Original Article

In Vitro the Impact of Surface Components and Environmental Factors on the Adhesion and Aggregation Ability of Oral *Lactobacillus fermentum*

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Abstract

Lactobacillus fermentum is considered as a beneficial member of the oral normal microbiota. Bacterial aggregation and/or adhesion are essential for their persistence and maintaining the balance in a healthy oral ecosystem. The coaggregation interaction with pathogenic strains is a key factor of the strains to exclude pathogens. The objective of this study was to investigate the environmental factors affecting the adhesion and autoaggregation of oral *L. fermentum* strains and their coaggregation ability with cariogenic pathogen, *Streptococcus mutans*. The bacterial cell surface components of these strains were evaluated. The results showed that the adhesion ability of oral *L. fermentum* to H357 keratinocyte cells was enhanced by cell surface protein, polysaccharides and S-layer protein, while aggregation ability was only mediated by a proteinaceous component. The tested factors including acid-base conditions, calcium ions, sugars and enzymes were analyzed. It was found that the adhesion and autoaggregation of these *L. fermentum* strains were not inhibited by any tested factors. Moreover, the acidic condition at pH 4.0 and calcium treatment significantly promoted adhesion abilities. Most of the tested factors showed no effect on the coaggregation between *L. fermentum* and *S. mutans*, except lactose and lysozyme treatments which decreased this ability. Overall results showed that oral *L. fermentum* strains had adaptive traits in various factors which were representative of various physico-chemical conditions in the oral cavity.

Keywords: Adhesion, Aggregation, Cell surface components, *Lactobacillus fermentum*, Oral cavity

Received Date: Aug 17, 2020 **Revised Date:** Sep 29, 2020 **Accepted Date:** Dec 21, 2020

Doi: 10.14456/jdat.2021.19

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Introduction

The oral cavity consists of a rich and diverse microbial population as the commensal microflora. The human oral cavity contains approximately 10^{10} bacteria and more than 700 bacterial species. The establishment of microbial ecology is directly influenced by the exposure to external environmental factors such as temperature, diet, pH level, enzymes and eating habits.[1] Physiologically interaction with these commensal bacteria is usually harmonious and beneficial for the host. Equilibrium of the commensal microbial community leads to a healthy oral cavity. However,

if an imbalance occurs, the oral ecosystem may contain an excess of pathological organisms, and this represents the first step of oral disease.[2,3]

L. fermentum has been reported to be the most predominant *Lactobacillus* species in the oral cavity.[4,5] The species may play an important role in maintaining an equilibrium between the commensals and oral pathogens. It has been shown that some *L. fermentum* strains provide benefits to human oral health by inhibiting the biofilm formation of harmful microflora such as cariogenic mutans streptococci.[6,7]

Adhesion, internalization by host tissues, and autoaggregation of bacteria are considered as an important first step of bacterial colonization to persist and form the biofilm within the host.[8,9] In addition, internalized into the host cells is one of the strategies of bacteria to survive by avoiding recognition by the host's immune system.[10] Aggregation between the same strains of microorganisms (autoaggregation) to form multicellular clumps can act as a barrier to protect the strains from environmental stress, while aggregation between the species (coaggregation) can inhibit the growth of pathogenic strains by direct coaggregation.[8,11] Bacterial adhesion and/or aggregation are complex processes that are influenced by many factors.[12] Studies of intestinal *L. plantarum* have shown that pretreating these strains with enzymes (pepsin, trypsin and lysozyme) and chemicals (lithium chloride) results in a significant decrease in their adhesion ability.[13] Our previous study reported a correlation between the adhesion ability of oral *L. fermentum* to human oral epithelial cells and the bacterial cell surface characteristics.[14] However, the environment of the oral cavity is an open, dynamic system, with diverse conditions that are influenced by several factors such as nutrient availability, salivary enzymes and variable conditions of pH. Little is known about the aggregation and adherence ability of this species, especially when influenced by the oral environment.

The objective of this study was to investigate the effects of these factors on the adhesion of *L. fermentum* to the H357 keratinocyte oral squamous carcinoma cell lines and evaluate their autoaggregation and coaggregation ability with *S. mutans* ATCC 25175.

Materials and Methods

Bacterial Strains and Culture Conditions

Twenty strains of *L. fermentum* obtained from a previous study[4] were kept at -80°C in the Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Thailand. Each clinical strain was isolated from a different child. The details of isolation and identification of the strains were previously described in the study of Piwat *et al.*[4] The bacteria were initially cultured for 18–24 hours on de Man Rogosa Sharpe (MRS) agar (Difco™; USA) under an anaerobic condition (80% N_2, 10% H_2 and 10% CO_2) at 37°C for 24 hours, and then transferred to MRS broth (Himedia®; India) for an additional 18–24 hours of incubation. The cariogenic pathogen, *S. mutans* ATCC 25175™, used for the coaggregation assay, was grown in a blood agar plate (BBL™; USA) for 18–24 hours, and transferred to a Brain Heart Infusion broth (Bacto™; USA) in an aerobic condition (5% CO_2) at 37°C for an additional 18–24 hours of incubation.

Biochemical Characterization of bacterial cell surface

The contribution of protein of the bacterial cell surface was examined by treating with proteinase K, while the contribution of the crystalline surface layer (S-layer) protein was characterized by lithium chloride. Metaperiodate solution was used for oxidizing cell surface carbohydrates.

After incubation, the bacterial cells were harvested by centrifugation at 3000 rpm for five minutes and washed twice with 5 mL of phosphate buffer saline (PBS) pH 7.0. To investigate the involvement of surface structures in the adhesion and aggregation ability, bacterial cells were subjected to surface treatment: 10 mM metaperiodate in 0.1 M citrate-phosphate buffer (pH 4.5), 5 M LiCl in distilled water and proteinase K in 50 mM Tris-HCl buffer (pH 7.5) at 37°C for 30 minutes. The bacteria in PBS solution (pH 7.0) was used as the control group.

Treated bacterial suspensions were mixed by vortexing (Vortex-Genie 2™) for 10 seconds. The optical

density was measured at 600 $_{nm}$ (OD600 nm) using a spectrophotometer (Ultrospec 2000™) to give viable counts of approximately 10^8 CFU/mL ($OD_{600\,nm}$ = 0.25±0.05) for adhesion assay and 10^{10} CFU/mL ($OD_{600\,nm}$ = 0.5±0.05) for aggregation assay.

Factors affecting adhesion and aggregation ability of bacterial cells

The effects of the factors were investigated according to the method of Lang *et al.*[15] with some modifications. The bacterial cells were treated for one hour at 37°C with several factors as follows: 1) PBS buffer adjusted to pH values 4.0 and 8.0, 2) 10 mM of calcium ions, 3) sugars (50 mM): glucose, sucrose and lactose and 4) enzyme solution (1 mg/mL): amylase in 15 mM PBS (pH 7.0), lysozyme in Tris EDTA sucrose buffer (pH 8.0), trypsin in 50 mM Tris-HCl buffer (pH 8.0), pepsin in 10 mM citrate-phosphate buffer (pH 4.5) and lipase in 50 mM Tris-HCl buffer (pH 8.0). The control in this study was PBS at pH 7.0. Treated bacterial suspensions were mixed by vortexing for 10 seconds and then adjusted to the optical density as suggested in a previous protocol.

Adhesion assay

The adhesion assay was performed using the method of Kintarak *et al.*[16] with modifications. The H357 keratinocyte cells, the oral squamous carcinoma cell line, were used in this study.

One milliliter of treated *L. fermentum* suspension (10^8 CFU/mL PBS, OD600 nm = 0.25±0.05) was inoculated into H357 keratinocyte cells in Dulbecco's modified Eagle's medium (DMEM) on 24-well plates. After incubation at 37°C in anaerobic condition for one hour, the non adherent bacteria were removed by washing twice with PBS pH 7.0 (1 mL/well). The numbers of bacterial cells adhering to monolayers plus intracellular bacteria were quantified as the total adhesion. For internalization assay, 1 mL of DMEM containing gentamicin solution (100 µg/mL) was added to the treated *L. fermentum* and H357 keratinocyte monolayer cells and incubated for two hours at 37°C to kill extracellular adhered bacteria.

To determine the amount of adherent bacterial cells, the H357 keratinocyte monolayers were treated with 0.05% trypsin 150 µl/well. 0.1% Triton X-100 (850µl/well) was added to lyse the keratinocyte cells. The ten-fold serial dilutions of the cell lysates were plated onto MRS agar and incubated at 37°C in anaerobic conditions for 48 hours to measure the number of viable bacterial colonies. The adhesion abilities including total adhesion, internalization and relative internalization (the internalization related to bacterial cells adhesion ability) were expressed as follows:

$$\text{Total adhesion (\%)} = (A_1/A_0) \times 100$$

$$\text{Internalization (\%)} = (A_2/A_0) \times 100$$

$$\text{Relative internalization (\%)} = (A_2/A_1) \times 100$$

where A_0 is the number of bacterial cells (CFU/mL) at the beginning, A_1 is the number of bacterial cells (CFU/mL) adhered totally, and A_2 is the number of bacterial cells (CFU/mL) internalized.

Autoaggregation and coaggregation assays

The specific cell–cell interactions were assessed. For autoaggregation assays, the method of Piwat *et al.*[14] was used. The treated *L. fermentum* suspension was adjusted to 0.5±0.05 OD_{600nm} (approximately 10^{10} CFU/mL). Cell suspensions (4 mL) were mixed by vortexing for 10 seconds. After incubation at 37°C for 24 hours, one milliliter of the upper suspension was carefully removed to measure absorbance at $OD_{600nm.}$ The autoaggregation was reported as percentages using the following formula:

$$\text{Autoaggregation (\%)} = (1- A_{time})/A_{initial}) \times 100$$

where A_{time} is the optical density of the upper layer of the suspensions at 24 hours after incubation and Ainitial is the optical density of the suspensions at baseline.

The coaggregation assays between *L. fermentum* and *S. mutans* ATCC 25175™ were performed according to Piwat *et al.*[14] Equal volumes (2 mL) of the *L. fermentum* and *S. mutans* suspensions were mixed together and subjected to vortexing for 10 seconds, and then incubated at 37°C for 24 hours. The controls were set up using 4 mL of each bacterial suspension on their own. One milliliter of the upper layer of each suspension was aspirated to measure the absorbance at OD600nm. The coaggregation was reported as a percentage using the following formula:

$$\text{Coaggregation (\%)} = [(A_{L.\ fermentum} + A_{S.\ mutans})/2] - (A_{mix})/[(A_{L.\ fermentum} + A_{S.\ mutans})/2] \times 100$$

where $A_{L.fermentum}$ is the optical density of *L. fermentum* suspensions at the initial time, $A_{S.\ mutans}$ is the optical density of *S. mutans* ATCC 25175™ suspensions at the initial time, Amix is the optical density of the mixed *L. fermentum* and *S. mutans* ATCC 25175™ suspensions at 24 hours after incubation.

Statistical Analysis

All experiments were independently conducted twice. The results were described as mean and standard deviation. Our data were non-parametric (normality test by the Shapiro-Wilks test). The multiple comparisons for non-parametric (the Kruskal-Wallis test) was performed and followed by post hoc tests, the Mann-Whitney U test, with a correction. The differences were considered significant when $P < 0.05$. The software package used for the analyses was the SPSS statistical program (SPSS Inc.; Chicago, IL) and differences were considered significant when the $p<0.05$.

Results and Discussion

Effects of Cell Surface Components on Bacterial Adhesion and Aggregation

The tested oral *L. fermentum* strains were able to adhere and to be internalized by keratinocyte cells at the level of 68.1 % and 47.7 % respectively. The effects of cell surface components on adhesion and aggregation abilities of oral *L. fermentum* are presented in Table 1. Adherence to H357 keratinocyte cells decreased significantly after proteinase K, metaperiodate and lithium chloride treatment (p=0.008, 0.040 and 0.011, respectively). These results indicated that the adhesion ability of oral *L. fermentum* was affected by the role of cell surface protein, polysaccharides and S-layer protein. Various cell surface components probably relate to multiple mechanisms which mediate the adherence of this species.[17] For internalization, the cells were only significantly (p=0.037) affected by metaperiodate, therefore the influence of cell surface carbohydrates was considered. Nevertheless, the rate of relative internalization was not affected by any of the treatments. It was found that the autoaggregation and coaggregation abilities were mediated mainly by a proteinaceous component due to a significant reduction (p=0.006 and 0.002, respectively) of these abilities after proteinase K treatment. This finding is in agreement with previous reports, which indicated that the adhesion and aggregation abilities of several *Lactobacillus* are involved by specific cell surface components[11,12,18] and is species and/or strain-dependent.[19] Archer *et al.*[18] reported that there were more than one cell surface protein involved in the adhesion process of *L. fermentum* strains obtained from infant feces. Baccigalupi *et al.*[20] indicated that at least two small (less than 3 kDa) cell surface proteins were involved in the adhesion of food isolated *L. fermentum* cells to Caco-2 cells. The results suggested that the cell surface components are essential for the aggregation and adhesion abilities of the strains, and the condition which affects these components may show a reduction in properties.

Table 1 *Effects of cell surface component on bacterial adhesion and aggregation of oral L. fermentum when treated by proteinase K, lithium chloride and metaperiodate*

Treatment	% Adhesion† (mean+SD)			% Aggregation (mean+SD)	
	Total adhesion	Internalization	Relative internalization	Autoaggregation	Coaggregation‡
Control	68.08 ± 9.12	47.69 ± 13.32	69.17 ± 13.09	60.99 ± 24.25	47.24 ± 12.23
Proteinase K	61.61 ± 7.83**	40.77 ± 11.98	65.58 ± 15.62	40.24 ± 9.86**	35.47 ± 7.42**
Lithium chloride	60.24 ± 13.29*	41.50 ± 16.01	67.77 ± 15.96	57.55 ± 25.53	45.24 ± 14.74
Metaperiodate	62.06 ± 8.95*	39.23 ± 14.30*	62.16 ± 16.82	53.75 ± 21.27	41.28 ± 8.57

†*Adhesion ability for L. fermentum strains with oral squamous carcinoma cell line, H357 keratinocyte cells*

‡*Coaggregation between L. fermentum strains and S. mutans ATCC 25175TM*

Asterisks indicate a statistically significant difference of treatments in comparison to the control (p < 0.05, ** p < 0.01).*

Factors affecting the adhesion ability to H357 keratinocyte cells

Mechanisms of adhesion have been studied with the H357 keratinocyte cells, oral squamous cell carcinoma. These cell lines were used as *in vitro* model for oral mucosa in this study due to their ability to grow indefinitely *in vitro* and retain most of the genetic properties as the oral squamous cell.[21] The environmental factors, which oral bacteria have been challenged, such as the acids/bases conditions, calcium ions, sugars and oral and GI tract enzymes, were evaluated (Fig. 1). The present study indicated that among the tested conditions, only acidic (pH 4.0) and calcium ions significantly ($p = 0.001$ and < 0.001, respectively) increased the adhesion to keratinocyte cells and also increased the internalization of *L. fermentum* by keratinocytes, whereas neither kind of sugar and enzyme, nor the basic condition significantly affected the adhesion. However, the rate of relative internalization, the internalization in relation to the bacterial cells adhesion, was not significantly different among the tested conditions. It was implied that the tested conditions directly affected the ability of bacteria to adhere to the keratinocytes, but did not have any effect on the rate of adhered bacteria to be internalized.

Adhesion is indeed the initial step to establish an intimate contact with host cells and subsequently to invade the host cells, which is considered as an important role in colonization.[9] In the oral cavity, adhesion ability prevents bacterial elimination by swallowing and provides a competitive advantage in the ecosystem.[9,22] The oral environment, which is constantly changing, may affect the adhering properties of these bacteria. The pH of saliva is usually in the range of 6.5–7.4[23] but may vary due to many factors. Acidity can be increased by the consumption of acidic beverages. Moreover, production of acids by plaque bacterial glycolysis of dietary sugar or fermentable carbohydrate can also create an acidic condition, while an alkaline condition may result from the consumption of fruit or vegetables.[24]

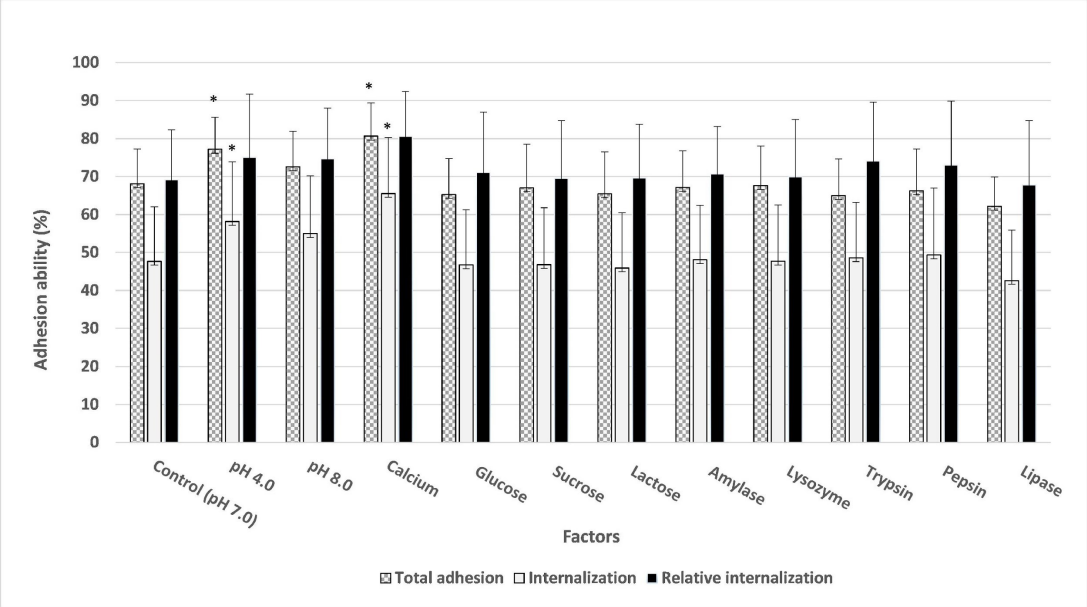

Figure 1 *Effects of pH, calcium ions, sugars and enzymes on adhesion abilities of oral L. fermentum to H357 keratinocyte cells. Asterisks indicate a statistically significant difference of treatments in comparison to the control (*p<0.05).*

Similar findings were observed in other studies where the acidic pH condition increased the adhesion ability of the strains of *L. acidophilus*, *L. rhamnosus* GG[12,25] and *L. gasseri*[26], whereas the levels of adherence were relatively consistent at basic pH.[12] That may explain how *L .fermentum* can survive under various acidic environments.[18,26,27] However, our previous study reported that a basic condition (at pH 8.0) is able to increase the adhesion ability of the oral

L. gasseri strain.[26] The specific adhesive protein located on the surface of bacterial strains may play a role in adhesion characteristics. The transcriptomic analysis by Bang *et al.*[25] revealed that the adherent ability of the *L. rhamnosus* strain under acid stress at pH 4.5 was enhanced through the induction of pilus-specific adhesion protein located on the bacterial surface.

Calcium is one of the main components of saliva electrolytes, which influences the balance between demineralization and remineralization of the exposed tooth surfaces. In the oral cavity, its concentration depends on the saliva flow rate and consumption of food containing calcium.[28] In addition, calcium is an essential component of the host environment that plays an important role in the adhesion and colonization of bacteria. The involvement of calcium ions can influence electrostatic interactions, and provide an ionic bridge between bacterial surfaces and host epithelial cells.[29,30,31] Calcium levels have been shown to regulate the invasion of bacteria that are entering a host.[31]

From these results, it can be implied that various oral and gastrointestinal tract conditions such as pH, ions, sugars and enzymes could not decrease and, in some way, increase the adhesion of *L. fermentum*, especially in the acidic condition and calcium treatment. These properties characterize a microorganism's adaptive capacity to establish and persist in the environment.[9] Therefore, it is not surprising that *L. fermentum* strains are found predominantly in the oral cavity.[4] This knowledge could be used to extrapolate or enhance the beneficial properties of this bacteria.

Factors affecting the aggregation ability

After 24 hours, the *L. fermentum* strain cells aggregation could be seen to be precipitated at the bottom of the tube at different amounts of sedimentation depending on the tested factors (Fig. 2). The autoaggregation and coaggregation abilities of *L. fermentum* when treated with pH, calcium ions, sugars and enzymes are shown in Figure 3. Autoaggregation was not significantly affected by any treatment, whereas coaggregation between *L. fermentum* and *S. mutans* ATCC 25175™ was significantly reduced from 47.2 % to 34.0 % and 0.03 % after treatment with lactose (p=0.008) and lysozyme (p<0.001), respectively. It could be assumed that *L. fermentum* maintains their autoaggregation ability in diverse environments which leads to their potential to form a barrier to prevent the colonization of pathogenic bacteria to a host tissue.[9] However, if these bacteria are under conditions with lactose and lysozyme, the coaggregation property with *S. mutans* may be reduced. The study of Park *et al.*[32] showed similar results in that lactose treatment was able to inhibit coaggregation between *L. rhamnosus* and *S. mutans* by up to 97 % and suggested that the inhibition of lactose may be related to the function of lectin on the surface of *Streptococcus* leading to inhibit both of the aggregation and coaggregation abilities of the oral streptococci. In addition, *S. mutans* were inhibited by lysozyme resulting in bacterial lysis[33], which may explain the near inability of *S. mutans* to auto- and coaggregate.

For further studies, it would be interesting to investigate the effects of the factors to these abilities in microscopic aspects which need SEM or fluorescent microscopy to show the detail of aggregation in each group. Moreover, the molecular mechanisms underlying the physiological characteristics with respect to the adhesiveness of oral *L. fermentum* and their corresponding receptors in the host cells should be further evaluated.

In conclusion, the surface-bound proteins, polysaccharides and S-layer proteins were involved in the adhesion of oral *L. fermentum* strains to the keratinocyte oral epithelial cells, and only surface proteins component mediated their aggregation ability. All of the sugars and enzymes tested showed no effect on the adhesion and aggregation, except for lactose and lysozyme which decreased the coaggregation with *S. mutans.* These results imply that oral *L. fermentum* strains have adaptive traits to establish in the oral environment and have the ability to coaggregate with the cariogenic *S. mutans* in various physico-chemical conditions.

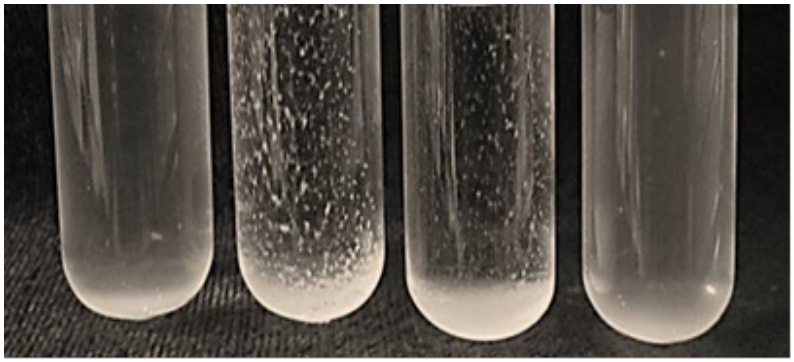

Figure 2 *Macroscopic co-aggregation assays between L. fermentum and S. mutans ATCC 25175TM in various situations*

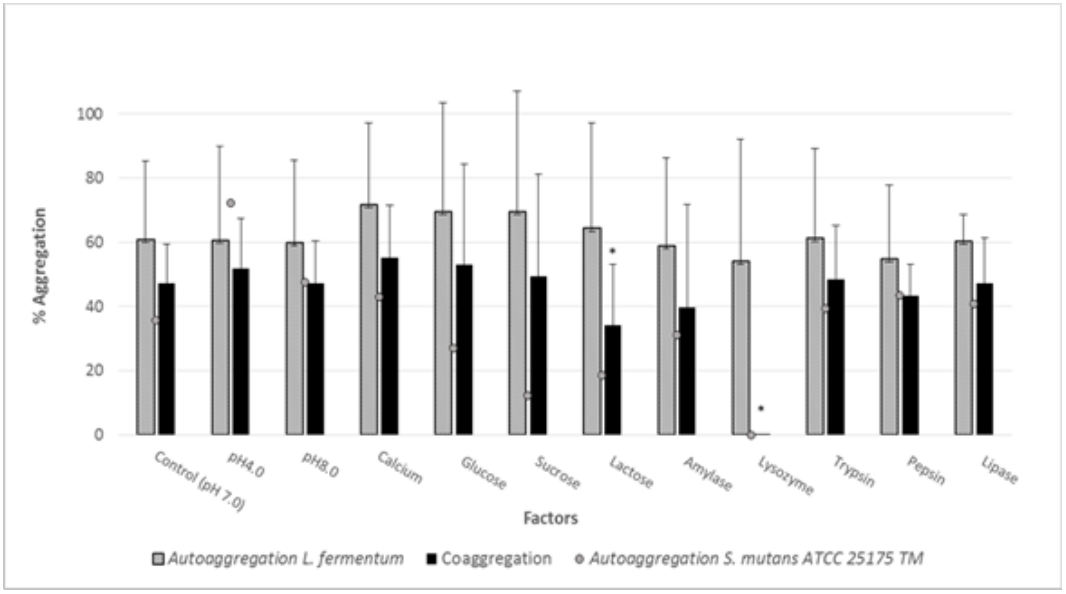

Figure 3 *Effects of pH, calcium ions, sugars and enzymes on the autoaggregation abilities of L. fermentum, S. mutans ATCC 25175^{TM}, and on the coaggregation between L. fermentum and S. mutans ATCC 25175^{TM}. Asterisks indicate a statistically significant difference of treatments in comparison to the control (*p<0.05).*

Acknowledgements

This study was supported by the Graduate School, Prince of Songkla University. The authors would like to thank staff members at the Oral Microbiology Laboratory, Faculty of Dentistry, Prince of Songkla University for their help in facilitating the use of equipment.

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