Original Article

The Inhibition of Dental Caries Pathogen by Using Prebiotic and Probiotic Combination

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Abstract

Dental caries is the most important global problem of oral disease. The local demineralization of tooth surface from an acid action is an initial step of the disease. The acids are produced when sugar from food has an interaction with bacteria in the dental plaque, which usually accumulates on the susceptible tooth surfaces. As more acidic condition, the aciduric and acidogenic bacteria can survive. *Streptococcus mutans* is a major contributor of tooth decay. Many strategies are recommended to protect the susceptible teeth from cariogenic bacteria. A probiotic application is one of techniques providing the health-beneficial microorganism to inhibit the cariogenic bacteria. Prebiotics are oligosaccharides which can promote the growth of probiotics in human bowel. The objective of this study was to evaluate the efficiency of the prebiotic (Galacto-oligosaccharides, (GOS)) to enhance the probiotic (*Lactobacillus acidophilus*) for inhibition of *S. mutans* and *L. acidophilus* were co-cultured with ratio of 1:20 in the de Man, Rogosa and Sharpe (MRS) media supplemented with different concentrations of GOS; 1, 2, 3 and 4 % (v/v). The efficiency of synbiotic against *S. mutans* was determined from their growth rate. The growth rate of *S. mutans* and *L. acidophilus* were similar (0.4848 and 0.4861 hr^{-1}, respectively) in the MRS agar without GOS. The growth rate of *S. mutans* insignificantly decreased when grew in 3 and 4 % of GOS (0.1719 and 0.3258 hr^{-1} respectively) compared with control group ($p > 0.05$), while the growth rate of *L. acidophilus* was constant (0.3443 and 0.3459 hr^{-1} respectively). The GOS was not an efficient prebiotic to enhance the function of *L. acidophilus* to inhibit growth of *S. mutans.*

Keywords: Probiotic, Prebiotic, Galacto-oligosaccharide, *L. acidophilus*, *S. mutans*, Dental caries

Received Date: APR 27, 2017 **Accepted Date:** JUL 17, 2017

doi:

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Introduction

Dental caries is a major oral infectious disease initiated with the local demineralization by acids which are produced from accumulated acidogenic bacteria in the dental plaque.[1] The dental plaque presents as a diverse community of microorganisms and extracellular matrix in an arranged biofilm. The microorganisms tightly attach each other to form an open architecture with channels and voids for the circulation of nutrients, waste products and gas.[2]

Acids are produced from the consumption of sugar by bacteria. It is a cause of the dissolution of the mineral composition of tooth leading to dental decay. The key pathogens in this mechanism are acidogenic bacteria such as *S. mutans*, *Actinomyces* spp. and *Lactobacillus* spp.

Streptococcus mutans is facultative anaerobic Gram-positive cocci. In human saliva, the number of them normally ranges from undetectable to 10^6 -10^7 CFU/ml. They can consume several kinds of sugar resulting in the production of several weak acids particularly lactic acid together with the linear-soluble and branched-insoluble exopolysaccharides (EPS), such as glucans and fructans, by glucosyltransferase (GTFs) and fructosyltransferase (FTFs) for cell adhesion.[2,3] These exopolysaccharides are used to facilitate the adhesion of the second colonizers, the stability of biofilm and the protection of bacteria from host defense mechanism.[1,2,3] The restriction of the number of *S. mutans* is one of plenty strategies for the caries prevention.[6,7]

Probiotics are living microorganisms providing health benefits to the hosts. They are naturally found in a human body and have an influence on other microorganisms by producing the specific antimicrobial substances.[5] In the oral cavity, the probiotics must first attach to oral tissue followed by creating a protective barrier to prevent the pathogenic microorganism colonization. They must increase in number in order to produce the effective capacity.[5,8] The most well-known probiotics are *Lactobacillus* spp. and *Bifidobacterium* spp. The consumption of probiotics approximately 10^7 – 10^9 CFU/ml of *Lactobacillus* spp. is adequate to modulate the benefit to intestine microflora.[9-11]

Lactobacillus spp. is Gram-positive, non-spore forming bacilli which normally isolated from gastrointestinal tract of human.[4,5] The optimal growth condition is between 30 – 40 °C, 5 % CO_2 and in the growth media pH 5.5–5.8. *Lactobacillus acidophilus* produce several kinds of bacteriocins; such as Lactacin B, Lactacin F, Brevicin 37 and Gassericin A, which affect specific strains in complex microbial biofilm.[4] *Lactobacillus casei* strain GG could produce various antimicrobial components such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins, and adhesion inhibitors against *Streptococcus* spp.[12,13] According to Kojima Y. *et al.* (2015), *Lactobacillus* spp. can also inhibit the insoluble glucan formation of *S. mutans*.[14] Some *Lactobacillus* spp. have been considered as potential probiotics.[15]

Prebiotics have been defined as non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth or activity of microorganisms in the human colon. The most common prebiotics are non-digestible polysaccharides such as lactosucrose, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and isomalto-oligosaccharides.[16] However, only FOS, GOS and inulin have been tested *in vivo* to meet all the requirements for current criteria of prebiotics.[17]

The probiotics have the negative effects on the cariogenic bacteria whilst the prebiotics selectively promote the growth or activities of the probiotics. The efficient combination between them is named synbiotics.[20] The synbiotics, an enhanced probiotics by prebiotics, provide beneficially effects on the hosts.[21] Culture *L. acidophilus* with conjac glucomanan as prebiotics was able to inhibit the growth of *S. mutans*.[22] According to Kondepudi *et al.* (2012), GOS increased growth rates of

Bifidobacterium breve, Bifidobacterium longum and *Bifidobacterium pseudocatenulatum*.[23] However, there are few studies of probiotics and prebiotics in dental caries modulation.[24] The aim of this study was to investigate the inhibitory effect of synbiotics between GOS and *L. acidophilus* (TISTR 2365T = DSMZ 20079T) on the growth of *Streptococcus mutans* (DSMZ 20523T).

Materials and Methods

1. Preparation of culture medium, prebiotic and microorganism

1.1 Culture medium preparation

Three culture media (Brain heart infusion broth (BHI), de Man Rogosa and Sharpe broth (MRS) and tryptic soy broth (TSB) were used for culture optimization. The composition of each culture media was prepared following the manufacturer's recommendation. For agar medium preparation, 1.5 % of agar powder was added into the culture broth. All prepared media were sterilized in an autoclave at 121°C, pressure 15 lb/inch^2 for 15 minutes.

1.2 Prebiotic preparation

The galacto-oligosaccharides (GOS) (Bornnet corporation Co., Ltd., Bangkok, Thailand) was prepared at concentrations 1 %, 2 %, 3 % and 4 % (v/v) in MRS broth.

1.3 Microorganism preparation

The microorganisms in this experiment were classified as cariogenic bacteria and probiotic. The cariogenic bacteria was *S. mutans* (DSMZ 20523T). The probiotic was *L. acidophilus* (TISTR 2365T or DSMZ 20079T). From the lyophilized stock, *S. mutans* and *L. acidophilus* were inoculated in the BHI and MRS broths respectively at 37°C, 5 % CO_2 for 18-24 hrs. The overnight cultures of each kind of bacteria were then inoculated on the BHI and MRS agars by the streak plate technique. An isolated colony was transferred to fresh media (BHI, MRS and TYE) and allowed to grow. Their growth patterns were recorded.

2. Determination of the efficacy of synbiotic on cariogenic bacteria

2.1 Determination of the suitable culture medium for co-culture

Five hundred microlitre of bacteria solution from section 1.3 was separately inoculated into 20 ml of three culture broths (BHI, MRS and TYE). The growth patterns of *S. mutans* and *L. acidophilus* in three culture broths were determined by the optical density (OD) at 600 nm along with serial dilution method for colony counting at 0, 1, 3, 6, 8, 10, 12, 14, 16, 18, 24, 36 and 48 hrs.

2.2 Determination of the proportion between *S. mutans* and *L. acidophilus* for co-culture.

S. mutans and *L. acidophilus* in the mid-log phase were used in the experiment. To determine the appropriate proportion for the equal number of *S. mutans* and *L. acidophilus*, they were co-cultured under various ratios; 1:5, 1:10, 1:20 and 1:40 in the MRS culture medium. Their growth patterns were investigated following section 2.1 in the MRS agar.

2.3 Effect of prebiotics on probiotics to inhibit *S. mutans*

The optimized proportion between *S. mutans* and *L. acidophilus* from the section 2.2 were co-cultured in MRS broth supplemented with different concentrations of GOS; 1, 2, 3 and 4 % (v/v). Their growth rates were calculated from the growth patterns, which were determined with the same fashion as section 2.1 in the MRS agar.

The bacteria growth rate was calculated from:

$\mu = ((\log 10\ N_t - \log 10\ N_0) \times 2.303)/ (t - t_0)$.[25]

μ : growth rate, N_t : the number of bacteria at t-log phase,

N_0 : the number of bacteria at time point 0,

t : time point reached the mid-log phase

3. Data analysis

The experiments were performed triplicate to investigate the concentration of GOS that had the highest efficacy to enhance the growth rate or activity of *L.*

acidophilus to inhibit the growth of *S. mutans*. The growth rates of *S. mutans* and *L. acidophilus* from the co-culture with GOS-supplemented MRS media were compared by Kruskal-Wallis Test followed by Mann Whitney U test ($p < 0.05$) with alpha correction.

Results

1. Determination of the suitable culture medium for co-culture

S. mutans and *L. acidophilus* were grown in BHI, MRS and TYE broth to find the most appropriate one for both of them. *S. mutans* grew well in the MRS medium, but they stopped growing in BHI after 14 hrs and in TYE after 10 hrs. *L. acidophilus* required more nutrients to maintain their viability. They grew efficiently only in the MRS medium (Fig. 1). Thus, MRS medium was selected for the next experiments.

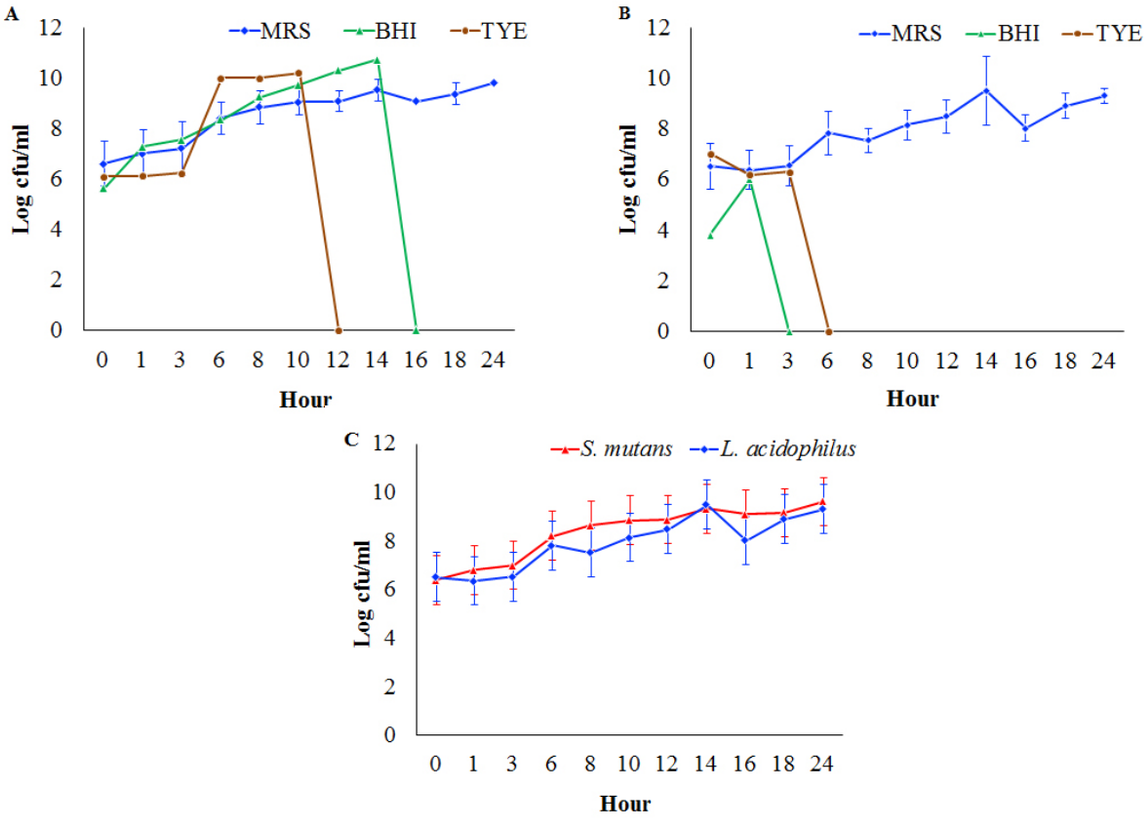

Figure 1 *The growth curves of S. mutans and L. acidophilus in MRS, BHI and TYE media are shown in 1a and 1b respectively. The individually growing capability of S. mutans and L. acidophilus in MRS broth is shown in 1C.*

2. Optimization of the inoculum ratio

From the section 1, the MRS medium was only one medium which both *S. mutans* and *L. acidophilus* could grow. When co-culture in the MRS broth and agar with the ratio 1:1, the number of living *S. mutans* was larger than that of *L. acidophilus* at the initial time point (data not shown). Therefore, the optimization for an equal number of both cells at the initial time point was performed.

S. mutans and *L. acidophilus* in the mid-log phase (OD 600 nm = 0.6) were selected. The various volume ratios between *S. mutans* and *L. acidophilus*;

1:5, 1:10, 1:20 and 1:40 were performed. The number of living *S. mutans* and *L. acidophilus* from the co-culture was similar (10^7 cells) in a ratio 1:20 and 1:40 at the initial time point. They had a similar growth pattern until 8 hrs. After that, *L. acidophilus* had the higher growth rate until 24 hrs (Fig. 2).

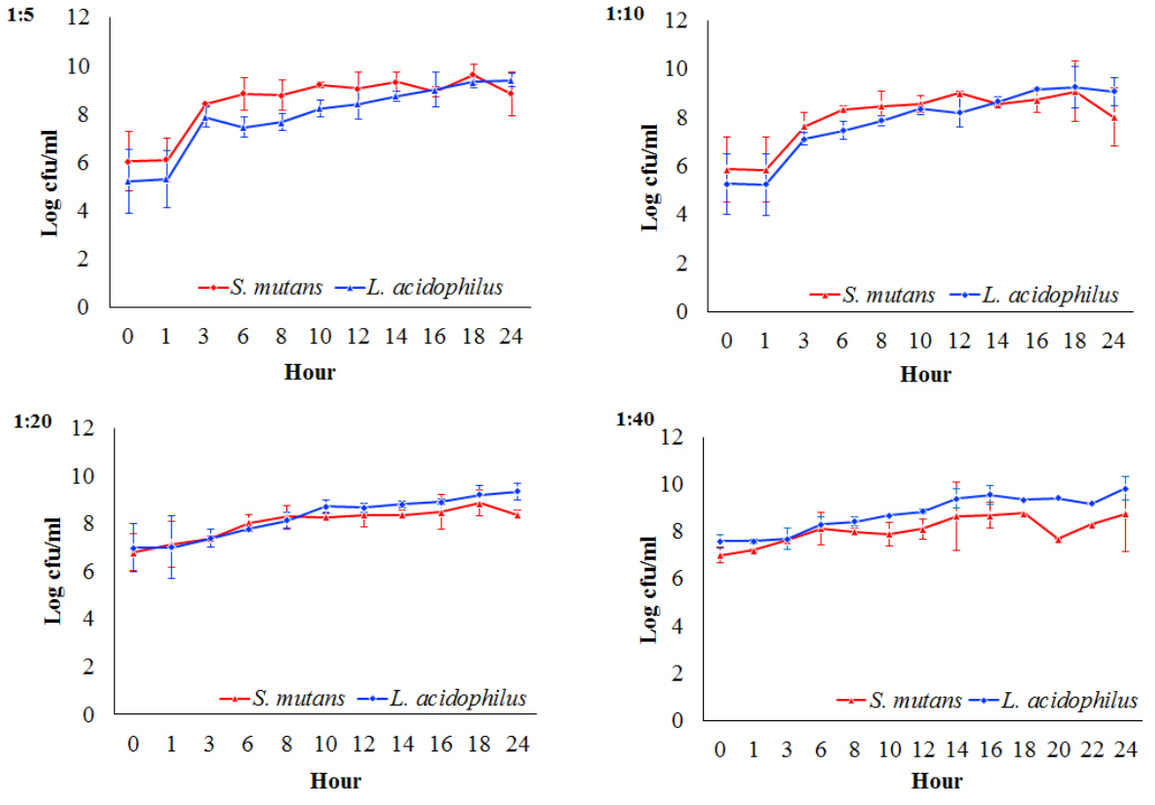

Figure 2 *The growth of S. mutans and L. acidophilus in different proportion*

S. mutans and *L. acidophilus* in the mid-log phase with the volume ratio 1:20 were selected for the next experiment since they had the equivalent number of living cells at the initial time point and their number of cells were more closely when compared with the ratio 1:40.

3. Determination of the inhibition effect of synbiotics towards *S. mutans*

S. mutans and *L. acidophilus* were cultured in the MRS broth supplemented with 1, 2, 3 and 4 % (v/v) of GOS. The growth patterns of them were determined by colony counting in the MRS agar. The maximum growth rate (at 6 hrs) was calculated from the growth patterns as in Fig. 3. Without GOS (control group), the growth rate of *S. mutans* and *L. acidophilus* were similar at 0.4848 and 0.4861 hr^{-1}, respectively.

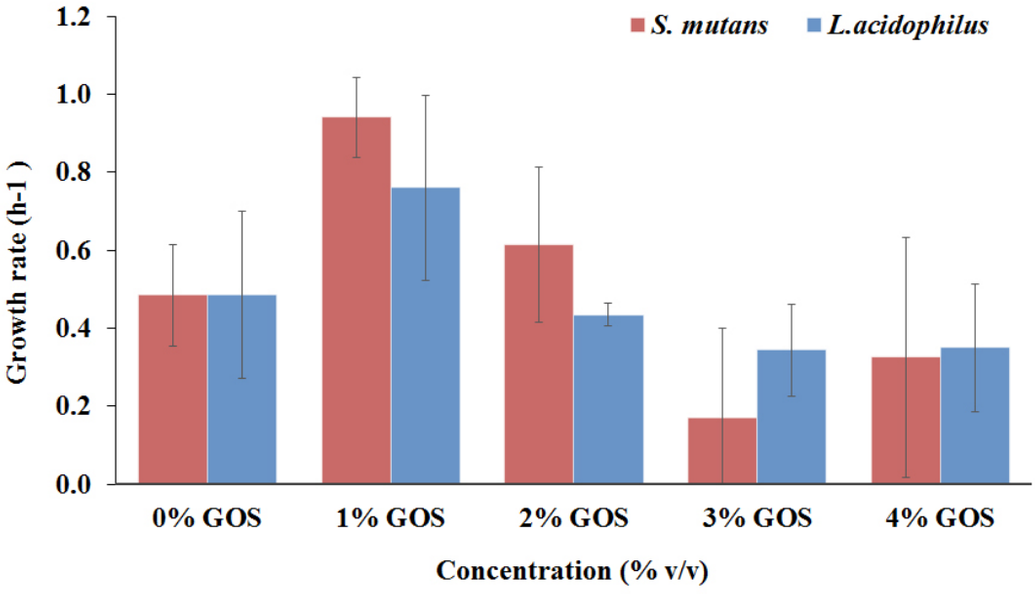

Figure 3 *The growth rates of S. mutans and L. acidophilus in different concentrations of GOS*

The growth rate of *S. mutans* increased in the culture presented 1 and 2 % of GOS (0.941 and 0.62 hr^{-1}). In 3 and 4 % of GOS, the growth rate of *S. mutans* decreased (0.1719 and 0.3258 hr^{-1}respectively) comparing to the control group (0.492 hr^{-1}) (*P*=0.057 and 0.857 respectively).

The growth rate of *L. acidophilus* slightly increased in the culture with 1 % of GOS (0.4861 hr^{-1}). When the concentration of GOS increased (2 %), the growth rates of *L. acidophilus* slightly decreased (0.4347 hr^{-1}) comparing to the control group and 1 % GOS group, but they were relatively constant at 3 and 4 % of GOS (0.3443 and 0.3499 hr^{-1} respectively).

Discussion

Prebiotics are oligosaccharides used to promote the functions of natural probiotics in human bowel. As in the oral cavity, dental caries is the major oral disease around the world. It has plenty of commensal microorganisms which are susceptible to function as pathogens with the alteration of ecology, similar to the human bowel. *L. acidophilus* has been known as probiotics in dental caries prevention. However there is no study to observe the efficiency of prebiotics in dental caries. This study investigated the effect of prebiotic (GOS) on *L. acidophilus* to inhibit growth of *S. mutans.* The suitable condition for co-culture needed to be determined. *L. acidophilus* normally prefer to grow in MRS broth while they can survive in BHI and TYE in just a short period of time. *L. acidophilus* require rich-amino acid and vitamin media. The MRS medium contains higher nutrients; such as peptone, beef extract, yeast extract including several salts (magnesium sulphate, manganese sulphate and dipotassium phosphate) to stimulate growth.[24] *S. mutans* can grow in either BHI or TYE and MRS broth while the maximum growth rate was observed in BHI medium at the first 14 hrs. Therefore MRS medium was selected for co-culture, *S. mutans* and *L. acidophilus.*

According to the growth curve of *S. mutans* and *L. acidophilus*, their logarithm phase were ranged from 6-8 hrs. At the mid-logarithm phase, the average number of *S. mutans* has shown about $2.7x10^8$ CFU/ml which was greater than *L. acidophilus* ($8.2x10^7$ CFU/ml). To determine the similar initial number of them, *S.*

mutans and *L. acidophilus* were co-culture in various ratios of 1:5, 1:10, 1:20 and 1:40. The number of *L. acidophilus* continuously increased to be larger than that of *S. mutans*. This result was corresponded with the previous studies of Singh *et al.* (2011)[26] and Nikawa *et al.* (2004).[27]

At 3 and 4 % of GOS in the co-culture, the number of *S. mutans* insignificantly decreased while there was fairly constant of that of *L. acidophilus.* The three percentages of GOS culture media was interesting for further studies in the synbiotic effect on *L. acidophilus.* Many studies found that *L. acidophilus* had ability to produce variety of antimicrobial substances such as Lactacin F and Lactacin B to compete growth of *S. mutans*.[24,28] The GOS might be able to activate the function (not the number) of *L. acidophilus* to inhibit the growth of *S. mutans.*

After 6 hrs. of the incubation, the number of *S. mutans* was gradually decrease while that of *L. acidophilus* was constant along with the pH of media drop to 5.3. *S. mutans* are the vital cariogenic initiators rather than *L. acidophilus*. When the number of *S. mutans* has been decreased, the dental caries might be difficult to occur. The pH of the co-culture medium dropped to 5.3 in this *in vitro* study. In the oral cavity, saliva has a buffering capacity to control the pH level. The pH level in the clinical situation is not likely to decline to the same level as the *in vitro* study. However the further studies are required.

GOS is a derivative of milk. It is commercially available as the ingredient in food for both infants and adults. It is not toxic when utilized for the clinical application. [29]

This is the preliminary study to investigate the synbiotic effect on the cariogenic bacteria. The prebiotic in this study showed some effects on the probiotic to against *S. mutans* even it was insignificant. The further studies were needed.

Conclusion

The GOS has no efficiency to enhance the function of *L. acidophilus* to inhibit the growth of *S. mutans* in this preliminary study.

Conclusion

This study was granted the FY2015 Thesis Grant for Master Degree Student from The National Research Council of Thailand. Many thanks to Dr. Pornsuda Nhawchai for statistic consultation.

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