the characterization of paracasin SD1 and fermencin SD11, a broad spectrum of antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and even anti-*Candida* was found. The bacteriocin activity of paracasin SD1 and fermencin SD11 were completely inactivated by trypsin and proteinase K. The activity of both bacteriocins were found to be resistant to α-amylase, catalase, lysozyme and whole saliva except paracasin SD1, it was relative loss of activity after treatment with lysozyme. Paracasin SD1 and fermencin SD11could be active in a broad pH range, although maximum activity was detected within a narrow pH range of 5.0-6.0. The antimicrobial activity of paracasin SD1 appeared to be relative heat-stable, since inhibitory activity was maintained upon heating at 100 °C for 5 min. In the other hand, fermencin SD11 was stable between 60-80 °C and retained more than 60 % of its activity after heating at 80 °C for 10 min, and no detectable activity was observed after heating at 100 °C for 10 min.

The optimum conditions for growth and bacteriocin production of *L. paracasei* SD1 and *L. fermentum* SD11 were cultured with acidic pHs of 5.0-6.0 at 37 °C or 40 °C under aerobic or anaerobic conditions for 12 h. In addition, it was found that storage of paracasin SD1 and fermencin SD11 for 6 months at 25 °C, 4 °C and -20 °C did not affect their activity. It is promising that *L. paracasei* SD1 and *L. fermentum* SD11 and their bacteriocin may be an alternative approach for promoting oral health or prevention of oral diseases e.g. dental caries and periodontitis, that would require further clinical trials.

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. ***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. Klaenhammer TR. Genetics of bacteriocins produced by lactic acid bacteria. ***FEMS Microbiol Rev*** 1993; 12: 39-85.
9. Parada LJ, Caron CR, Medeiros AB, Soccol CR. Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. ***Braz Arch Biol Techn*** 2007; 50: 521-42.
10. Deegan LH, Cotter PD, Hill C, Ross P. Bacteriocins: biological tools for bio-preservation and shelf-life extension. ***Int Dairy J*** 2006; 16: 1058–71.
11. Dover SE, Aroutcheva AA, Faro S, Chikindas ML. Safety study of an antimicrobial peptide lactocin 160, produced by the vaginal *Lactobacillus rhamnosus*. ***Infect Dis Obstet Gynecol*** 2007: 1-6.
12. Reddy KV, Aranha C, Gupta SM, Yedery RD. Evaluation of antimicrobial peptide nisin as a safe vaginal contraceptive agent in rabbits: in vitro and in vivo studies. ***Reproduction*** 2004; 128: 117-26.

13. Biswas SR, Ray P, Johnson MC, Ray B. Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. ***Appl Environ Microbiol*** 1991; 57: 1265-7.

14. Leroy F, de Vuyst L. Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. ***Appl Environ Microbiol*** 1999; 65: 974-81.

15. Herranz C, Martinez JM, Rodriguez JM, Hernandez PE, Cintas LM. Optimization of enterocin P production by batch fermentation of *Enterococcus faecium* P13 at constant pH. ***Appl Microbiol Biotechnol*** 2001; 56: 378-83.

16. Parente E, Ricciardi A. Influence of pH on the production of enterocin 1146 during batch fermentation. ***Lett Appl Microbiol*** 1994; 19: 12-5.

17. 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.

18. Pascual LM, Daniele MB, Giordano W, Pajaro MC, Barberis IL. Purification and partial characterization of novel bacteriocin L23 produced by *Lactobacillus fermentum* L23. ***Curr Microbiol*** 2008; 56: 397-402.

19. 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.

20. Palacios J, Vignolo G, Farias ME, de Ruiz Holgado AP, Oliver G, Sesma F. Purification and amino acid sequence of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. ***Microbiol Res*** 1999; 154: 199-204.

21. Bogovic-Matijasic B, Rogelj I, Nes IF, Holo H. Isolation and characterization of two bacteriocins of *Lactobacillus acidophilus* LF221. ***Appl Microbiol Biotechnol*** 1998; 49: 606-12.

22. Amortegui J, Rodriguez-Lopez A, Rodriguez D, Carrascal AK, Almeciga-Diaz CJ, Melendez Adel P, *et al*. Characterization of a new bacteriocin from *Lactobacillus plantarum* LE5 and LE27 isolated from ensiled corn. ***Appl Biochem Biotechnol*** 2014; 172: 3374-89.

23. Sahoo TK, Jena PK, Patel AK, Seshadri S. Purification and molecular characterization of the novel highly potent bacteriocin TSU4 produced by *Lactobacillus animalis* TSU4. ***Appl Biochem Biotechnol*** 2015; 177: 90-104.

24. 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.

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. Ahola AJ, Yli-Knuuttila H, Suomalainen T, Poussa T, Ahlstrom A, Meurman JH, *et al*. Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. ***Arch Oral Biol*** 2002; 47: 799-804.

27. 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.

28. Koll P, Mandar R, Marcotte H, Leibur E, Mikelsaar M, Hammarstrom L. Characterization of oral lactobacilli as potential probiotics for oral health. ***Oral Microbiol Immunol*** 2008; 23: 139-47.

29. Gilliland SE. Health and nutritional benefits from lactic acid bacteria. ***FEMS Microbiol Rev*** 1990; 7: 175-88.

30. Reid G, Bruce AW, McGroarty JA, Cheng KJ, Costerton JW. Is there a role for lactobacilli in prevention of urogenital and intestinal infections? ***Clin Microbiol Rev*** 1990; 3: 335-44.

31. Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld JH. Overview of gut flora and probiotics. ***Int J Food Microbiol*** 1998; 41: 85-101.

32. Schillinger U, Lucke FK. Antibacterial activity of *Lactobacillus sake* isolated from meat. ***Appl Environ Microbiol*** 1989; 55: 1901-6.

33. Prakash S, Jones ML. Artificial cell therapy: new strategies for the therapeutic delivery of live bacteria. ***J Biomed Biotechnol*** 2005; 2005: 44-56.

34. Saraf K, Shashikanth MC, Priy T, Sultana N, Chaitanya NC. Probiotics--do they have a role in medicine and dentistry? ***J Assoc Physicians India*** 2010; 58: 488-90, 95-6.

35. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. ***J Clin Microbiol*** 2005; 43: 5721-32.

36. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. ***Periodontology 2000*** 1994; 5: 78-111.

37. Badet C, Thebaud NB. Ecology of lactobacilli in the oral cavity: a review of literature. ***Open Microbiol J*** 2008; 2: 38-48.

38. Ahumada MdC, Bru E, Colloca M, Lopez M, Nader-Macias M. Lactobacilli Isolation from dental plaque and saliva of a group of patients with caries and characterization of their surface properties. ***Anaerobe*** 2001; 7: Colloca ME71-7.

39. Ishikawa H, Aiba Y, Nakanishi M, Oh-Haashi Y, Koga Y. Suppression of periodontal pathogenic bacteria in the saliva of human by the administration of *lactobacillus salivarius*II2711. ***J Jap Soc Periodontol*** 2003; 45: 105-12.

40. Petti S, Tarsitani G, D'Arca AS. A randomized clinical trial of the effect of yoghurt on the human salivary microflora. ***Arch Oral Biol*** 2001; 46: 705-12.

41. Busscher HJ, Mulder AF, van der Mei HC. In vitro adhesion to enamel and in vivo colonization of tooth surfaces by lactobacilli from a bio-yoghurt. ***Caries Res*** 1999; 33: 403-4.

42. Chung J, Ha ES, Park HR, Kim S. Isolation and characterization of *Lactobacillus* species inhibiting the formation of *Streptococcus mutans* biofilm. ***Oral Microbiol Immunol*** 2004; 19: 214-6.

43. Blom H, Mortvedt C. Anti-microbial substances produced by food associated micro-organisms. ***Biochem Soc Trans*** 1991; 19: 694-8.

44. Pederson CS. Microbiology in food fermentations. AVI Publishing Co, Westport, Conference, pp 1971: 283.

45. De Vuyst L, Vandamme EJ. Antimicrobial potential of lactic acid bacteria. In: De Vuyst, L., Vandamme, E.J. (Eds.), bacteriocins of lactic acid bacteria. Blackie Academic and Professional, London, pp 1994: 91-142.

46. Ouwehand A, Vesterlund S. Antimicrobial components from lactic acid bacteria. Microbiological and Functional aspects, third edition Marcel Dekker, New York, pp 2004: 375-95.

47. Ferreira CL, Gilliland SE. Bacteriocin involved in premature death of *Lactobacillus acidophilus* NCFM during growth at pH 6. ***J Dairy Sci*** 1988; 71: 306-15.

48. Caplice E, Fitzgerald GF. Food fermentations: role of microorganisms in food production and preservation. ***Int J Food Microbiol*** 1999; 50: 131-49.

49. Davies JE. Origins, acquisition and dissemination of antibiotic resistance determinants. ***Ciba Found Symp*** 1997; 207: 15-27; discussion -35.

50. Sheu CW, Konings WN, Freese E. Effects of acetate and other short-chain fatty acids on sugar and amino acid uptake of *Bacillus subtilis*. ***J Bacteriol*** 1972; 111: 525-30.

51. Sandine WE, Muralidhara KS, Elliker PR, England DC. Lactic acid bacteria in food and health: a review with special reference to enteropathogenic *Esherichia coli* as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. ***J Milk Food Technol*** 1972; 35: 691-702.

52. Speck ML. Interactions among lactobacilli and man. ***J Dairy Sci*** 1976; 59: 338-43.

53. Gilliland SE, Speck ML. Interactions of food starter cultures and foodborne pathogens: lactic streptococci versus staphylococci and salmonellae. ***J Milk Food Technol*** 1972; 35: 307-10.

54. Haller D, Colbus H, Ganzle MG, Scherenbacher P, Bode C, Hammes WP. Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: a comparative in vitro study between bacteria of intestinal and fermented food origin. ***Syst Appl Microbiol*** 2001; 24: 218-26.

55. Alakomi HL, Skytta E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM. Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. ***Appl Environ Microbiol*** 2000; 66: 2001-5.

56. Aiba Y, Suzuki N, Kabir AM, Takagi A, Koga Y. Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. ***Am J Gastroenterol*** 1998; 93: 2097-101.

57. Lavermicocca P, Valerio F, Lonigro SL, Di Leo A, Visconti A. Antagonistic activity of potential probiotic lactobacilli against the ureolytic pathogen *Yersinia enterocolitica*. ***Curr Microbiol*** 2008; 56: 175-81.

58. Sgouras D, Maragkoudakis P, Petraki K, Martinez-Gonzalez B, Eriotou E, Michopoulos S, *et al*. In vitro and in vivo inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota. ***Appl Environ Microbiol*** 2004; 70: 518-26.

59. Condon S. Responses of lactic acid bacteria to oxygen. ***FEMS Microbiol Rev*** 1987; 46: 269-80.

60. Dahiya RS, Speck ML. Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. ***J Dairy Sci*** 1968; 51: 1568-72.

61. Fontaine EA, Claydon E, Tayler-Robinson D. Lactobacilli from women with of without bacterial vaginosis and observations on the significance of hydrogen peroxide. ***Microb Ecol Health Dis*** 1996; 9: 135-41.

62. Martin R, Jimenez E, Olivares M, Marin ML, Fernandez L, Xaus J, *et al. Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. ***Int J Food Microbiol*** 2006; 112: 35-43.

63. Otero MC, Nader-Macias ME. Inhibition of *Staphylococcus aureus* by H2O2-producing *Lactobacillus gasseri* isolated from the vaginal tract of cattle. ***Anim Reprod Sci*** 2006; 96: 35-46.

64. Pridmore RD, Pittet AC, Praplan F, Cavadini C. Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. ***FEMS Microbiol Lett*** 2008; 283: 210-5.

65. Glynn AA, O'Donnell ST, Molony DC, Sheehan E, McCormack DJ, O'Gara JP. Hydrogen peroxide induced repression of icaADBC transcription and biofilm development in *Staphylococcus epidermidis*. ***J Orthop Res*** 2009; 27: 627-30.

66. Lindgren SE, Dobrogosz WJ. Antagonistic activities of lactic acid bacteria in food and feed fermentations. ***FEMS Microbiol Rev*** 1990; 7: 149-63.

67. King ADJ, Nagel CW. Influence of carbon dioxide on the growth of spoilage bacteria. ***Lebensm Wiss Technol*** 1975; 40: 362-6.

68. Jay JM. Antimicrobial properties of diacetyl. ***Appl Environ Microbiol*** 1982; 44: 525-32.

69. Jyoti B, Suresh AK, Venkatesh KV. Diacetyl production and growth of *Lactobacillus rhamnosus* on multiple substrates. ***World J Microbiol Biotechnol*** 2003; 19: 509–15.

70. Ross RP, Morgan S, Hill C. Preservation and fermentation: past, present and future. ***Int J Food Microbiol*** 2002; 79: 3-16.

71. Chen H, Hoover DG. Bacteriocins and their food applications. ***Comp Rev Food Sci Food Saf*** 2003; 2: 82-100.

72. Riley MA, Wertz JE. Bacteriocins: evolution, ecology, and application. ***Annu Rev Microbiol*** 2002; 56: 117-37.

73. Gratia A. Sur un remarquable exemple d'antagonisme entre deux souches de colibacille. ***Compt Rend Soc Biol*** 1925; 93: 1040–1.

74. Tagg JR, Dajani AS, Wannamaker LW. Bacteriocins of Gram-positive bacteria. ***Bacteriol Rev*** 1976; 40: 722-56.

75. Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. ***Lancet Infect Dis*** 2001; 1: 156-64.

76. Zasloff M. Innate immunity, antimicrobial peptides, and protection of the oral cavity. ***Lancet*** 2002; 360: 1116-7.

77. Papagianni M. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. ***Biotechnol Adv*** 2003; 21: 465-99.

78. Kanatani K, Oshimura M. Plasmid-associated bacteriocin production by a *Lactobacillus plantarum* strain. ***Biosci Biotechnol Biochem*** 1994; 58: 2084-6.

79. Holck AL, Axelsson L, Huhne K, Krockel L. Purification and cloning of sakacin 674, a bacteriocin from *Lactobacillus sake* Lb674. ***FEMS Microbiol Lett*** 1994; 115: 143-9.

80. Upreti GC, Hinsdill RD. Isolation and characterization of a bacteriocin from a homofermentative *Lactobacillus*. ***Antimicrob Agents Chemother*** 1973; 4: 487-94.

81. Ennahar S, Sonomoto K, Ishizaki A. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. ***J Biosci Bioeng*** 1999; 87: 705-16.

82. Fimland G, Pirneskoski J, Kaewsrichan J, Jutila A, Kristiansen PE, Kinnunen PK, *et al*. Mutational analysis and membrane-interactions of the beta-sheet-like N-terminal domain of the pediocin-like antimicrobial peptide sakacin P. ***Biochim Biophys Acta*** 2006; 1764: 1132-40.

83. Bilkova A, Sepova HK, Bilka F, Balazova A. Bacteriocins produced by lactic acid bacteria. ***Ceska Slov Farm*** 2011; 60: 65-72.

84. Moreno I, Lerayer ASL, Leitao MFF. Detection and characterization of bacteriocin producing *Lactococcus lactis* strains. ***Rev Microbiol*** 1999; 30: 130-6.

85. Tahara T, Kanatani K. Isolation, partial characterization and mode of action of acidocin J1229, a bacteriocin produced by *Lactobacillus acidophilus* JCM 1229. ***J Appl Bacteriol*** 1996; 81: 669-77.

86. Lakshminarayanan B, Guinane CM, O'Connor PM, Coakley M, Hill C, Stanton C, *et al*. Isolation and characterization of bacteriocin-producing bacteria from the intestinal microbiota of elderly Irish subjects. ***J Appl Microbiol*** 2013; 114: 886-98.

87. Ndlovu B, Schoeman H, Franz CM, du Toit M. Screening, identification and characterization of bacteriocins produced by wine-isolated LAB strains. ***J Appl Microbiol*** 2015; 118: 1007-22.

88. Yang R, Johnson MC, Ray B. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. ***Appl Environ Microbiol*** 1992; 58: 3355-9.

89. Garver KI, Muriana PM. Purification and partial amino acid sequence of curvaticin FS47, a heat-stable bacteriocin produced by *Lactobacillus curvatus* FS47. ***Appl Environ Microbiol*** 1994; 60: 2191-5.

90. Muriana PM, Klaenhammer TR. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. ***Appl Environ Microbiol*** 1991; 57: 114-21.

91. Nissen-Meyer J, Larsen AG, Sletten K, Daeschel M, Nes IF. Purification and characterization of plantaricin A, a *Lactobacillus plantarum* bacteriocin whose activity depends on the action of two peptides. ***J Gen Microbiol*** 1993; 139: 1973-8.

92. Larsen AG, Vogensen FK, Josephsen J. Antimicrobial activity of lactic acid bacteria isolated from sour doughs: purification and characterization of bavaricin A, a bacteriocin produced by *Lactobacillus bavaricus* MI401. ***J Appl Bacteriol*** 1993; 75: 113-22.

93. Busarcevic M, Kojic M, Dalgalarrondo M, Chobert JM, Haertle T, Topisirovic L. Purification of bacteriocin LS1 produced by human oral isolate *Lactobacillus salivarius* BGHO1. ***Oral Microbiol Immunol*** 2008; 23: 254-8.

94. Song DF, Zhu MY, Gu Q. Purification and characterization of plantaricin ZJ5, a new bacteriocin produced by *Lactobacillus plantarum* ZJ5. ***PloS One*** 2014; 9: e105549.

95. Todorov SD, van Reenen CA, Dicks LM. Optimization of bacteriocin production by *Lactobacillus plantarum* ST13BR, a strain isolated from barley beer. ***J Gen Appl Microbiol*** 2004; 50: 149-57.

96. 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.

97. Ambalam P, Prajapati JB, Dave JM, Nair BM, Ljungh A, Vyas BRM. Isolation and characterization of antimicrobial proteins produced by a potential probiotics strain of human *Lactobacillus rhamnosus* 231 and its effect on selected human pathogens and food spoilage organisms. ***Microb Ecol Health Dis*** 2009; 21: 211-20.

98. Deraz SF, Karlsson EN, Hedstrom M, Andersson MM, Mattiasson B. Purification and characterisation of acidocin D20079, a bacteriocin produced by *Lactobacillus acidophilus* DSM 20079. ***J Biotechnol*** 2005; 117: 343-54.

99. Moretro T, Aasen IM, Storro I, Axelsson L. Production of sakacin P by *Lactobacillus sakei* in a completely defined medium. ***J Appl Microbiol*** 2000; 88: 536-45.

100. Barefoot SF, Klaenhammer TR. Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. ***Antimicrob Agents Chemother*** 1984; 26: 328-34.

101. Parente E, Ricciardi A, Addario G. Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC during batch fermentation. ***Appl Microbiol Biotechnol*** 1994; 41: 388-94.

102. Tahara T, Kanatani K, Yoshida K, Miura H, Sakamoto M, Oshimura M. Purification and some properties of acidocin 8912, a novel bacteriocin produced by *Lactobacillus acidophilus* TK8912. ***Biosci Biotechnol Biochem*** 1992; 56: 1212-5.

103. De Vuyst L, Vandamme EJ. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. ***J Gen Microbiol*** 1992; 138: 571-8.

104. Joerger MC, Klaenhammer TR. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. ***J Bacteriol*** 1986; 167: 439-46.

105. Lyon WJ, Glatz BA. Isolation and purification of propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoenii*. ***Appl Environ Microbiol*** 1993; 59: 83-8.

106. Schved F, Lalazar A, Henis Y, Juven BJ. Purification, partial characterization and plasmid-linkage of pediocin SJ-1, a bacteriocin produced by *Pediococcus acidilactici*. ***J Appl Bacteriol*** 1993; 74: 67-77.

107. 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.

108. 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.

109. Chumchalova J, Stiles J, Josephsen J, Plockova M. Characterization and purification of acidocin CH5, a bacteriocin produced by *Lactobacillus acidophilus* CH5. ***J Appl Microbiol*** 2004; 96: 1082-9.

110. 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.

111. Farias LM, Totola AH, Miranda CM, Carvalho MA, Damasceno CA, Tavares CA, *et al*. Extraction, partial purification and characterization of a bacteriocin (fragicilin) produced by a strain of *Bacteroides fragilis* isolated from *Callithrix penicillata*. ***Res Microbiol*** 1994; 145: 9-16.

112. 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.

113. FAO/WHO. Guidelines for the evaluation of probiotics in food. Food and Agricultural Organization of the United Nations and World Health Organization Working Group Report 2002.

114. 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.

115. Yan TR, Lee CS. Characterization of a partially purified bacteriocin, fermencin B, from *Lactobacillus fermentum*. ***Biotechnol Lett*** 1997; 19: 741-4.

116. 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.

117. Sabia C, Anacarso I, Bergonzini A, Gargiulo R, Sarti M, Condo C, *et al*. Detection and partial characterization of a bacteriocin-like substance produced by *Lactobacillus fermentum* CS57 isolated from human vaginal secretions. ***Anaerobe*** 2014; 26: 41-5.

118. 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.

119. Juarez Tomas MS, Bru E, Wiese B, de Ruiz Holgado AA, Nader-Macias ME. Influence of pH, temperature and culture media on the growth and bacteriocin production by vaginal *Lactobacillus salivarius* CRL 1328. ***J Appl Microbiol*** 2002; 93: 714-24.

120. Zhang J, Zhang Y, Liu SN, Han Y, Zhou ZJ. Modelling growth and bacteriocin production by *Pediococcus acidilactici* PA003 as a function of temperature and pH value. ***Appl Biochem Biotechnol*** 2012; 166: 1388-400.

121. Zhou K, Zeng YT, Han XF, Liu SL. Modelling growth and bacteriocin production by *Lactobacillus plantarum* BC-25 in response to temperature and pH in batch fermentation. ***Appl Biochem Biotechnol*** 2015; 176: 1627–37.

122. De Vuyst L, Callewaert R, Crabbe K. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. ***Microbiology*** 1996; 142: 817-27.

123. Wannun P, Piwat S, Teanpaisan R. Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. ***Anaerobe*** 2014; 27: 17-21.

124. 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. ***Songkhlanakharin J Sci Technol*** 2012; 34: 241-5.

125. Teanpaisan R, Chooruk A, Kampoo T. Survival of free and microencapsulated human-derived oral probiotic *Lactobacillus paracasei* SD1 in orange and aloe vera juices. ***Songkhlanakharin J Sci Technol*** 2015; 37: 265-70.

126. Ritthagol 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: 257-63.

127. 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.

128. Wattanarat O, Makeudom A, Sastraruji T, Piwat S, Tianviwat S, Teanpaisan R, *et al*. Enhancement of salivary human neutrophil peptide 1-3 levels by probiotic supplementation. ***BMC Oral Health*** 2015; 15: 1-11.

129. Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: safe, natural antimicrobials for food preservation. ***Int J Food Microbiol*** 2001; 71: 1-20.

130. Yang SC, Lin CH, Sung CT, Fang JY. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. ***Front Microbiol*** 2014; 5: 241.

APPENDICES

APPENDIX A

Phirawat Wannun, Supatcharin Piwat and Rawee Teanpaisan. Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. ***Anaerobe*** 2014; 27: 17-21.

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

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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

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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 μg^{-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 μg^{-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 μg^{-1} (in the supernatant) to 46,875 AU μg^{-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

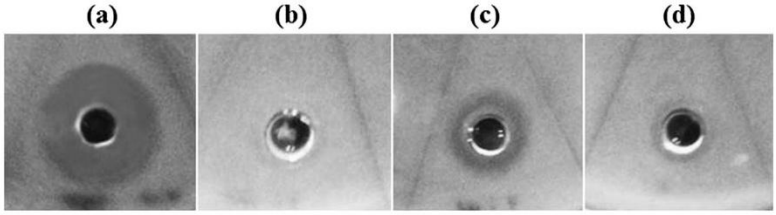

Fig. 1. The inhibitory activity of the culture supernatant of *L. paracasei* SD1 against *S. mutans* ATCC 25175 treated with various enzymes. (a) untreated supernatant, (b) treated with proteinase K, (c) treated with catalase, and (d) treated with lysozyme.

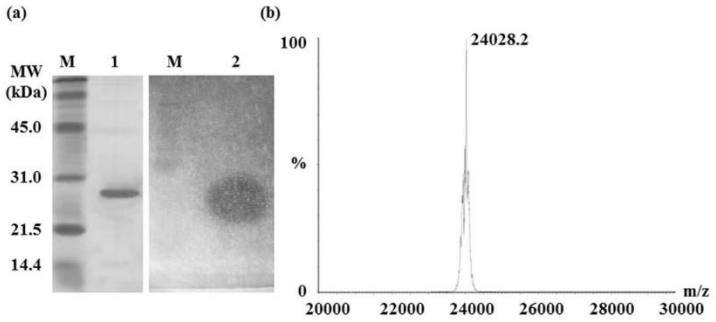

Fig. 2. (a) Tricine-SDS PAGE analysis of purified bacteriocin from *L. paracasei* SD1: (Lane 1) Coomassie brilliant blue stained gel, (Lane 2) gel overlaid with soft agar containing *S. mutans* ATCC 25175, and (Lane M) molecular weight standard, low range (Bio-Rad), and (b) Liquid chromatography mass 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 α-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

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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.

APPENDIX B

Phirawat Wannun, Supatcharin Piwat and Rawee Teanpaisan. Purification, Characterization and Optimum Conditions of Fermencin SD11, a Bacteriocin Produced by Human Orally *Lactobacillus fermentum* SD11. ***Appl Biochem Biotechnol*** 2016; 179: 572-582.

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Purification, Characterization, and Optimum Conditions of Fermencin SD11, a Bacteriocin Produced by Human Orally *Lactobacillus fermentum* SD11

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Abstract Fermencin SD11, a bacteriocin produced by human orally *Lactobacillus fermentum* SD11, was purified, characterized, and optimized in conditions for bacterial growth and bacteriocin production. Fermencin SD11 was purified using three steps of ammonium sulfate precipitation, gel filtration chromatography, and reverse-phase high-performance liquid chromatography. The molecular weight was found to be 33,000 Da using SDS-PAGE and confirmed as 33,593.4 Da by liquid chromatography-mass spectrometry. Fermencin SD11 exhibited activity against a wide range of oral pathogens including cariogenic and periodontogenic pathogens and *Candida*. The active activity was stable between 60 - 80 °C in a pH range of 3.0 to 7.0. It was sensitive to proteolytic enzymes (proteinase K and trypsin), but it was not affected by α-amylase, catalase, lysozyme, and saliva. The optimum conditions for growth and bacteriocin production of *L. fermentum* SD11 were cultured at acidic with pH of 5.0–6.0 at 37 or 40 °C under aerobic or anaerobic conditions for 12 h. It is promising that *L. fermentum* SD11 and its bacteriocin may be an alternative approach for promoting oral health or prevention of oral diseases, e.g., dental caries and periodontitis, which would require further clinical trials.

Keywords *Lactobacillus fermentum* · Fermencin SD11 · Bacteriocin · Purification · Characterization · Optimum conditions · Oral diseases

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Introduction

Lactobacillus species are considered to be important organisms; they have been proposed as probiotics for promoting and maintaining human health including in the gastrointestinal tract, genitourinary tract, and oral cavity [1–3]. A selection of probiotic strains is generally based on their ability to suppress growth of pathogens in target areas. One of the main mechanisms for *Lactobacillus* strains to inhibit growth of pathogens is a production of antimicrobial agents such as organic acids, hydrogen peroxide, and bacteriocins or related substances [4, 5].

Bacteriocins are small bacterial proteins or peptides synthesized by ribosomes, and they exhibit a bacteriostatic or bactericidal activity against genetically closely related bacteria [6]. Bacteriocin-producing bacteria as well as bacteriocins are increasing interests because they can be used as biological controls in the manufacture of beverages and ferment products [7, 8]. In addition, it may be used as a pharmaceutical product for local applications, e.g., in the vagina [9, 10]. A number of studies have reported that physicochemical factors, e.g., pH, temperature, and aeration, have a dramatic effect on the production of bacteriocins [11–14]. Thus, the optimization for biomass production to improve the yield of bacteriocins is necessary.

Lactobacillus fermentum has been considered as a commensal in human oral cavities, which was commonly found in saliva and dental plaque [15]. Some studies previously reported that it was the most predominant species found in saliva of caries-free subjects [15, 16]. It has been stated that the effects of probiotic strains are strain-specific [17]; not all the *Lactobacillus* are probiotics that possess the ability to confer health benefits for the host. Our previous study revealed that *Lactobacillus paracasei* SD1, a strain derived from a caries-free subject, could produce paracasin SD1, a specific antimicrobial protein, against various oral pathogens [18].

The objective of this study was to investigate the bacteriocin protein of *L. fermentum* strains isolated from caries-free subjects. The study included purification, characterization, and optimum conditions for bacteriocin production.

Materials and Methods

Screening Examination for Selection of Bacterial Strains

A total 30 *L. fermentum* strains, isolated from 30 caries-free subjects, were obtained from the previous study of Piwat et al. [15], where 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 had been identified to the species level using a restriction fragment length polymorphism (RFLP) analysis of a polymerase chain reaction (PCR) [19], and 16S-rRNA sequencing was carried out to confirm the initial identification.

The strains were selected by deferred antagonism against *Streptococcus mutans* ATCC 25175 according to the method of Wannun et al. [18]. Briefly, the cell-free supernatant of each tested strain was screened for inhibitory activity against *S. mutans* ATCC 25175. After recovery from storage, each strain was inoculated into a de Man Rogosa Sharpe (MRS) broth at 37 °C for 24 h. The supernatant was separated from the bacterial cells by centrifugation at $8000 \times g$ for 10 min. Then, it was adjusted to pH 6.5 with 1 M NaOH and treated with proteinase K, catalase, and lysozyme at a final enzyme concentration of 1 mg/mL. The treated supernatants were tested for antimicrobial activity using an agar well diffusion assay.

Purification of the Bacteriocin of *L. fermentum* SD11

The result of the screening examination indicated that only the *L. fermentum* SD11 strain had an inhibitory protein in the supernatant. Thus, the strain was subjected for bacteriocin purification according to the modified method of Wannun et al. [18], which comprised three steps: ammonium sulfate precipitation, gel filtration chromatography, and reverse-phase high-performance liquid chromatography (HPLC) (Table 1).

One liter of the supernatant of *L. fermentum* SD11 was adjusted to pH 6.5 and precipitated with 40 % (*w*/*v*) 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 phosphate-buffered saline (PBS, pH 7.0). The precipitated proteins (10 mL) were desalted twice using a 10-kDa-molecular-weight cutoff dialysis membrane in 1 L of 0.05 M PBS (pH 7.0) at 4 °C for 24 h. 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 0.1 M PBS.

The sample (0.5 mL) was loaded on a Fractogel® EMD BioSEC (1.5 × 90 cm) column (Merck, Germany). The column was equilibrated and eluted by a 50-mM sodium phosphate buffer at pH 6.5 with the addition of 0.15 M NaCl at a flow rate of 0.5 mL/min, with a time interval of 20 min after the samples were injected. The individual fraction was desalted and lyophilized under pressure (25 mHg) at −110 °C of freeze-drying (CoolSafe 110-4 Pro, ScanLaf, Denmark) for 24 h. Each fraction was then tested for bacteriocin activity using a broth microdilution assay.

For further purification, the active eluted fraction (100 μL) was purified by reverse-phase HPLC on a C_{18} Sep-Pak column (Millipore Corp., Milford, MA) at a flow rate of 0.5 mL/min. Elution was carried out by applying a linear gradient of acetonitrile with deionized water from 15 to 100 % within 30 min. Absorbencies were recorded between 210 and 280 nm using a photodiode array detector (model 996; Waters). All collected fractions were evaporated and then tested for bacteriocin activity. Protein concentration was determined using Bradford's assay [20].

The purified bacteriocin was confirmed by liquid chromatography-mass spectrometry (LC-MS, model V BIO-Q triple-quadrupole; Biotech, Altrincham, UK). The bacteriocin size was demonstrated using 12 % tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE), and antimicrobial activity of the specific protein was examined using a bioautographic assay [18].

The inhibitory activity of a tested sample was defined as >80 % inhibition growth of *S. mutans* ATCC 25175 using a microdilution assay compared to the control. Antimicrobial protein titers were reported as arbitrary units (AU) per milliliter or microgram, and one AU

Table 1 Purification of bacteriocin produced by *L. fermentum* SD11

Purification stage	Volume (ml)	Total proteins (μg)	Total activity (Au)	Specific activity (AU/μg)	Purification (fold)
Culture supernatant	1000	33,000	606,060.6	18.36	1
Ammonium sulfate precipitation	50	850	88,235.29	103.8	5.61
Gel filtration chromatography	1	2.2	13,636.36	6,198.34	337.60
Reverse-Phase HPLC	1	0.16	125,000	781,250	42,551.74

was defined as the reciprocal of the highest dilution showing inhibitory action towards the sensitive strains.

Characterization of Bacteriocin Protein of *L. fermentum* SD11

The effects of enzymes, pH, and spectrum of bacteriocin activity were investigated. The purified protein (1 mg/mL) was tested by incubating with various enzymes (final concentration 1 mg/mL), proteinase K and trypsin, α-amylase, catalase, lysozyme, and whole saliva at 37 °C for 3 h. The effect of pH on bacteriocin activity was determined by incubating in the pH range of 3.0–9.0 for 4 h at 37 °C. Thermal sensitivity was evaluated by heating purified bacteriocin in a water bath at 60, 80, 100, and 120 °C or autoclaved for 10–20 min. Antimicrobial activity was determined using the microdilution assay compared to the untreated purified protein as the control.

A spectrum of antimicrobial activity of the purified bacteriocin was determined against several indicators (details in Table 2). The representative strains were chosen on the basis of their relatedness to common oral diseases, e.g., dental caries, periodontal diseases etc., and they were grown overnight in MRS, BHI, and sabouraud dextrose broth depending on the strains as appropriate. The bacteriocin activity against the indicators was tested using a broth microdilution assay, and it was presented as arbitrary units (AU/mL).

Determination of Optimum Growth and Bacteriocin Production of *L. fermentum* SD11

The experiment was performed using a biofermenter (BioFlo®/CelliGen 115, Germany) where MRS broth (2 L) was inoculated with 100 mL of *L. fermentum* SD11 culture for 12–18 h at 37 °C to an initial cell density of 10^8 CFU/mL.

Initially, the experiments were set up to explore the optimum growth and bacteriocin production by incubating at 37 °C under aerobic or anaerobic conditions with different pH of 5.0, 6.0, 7.0, and 8.0. To monitor the bacterial growth, an aliquot of culture was removed at 0, 3, 6, 12, 24, and 48 h time interval and measured at 600 nm absorbance. After concentrating the supernatant with a SpeedVac concentrator and adjusting pH to 6.5, bacteriocin activity against *S. mutans* ATCC 25175 was determined using a microdilution assay.

The optimum growth and bacteriocin activity were evaluated by comparing the growth rate and bacteriocin activity between times that were calculated as follows:

Growth rate or bacteriocin activity $= X_2 - X_1 / t_2 - t_1$, where X_1 and X_2 were growth (OD) or bacteriocin activity (AU) at time point 1 (t_1) and time point 2 (t_2), respectively.

The results indicated that at pH 5.0 and 6.0 under either aerobic or anaerobic condition, it provided the optimum growth and bacteriocin production. However, it was more convenient to conduct the experiment under aerobic condition to explore for the optimum temperature. Thus, the biofermenter was set to pH 6.0 under aerobic condition at different temperatures of 25, 37, and 40 °C, and the bacterial growth rate and bacteriocin production was monitored as mentioned above.

Statistics Analysis

The results were expressed as the means ± standard deviation. The difference between treatments (pH, temperatures, and aeration) were analyzed using the Mann–Whitney *U* Test. Data

Table 2 Bacterial strains used in this study and their sensitivity to fermencin SD11 produced by *L. fermentum* SD11

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

[a] Antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + +, antimicrobial activity ≥125,000 AU/mL; + +, antimicrobial activity ≥13,636.36 AU/mL; +, antimicrobial activity ≥9,090.9 AU/mL. All tests were performed in triplicate

were analyzed by the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL), and significant differences were considered as $p < 0.05$.

Results and Discussion

Screening Examination for Selection of Bacterial Strains

Initially, 30 strains of *L. fermentum* from caries-free subjects were screened for antimicrobial production against *S. mutans* ATCC 25175, and a bacteriocin-like antagonism against an indicator strain was observed in the supernatants of all strains tested. However, after adjusting each culture filtrate to pH 6.5, *L. fermentum* SD11 still showed inhibitory activity. Its activity revealed partial disappearing and complete loss after treating with catalase and proteinase K, respectively, which indicates a protein nature (data not shown).

This study supports the previous reports in which inhibitory activity of certain *Lactobacillus* strains could be found in subjects with good oral health. It was demonstrated that the effective inhibition of *Lactobacillus* against cariogenic *S. mutans* strains was found to relate to caries-free subjects [4]. Koll-Klais et al. also showed that *Lactobacillus gasseri* from healthy persons had a stronger inhibitory activity against periodontogenic *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* than did the strains from patients with chronic periodontitis [21]. The present study screened for a bacteriocin-producing strain from caries-free subjects, and only one from 30 strains showed a bacteriocin-like activity. This finding confirmed that the probiotic property is strain-specific [17]. Thus, *L. fermentum* SD11 was used for further study.

Purification of the Bacteriocin of *L. fermentum* SD11

The bacteriocin was purified using the following three steps: 40 % ammonium sulfate precipitation, gel filtration chromatography, and reverse-phase HPLC. The antimicrobial activity of bacteriocin and purification procedures are shown in Table 1; the specific activity dramatically increased from 103.8, to 6198.34, to 781,250.0 AU/μg, respectively. The tricine SDS-PAGE and bioautographic analysis revealed one active protein against *S. mutans* ATCC 5175, having an estimated molecular mass of approximately 33,000 Da (Fig. 1a), and it was confirmed as having a molecular mass of 33,593.4 Da by LC-MS (Fig. 1b).

Most reports of bacteriocin purification mainly derived from various *Lactobacillus* strains, e.g., *L. paracasei* [18], *L. acidophilus* [22], *L. salivarius* [23], *L. animalis* [24], etc.; only a few relating to *L. fermentum* were mentioned. Of those, two strains, *L. fermentum* L23 and *L. fermentum* CS57, originated from vagina [25, 26] and one strain, *L. fermentum* Beijerinck CCRC 14018, originated from food [27]. Although the purification of bacteriocins of those *L. fermentum* strains was carried out, only an estimated size of the bacteriocins *L. fermentum* L23 (<7 kDa), *L. fermentum* CS57 (>30 kDa), and *L. fermentum* Beijerinck CCRC 14018 (1–3 kDa) were reported.

This may be the first report of any purified bacteriocin of *L. fermentum* SD11, a strain originating from a human oral cavity, designated as "fermencin SD11." It had a molecular weight of 33,593.4 Da, which is quite close to the weight for bacteriocin of *L. fermentum* CS57 [26]. It indicated that *L. fermentum* strains from different sources could produce various sizes of bacteriocin proteins.

Characterization of Bacteriocin Protein of *L. fermentum* SD11

Fermencin SD11 presented a wide range of inhibitory spectrum against the indicators listed in Table 2, having the strongest antagonistic activity toward gram-positive bacteria followed by the *Candida* strain. In general, bacteriocins from lactic acid bacteria (LAB) are active toward closely related organisms (gram-positive bacteria) [6]. However, some bacteria produced bacteriocins that exhibited broad-spectrum activity [24–27]. In this study, *L. fermentum*

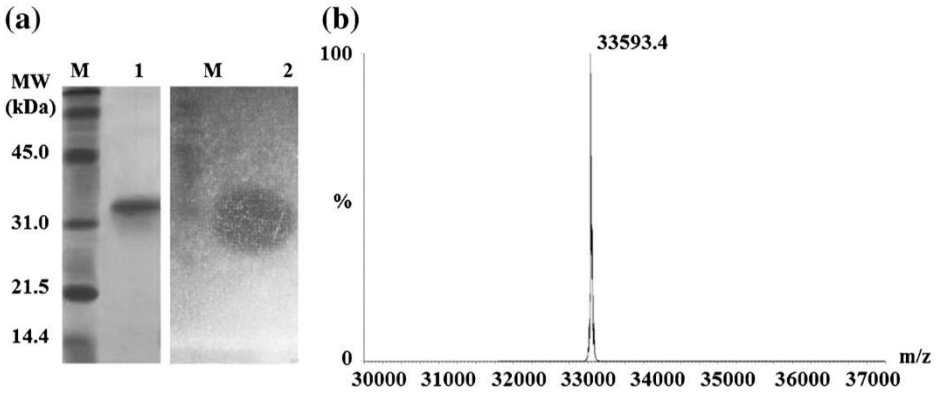

Fig. 1 **a** Tricine-SDS-PAGE analysis of purified bacteriocin from *L. fermentum* SD11: lane *1*, Coomassie brilliant blue stained gel; lane *2*, gel overlaid with soft agar containing *S. mutans* ATCC 25175; and lane *M*, molecular weight standard (low range of Bio-Rad). **b** Liquid chromatography-mass spectrometry of fermencin SD11

SD11 could produce bacteriocin against a wide range of microorganisms, focusing on oral pathogens such as cariogenic bacteria (*S. mutans* and *S. sobrinus*), periopathogenic bacteria (*A. actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*), and *C. albicans*, thus allowing *L. fermentum* SD11 to be considered as a potential probiotic strain.

The antimicrobial activity of fermencin SD11 was completely abolished by proteinase K and trypsin, confirming that the inhibitory material was proteinaceous. Catalase, lysozyme, amylase, and saliva had no effect on the activity. The bacteriocin activity was stable at a wide range of non-alkaline pH levels (3.0–7.0) with optimal activity at pH 5.0 and 6.0 (Table 3).

Table 3 Effects of enzyme, pH, and heat treatment on the antimicrobial protein activity produced by *L. fermentum* SD11

Effect of treatment on antimicrobial activity	Antimicrobial activity[a]
Enzyme	
α-Amylase	+ + +
Catalase	+ + +
Lysozyme	+ + +
Proteinase K	−
Trypsin	−
Saliva	+ + +
pH	
3.0	+
4.0	+ +
5.0	+ + +
6.0	+ + +
7.0	+ +
8.0	−
9.0	−
Temperature (°C)	
5 min	
60	+ + +
80	+ + +
100	−
120	−
10 min	
60	+ + +
80	+ +
100	−
120	−
20 min	
60	+ + +
80	+
100	−
120	−

[a] Antimicrobial activity was determined by the microdilution assay against an oral pathogenic *S. mutans*. + + +, antimicrobial activity ≥125,000 AU/mL; + +, antimicrobial activity ≥13,636.36 AU/mL; +, antimicrobial activity ≥9,090.9 AU/mL; −, non-detected antimicrobial activity. All tests were performed in triplicate

This finding was in agreement with previous studies reporting that bacteriocin activity from LAB was most stable when acidic and neutral [25–27].

Fermencin SD11 was stable between 60 - 80 °C and retained more than 60 % of its activity after heating at 80 °C for 10 min (Table 3). No detectable activity was observed after heating at 100 °C for 10 min, suggesting that the antimicrobial substance was a heat-labile protein. According to Klaenhammer's classification, fermencin SD11 should be classified into bacteriocin class III due to its properties of large molecules (>30 kDa) and sensitivity to heat [6].

Determination of Optimum Growth and Bacteriocin Production of *L. fermentum* SD11

Initially, the experiments were conducted to explore the optimum growth and bacteriocin production by incubating at 37 °C under aerobic or anaerobic conditions with different pH of 5.0, 6.0, 7.0, and 8.0.

Results demonstrated that the increase of bacteriocin activity depended on the increase of the growth rate. After considering all the different conditions used, the maximum growth rate (approximately 0.11/h) and bacteriocin production (approximately 293 AU/h) were achieved between 6 - 12 h of culturing in MRS broth at 37 °C. Both pH, 5.0 and 6.0, gave significantly higher growth rate and bacteriocin production than pH 7.0. There were no significant differences of growth rate and bacteriocin production when culturing under aerobic or anaerobic conditions (Fig. 2). It indicated that the maximum fermencin SD11 occurred during the exponential phase when bacterium was actively dividing. This concurs with earlier findings where bacteriocin activity generally increased rapidly during the exponential growth phase [28–30]. Only in a few cases could bacteriocin production occur during the stationary phase. Bacteriocin activity frequently decreased in the late stages of the culture; this may be due to proteolytic degradation, aggregation, or adsorption to cells [31]. No bacterial growth and bacteriocin activity was found under pH 8.0 (data not shown).

After trials for the optimum temperature at 25, 37, and 40 °C, trials at 37 and 40 °C provided the higher bacterial growth and bacteriocin production compared with that at 25 °C (Fig. 3). The results indicate that the optimum conditions for growth and bacteriocin production of *L. fermentum* SD11 were cultured with acidic pH of 5.0–6.0 at 37 or 40 °C under aerobic or anaerobic conditions for 12 h.

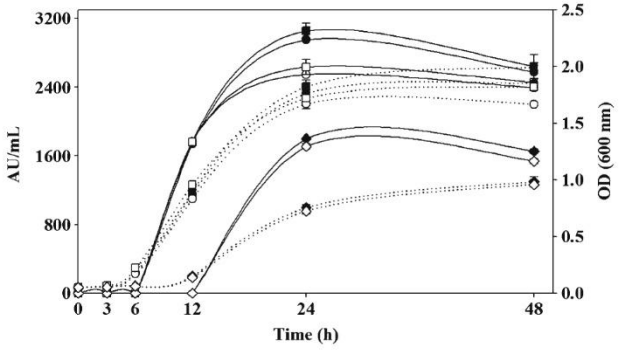

Fig. 2 The bacteriocin activity (*solid line*) and growth (*dotted line*) of *L. fermentum* SD11 in MRS broth at 37 °C with different pH: pH 5.0 (●), pH 6.0 (■), and pH 7.0 (♦) under aerobic condition; pH 5.0 (○), pH 6.0 (□), and pH 7.0 (◊) under anaerobic condition

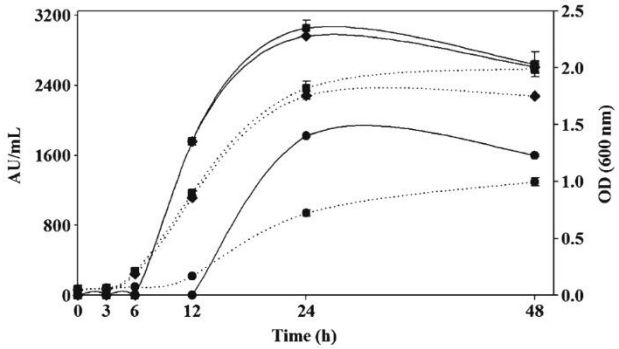

Fig. 3 The bacteriocin activity (*solid line*) and growth (*dotted line*) of *L. fermentum* SD11 in MRS broth at pH 6.0 under aerobic condition with different temperatures: 25 °C (●), 37 °C (■), and 40 °C (♦)

Probiotics have been defined as live microorganisms that can be administered to improve or promote human health. Along with the live bacteria, bacteriocins, antagonistic substances against pathogens, could be an alternative approach for controlling the ecological balance of the microflora of the host.

Our research group is interested in the development of probiotics to promote oral health and to prevent oral diseases. Our previous studies reported that *L. paracasei* SD1, a selected strain, could produce the bacteriocin named paracasin SD1 [18]. The strain has been incorporated into various products, e.g., milk powder, yogurt, and fruit juices [32, 33], and the product containing probiotic strain has been studied in clinical trials. It has been proven that *L. paracasei* SD1 is a useful probiotic strain for caries prevention based on the finding of significant reduction of cariogenic bacteria (mutans streptococci) and less caries lesions among the volunteers who received the probiotic milk powder compared to the controls [34–36].

One clinical study reported that *L. fermentum* was effective in decreasing intestinal pathogens and increasing the ratio of probiotic bacteria in healthy human [37]. *L. fermentum* strains are commensal and so are found predominately in oral cavities of human [15]. In this study, *L. fermentum* SD11 with its beneficial properties was selected, and we attempted to determine the optimum conditions in order to obtain both the highest number of viable bacteria and the highest level of bacteriocin production. Here, we have described an investigation of bacteriocin purification and the conditions in which the highest growth and bacteriocin production were obtained. Such information may be useful to improving the yield of fermencin SD11 for biomass production.

Conclusion

L. fermentum SD11, a strain derived from a caries-free subject, could produce an antimicrobial substance, from which later the purified protein, namely, fermencin SD11, with molecular weight of 33,593.4 Da, was obtained. It was active over a pH range of 3–7 with a broad spectrum against various oral pathogens. The optimum conditions for growth and bacteriocin production of *L. fermentum* SD11 were the cultivation at acidic pH of 5.0–6.0 at 37 or 40 °C under aerobic or anaerobic conditions for 12 h. It is promising that *L. fermentum* SD11 and its bacteriocin may be an alternative approach for promoting oral health or prevention of oral diseases, e.g., dental caries and periodontitis, which would require further clinical trials.

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References

1. Parvez, S., Malik, K. A., Ah Kang, S., & Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology, 100*, 1171–1185.
2. Dover, S. E., Aroutcheva, A. A., Faro, S., & Chikindas, M. L. (2008). Natural antimicrobials and their role in vaginal health: a short review. *International Journal of Probiotics & Prebiotics, 3*, 219–230.
3. Meurman, J. H., & Stamatova, I. (2007). Probiotics: contributions to oral health. *Oral Diseases, 13*, 443–451.
4. Simark-Mattsson, C., Emilson, C. G., Hakansson, E. G., Jacobsson, C., Roos, K., & Holm, S. (2007). *Lactobacillus*-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. *European Journal of Oral Science, 115*, 308–314.
5. Teanpaisan, R., Piwat, S., & Dahlen, G. (2011). Inhibitory effect of oral *Lactobacillus* against oral pathogens. *Letters in Applied Microbiology, 53*, 452–459.
6. Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews, 12*, 39–85.
7. Parada, L. J., Caron, C. R., Medeiros, A. B., & Soccol, C. R. (2007). Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. *Brazilian Archives of Biology and Technology, 50*, 521–542.
8. Deegan, L. H., Cotter, P. D., Hill, C., & Ross, P. (2006). Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International Dairy Journal, 16*, 1058–1071.
9. Dover, S. E., Aroutcheva, A. A., Faro, S., & Chikindas, M. L. (2007). Safety study of an antimicrobial peptide lactocin 160, produced by the vaginal *Lactobacillus rhamnosus*. *Infectious Diseases in Obstetrics and Gynecology, 78248*, 1–6.
10. Reddy, K. V., Aranha, C., Gupta, S. M., & Yedery, R. D. (2004). Evaluation of antimicrobial peptide nisin as a safe vaginal contraceptive agent in rabbits: in vitro and in vivo studies. *Reproduction, 128*, 117–126.
11. Biswas, S. R., Ray, P., Johnson, M. C., & Ray, B. (1991). Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. *Applied and Environmental Microbiology, 57*, 1265–1267.
12. Leroy, F., & De Vuyst, L. (1999). Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. *Applied and Environmental Microbiology, 65*, 974–981.
13. Herranz, C., Martinez, J. M., Rodriguez, J. M., Hernandez, P. E., & Cintas, L. M. (2001). Optimization of enterocin P production by batch fermentation of *Enterococcus faecium* P13 at constant pH. *Applied Microbiology and Biotechnology, 56*, 378–383.
14. Parente, E., & Ricciardi, A. (1994). Influence of pH on the production of enterocin 1146 during batch fermentation. *Letters in Applied Microbiology, 19*, 12–15.
15. Piwat, S., Teanpaisan, R., Thitasomakul, S., Thearmontree, A., & Dahlen, G. (2010). *Lactobacillus* species and genotypes associated with dental caries in Thai preschool children. *Molecular Oral Microbiology, 25*, 157–164.
16. Ahumada, M. C., Bru, E., Colloca, M. E., Lopez, M. E., & Nader-Macias, M. E. (2003). Evaluation and comparison of lactobacilli characteristics in the mouths of patients with or without cavities. *Journal of Oral Science, 45*, 1–9.
17. FAO/WHO. (2002). Guidelines for the evaluation of probiotics in food. *Food and Agricultural Organization of the United Nations and Wrold Health Organization Working Group report*. London, Ontario, Canada: FAO.
18. Wannun, P., Piwat, S., & Teanpaisan, R. (2014). Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. *Anaerobe, 27*, 17–21.
19. Teanpaisan, R., & 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.
20. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

21. Koll-Klais, P., Mandar, R., Leibur, E., Marcotte, H., Hammarstrom, L., & Mikelsaar, M. (2005). Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. *Oral Microbiology and Immunology, 20*, 354–361.
22. Chumchalova, J., Stiles, J., Josephsen, J., & Plockova, M. (2004). Characterization and purification of acidocin CH5, a bacteriocin produced by *Lactobacillus acidophilus* CH5. *Journal of Applied Microbiology, 96*, 1082–1089.
23. Busarcevic, M., Kojic, M., Dalgalarrondo, M., Chobert, J. M., Haertle, T., & Topisirovic, L. (2008). Purification of bacteriocin LS1 produced by human oral isolate *Lactobacillus salivarius* BGHO1. *Oral Microbiology and Immunology, 23*, 254–258.
24. Sahoo, T. K., Jena, P. K., Patel, A. K., & Seshadri, S. (2015). Purification and molecular characterization of the novel highly potent bacteriocin TSU4 produced by *Lactobacillus animalis* TSU4. *Applied Biochemistry and Biotechnology, 177*, 90–104.
25. Pascual, L. M., Daniele, M. B., Giordano, W., Pajaro, M. C., & Barberis, I. L. (2008). Purification and partial characterization of novel bacteriocin L23 produced by *Lactobacillus fermentum* L23. *Current Microbiology, 56*, 397–402.
26. Sabia, C., Anacarso, I., Bergonzini, A., Gargiulo, R., Sarti, M., Condo, C., Messi, P., de Niederhausern, S., Iseppi, R., & Bondi, M. (2014). Detection and partial characterization of a bacteriocin-like substance produced by *Lactobacillus fermentum* CS57 isolated from human vaginal secretions. *Anaerobe, 26*, 41–45.
27. Yan, T. R., & Lee, C. S. (1997). Characterization of a partially purified bacteriocin, Fermencin B, from *Lactobacillus fermentum. Biotechnology Letters, 19*, 741–744.
28. Juarez Tomas, M. S., Bru, E., Wiese, B., de Ruiz Holgado, A. A. P., & Nader-Macias, M. E. (2002). Influence of pH, temperature and culture media on the growth and bacteriocin production by vaginal *Lactobacillus salivarius* CRL 1328. *Journal of Applied Microbiology, 93*, 714–724.
29. Zhang, J., Zhang, Y., Liu, S. N., Han, Y., & Zhou, Z. J. (2012). Modelling growth and bacteriocin production by *Pediococcus acidilactici* PA003 as a function of temperature and pH value. *Applied Biochemistry and Biotechnology, 166*, 1388–1400.
30. Zhou, K., Zeng, Y. T., Han, X. F., & Liu, S. L. (2015). Modelling growth and bacteriocin production by *Lactobacillus plantarum* BC-25 in response to temperature and pH in batch fermentation. *Applied Biochemistry and Biotechnology, 176*, 1627–1637.
31. De Vuyst, L., Callewaert, R., & Crabbe, K. (1996). Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology, 142*, 817–827.
32. Teanpaisan, R., Chooruk, A., Wannun, A., Wichienchot, S., & Piwat, S. (2012). Survival rates of human-derived probiotic *Lactobacillus paracasei* SD1 in milk powder using spray drying. *Songklanakarin Journal of Science and Technology, 34*, 241–245.
33. Teanpaisan, R., Chooruk, A., & Kampoo, T. (2015). Survival of free and microencapsulated human-derived oral probiotic *Lactobacillus paracasei* SD1 in orange and aloe vera juices. *Songklanakarin Journal of Science and Technology, 37*, 265–270.
34. Ritthagol, W., Saetang, C., & Teanpaisan, R. (2014). Effect of probiotics containing *Lactobacillus paracasei* SD1 on salivary mutans streptococci and lactobacilli in orthodontic cleft patients: a double-blinded, randomized, placebo-controlled study. *The Cleft Palate-Craniofacial Journal, 51*, 257–263.
35. Teanpaisan, R., & Piwat, S. (2014). *Lactobacillus paracasei* SD1, a novel probiotic, reduces mutans streptococci in human volunteers: a randomized placebo-controlled trial. *Clinical Oral Investigations, 18*, 857–862.
36. Teanpaisan, R., Piwat, S., Tianviwat, S., Sophatha, B., & Kampoo, T. (2015). Effect of long-term consumption of *Lactobacillus paracasei* SD1 on reducing mutans streptococci and caries risk: a randomized placebo-controlled trial. *Dentistry Journal, 3*, 43–54.
37. Shieh, M. J., Shang, H. F., Liao, F. H., Zhu, J. S., & Chien, Y. W. (2011). *Lactobacillus fermentum* improved intestinal bacteria flora by reducing *Clostridium perfringens. e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism, 6*, e59–e63.

APPENDIX C

Wannun A. and Teanpaisan R. Antimicrobial protein produced by oral *Lactobacillus* against dental caries pathogen. The 2nd Current Drug Development International Conference; 2012 May 2-4; Phuket Graceland Resort & Spa Hotel, Phuket, Thailand. Quintessence Publishing; 2012. p. 179-180.

Proceedings

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Faculty of Pharmaceutical Sciences
Prince of Songkla University

Antimicrobial protein produced by oral *Lactobacillus* against dental caries pathogen

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Abstract- *Lactobacillus* species are found to associate with the presence and progression of dental caries. Our previous study has reported that certain *Lactobacillus* species may be useful to use as probiotics in prevention of dental caries due to their inhibitory activities against cariogenic *Streptococcus* spp. The objective of this study was to examine the antimicrobial proteins from oral *Lactobacillus* against *S. mutans* ATCC25175. The antimicrobial proteins were extracted from culture supernatants of *L. paracasei* SD1 and *L. fermentum* SD11, precipitated with ammonium sulfate, and performed the native-polyacrylamide gel electrophoresis. Antimicrobial activity was examined using methods of broth microdilutions and agar overlay. Both *L. paracasei* SD1 and *L. fermentum* SD11 presented antimicrobial proteins against *Streptococcus mutans* ATCC 25175. Antimicrobial proteins from *L. paracasei* SD1 and *L. fermentum* SD11 had a molecular weight of 49.1 and 97.6 kDa respectively, and the minimum inhibitory concentration (MIC) value of both proteins against *S. mutans* were 5.52 and 4.83µg/ml respectively. In conclusion, the antimicrobial proteins from *L. paracasei* SD1 and *L. fermentum* SD11 had the ability to inhibit growth of *S. mutans*, suggesting that there may be promising alternative chemotherapeutic agents for prevention of dental caries.

Introduction

Lactobacillus are part of the normal oral microflora, and it has been recognized for decades as a major contributor in the caries process (1). Our previous reports have shown that certain *Lactobacillus* species associated with the presence and progression of dental caries in Thai children (2). Also, our group has previously demonstrated that various *Lactobacillus* species e.g. *L. fermentum*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. salivarius* gave strong antimicrobial activities against putative oral pathogens (3). Thus, the present study was to examine the antimicrobial proteins from oral *Lactobacillus* against *S. mutans* ATCC25175.

Materials and methods

1)Bacterial strains and culture conditions: L. paracasei SD1 and *L. fermentum* SD11 were selected from a bacterial collection of the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Thailand and *Streptococcus mutans* ATCC 25175 was obtained from Department of Oral Microbiology, Faculty of Odontology, Gothenburg University, Sweden. *Lactobacillus* strains were cultured in MRS broth for 24-48 h, at 37°C in an anaerobic condition (80% N_2, 10%H_2 and 10% CO_2). *S. mutans* was cultured in BHI broth for 24 h, at 37°C in an anaerobic condition.

2)Preparation of proteins: The supernatant of *Lactobacillus* strains were adjusted to pH 6.5 with NaOH solution and precipitated with 40% ammonium sulfate an overnight at 4°C. The mixture was then centrifuged at 10,000 rpm at 4°C for 20 min to collect the proteins, and resuspended in sterile 0.1 M sodium phosphate buffer pH 7.0.

3)Antimicrobial assays and determination the size of proteins:

4)Broth microdilution test: S. mutans ATCC25175 was incubated overnight in BHI broth at 37°C. One hundred microliters of 1×10^8 CFU/ml bacteria suspension (0.5 McFarland) was inoculated into 96-well microtiterplates which contained 100 µl as serial-2 fold dilution proteins. The final volume of each well was 200 µl. Each plate included positive control (bacteria without protein) and negative control (broth only). All samples were incubated overnight at 37°C. The MIC was determined as the lowest concentration of antimicrobial proteins that inhibited the visible growth of bacteria after incubated overnight. The experiment was performed in duplicate.

5)Agar overlay test: The NATIVE-PAGE was performed antimicrobial activities of proteins and the size of proteins was estimated using silver staining compared with protein marker (Bio-Rad Laboratories, Hercules, CA)

Results and Discussions

This study showed that both *L. paracasei* SD1 and *L. fermentum* SD11 presented antimicrobial proteins against *Streptococcus mutans* ATCC 25175. Antimicrobial proteins from *L. paracasei* SD1 and *L. fermentum* SD11 had a molecular weight of 49.1 and 97.6 kDa respectively (Figure 1), and the minimum inhibitory concentration (MIC) value of both proteins against *S. mutans* were 5.52 and 4.83 µg/ml respectively. This is in agreement of Ishihara *et al*. (4) reported that a water-soluble extract of *L. fermentum* completely inhibited the growth of *S. mutans*. Also, Chung *et al*. (5) reported that the cultured supernatant of *L. fermentum* S11, isolated from the saliva of a free caries child, significantly inhibited the formation of the insoluble glucan produced by *S. mutans*.

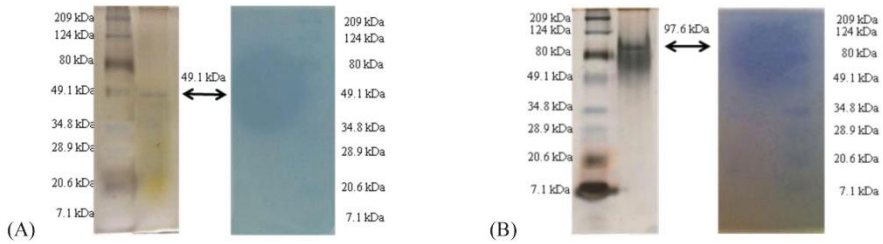

Figure1. NATIVE-PAGE of ammonium sulfate precipitated antimicrobial proteins of *L. paracasei* SD1 (A), and *L. fermentum* SD11 (B). The size of protein stained with silver was shown on the left column indicated with the protein markers and antimicrobial activity of proteins demonstrated by agar overlay method on the right column.

Conclusion

In conclusion, the antimicrobial proteins from *L. paracasei* SD1 and *L. fermentum* SD11 had the ability to inhibit growth of *S. mutans*, suggesting that there may be promising alternative chemotherapeutic agents for prevention of dental caries.

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Reference(s)

[1] Van Houte J. Bacterial specificiry in the ctiology of dental caries. Int Dent J 1980;30:305-26.
[2] 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 Apr;25(2):157-64.
[3] Teanpaisan R, Piwat S, Dahlen G. Inhibitory effect of oral Lactobacillus against oral pathogens. Lett Appl Microbiol 2011 Oct;53(4):452-9.
[4] Ishihara K, Miyakawa H, Hasegawa A, Takazoe I, Kawai Y. Growth inhibition of Streptococcus mutans by cellular extracts of human intestinal lactic acid bacteria. Infect Immun 1985 Sep;49(3):692-4.
[5] Chung J, Ha ES, Park HR, Kim S. Isolation and characterization of Lactobacillus species inhibiting the formation of Streptococcus mutans biofilm. Oral Microbiol Immunol 2004 Jun;19(3):214-6.

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List of Publication and Proceeding

Phirawat Wannun, Supatcharin Piwat and Rawee Teanpaisan. Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. ***Anaerobe*** 2014; 27: 17-21.

Phirawat Wannun, Supatcharin Piwat and Rawee Teanpaisan. Purification, Characterization and Optimum Conditions of Fermencin SD11, a Bacteriocin Produced by Human Orally *Lactobacillus fermentum* SD11. ***Applied Biochemisty and Biotechnology*** 2016; 179: 572-582.

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