Aggregation Abilities and Surface Charges of Oral *Lactobacillus*

Kamonchanok Pongpanit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Oral Health Sciences

Prince of Songkla University

2014

Thesis Title Aggregation abilities and surface charges of oral *Lactobacillus*

Author Miss Kamonchanok Pongpanit

Major Program Pediatric dentistry

Major Advisor :

…….....

(Dr.Supatcharin Piwat)

Co-advisor

…….....

(Prof.Dr.Rawee Teanpaisan)

……..……..

(Miss.Nuchnaree Akkarachaneeyakorn)

Examining Committee :

……..Chairperson

(Prof.Dr.Sittichai Koontongkaew)

……...

(Assoc.Prof.Dr.Siroj Jitsuronk)

……...

(Dr.Supatcharin Piwat)

……

(Prof.Dr.Rawee Teanpaisan)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Oral and Maxillofacial Surgery.

…….……..……..

(Assoc.Prof.Dr.Teerapol Srichana)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……..Signature

(Dr. Supatcharin Piwat)

Major advisor

……..Signature

(Kamonchanok Pongpatit)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……...Signature

(Kamonchanok Pongpanit)

Candidate

ชื่อวิทยานิพนธ์ ความสามารถในการเกาะกลุ่มและประจุพื้นผิวของเชื้อแลคโตบาซิลลัสจากช่องปาก

ชื่อผู้เขียน นางสาวกมลชนก พงษ์พานิช

สาขาวิชา วิทยาศาสตร์สุขภาพช่องปาก

ปีการศึกษา 2556

บทคัดย่อ

การวิจัยนี้เป็นการศึกษาความสามารถในการเกาะกลุ่มและประจุพื้นผิวของแลคโตบาซิลลัส (*Lactobacillus* (*L*)) ในช่องปาก 10 ชนิด *คือ L. fermentum, L. salivarius, L. gasseri, L. plantarum, L. rhamnosus, L. mucosae, L. casei, L. oris, L. paracasei* และ *L. vaginalis* และศึกษาความสัมพันธ์ระหว่างความสามารถดังกล่าวกับสภาวะสุขภาพช่องปาก

วิธีการศึกษา คือ นำเชื้อแลคโตบาซิลลัสที่แยกได้จากช่องปากจำนวน 198 strains คือ *L. fermentum* (62), *L. salivarius* (29), *L. gasseri* (17), *L. plantarum* (12), *L. rhamnosus* (17), *L. mucosae* (12), *L. casei* (11), *L. oris* (8), *L. paracasei* (22) และ *L. vaginalis* (8) มาศึกษาประจุพื้นผิวโดยวิธี MATH และ ความสามารถในการเกาะกลุ่มทั้งกับตัวเชื้อแลคโตบาซิลลัสเอง และกับเชื้อก่อโรคฟันผุในช่องปาก คือ *Streptococcus mutans*

ผลการศึกษาพบว่า เชื้อแลคโตบาซิลลัสที่แยกได้จากช่องปากทุกสายพันธ์จากกลุ่มตัวอย่างมีค่าประจุพื้นผิวแบบให้อิเลกตรอนในระดับสูง ค่าประจุพื้นผิวแบบรับอิเลคตรอนในระดับน้อยถึงปานกลาง และค่า hydrophobicity ในระดับปานกลาง ยกเว้น *L. gasseri* มีค่า hydrophobicity ต่ำ เชื้อแลคโตบาซิลัสในกลุ่มตัวอย่างที่มีฟันผุสูงจะมีค่า hydrophobicity สูงกว่ากลุ่มตัวอย่างที่มีฟันผุต่ำอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) นอกจากนี้ยังพบว่า ความสามารถในการเกาะกลุ่มของเชื้อแลคโตบาซิลลัสสัมพันธ์กับระยะเวลา กล่าวคือ เมื่อเวลานานขึ้นความสามารถในการเกาะกลุ่มของเชื้อก็มากขึ้น นอกจากนี้ยังพบว่า ความสามารถในการเกาะกลุ่มของเชื้อแลคโตบาซิลลัสในกลุ่มฟันผุสูงและต่ำไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ

กล่าวโดยสรุปได้ว่า เชื้อแลคโตบาซิลลัสที่แยกได้จากช่องปากส่วนใหญ่แสดงให้เห็นว่ามีคุณสมบัติในการเกาะกลุ่มและมีค่าประจุพื้นผิวแบบให้อิเลกตรอนสูง มีค่าประจุพื้นผิวแบบรับอิเลกตรอนน้อยถึงปานกลาง และค่า hydrophobicity ปานกลาง นอกจากนี้มีเฉพาะค่า hydrophobicity เท่านั้นที่มีความต่างอย่างนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเปรียบเทียบระหว่างกลุ่มฟันผุสูงและต่ำ

Thesis Title Aggregation abilities and surface charges of oral *Lactobacillus*

Author Miss Kamonchanok Pongpanit

Major Program Oral Health Sciences

Academic Year 2013

ABSTRACT

Objective: The aims of the study were to compare aggregation ability and surface charges of oral *Lactobacillus* (*L*) 10 species namely *L. fermentum*, *L. salivarius*, *L. gasseri*, *L. plantarum*, *L. rhamnosus*, *L. mucosae*, *L. casei*, *L. oris*, *L. paracasei* and *L. vaginalis*. The relation between such ability with oral health status (dt).

Methods: Total 198 strains of *L. fermentum* (62), *L. salivarius* (29), *L. gasseri* (17), *L. plantarum* (12), *L. rhamnosus* (17), *L. mucosae* (12), *L. casei* (11), *L. oris* (8), *L. paracasei* (22) and *L. vaginalis* (8) were investigated for their cell surface properties (The microbial adhesion to hydrocarbon test) and measured their autoaggregation and pathogenic coaggregation properties with *Streptococcus mutans*.

Results: Most species showed high basic surface (electron donor) charges, low to moderate acid surface charges (electron acceptor) and moderate to high hydrophobicity. *L. gasseri* was low hydrophobicity. Lactobacillus from high caries showed a greater ability to adhere to hydrophobic substances compare to *Lactobacillus* that obtained from low caries. The aggregation abilities (autoaggregation and coaggregation with *S.mutans*) were depended on incubation, Time variation. *L. gasseri* was the highest aggregation ability. However, there were no significant in aggregation ability between Lactobacillus obtained from high and low caries subject.

Conclusion: Most of oral *Lactobacillus* species showed adhesion relate properties included high electron donor, low to moderate electron acceptor and moderate hydrophobicity. However, only hydrophobicity was statistically significant difference ($p < 0.05$) between the groups of high and low caries subject.

ACKNOWLEDGEMENT

Foremost, I offer my sincerest gratitude to my principal supervisor Dr. Supatcharin Piwat, co-supervisor Prof. Dr. Rawee Teanpaisan and Dr. Nuchnaree Akkarachaneeyakorn who have supported me throughout my thesis with their patience and knowledge. I attribute the level of my Master of Science program in oral health sciences (Major in Pediatric dentistry) degree to their encouragement and effort and without them this thesis, too, would not have been completed or written. I am grateful to all the Preventive Dentistry Department staffs who promoted a welcoming academic and social environment.

I would like to acknowledge Miss Benchamat Sophatha for their assistance in bacterial cultivation. I also would like to acknowledge Mr. Kemarajt Kemavongse and all the staff at the Research Center, Faculty of Dentistry, Prince of Songkla University, for providing the proper setting and introduction to the laboratory tools application. Many thanks are extended to all my fellow postgraduate students. I would like to thank my fellow, Mr. Chaimes Soonhuae, who kindly took care of my clinical works whenever I needed help.

Last, but by no means least, I thank my parents for love, encouragement and supporting with their best wishes throughout all my studies at the university. My studies and research would not have been possible without them.

Kamonchanok Pongpanit

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

AB	=	Acid-Base
BHI	=	Brain Heart Infusion
CFU	=	Colony Forming Unit
DLVO	=	Derjaguin-Landau-Verwey-Overbeek Theory
dt	=	Decayed teeth
et al	=	And others
FAO	=	Food and Agriculture Organization of the United Nations
Fig.	=	Figure
hrs.	=	Hour
i.e.	=	id est
LW	=	Lifshitz-van der Waals
MATH	=	The microbial adhesion to hydrocarbon test
ml.	=	Milliliter
MRS	=	Man Rogosa Sharpe
nm.	=	Nanometer
No.	=	Number
OD	=	Optical density
PBS	=	Phosphate buffered saline
pH	=	Potential of Hydrogen ion
SPSS	=	Statistical Package for the Social Sciences
vdW	=	van der Waals
*V*R	=	repulsive interactions
WHO	=	World Health Organization

CHAPTER 1

Introduction

Lactobacilli are gram-positive, non spore forming, rods or coccobacilli microorganisms. In environments where carbohydrates are available such as food (dairy products, fermented meat, vegetables, fruits, beverages), gastrointestinal, respiratory and genital tracts of humans and animals, and in sewage and plant material usaually found them[1].

Lactobacilli normally comprise less than 1% of the total cultivable microbiota in the oral cavity. Commonly isolated species include *L. paracasei, L. plantarum, L. rhamnosus* and *L. salivarius*[2]. Lactobacilli are considered as part of the normal oral microbiota. They are the most acidogenic among the lactic acid bacteria and are associated with the progression of dental caries. Sometimes, the number of lactobacilli in saliva has been suggested to use as a part of caries risk estimation[3]. The species associated with caries are still ambiguous, however the differences between *Lactobacillus* species from caries-active and from healthy subjects have been reported[4]. Piwat *et al*.[5] isolated salivary lactobacilli from 59 children. They found that *L. fermentum* and *L. salivarius* were the predominant species, but in the moderate to high caries group *L. salivarius* found *L. salivarius* significantly higher numbers. In some cases, lactobacilli could also play a useful role by inhibiting the growth of some cariogenic bacteria. Michalek *et al*.[6] found that the presence of *L. casei* in plaque may reduce cariogenic pathogen (*S. mutans*) in gnotobiotic rats. Some *Lactobacillus* species such as *L. paracasei L. rhamnosus L. plantarum* and *L. salivarius* which isolated from the oral cavities of sound subjects have capability of having an antimicrobial activity against streptococci[7–9]. However, some *Lactobacillus* species have been proposed as probiotics in the prevention of dental caries, mainly because of their inhibitory activities against cariogenic *Streptococcus* spp. and contributing to the balance of microflora in the oral cavity[4].

Aggregation has been related with adhesion, which is a prerequisite for colonization and infection by pathogens. Autoaggregation is defined as the adherence of bacteria themselves. The bacteria that can autoaggregate conveys a selective advantage over non-autoaggregation species by enhancing attachment within a developing biofilm[10]. Bacterial coaggregation is a result of two or more different species of bacteria interacting to form a stable composite aggregation[11]. Coaggregation was first presented to the bacteria isolated from human

dental plaque, which are found living together of many bacterial species. Several studies demonstrated that coaggregation was a common phenomenon between broad ranges of genera from dental plaque. These investigations showed that coaggregation between pairs of bacteria was highly specific[12,13]. Coaggregation is a process by which genetically distinct bacteria become attached to one another via specific molecules. Cumulative evidence suggests that such adhesion influences the development of complex multi-species biofilms. When considering the benefits coaggregation confers on bacterial partnerships, it is probable that the strength and specificity of the interactions will be subject to natural selection[13]. The ability to penetrate dense pathogen biofilms could also be supported by biosurfactant production if adhesion forces of lactobacilli with pathogens are more than those other binding the pathogens[14], but thereafter lactobacilli integration into the multilayered structure and formation of coaggregates with the pathogens would allow their antimicrobial molecules to disturb the biofilms and reduce pathogen viability[15–17].

Surface charge is the study of the acid - base on the surface of bacteria using chloroform (polar acidic solvent) as the electron donor and ethyl acetate (polar basic solvent) as electron acceptor. Surface charge can affect the ability of aggregations and hydrophobicity of bacterial cells[18]. Hydrophobicity of bacterial cell surface is one of important factor that control bacterial adhesion to various surfaces such as air/water interface, oil/water interface, biomaterial, teeth, animal cell, activate sludge and different solid surfaces[19]. Hydrophobic interactions define the strong attraction between hydrophobic molecules and surfaces in water. In biological systems hydrophobic interactions are the strongest long-range non-covalent interactions and are considered a determining factor in microbial adhesion to surfaces[20,21].

Aggregation abilities test together with cell surface abilities could be used for preliminary screening in identifying potentially adhered to each bacteria[2]. The aims of this study are to define aggregation abilities and surface characteristics; cell charge and hydrophobicity of oral *Lactobacillus* and to relate them with oral health status (dt).

Review of Literature

Lactobacilli

Lactobacilli are gram-positive, non spore forming, rods or coccobacilli microorganisms. They are fermentative, facultative anaerobe or microaerophylic and chemo-

organotrophic. They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains. They are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, gastrointestinal and genital tracts of humans and animals, and in sewage and plant material[1].

Lactobacillus in oral cavity

Lactobacilli usually comprise less than 1% of the total cultivable microbiota in the oral cavity. Commonly isolated species include *L. paracasei, L. plantarum, L. rhamnosus* and *L. salivarius*[2]. Lactobacilli are considered as part of the normal oral microbiota. They are the most bacteria that produce lactic acid and are associated with the progression of dental caries. Sometimes, the number of lactobacilli in saliva has been suggested to used as a part of caries risk estimation[3]. The species associated with caries are still ambiguous, however the differences between *Lactobacillus* species from caries-active and from healthy subjects have been reported[4]. Piwat *et al.*[5] isolated salivary lactobacilli from 59 children. They found that lactobacilli levels in children's saliva with low caries prevalence were significantly lower than lactobacilli levels in children's saliva with moderate to high caries prevalence. *L. fermentum* and *L. salivarius* were the predominant species but *L. salivarius* was found significantly in the moderate to high caries group found. Nonetheless, some *Lactobacillus* species have been proposed as probiotics in caries prevention, mainly because of their inhibitory activities against cariogenic *Streptococcus spp.* and contributing to the balance of microflora in the oral cavity[4].

Probiotic

Not all bacteria are harmful to the human body, some types of microorganisms that can provide a beneficial health effect on the host. Such that the live microorganisms called probiotics. "Probiotic" term, as opposed to "antibiotic", was initially proposed by Lilley and Stillwell in 1965. According to a WHO/FAO report (2002), probiotics are "Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host"[22]. Metchnikoff was the first to state a health benefit of probiotics, and proposed that Bulgarian people had a longer longevity because of viable bacteria in fermented milk. *L. acidophilus* was First probiotic species introduced in research by Hull *et al*. in 1984; followed by *B. bifidum* by Holcombh *et al* in 1991[23].

Probiotics can improve the condition of patients in medical disorders such as diarrhea, gastroenteritis, short-bowel syndrome, inflammatory intestinal diseases (Crohn's disease and ulcerative colitis), cancer, immunodepressive states, inadequate lactase digestion, pediatric allergies, growth retardation, hyperlipidemia and liver diseases, etc.; improved results in medical disorders after using probiotics have been supported by several researchs [23,24].

In oral cavity, probiotics can create a biofilm that role as a protective coating for oral tissues against oral diseases. like a biofilm protect oral tissues from bacterial pathogens and competing with cariogenic bacteria and periodontal pathogens growth[23–25].

Bussher *et al*.[26] reported that "*L. acidophilus* and *L. casei* presented in yoghurts can colonize the oral cavity because their ability to adhere to enamel". A one week consumption of this yoghurt caused a removal of other lactobacilli in dental plaque and in saliva. Näse *et al*.[27] showed that "long-term consumption of milk containing *L. rhamnosus GG* caused a significant lowering in caries risk in day care children".

Petti *et al*.[28] also found that "the regular consumption of *L. rhamnosus* GG yoghurt could decrease the salivary lactobacilli and *S. mutans* count, while *L. bulgaricus* contained in that product did not colonize the oral cavity. However effect of *L. rhamnosus* GG disappeared when its intake has been ended. A short-term consumption of cheese contained *L. rhamnosus* GG and *L. rhamnosus* LC 705 has been conducted whether this could diminished caries-associated salivary microbial counts in young adults". This cheese seemed to reduce counts of *S. mutans* and yeasts loading to reduce the carious risk[7,23,29].

Aggregation

Aggregation has been correlated with adhesion, which is known to be a prerequisite for colonization and infection by pathogens. Autoaggregation is defined as the adherence of bacteria themselves. The bacteria that can autoaggregate conveys a selective advantage over non-autoaggregation species by enhancing attachment within a developing biofilm[10].

Bacterial coaggregation is a result of interaction between two or more different species of bacteria to form a stable composite aggregation (Fig. 1)[11,13]. Coaggregation was first recognized among bacteria isolated from human dental plaque and the residue is defined as the process of bacterial adhesion between pairs of genetic difference. Several papers published in the 1970s

indicated that coaggregation was a common spectacle between broad ranges of genera from dental plaque. These early investigations showed that coaggregation between pairs of bacteria was highly specific and was typically mediated by a protein 'adhesin' on one cell type and a complementary saccharide 'receptor' on the other[12,13].

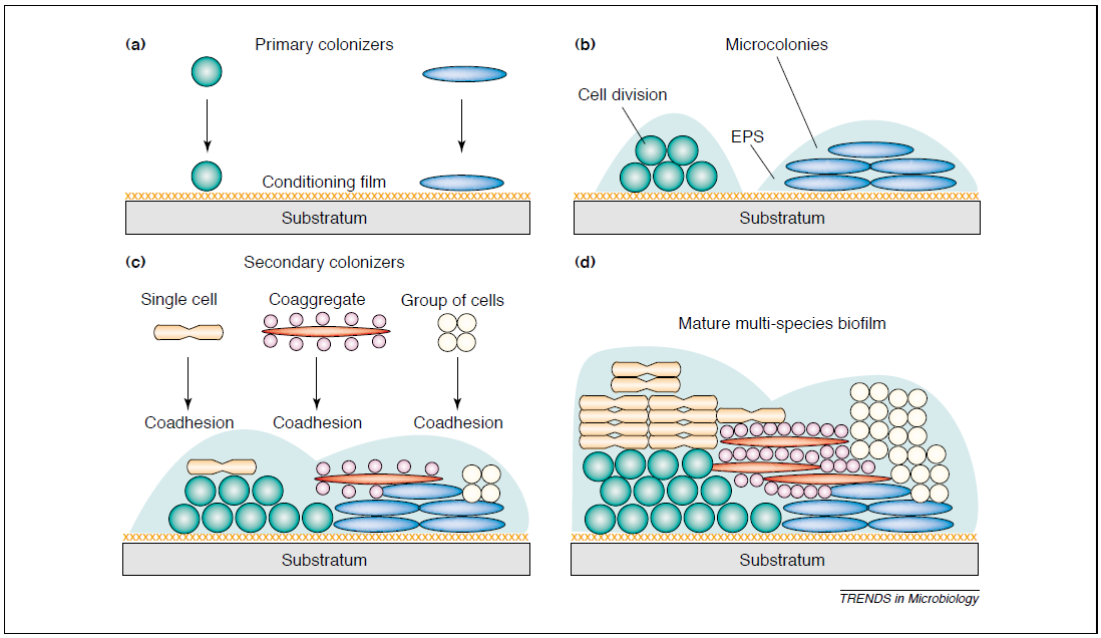

Fig. 1 According to Rickard *et al.*[13] "Diagram illustrating the possible roles of coaggregation in the development of multi-species biofilms. (a) Primary colonization of a substratum covered in a 'conditioning film' composed of polysaccharides and proteins; (b) cell growth, division and production of extracellular polysaccharide (EPS) leading to the development of microcolonies; (c) coadhesion of single cells, coaggregated cells and groups of identical cells into the young multi-species biofilm; and (d) maturation and the formation of clonal mosaics within the multi-species biofilm".

Collado *et al.*[30] have studied adhesion and aggregation of commercial probiotic strains compare with enteric pathogens, the pathogen strains showed lower autoaggregation abilities than probiotic strains. Highest autoaggregation are *L. fermentum* ME-3 at 20 and 37 °C. Coaggregation abilities of probiotic strains and pathogen strains were demonstrated to be strain-specific and dependent on time and incubation conditions. The highest coaggregation was obtained between *B. vulgatus* with *L. rhamnosus* LC-705 and with *L. fermentum* ME-3, respectively. The

strains with less coaggregation ability with *B. vulgatus* were *B. lactis* 420, *B. longum* 46 and *P. freudenreichii* JS.

Kos *et al.*[31] has investigated aggregation and adhesiveness of *L. acidophilus* M92, they found the relationship between autoaggregation and adhesiveness ability of *L. acidophilus* M92 which mediated by proteinaceous components on the cell surface.

Physiochemical characteristic of cell surface properties

Surface charge effect

Surface charge is the study of the acid - base on the surface of bacteria using chloroform (polar acidic solvent) as the electron donor and ethyl acetate (polar basic solvent) as electron acceptor. Surface charge can effect the ability of aggregations and hydrophobicity of bacterial cells.

Rijinaarts *et al.*[32] insisted that "long-range forces, mainly electrostatic interactions due to the overlapping of diffuse layers and van der Waals forces, i.e. DLVO forces, are a very important factor of adhesion onto substratum surfaces at relatively lower ionic concentrations". Bos *et al.*[33] revealed that "substratum hydrophobicity is a major determinant of bacterial cell retention although it hardly influences bacterial adhesion".

According to van Oss[34], "Hydrophobic interactions in biological systems are commonly the strongest of all long-range non-covalent interactions, can be designated as the attraction between apolar or slightly polar molecules, particles or cells immersed in water. The main driving force is the hydrogen bonding (AB forces or Lewis Acid-Base) energy of cohesion between the surrounding water molecules. This means that the AB forces, if strongly asymmetrical or monopolar, are responsible for the orientation of water molecules adsorbed on the surfaces". As a result of orientation of water molecules on the one particle's surface will repel orientation of water molecules in the same manner on the surface of an adjacent particle[35]. If the orientation of the water molecules is sufficiently strong, the two particles will not approach each other. If on the other hand the surface is more weakly apolar, its capacity for orientation of the most closely adsorbed water molecules is less pronounced and the particles will approach each other under the influence of their net Lifshitz-van der Waals (LW) attraction. "Hydrophobic" compounds or surfaces do not repel water rather they attract water with a substantial binding energy, although not quite strongly as very hydrophilic compounds or surfaces [36]. It should be emphasized that hydrophobic attractions can prevail between one

hydrophobic and one hydrophilic site immersed in water, as well as between two hydrophobic entities.

Colloca *et al.*[37] has researched surface properties of lactobacilli that isolated and identified from teeth, tongue, saliva and gum of healthy patients, it was found that strains from saliva and tongue had high electron donor. And lactobacilli that isolates from the tongue showed low solvents affinity that concurs with low hydrophobicity results. They summarized that the bacterial cell surface charge of lactic acid bacteria were different. And that could be involved in their adhesion to hard or soft tissues of the mouth, or in attachment to other bacteria.

L. casei, L. paracasei, and *L. rhamnosus* showed strong electron donor. In contrast they were low electron acceptor, as confirmed by their hydrophilic cell surface properties[38].

On the other hand, previous investigations on the physicochemistry of microbial cell surfaces have shown correlations between hydrophobicity, surface charges and elemental surface compositions of the cells[39]. They indicate that higher hydrophobicity result from the presence of (glyco-) proteinaceous material at the cell surface[39], whereas a hydrophilic surface was associated with the presence of polysaccharides. Lipoteichoic acids and other outer cell wall substances might have an effect on hydrophobicity as well, but it is still unclear.

DLVO theory

According to Hermansson[40] "The DLVO theory has been used to describe the net interaction between a cell and a flat surface (substratum) as a balance between two additive factors, van der Waals (vdW) interactions (generally attractive) and repulsive interactions (V_R) from the overlap between the electrical double layer of the cell and the substratum (generally repulsive, due to the negative charge of cells and substrata) (Fig. 2)".

"The vdW interaction is a material property that describes the strength of the interaction between a surfaces and the medium, as well as between two interacting bodies in a medium. It spends on the dielectric properties of the medium, the substratum and the cell. The only significant attractive force known to be present is van der Waals force which is due to an interaction between oscillating dipoles on the surface molecules. This force is unaffected by ionic strength. Van der Waals attractive force is a very powerful force but it only operates over a small distance. Significantly, van der Waals attractive force reveal force less than the repulsive force due to overlapping electric double layers. It is, however, very strong and if the cell can get close

enough to the surface, the van der Waals force will hold it very tightly. It is showed that, when the cell gets very close to the surface the van der Waals force starts to get very big indeed. In fact, at very small distances the van der Waals attractive force is enormously bigger than the electrostatic repulsion"[40].

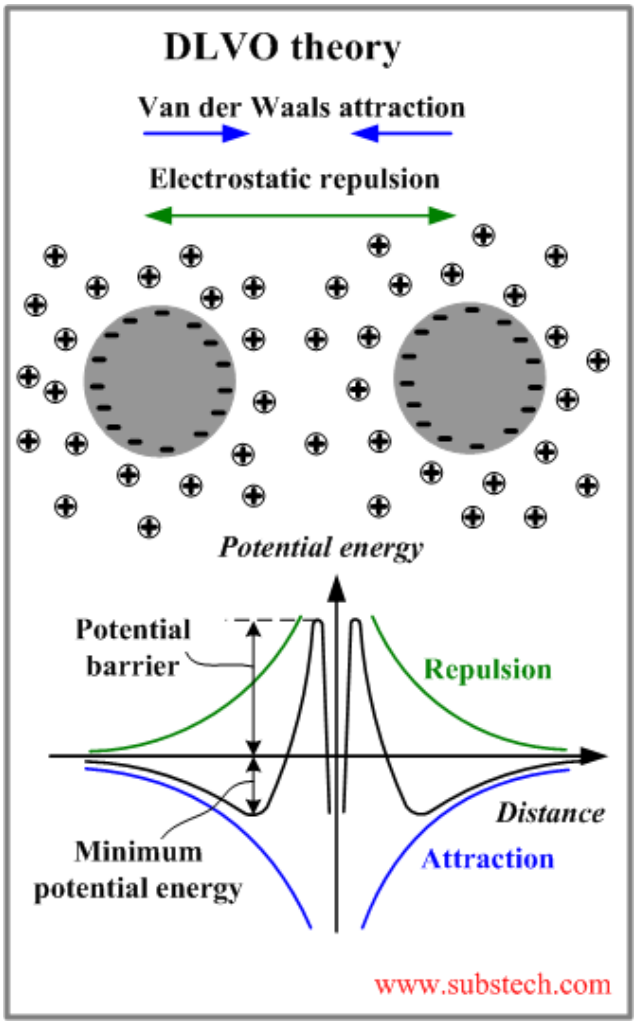

Fig. 2 The graphs describing the potential energy of the interaction between two particles.

"The double layer interaction (*V*R) originates from the Coulomb interaction between charged, molecules, and its strength and range. It is strongly affected by the presence of surrounding ions. Independent of charging mechanism of any surface, the surface charge is balanced (electroneutrality) by an equal but oppositely charged region of counterions"[40].

The theory states that the stability of colloidal is determined by the potential energy of the particles (V_T) outlining two potential energy of the interaction of interest due to van der Waals force V_A and potential energy of the repulsive electrostatic interaction V_R:

$$\mathbf{V_T = V_A + V_R}$$

"The minimum of the potential energy determines the distance between two particles corresponding to their stable equilibrium. The two particles form a loose aggregate, which can be easily re-dispersed. The strong aggregate may be formed at a shorter distance corresponding to the primary minimum of the potential energy (not shown in the picture). In order to approach to the distance of the primary minimum the particle should overcome the potential barrier"[40].

Hypothesis of the study

1. Aggregation abilities and surface charge of oral *Lactobacillus* are interrelated.
    a. Autoaggregation and hydrophobicity of oral *Lactobacillus* are interrelated.
2. Aggregation ability of oral *Lactobacillus* is related caries status.
    a. Aggregation abilities of oral *Lactobacillus* is related to high caries.

Objective of the study

1. To study the autoaggregation abilities of oral *Lactobacillus*.
2. To study the coaggregation abilities between oral *Lactobacillus* species and *S. mutans*.
3. To study the surface charge of oral *Lactobacillus*.
4. To study relationship between aggregation abilities and surface charge of oral *Lactobacillus*.
5. To study relationship between aggregation abilities/surface charge and caries status.

CHAPTER 2

Materials and Methods

Bacteria strains

One hundred and ninety-eight strains of oral *Lactobacillus* obtained from the culture collection of Department of Stomatology, Faculty of Dentistry, Prince of Songkla University were studied including 62 *L. fermentum* strains, 29 *L. salivarius* strains, 17 *L. gasseri* strains, 12 *L. plantarum* strains, 17 *L. rhamnosus* strains, 12 *L. mucosae* strains, 11 *L. casei* strains, 8 *L. oris* strains, 22 *L. paracasei* strains, 8 *L. vaginalis* strains and 10 *Lactobacillus* reference strains were included. The tested reference strains were *L. fermentum* ATCC 14931^T, *L. salivarius* ATCC 11741^T, *L. gasseri* ATCC 33323^T, *L. plantarum* ATCC 14917^T, *L. rhamnosus* ATCC 7469^T, *L. mucosae* CCUG 43179^T, *L casei* ATCC 393^T, *L .oris* CCUG 37396^T, *L. paracasei* CCUG 32212^T and *L. vaginalis* CCUG 31452^T. These clinical strains were isolated from saliva sample of 59 two-year-old children. Each tested strains were selected from different child and different genotype within the same species of *Lactobacillus*. The study design, selection of patients, isolation and identification procedures have been described in the study of Teanpaisan *et al.*[41]. The oral pathogen used for coaggregation test in this study was *Streptococcus mutans* ATCC 25175™. The bacterial strains were kept in a freezer at -80°C until required.

Bacterial cultivation

Before the experiment, the *Lactobacillus* strains were cultivated in Man Rogosa Sharpe (MRS) broth (LAB scan, India) in an anaerobic condition (80% N_2, 10% H_2 and 10% CO_2) at 37^{o}C for 24 hrs. The streptococcus strains were grown in Brain Heart Infusion (BHI) broth (Difco, France) under conditions of low-oxygen (5% CO_2) at 37^{o}C for 24 hrs. After incubation, the bacteria were harvested by centrifugation at 10,000 rpm for 15 min at 4ºC. Cells were washed twice in phosphate buffered saline (PBS).

Aggregation of *Lactobacillus*

Autoaggregation: Autoaggregation assays were performed according to Del Re *et al.*[42]. The cell density was monitored by measuring the optical density at 600 nm (OD_{600}) using a

spectrophotometer (Multiskan GO, Thermo Scientific, USA) throughout the study. The bacterial cells were suspended in PBS buffer to give an OD_{600} of 0.5 (approximately 10^8 CFU ml^{-1} cells density). Cell suspensions (6 ml.) were mixed by vortexing for 10 sec and left undisturbed at room temperature. One milliliter of upper layer of each tube was carefully removed after 1, 4 and 24 hrs. Absorbance of the supernatant was measured at 600 nm. The autoaggregation percentage was calculated by the formula:

$$\% \text{ Autoaggregation} = (OD_0\text{-}OD_t)/OD_0 \times 100$$

Where OD_t represents the absorbance at time t = 1, 4 or 24 hrs. and OD_0 represents the absorbance at t = 0.

Coaggregation: Coaggregation assays between *Lactobacillus* and *S. mutans* ATCC 25175™ were also performed according to Del Re *et al.*[42]. The bacterial cells were suspended in PBS buffer to OD_{600} of 0.5 (approximately 10^8 CFU ml^{-1} cells for *Lactobacillus* and 10^8 CFU ml^{-1} cells for *S. mutans*). Equal volumes (3 ml. each) of the *Lactobacillus* and *S. mutans* suspensions were mixed together by vortexing for 10 sec. Control tubes were set up at the same time, containing 6 ml. of each bacterial suspension on its own. The preincubation OD value of control and mixed suspension was measured. After incubation at room temperature without agitation for 1, 4 and 24 hrs. to allow coaggregation occurred, 1 ml. of upper layer of the supernatant was carefully removed. Absorbance was measured. The percentage of coaggregation was calculated using the equation[43]:

$$\% \text{ Coaggregation} = \{[(OD_x + OD_y)/2 - OD_{(x+y)}] / (OD_x + OD_y)/2\} \times 100$$

Where OD_x and OD_y represent preincubation OD value of each of the two strains in the control tubes, and $OD_{(x+y)}$ represents OD value of the mixture at time 1, 4 or 24 hrs.

Surface charge of *Lactobacillus*

The microbial adhesion to hydrocarbon test (MATH)[44] was determined following the modified method described by Geertsema *et al.*[45]. The adhesion of bacteria to the different hydrocarbon solution, including xylene (nonpolar), chloroform (monopolar and electron-acceptor) and ethyl acetate (monopolar and electron-donor) were measured. The bacterial cells suspended in PBS solution were adjusted to OD_{600} of 0.2 (approximately 10^6 CFU ml^{-1} cell density). After homogenization, 3 ml. of the suspension were pipetted into a test tube. Subsequently, 1 ml. of hydrocarbon solution was added and then the mixture was vortexed at maximum speed for 60 sec.

The OD of both the initial and the extracted solution was determined at 600 nm. using a spectrophotometer (Multiskan GO, Thermo Scientific, USA) and disposable polystyrene cuvettes with an effective volume of 1 ml. A blank value was determined using the PBS buffer without added bacteria. After a waiting period of 15 min. employed to achieve complete phase separation between the sample and hydrocarbon phases (Fig. 3), the aqueous phase was carefully collected and its optical density at 600 nm. was measured. The percentage of bacterial adhesion to solvent was calculated as:

$$\% \text{ Microbial adhesion to hydrocarbon (MATH)} = (OD_{before}\text{-}OD_{after})/OD_{before} \times 100$$

Lactobacillus strains were classified in three groups depending on their % MATH: those with low (0–35%), moderate (36–70%), and high of charge surface (71–100%)[37].

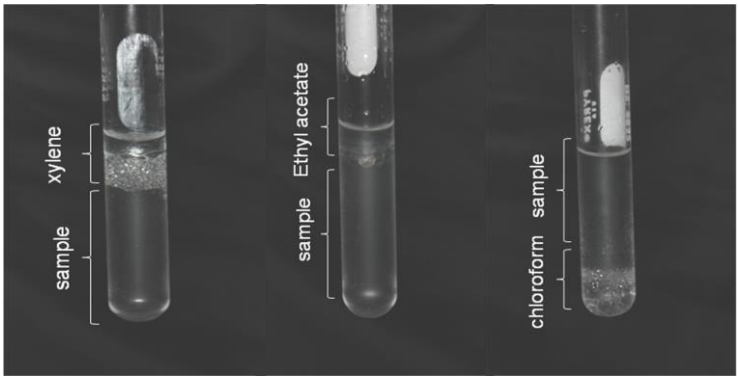

Fig. 3 The phase separation between the sample and hydrocarbon

Statistical analysis

Each experiment was carried out in duplicate using independently fermented cultures. The data were descriptive by mean value and standard deviation. Spearman rank correlation coefficient and relative R^2 was calculated between aggregation ability and physicochemical properties. The Mann-Whitney U test was determined for the comparison between the data from low caries and high caries group. The analyses were performed with the SPSS statistical program (SSPS Inc., Chicago, IL). The differences were considered significant when $p < 0.05$.

CHAPTER 3

Result

Bacteria strains

A total of 198 strains of oral *Lactobacillus* and their oral health status were shown in Table 1. The strains were divided into two groups according to the caries status of the children: low caries (the numbers of decayed teeth (dt) ≤5), high caries (dt >5)[5].

Table 1 Oral *Lactobacillus* with their oral health status

	No. of strains	
Species(n)	Low caries (dt≤5)	High caries (dt>5)
L. casei (11)	6	5
L. fermentum (62)	28	34
L. gasseri (17)	13	4
L. mucosae (12)	0	12
L. oris (8)	5	3
L. paracasei (22)	9	13
L. plantarum (12)	1	11
L. rhamnosus (17)	10	7
L. salivarius (29)	3	26
L. vaginalis (8)	8	0
Total (198)	75	123

Aggregation of oral *Lactobacillus*

The autoaggregation of oral *Lactobacillus* (Fig. 4A) and coaggregation with *S. mutans* (Fig. 4B) are shown. Autoaggregation and coaggregation ability of *Lactobacillus* strains depended on time of incubation and varied among the species. The highest autoaggregation and coaggregation values were presented at 24 hrs. *L. gasseri* showed the highest autoaggregation and coaggregation abilities while *L. oris* and *L. mucosae* showed the lowest ability compared with

other strains. The correlation between autoaggregation and coaggregation of all species was found at a significant of $p < 0.05$ as shown in Fig. 5 and Fig. 6.

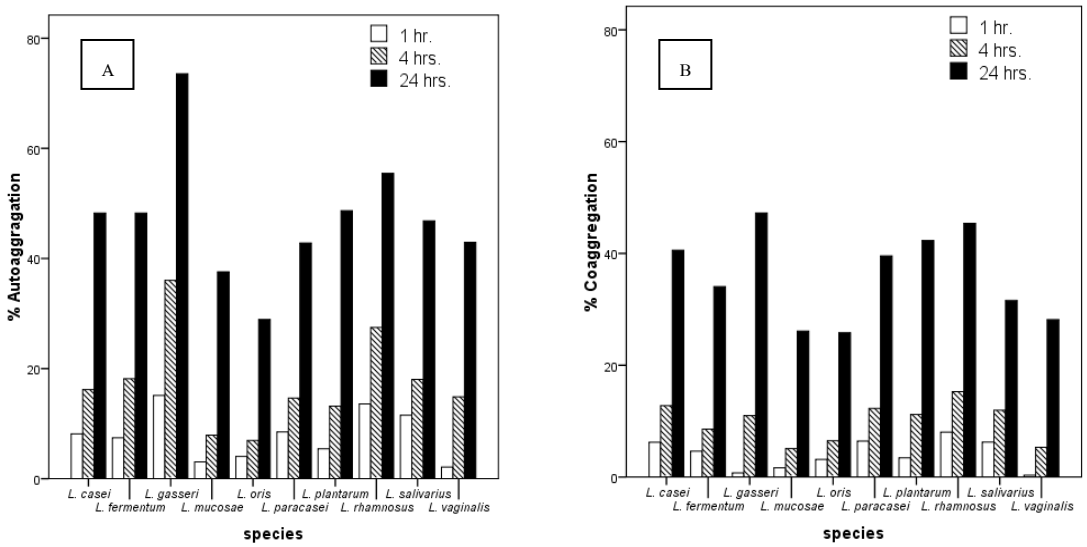

Fig. 4 Autoaggregation of oral *Lactobacillus* (A) and their coaggregation abilities with *S.mutans* (B) at 1, 4 and 24 hrs.

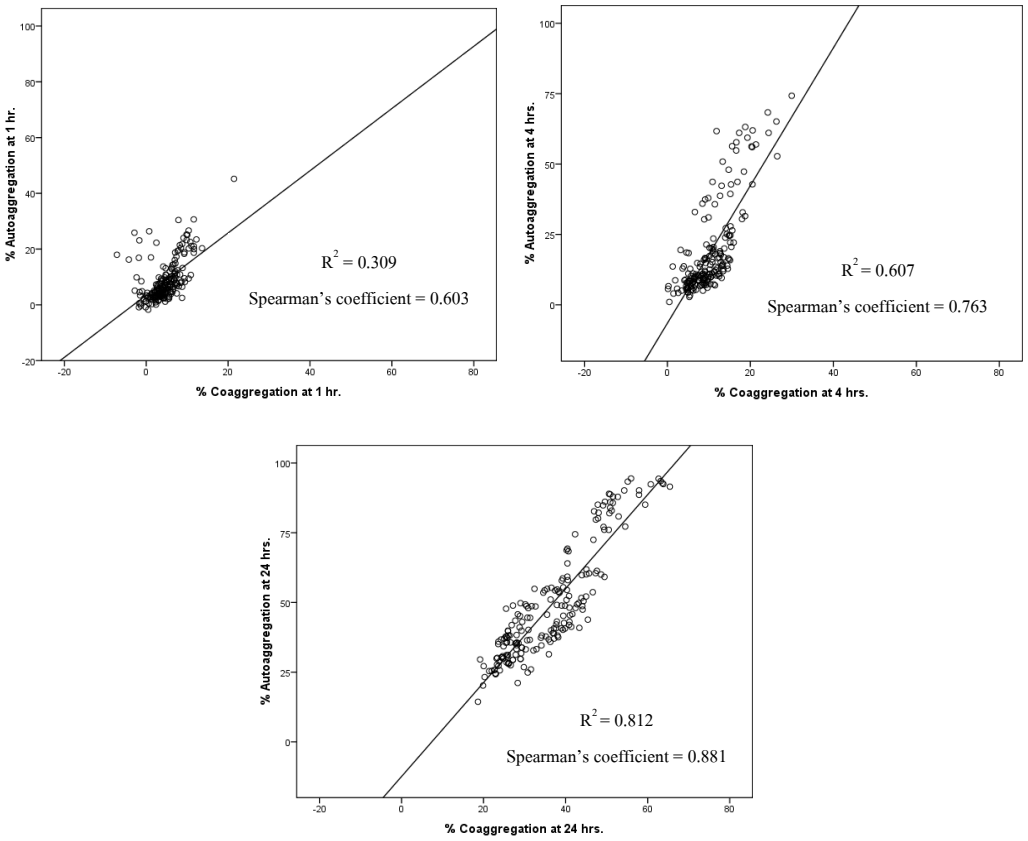

Fig. 5 Scatter graph of Spearman's correlations and relative R^2 values between autoaggregation and coaggregation properties of oral *Lactobacillus* at 1, 4 and 24 hrs.

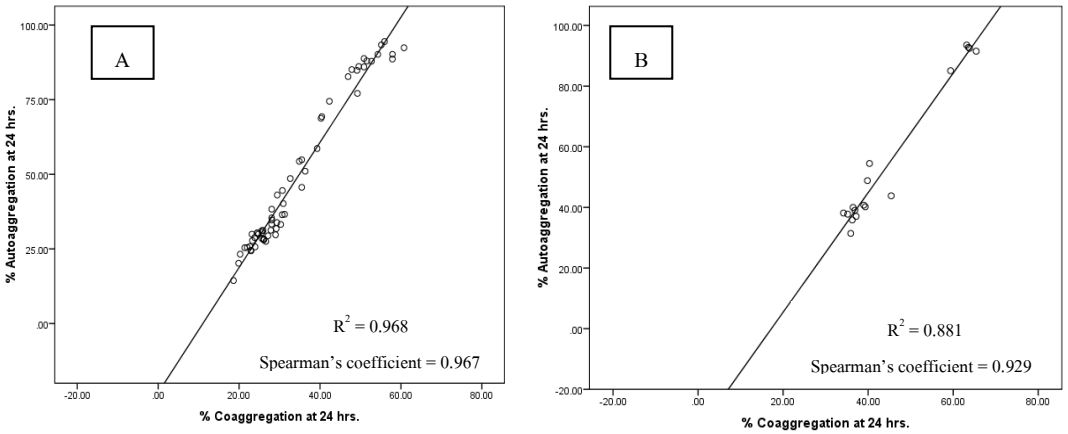

Fig. 6 Scatter graph of Spearman's correlations and relative R^2 values between autoaggregation and coaggregation properties of *L. fermentum* (A) and *L. paracasei* (B) at 24 hrs.

Surface charge of oral *Lactobacillus*

The adhesive characteristics of oral *Lactobacillus* to xylene, chloroform and ethyl acetate are shown in Fig. 7. Most *Lactobacillus* strains showed high affinity (> 85%) with chloroform (electron donor). All strains show moderate to high affinity with xylene (hydrophobicity) except *L. gasseri* and *L. vaginalis* showed statistically significant low hydrophobicity (< 35%) at $p < 0.05$. The bacterial cell adhesion to ethyl acetate, a strain basic solvent and electron donor, was moderate except *L. gasseri* has statistically significant the lowest value (10.9%).

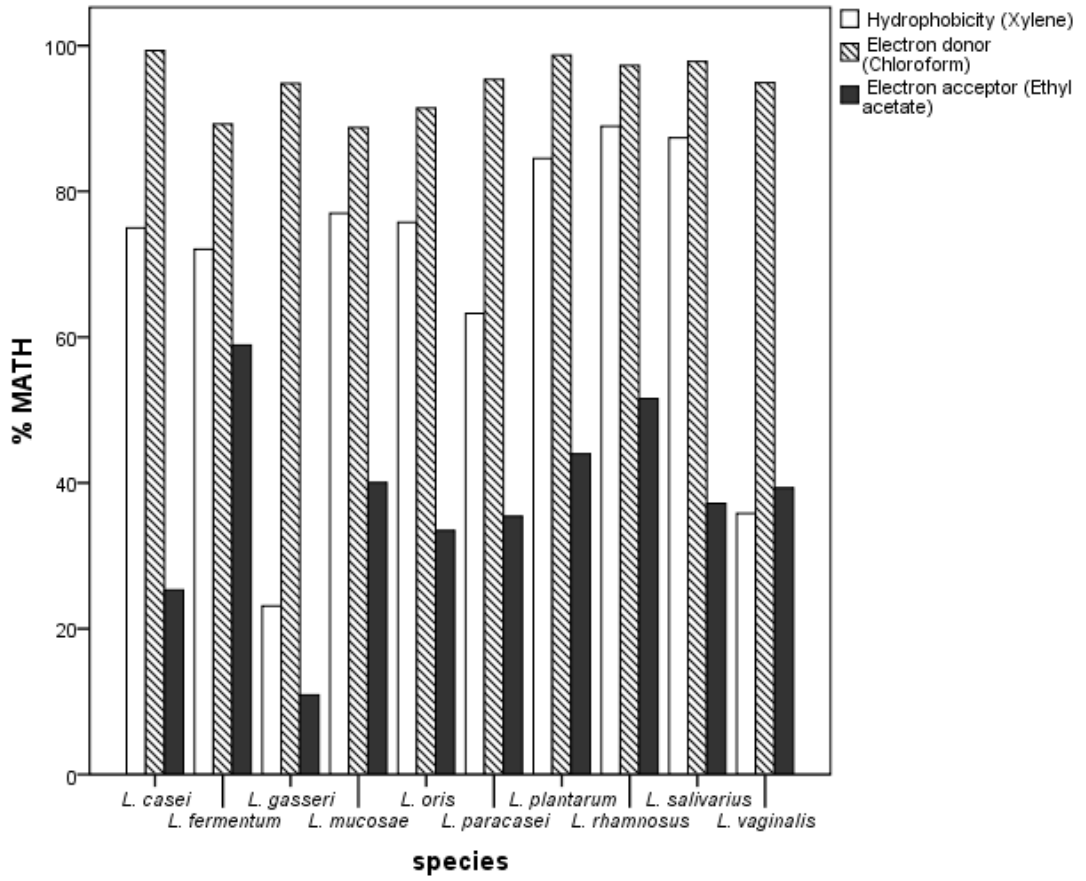

Fig. 7 Cell surface hydrophobicity, surface charge characteristic of oral *Lactobacillus*

Aggregation ability and surface charges

The correlation between autoaggregation/coaggregation of oral *Lactobacillus* and surface charge (hydrophobicity, electron donor, electron acceptor) are significantly correlated ($p < 0.05$) (Fig. 8).

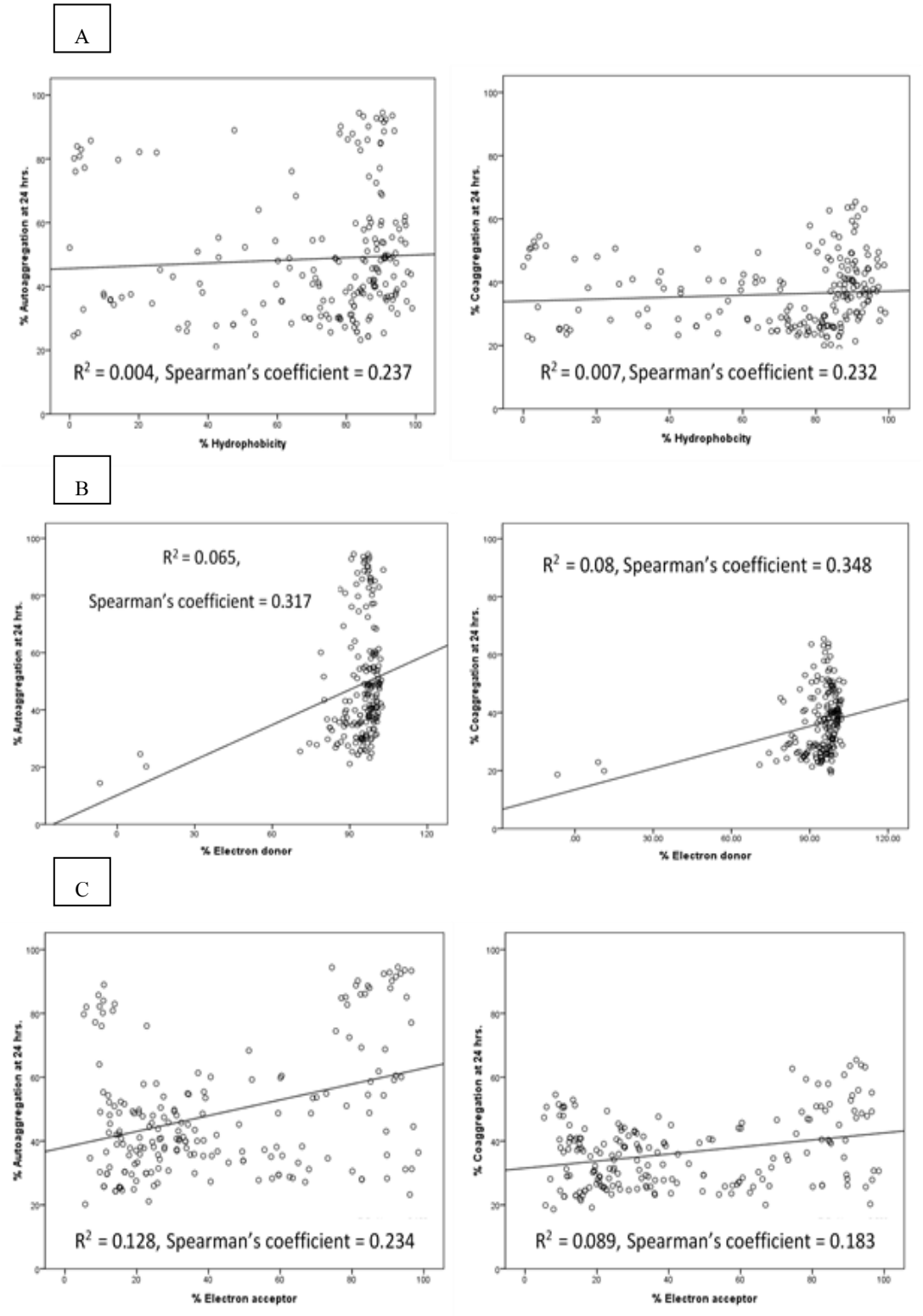

Fig. 8 Scatter graph of Spearman's correlations and relative R^2 values between autoaggregation/coaggregation at 24 hrs. and hydrophobicity (A), electron donor (B) and electron acceptor (C) characteristics of cell surface

Aggregation ability and surface charges with oral health status (dt)

No correlation was found between autoaggregation/coaggregation and oral health status and between surface charge and oral health status (Fig. 9A and 9B). The surface hydrophobicity was significantly lower in the strains from low caries group, compared with the strains from high caries group ($p<0.05$) (Fig. 10).

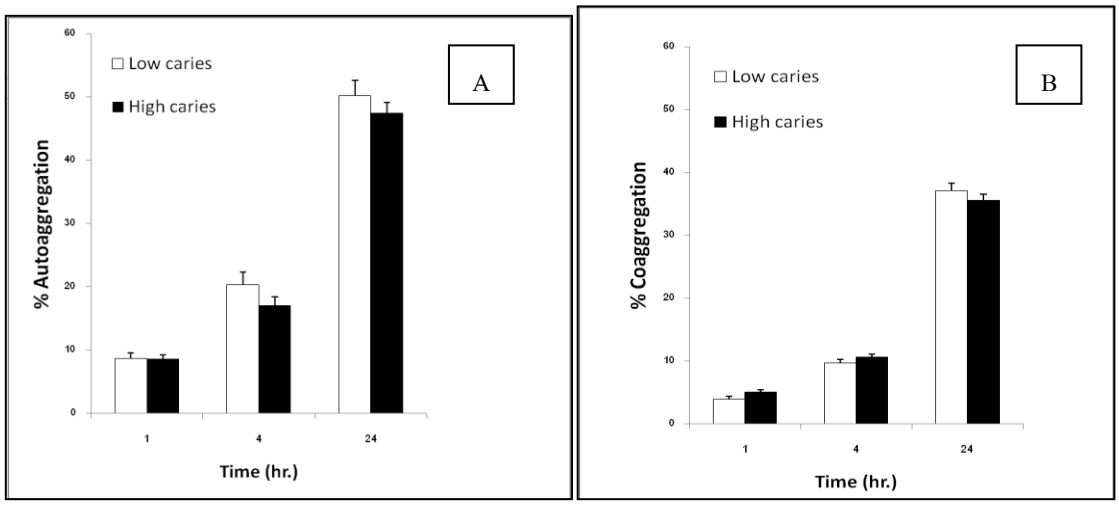

Fig. 9 Bar graph of autoaggregation/coaggregation at 24 hrs. enhanced by low caries and high caries.

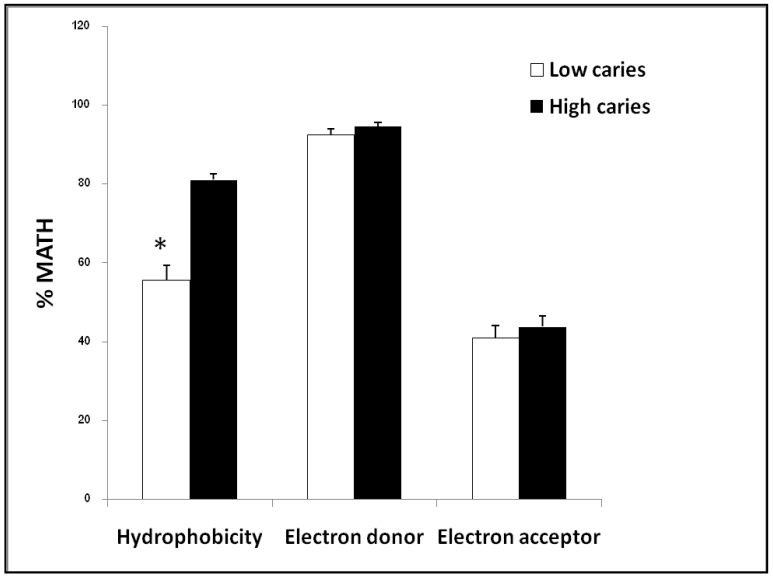

Fig. 10 Bar graph of surface charge at 24 hrs. enhanced by low caries and high caries. Asterisk indicates significant difference between cell surface hydrophobicity of the strains from low caries and from high caries**.**

CHAPTER 4

Discussion

Cell adhesion is a complex process involving contact between the bacterial cell membrane and interacting surfaces. The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains in several ecological niches[30,46] especially in the oral cavity. This specific interaction selectively integrates bacteria of different species into a biofilm. Autoaggregation ability is one of the key factors that determine the ability of bacterial strains to adhere, which is known to be a prerequisite for colonization not only for the pathogens but also for the probiotics strains.

The microbial adhesion to hydrocarbon test has been extensively used for measuring cell surface characteristics in lactic acid bacteria. Xylene, chloroform and ethyl acetate were used to assess the hydrophobic/hydrophilic, electron donor (basic) and electron acceptor (acidic) characteristics of bacterial surface. Many authors have reported that hydrophobicity and surface charge of bacterial are related to cell adherence properties[47,48].

Our results demonstrated that most oral lactobacilli strains tested showed relatively high autoaggregation at 24 hrs. Bacterial aggregation has been observed widely among oral bacteria. The non-aggregating bacteria cannot be incorporated in the multi-generic aggregates formed in cell suspensions and are eventually washed out along with swollen saliva[13]. In this study, the bacterial affinities to ethyl acetate were relatively low when compared to chloroform, indicating oral *Lactobacillus* have the strong electron donor and poor electron acceptor property. Hydrophobic cell surface is demonstrated by high affinity to xylene, an apolar solvent. A high percentage adhered to xylene was found in this study, demonstrated that most of oral *Lactobacillus* had high hydrophobicity except *L. gasseri* and *L. vaginalis*. Cuperus *et al.*[39] studied the physicochemical surface characteristics of *Lactobacillus* and reported that the presence of (glycol-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides. Pan *et al.*[47] reported the relationship between the higher hydrophobic strains and the stronger adhesive capability which is a major role in initial interaction with host tissue. Microbial with high aggregation ability and hydrophobic cell surface could have more chance for adhesion and colonization[49].

L. fermentum, *L. salivarius, L. casei, L. plantarum* and *L. paracasei* were demonstrated high autoaggregate and hydrophobic cell surface in our study. Several studies reported high prevalence of these species in the oral cavity[5,50]. Our previous study found the relation between the presence of *L. salivarius* and high caries while *L. fermentum* was found in both low and high caries status[5]. In this study, autoaggregation/coaggregation and basic/acid surface characteristic were no correlation with dental health status. However, the correlation between dental health status and hydrophobicity was statistically significant. The strains from high caries group were high hydrophobicity. These results do not coincide with the study of Colloca *et al.*[37] and Ahumanda *et al*.[50]. They reported that *Lactobacillus* species isolated from healthy mouth have high hydrophobicity, while the strains from caries active mouths, showed lower ability.

In probiosis for oral health, coaggregation ability of *Lactobacillus* strains with cariogenic pathogens are of importance for prevent colonization by pathogens[51]. The current study found that the coaggregation ability with *S. mutans* of oral *Lactobacillus* was varies among the species. For probiotic candidate, not only the coaggregation ability but also the other properties such as inhibitory effect against pathogens, acidogenicity and aciduricity should be considered. Our previous studies reported the higher inhibitory effect against *S. mutans* of *L. paracasei*, *L. plantarum*, *L. rhamnosus*[52] and the lowest acid production of the *L. paracasei* strains[53]. Although *L. gasseri* showed highest pathogenic coaggregation abilities and the greatest autoaggregation, they had low hydrophobicity and low affinity to ethyl acetate. Piwat *et al*.[5] and Koll-Klais *et al*.[9] found that *L. gasseri* had really low antimicrobial activity against *S. mutans*, and the presence of this species was quite low in the oral cavity. Adhesion is a complex process involving non-specific (hydrophobicity and hydrophilic) and specific ligand-receptor mechanisms. The information in the present study can be used as the preliminary data for select the strains that suit for use as oral probiotics.

CHAPTER 5

Conclusion

In conclusion, most oral lactobacilli strains showed adhesion related properties included high electron donor, low to moderate electron acceptor and moderate hydrophobicity. However, only hydrophobicity was statistically significant difference ($p < 0.05$) between the groups of high and low caries subject. Bacterial binding capabilities (autoaggregation and coaggregation) and surface characteristics (adhesion to hydrocarbons) is preliminary screening in order to identify potentially bacteria adhesion. Further studies are needed to understand mechanism of surface charge characteristic and correlation between aggregation ability/surface charges with the other microorganisms.

BIBLIOGRAPHY

1. Felis GE, Dellaglio F. Taxonomy of lactobacilli and bifidobacteria. ***Curr Issues Intest Microbiol*** 2007; 8(2): 44–61.
2. Haukioja A. Probiotic lactobacilli and bifidobacteria in the mouth – in vitro studies on saliva-mediated functions and acid production [dissertion]. Turku: University of Turku; 2009.
3. Larmas M. Saliva and dental caries: diagnostic tests for normal dental practice. ***Int Dent J*** 1992; 42(4): 199–208.
4. S Simark-Mattsson C, Emilson C-G, Håkansson 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(4): 308–14.
5. Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlén G. *Lactobacillus* species and genotypes associated with dental caries in Thai preschool children. ***Mol Oral Microbiol*** 2010; 25(2): 157–64.
6. Michalek SM, Hirasawa M, Kiyono H, Ochiai K, McGhee JR. Oral ecology and virulence of *Lactobacillus casei* and *Streptococcus mutans* in gnotobiotic rats. ***Infect Immun*** 1981; 33(3): 690–6.
7. Badet C, Thebaud N. Ecology of lactobacilli in the oral cavity: a review of literature. ***Open Microbiol J*** 2008; 29(2): 38–48.
8. 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(2): 172–9.
9. Kõll-Klais P, Mändar R, Leibur E, Marcotte H, Hammarström L, Mikelsaar M. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. ***Oral Microbiol Immunol*** 2005; 20(6): 354–61.
10. Elliott DR, Wilson M, Buckley CMF, Spratt DA. Aggregative behavior of bacteria isolated from canine dental plaque. ***Appl Environ Microbiol*** 2006; 72(8): 5211–7.
11. Chen F. Bacterial auto-aggregationand co-aggragation in activated slude [dissertion]. South Carolina: Clemson University; 2007.

12. Gibbons RJ, Nygaard M. Interbacterial aggregation of plaque bacteria. ***Arch Oral Biol*** 1970; 15(12): 1397–400.

13. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial coaggregation: an integral process in the development of multi-species biofilms. ***Trends Microbiol*** 2003; 11(2): 94–100.

14. Velraeds MM, van de Belt-Gritter B, van der Mei HC, Reid G, Busscher HJ. Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. ***J Med Microbiol*** 1998; 47(12): 1081–5.

15. McMillan A, Dell M, Zellar MP, Cribby S, Martz S, Hong E, *et al*. Disruption of urogenital biofilms by lactobacilli. ***Colloids Surf B Biointerfaces*** 2011; 86(1): 58–64.

16. Jones SE, Versalovic J. Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors. ***BMC Microbiol*** 2009; 9(1): 35.

17. Saunders S, Bocking A, Challis J, Reid G. Effect of *Lactobacillus* challenge on *Gardnerella vaginalis* biofilms. ***Colloids Surf B Biointerfaces*** 2007; 55(2): 138–42.

18. Xu H, Jeong HS, Lee HY, Ahn J. Assessment of cell surface properties and adhesion potential of selected probiotic strains. ***Lett Appl Microbiol*** 2009; 49(4): 434–42.

19. Zita A, Hermansson M. Determination of bacterial cell surface hydrophobicity of single cells in cultures and in wastewater in situ. ***FEMS Microbiol Lett*** 1997; 152(2): 299–306.

20. Sanin SL, Sanin FD, Bryers JD. Effect of starvation on the adhesive properties of xenobiotic degrading bacteria. ***Process Biochem*** 2003; 38(6): 909–14.

21. Kadam TA, Rupa L, Balhal DK, Totewad ND, Gyananath G. Determination of the degree of hydrophobicity - a technique to assess bacterial colonization on leaf surface and root region of lotus plant. ***Asian J Exp Sci*** 2009; 23(1): 135–9.

22. Reid G, Jass J, Sebulsky MT, McCormick JK. Potential uses of probiotics in clinical practice. ***Clin Microbiol Rev*** 2003; 16(4): 658–72.

23. Flichy-Fernandez A, Alegre-Domingo T, Penarrocha-Oltra D, Penarrocha-Diago M. Probiotic treatment in the oral cavity: an update. ***Med Oral Patol Oral Cirugia Bucal*** 2010; 15(5): 677–680.

24. Caglar E, Kargul B, Tanboga I. Bacteriotherapy and probiotics' role on oral health. ***Oral Dis*** 2005; 11(3): 131–7.

25. Comelli EM, Guggenheim B, Stingele F, Neeser J-R. Selection of dairy bacterial strains as probiotics for oral health. ***Eur J Oral Sci*** 2002; 110(3): 218–24.

26. 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(5): 403–4.

27. Näse L, Hatakka K, Savilahti E, Saxelin M, Pönkä 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(6): 412–20.

28. 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(8): 705–12.

29. Ahola AJ, Yli-Knuuttila H, Suomalainen T, Poussa T, Ahlström 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(11): 799–804.

30. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. ***Eur Food Res Technol*** 2008; 226(5): 1065–73.

31. Kos B, Suskoviċ J, Vukoviċ S, Simpraga M, Frece J, Matosiċ S. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. ***J Appl Microbiol*** 2003; 94(6): 981–7.

32. Rijnaarts HHM, Norde W, Lyklema J, Zehnder AJB. DLVO and steric contributions to bacterial deposition in media of different ionic strengths. ***Colloids Surf B Biointerfaces*** 1999; 14(1-4): 179–95.

33. Bos R. Retention of bacteria on a substratum surface with micro-patterned hydrophobicity. ***FEMS Microbiol Lett*** 2000; 189(2): 311–5.

34. Van Oss CJ. Hydrophobicity and hydrophilicity of biosurfaces. ***Curr Opin Colloid Interface Sci*** 1997; 2(5): 503–12.

35. Leikin S, Parsegian VA, Rau DC, Rand RP. Hydration Forces. ***Annu Rev Phys Chem*** 1993; 44(1): 369–95.

36. Van Oss CJ. Hydrophobicity of biosurfaces - origin, quantitative determination and interaction energies. ***Colloids Surf B Biointerfaces*** 1995; 5(3-4): 91–110.

37. Colloca ME, Ahumada MC, López ME, Nader-Macías ME. Surface properties of lactobacilli isolated from healthy subjects. ***Oral Dis*** 2000; 6(4): -227–33.

38. Pelletier C, Bouley C, Cayuela C, Bouttier S, Bourlioux P, Bellon-Fontaine MN. Cell surface characteristics of *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, and *Lactobacillus rhamnosus strains*. ***Appl Environ Microbiol*** 1997; 63(5): 1725–31.

39. Cuperus PL, van der Mei HC, Reid G, Bruce AW, Khoury AH, Rouxhet PG, *et al*. Physicochemical surface characteristics of urogenital and poultry lactobacilli. ***J Colloid Interface Sci*** 1993; 156(2): 319–24.

40. Hermansson M. The DLVO theory in microbial adhesion. ***Colloids Surf B Biointerfaces*** 1999; 14(1-4): 105–19.

41. Teanpaisan R, Thitasomakul S, Piwat S, Thearmontree A, Pithpornchaiyakul W, Chankanka O. Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3–24 month old Thai children. ***Int Dent J*** 2007; 57(6): 445–51.

42. Del Re B, Sgorbati B, Miglioli M, Palenzona D. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. ***Lett Appl Microbiol*** 2000; 31(6): 438–42.

43. Handley PS, Harty DW, Wyatt JE, Brown CR, Doran JP, Gibbs AC. A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of *Streptococcus salivarius*. ***J Gen Microbiol*** 1987; 133(11): 3207–17.

44. Rosenberg M. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. ***FEMS Microbiol Lett*** 1984; 22(3): 289–95.

45. Geertsema-Doornbusch GI, van der Mei HC, Busscher HJ. Microbial cell surface hydrophobicity. The involvement of electrostatic interactions in microbial adhesion to hydrocarbons (MATH). ***J Microbiol Methods*** 1993; 18(1): 61–8.

46. Jankovic I, Ventura M, Meylan V, Rouvet M, Elli M, Zink R. Contribution of aggregation-promoting factor to maintenance of cell shape in *Lactobacillus gasseri* 4B2. ***J Bacteriol*** 2003; 185(11): 3288–96.

47. Pan W-H, Li P-L, Liu Z. The correlation between surface hydrophobicity and adherence of *Bifidobacterium* strains from centenarians' faeces. ***Anaerobe*** 2006; 12(3): 148–52.

48. Pérez PF, Minnaard Y, Disalvo EA, De Antoni GL. Surface properties of bifidobacterial strains of human origin. ***Appl Environ Microbiol*** 1998; 64(1): 21–6.

49. Martín R, Olivares M, Marín ML, Fernández L, Xaus J, Rodríguez JM. Probiotic potential of 3 lactobacilli strains isolated from breast milk. ***J Hum Lact Off J Int Lact Consult Assoc*** 2005; 21(1): 8–17.

50. Ahumada M del C, Bru E, Colloca ME, López ME, Nader-Macías ME. Evaluation and comparison of lactobacilli characteristics in the mouths of patients with or without cavities. ***J Oral Sci*** 2003; 45(1): 1–9.

51. Anwar A. Abdulla, Thikra A. Abed, A.M.Saeed. Adhesion, autoaggregation and hydrophonicity of six *Lactobacillus* strains. ***Br Microbiol Res J*** 2014; 4(4): 1-9.

52. Teanpaisan R, Piwat S, Dahlén G. Inhibitory effect of oral *Lactobacillus* against oral pathogens. ***Lett Appl Microbiol*** 2011; 53(4): 452–9.

53. Piwat S, Teanpaisan R, Dahlén G, Thitasomakul S, Douglas CWI. Acid production and growth by oral *Lactobacillus* species in vitro. ***J Investig Clin Dent*** 2012; 3(1): 56–61.

APPENDIX

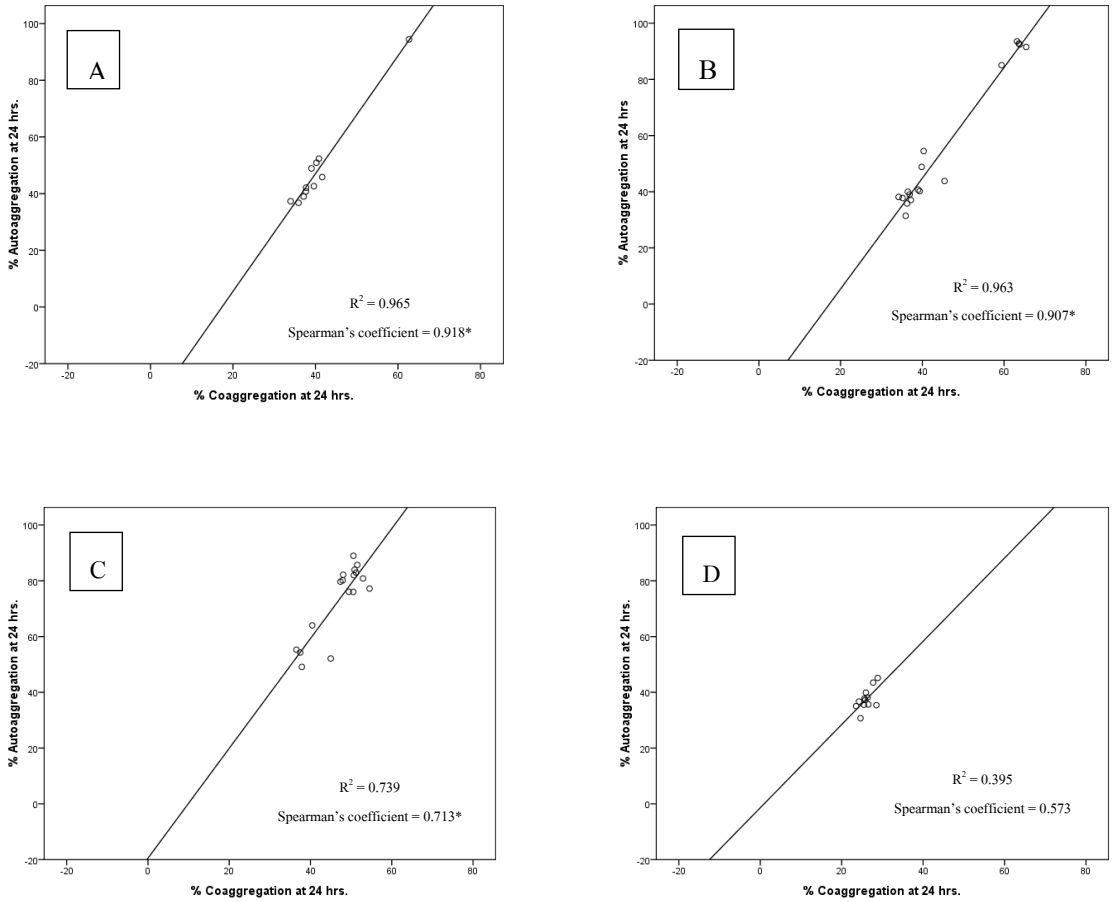

Fig. 11 Scatter graph of Spearman's correlations and relative R^2 values between autoaggregation and coaggregation properties of *L. casei* (A), *L. rhamnosus* (B), *L. gasseri* (C) and *L. mucosae* (D) at 24 hrs. Asterisk indicates correlated significant at $p < 0.05$.

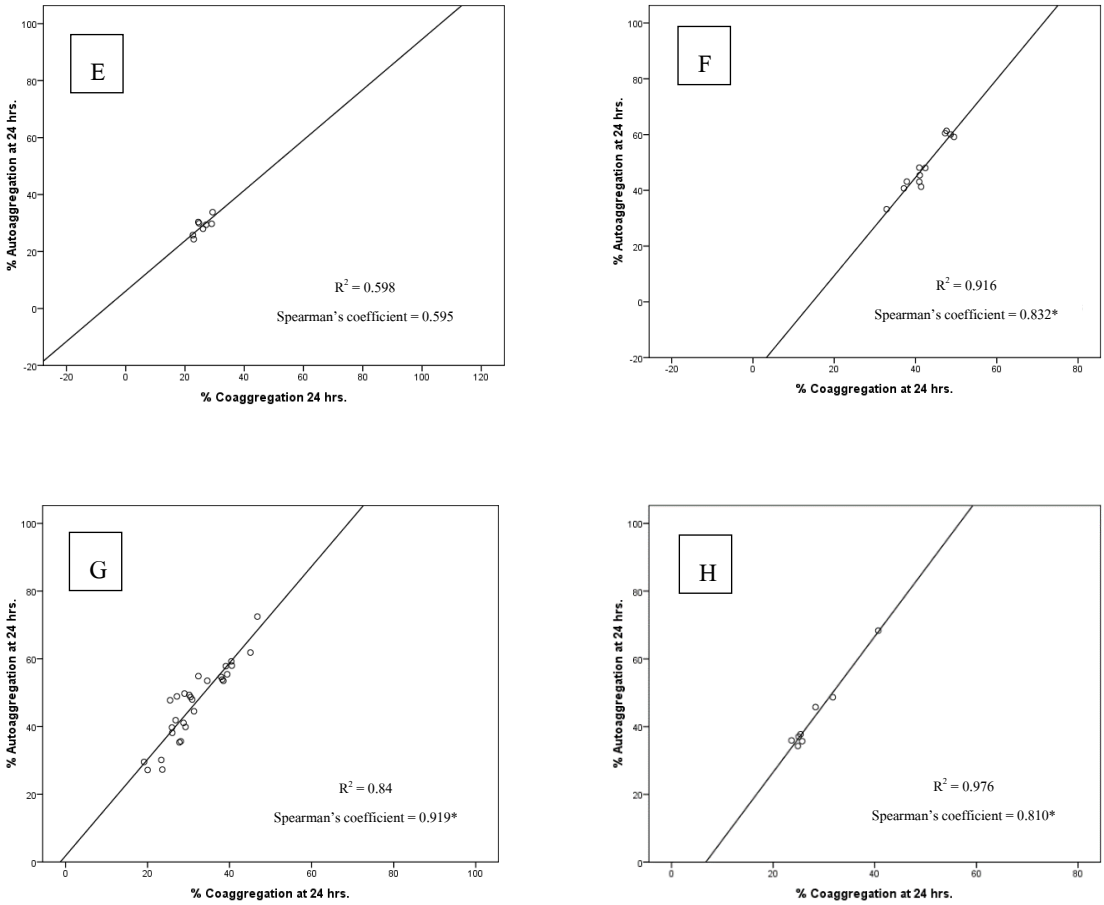

Fig. 12 Scatter graph of Spearman's correlations and relative R^2 values between autoaggregation and coaggregation properties of *L. oris* (E), *L. plantarum* (F), *L. salivarius* (G) and *L. vaginalis* (H) at 24 hrs. Asterisk indicates correlated significant at $p < 0.05$.

ที่ ทม 1204/ 512

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
ตู้ไปรษณีย์เลขที่ 17
ที่ทำการไปรษณีย์โทรเลขคอหงส์
อ. หาดใหญ่ จ. สงขลา 90112

หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง การศึกษาระยะยาวถึงปัจจัยที่มีผลต่อพัฒนาการของฟันและใบหน้า และการเกิดโรคต่าง ๆ ในช่องปากของเด็กอายุ 0-3 ปี (โครงการเฉพาะ 1 ปีแรก)

หัวหน้าโครงการ ทพ.ทรงชัย ฐิตโสมกุล

สังกัดหน่วยงาน ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคน ของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ แล้ว

ให้ไว้ ณ วันที่ 23 พฤษภาคม 2544

(ทพ.ณรงค์ สุขสุอรรถ)
คณบดี
ประธานกรรมการ

.....กรรมการ
(ศ.นพ.นครชัย เผื่อนปฐม)

.....กรรมการ
(ผศ.ทพ.สุทธิพงศ์ เชาวนาดิศัย)

.....กรรมการ
(รศ.ช่อลดา พันธุเสนา)

.....กรรมการ
(นางสาวเครือสิริ ดิสรพงศ์)

.....กรรมการ
(ผศ. ทพญ.สรียา ศรีสินทร)

Certificate of Attendance

This is to certify that

Kamonchanok Pongpanit

has participated as an

Presenter

in

The 11th Dental Faculty Consortium of Thailand
Academic Meeting and Research Presentation (DFCT 2013)
and 30th Anniversary of the Dental Faculty Consortium of Thailand

7 - 9 May 2013
at Pullman Pattaya Hotel, Chonburi, Thailand

Prof.Dr. Sittichai Koontongkaew
Dean, Faculty of Dentistry, Thammasat University
Conference Chair

CONFERENCE PAPER

Pongpanit K, Piwat S, Teanpaisan R, Akkarachaneeyakorn N. (2013) Aggregation abilities and cell surface characteristic of *oral Lactobacillus fermentum* and *Lactobacillus salivarius*. Proceeding of the 11th Dental Faculty Consortium of Thailand Academic Meeting and Research Presentation (DFCT2013). May 7-9, 2013; Chonburi, Thailand. 2013.

PP14

Aggregation abilities and cell surface characteristics of oral Lactobacillus fermentum and Lactobacillus salivarius

Kamonchanok Pongpanit, Supatcharin Piwat, Rawee Teanpaisan and Nuchnaree Akkarachaneeyakorn*

The bacterial aggregation which correlated with the adhesion ability, depend mainly on physicochemical interactions of cell surface characteristics; hydrophobicity and surface charge. Autoaggregation appeared to be necessary for bacterial colonization and coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms. Objectives: The aims of the study were to compare aggregation ability of oral Lactobacillus fermentum and Lactobacillus salivariusand to relate such ability with oral health status (dmft). Methods: Total 21 strains of L. fermentum (10) and L. salivarius (11) were investigated for theircell surface properties, measured autoaggregation, pathogeniccoaggregation properties with Streptococcus mutans. Results: It was shown that the aggregation abilities (autoaggregation and coaggregation with S.mutans) of L.fermentum were statistically significant higher than L.salivarius. Surface charge with ethyl acetate of L.fermentum was higher than ofL.salivarius.Itwas found that autoaggregation and surface charge with acetyl acetate of both L. fermentum and L. salivarius were higher in the high caries group (dmft>5) compared to the low caries group (dmft≤ 5).The coaggregation of L. fermentum was high in the high caries group, whereas, the high coaggregation L. salivariuswas found in the low caries group.For hydrophobicity, there was no difference of L. fermentum between high and low caries group, however, the higher hydrophobicity of L. salivariuswas observed inhigh caries group. The surface charge with chloroform was similar in general. Conclusion: This preliminary study demonstrated that L. fermentumand L. salivarius showed low to moderate autoaggregation and coaggregation with S. mutansdepended on the strains. The association of hydrophobicity and caries status was found in L. salivarius. Further study will include more strains and spe cies in the study.

Aggregation abilities and cell surface characteristics of *oral Lactobacillus fermentum* and *Lactobacillus salivarius*

Kamonchanok Pongpanit[a], Supatcharin Piwat[a], Rawee Teanpaisan[a], Nuchnaree Akkarachaneeyakorn[a]

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

Abstract

The bacterial aggregation which correlated with the adhesion ability, depend mainly on physicochemical interactions of cell surface characteristics; hydrophobicity and surface charge. Autoaggregation appeared to be necessary for bacterial colonization and coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms. ***Objectives****: The aims of the study were to compare aggregation ability of oral Lactobacillus fermentum and Lactobacillus salivariusand to relate such ability with oral health status (dmft).* ***Methods:*** *Total 21 strains of L. fermentum (10) and L. salivarius (11) were investigated for theircell surface properties, measured autoaggregation, pathogeniccoaggregation properties with Streptococcus mutans.* ***Results:*** *It was shown that the aggregation abilities (autoaggregation and coaggregation with S.mutans) of L.fermentum were statistically significant higher than L.salivarius. Surface charge with ethyl acetate of L.fermentum was higher than ofL.salivarius.Itwas found that autoaggregation and surface charge with acetyl acetate of both L. fermentum and L. salivarius were higher in the high caries group (dmft>5) compared to the low caries group (dmft≤ 5).The coaggregation of L. fermentum was high in the high caries group, whereas, the high coaggregation L. salivariuswas found in the low caries group.For hydrophobicity, there was no difference of L. fermentumbetween high and low caries group, however, the higher hydrophobicity of L. salivariuswas observed inhigh caries group. The surface charge with chloroform was similar in general.* ***Conclusion:*** *This preliminary study demonstrated that L. fermentumand L. salivarius showed low to moderate autoaggregation and coaggregation with S. mutansdepended on the strains. The association of hydrophobicity and caries status was found in L. salivarius. Further study will include more strains and species in the study.*

Keyword:

Aggregation, hydrophobicity, surface charges, oral Lactobacillus

Introduction

Lactobacilli are known as a part of the normal oral microflora. They have also been reported their association with the presence and progression of dental caries [1–3]. However, the relation between certain species of *Lactobacillus* and caries is unclear. The role of *Lactobacillus* in oral cavity may vary between their species. Piwat et al. [4] reported that *Lactobacillus salivarius* was more prevalent in children with moderate to high caries prevalence compared with children with low caries prevalence, while *Lactobacillus fermentum* was the most predominant species in all study groups. On the other hand, some *Lactobacillus* species, such as *Lactobacillus rhamnosus*, *Lactobacillus reuteri* and *Lactobacillus paracasei*, have been selected for used as probiotics for the oral health. They play a beneficial role either by inhibiting the growth of some pathogenic bacteria or by their adhesive ability to complete with oral pathogens [5–7].

Bacterial aggregation and adhesion are key factors for colonization of bacterial strains in the oral cavity, preventing their immediate elimination by swallowing and providing a competitive advantage in this ecosystem [8]. Piette and Idzı´ak [9] reported that the cell surface charge and hydrophobicity have influenced the strength of adhesion. Autoaggregation has been correlated with adhesion, which is a required condition for colonization of the oral pathogens. The coaggregation ability of probiotic strains with pathogens as well as their ability to displace pathogens is the significant mechanism for therapeutic manipulation of the pathogenic oral bacteria [10]. Aggregtion abilites test together with cell surface abilites could be used for preliminary screening in identifying potentially adhered to each bacteria [11]. The aims of this study are to define aggregation abilities and surface characteristics; surface charge and hydrophobicity of oral *Lactobacillus* and to relate them with oral health status (dmft).

Materials and Methods

Bacteria strains

Twenty one strains of oral *Lactobacillus* obtained from the culture collection of Department of Stomatology Faculty of Dentistry, Prince of Songkla University were studied including 10 *L. fermentum* strains and 11 *L. salivarius* strains. These strains were isolated from saliva sample of two years old children. Each tested strains were selected from different child and different genotype within the same species of *Lactobacillus*. The study design, selection of patients, their dmft score, and isolation and identification of *Lactobacillus* have been described in the study of Piwat et al.[4] The oral pathogen used for co-aggregation test in this study was *Streptococcus mutans* ATCC® 25175™. The bacterial strains were kept in a freezer at -80°C until required.

Bacterial cultivation

Before the experiment, the lactobacilli were cultivated in Man Rogosa Sharpe (MRS) broth (LAB scan, India) in an anaero-

bic condition (80% N_2, 10% H_2 and 10% CO_2) at 37°C for 24 h. The streptococci were grown in Brain Heart Infusion (BHI) broth (Difco, France) under conditions of low-oxygen (5% CO_2) at 37°C for 24 h. After incubation, the bacteria were harvested by centrifugation at 10,000 rpm for 15 min at 4°C. Cells were washed twice in phosphate buffered saline (PBS).

Aggregation of Lactobacillus

Autoaggregation

Autoaggregation assays were performed according to Del Re *et al.*, (2000). The cell density was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Multiskan GO, Thermo Scientific, USA) throughout the study. The bacterial cells were suspended in PBS buffer to give an OD_{600} of 1.0 (approximately 10^9CFU ml^{-1} cell density). Cell suspensions (6 ml) were mixed by vortexing for 10 sec and left undisturbed at room temperature. Upper layer was carefully removed after 4 h. at room temperature and absorbance (OD) was measured at 600 nm. The autoaggregation percentage was calculated by the formula:

$$\% \text{ Autoaggregation} = (OD_0 - OD_t)/OD_0 \times 100$$

Where OD_t represents the absorbance at time t = 4 h. and OD_0 the absorbance at t = 0.

Coaggregation

Coaggregation assays were also performed according to Del Re et al., (2000). The bacterial cells were suspended in PBS buffer to OD_{600} of 1.0 (approximately 10^9 CFU $ml^{-1}$for *Lactobacillus* and *S. mutans*). Equal volumes (3 ml each) of the *Lactobacillus* and *S. mutans* strains were mixed together by vortexing for 10 sec. Control tubes were set up at the same time, containing 6 ml of each bacterial suspension on its own. The preincubation OD value of control and mixed suspension were measured. After incubation at room temperature without agitation for 4 h. to allow coaggregation occurred, 1 ml of the supernatant of the mixed suspensions was transferred to another tube and the OD was measured. Samples were taken in the same way as in the autoaggregation assay. The percentage of coaggregation was calculated using the equation (Handley et al. 1987):

$$\% \text{ co-aggregation} = \{[(OD_x + OD_y)/2 - OD_{(x+y)}] / (OD_x + OD_y)/2\} \times 100$$

Where OD_x and OD_y represent preincubation OD value of each of the two strains in the control tubes, and $OD_{(x+y)}$ represents OD value of the mixture at time 4 h.

Cell surface characteristics of *Lactobacillus*

The microbial adhesion to hydrocarbon test (MATH) (Rosenberg, 1984) was determined following the modified method described by Geertsema *et al* (1993). The adhesion of bacteria to the different hydrocarbon solution, including xylene (nonpolar), chloroform (monopolar and electron-accepter) and ethyl acetate (monopolar and electron-donor) were measured. The bacterial cells suspended in PBS solution were adjusted to OD_{600} of 0.2 (approximately 10^8 CFU ml^{-1} cell density). After homogenization, 3 ml of the suspension will be pipetted into a test tube. Subsequently, 1ml of hydrocarbon solution was added and then the mixture was vortexed at maximum speed for 60 sec. The OD of both the initial and the extracted solution were determined at 600 nm using a spectrophotometer (Multiskan GO, Thermo Scientific, USA) and disposable polystyrene cuvettes with an effective volume of 1 ml. A blank value was determined for the PBS buffer without added bacteria. After a waiting period of 15 min employed to achieve complete phase separation between the water and hydrocarbon phases, the aqueous phase was carefully removed and its optical density at 600 nm was measured. The percentage of microbial adhesion to hydrocarbon was calculated as:

$$\% \text{ MATH} = (OD_{before} - OD_{after})/OD_{before} \times 100$$

MATH value was classified in three groups: those with low MATH (0–35%), moderate MATH (36–70%) and high MATH (71–100%) [12]

Experimental design and data analysis

All experiments were conducted in duplicate on two different occasions. The data from both experiments were descriptive by mean values and standard deviation. Aggregation ability and surface charge of *Lactobacillus* species were analysed by Student's t-test with $p < 0.05$

Results

In the present study, it was shown that the aggregation abilities (autoaggregation and coaggregation with *S.mutans*) of *L.fermentum* was statistically significant higher than *L.salivarius* (figure 1).

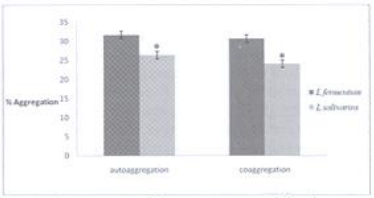

Figure 1- Comparison of the aggregation ability between L fermentum and L. salivarius. Data correspond to the mean o two independent replicates (n=10 in *L.fermentum*, n=11 ir *L.salivarius*). Asterisks indicate value that show significan difference between *L.fermentum* and *L.salivarius* of autoaggregation and coaggregation.(Student's t-test; $p<0.05$)

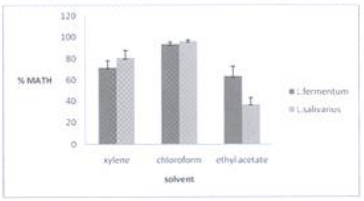

Figure2 - Cells surface charge of L. fermentum and L. salivarius to xylene, chloroform and ethyl acetate. Error bars represent standard error of the mean values of results.

Table 1 – Relation of caries status on cell surface properties and aggregation ability of L. fermentum and L. salivarius

Species	dmft(number of strain)	% Autoaggregaion ±SE	% Coaggregation ±SE	% Hydrophobicity ±SE	% Surface charge±SE	
					Chloroform	Ethyl acetate
L.fermentum	<5(4)	15.1±3.3	22.2±1.9	73.6±4.6	93.8±2.5	45.7±12.4
	≥5(6)	42.8±12.4	36.3±6.7	70.0±11.1	93.5±1.8	74.9±9.8
L.salivarius	<5(2)	22.1±11.2	27.9±7.2	36.1±6.9	91.1±0.7	27.0±10.4
	≥5(9)	27.3±3.2	23.2±2.4	90.3±3.3	97.4±0.9	39.1±7.4

Hydrophobicity and surface charge with chloroform of both *L. fermentum* and *L. salivarius* were more the same (figure 2). Surface charge with ethyl acetate of *L.fermentum* was higher than *L.salivarius*. However, it was not statistically significance since the only few strain were studied.

When the dental health status (dmft) was considered together with aggregation abilities and cell surface characteristics (table1), it was found that autoaggregation and surface charge with ethyl acetate of both *L. fermentum* and *L. salivarius* were higher in the high caries group (dmft > 5) compared to the low caries group (dmft ≤ 5).The coaggregation of *L. fermentum* was high in the high caries group, whereas, the high coaggregation of *L. salivarius* was found in the low caries group. in contrast to *L. salivarius*, the high coaggregation was found in the low caries group. However, the difference was statistic insignificant. For hydrophobicity, there was no difference of *L. fermentum* between high and low caries group, however, the higher hydrophobicity of *L. salivarius*was observed inhigh caries group. The surface charge with chloroform was similar in general.

Discussion

Aggregation has been demonstrated to correlate with adhesion and hydrophobicity, which is known to be a prerequisite for colonization and infection of pathogens. Autoaggregation is defined as the adherence of bacteria themselves [13]. Bacterial coaggregation is a result of two or more different species of bacteria interacting to form a stable composite aggregation [14]. Generally, both *L. fermentum*and *L. salivarius*showed-low to moderate autoaggregation and coaggregation with *S. mutans*.This is inagreement with the others which reported the similar results[12]. The surface charge with ethyl acetate of *L. fermentum* tended to be higher than of *L. salivarius* which indicated that *L. fermentum* was electron acceptor.

The relationship between dental health status (dmft) and aggregation abilities and cell surface characteristics was analyzed. Hydrophobicity and surface charges are known to influence the adhesion ability of organisms to host cells [8]. The strains with high hydrophobicity can adhere well to the cells. In our study, the percentage of hydrophobicity of *L. fermentum* strains either in the high caries group or in the low caries group were high. This may be the reason to explain that *L. fermentum* was commonly found in oral cavity. It is noticed that *L. salivarius* strains with the high hydrophobicity were found in the high caries groups. *L. salivarius* has been reported to be active acid producer. Thus, the strains with the high adhesion ability may have more chance to adhere and produce the acid. However, the further studies should be performed in the larger number of stains and species.

Conclusion

This preliminary study demonstrated that *L. fermentum* and *L. salivarius* showed low to moderate autoaggregation and coaggregation with *S. mutans* depended on the strains. The association of hydrophobicity and caries status was found in *L. salivarius*. Further study will include more strains and species in the study.

Acknowledgments

This work was supported by the National Research Council of Thailand.

References

[1] Teanpaisan R, Thitasomakul S, Piwat S, Thearmontree A, Pithpornchaiyakul W, Chankanka O. Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3–24 month old Thai children. International Dental Journal. 2007;57(6):445–51.

[2] Badet C, Thebaud N. Ecology of lactobacilli in the oral cavity: a review of literature. Open Microbiol J. 2008 Apr 29;2:38–48.

[3] Steinle CJ, Madonia JV, Bahn AN. Relationship of lactobacilli to the carious lesion. Journal of Dental Research. 1967 Jan 1;46(1):191–6.

[4] Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlén G. *Lactobacillus* species and genotypes associated with dental caries in Thai preschool children. Mol Oral Microbiol. 2010 Apr;25(2):157–64.

[5] Caglar E, Kargul B, Tanboga I. Bacteriotherapy and probiotics' role on oral health. Oral Dis. 2005 May;11(3):131–7.

[6] Näse L, Hatakka K, Savilahti E, Saxelin M, Pönkä 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 Dec;35(6):412–20.

[7] Shimauchi H, Mayanagi G, Nakaya S, Minamibuchi M, Ito Y, Yamaki K, et al. Improvement of periodontal condition by probiotics with *Lactobacillus salivarius* WB21: a randomized, double-blind, placebo-controlled study. J. Clin Periodontol. 2008 Oct;35(10):897–905.

[8] Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol. 2008 Mar 1;226(5):1065–73.

[9] Piette JP, Idziak ES. A model study of factors involved in adhesion of *Pseudomonas fluorescens* to meat. Appl Environ Microbiol. 1992 Sep;58(9):2783–91.

[10] Twetman L, Larsen U, Fiehn N-E, Stecksén-Blicks C, Twetman S. Coaggregation between probiotic bacteria and caries-associated strains: an in vitro study. Acta Odontologica Scandinavica. 2009 Jan;67(5):284–8.

[11] E Vlková VR. Auto-aggregation and co-aggregation ability in bifidobacteria and clostridia. Folia microbiologica. 2008;53(3):263–9.

[12] Colloca ME, Ahumada MC, López ME, Nader-Macías ME. Surface properties of lactobacilli isolated from healthy subjects. Oral Dis. 2000 Jul;6(4):227–33.

[13] Elliott DR, Wilson M, Buckley CMF, Spratt DA. Aggregative behavior of bacteria isolated from canine dental plaque. Appl Environ Microbiol. 2006 Aug 1;72(8):5211–7.

[14] Fei Chen. Bacterial auto-aggregation and co-aggregation in activated sludge. Clemson: Clemson University; 2007.

Address for correspondence

Supatcharin Piwat Tel.: +66 74 42987, E-mail address: supacharin@hotmail.com

VITAE

Name Kamonchanok Pongpanit

Student ID 5510820021

Education Attainment

Degree	Name of Institution	Year of Graduation
Doctor of Dental Surgery	Prince of Songkla University	2010

Work-Position and address

The Dental unit, Dental hospital Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkla, Thailand, 90110

Proceedings (Poster presentation)

Pongpanit K, Piwat S, Teanpaisan R, Akkarachaneeyakorn N. (2013) Aggregation abilities and cell surface characteristic of oral *Lactobacillus fermentum* and *Lactobacillus salivarius*. Proceeding of the 11th Dental Faculty Consortium of Thailand Academic Meeting and Research Presentation (DFCT2013). May 7-9, 2013; Chonburi, Thailand. 2013.