



## The enhancement of prebiotic on probiotic for inhibiting growth of dental caries pathogen

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### Abstract

Dental caries is a major oral problem in almost all countries around the world. The local demineralization of tooth surface from an acid action is an initial step of disease. The acid is produced when sugar from food has an interaction with bacteria in dental plaque that usually accumulates on the susceptible tooth surface. As more acidic condition, some commensal oral bacteria are driven to become the cariogenic bacteria. *Streptococcus mutans* is a major contributor of dental decay. Many strategies are recommended to protect the susceptible teeth from cariogenic bacteria. A probiotic application is one technique providing the health-beneficial microorganism to inhibit the cariogenic bacteria. Prebiotics are oligosaccharides which can promote the growth of probiotics in human bowel.

**Objective:** to evaluate the efficiency of the prebiotic (Galacto-oligosaccharides, (GOS)) to enhance the probiotic (*Lactobacillus acidophilus*) for inhibition of *S. mutans*.

**Methods:** *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 at 1, 2 and 3% (v/v). The efficiency of probiotic against *S. mutans* was determined by colony count at 0, 3, 6, 9, 12, 15, 18 and 24 hrs and growth rate interpretation.

**Results:** the growth rate of *S. mutans* increased when co-cultured in 1 and 2% (v/v) of GOS (0.6885 and 0.6142 hr<sup>-1</sup>, respectively). Then the growth rate of *S. mutans* (0.0109 hr<sup>-1</sup>) significantly decreased in 3% (v/v) of GOS. The growth rate of *L. acidophilus* in the co-culture was constant compared between 2 and 3% (v/v) of GOS (0.4347 and 0.4282 hr<sup>-1</sup>).

**Conclusion:** the 3% of GOS provided a promised efficiency to inhibit the growth of *S. mutans* while it did not effect on the growth of *L. acidophilus*.

**Keywords:** probiotic, prebiotic, galacto-oligosaccharides, *L. acidophilus* and *S. mutans*

### INTRODUCTION

Dental caries is a result of an imbalance in physiological equilibrium of tooth mineral within a complex microorganism community that is called dental biofilm (Fejerskov & Kidd, 2008, p. 4). Biofilms can be found on both soft and hard dental tissue. They consist of extracellular matrix and microorganisms. Pathogenic bacteria adhere to the tooth surface where they multiply and produce extracellular matrix to form a plaque community. The metabolic acids are released from the consumption of sugar by bacteria. These acids can cause tooth decay and dental erosion. The key pathogens in this mechanism are acidogenic bacteria that consisted of *S. mutans*, *Actinomyces* spp. and *Lactobacillus* spp., *Streptococcus mutans* is a Gram-positive, a facultative anaerobe, cocci shape which usually known as human oral normal flora and cariogenic pathogen. In human saliva, its number is normally ranges from undetectable to 10<sup>6</sup> - 10<sup>7</sup> CFU/ml. It can use several kinds of sugar resulting in the production of weak acids such



as lactic acid, formic acid and acetic acid. Moreover, *S. mutans* can produce both linear-soluble and branched-insoluble exopolysaccharides (EPS) by glucosyltransferase (GTFs) and fructosyltransferase (FTFs) such as glucans and fructans from sucrose. These exopolysaccharides will be used to facilitate the second colonizer adhesion, stabilize biofilm and protect bacteria from host defense mechanism and reserve energy source (Batt, Robinson, & Patel, 2000, p. 1134- 1157; Jenkinson & Lamont, 2010, p. 34-35; Nair & Takeda, 2011, p. 87-109). Since the reduction of *S. mutans* is recommended for preventing and slowdown disease progression (Bajwa, Jingrwar, & Pathak, 2014, p. ZE04-ZE08). At the present, several studies attempt to replace *S. mutans* with probiotics which are beneficial living microorganism. Probiotics have an influence on both microorganisms and host cells in several mechanisms that include specific antimicrobial substances production, host gene modulation expression and pathogen exclusion (Nair & Takeda, 2011, p. 87-109). In oral cavity, probiotics must first attach to oral tissue. Then, they create a protective barrier preventing pathogenic microorganism colonization. Finally, they must increase in number in order to survive and produce effective capacity (Essche, Quirynen, Sliepen, & Teughels, 2000, p. 111-147; Nair & Takeda, 2011, p. 87-109). The most well-known probiotics are *Lactobacillus* species and *Bifidobacterium* species. From previous studies showed that consumption approximately  $10^7 - 10^9$  CFU/ml of *Lactobacillus* spp. are adequate to modulate the benefit to intestine microflora (Barbés, Boris, & Fernández, 2003, p. 449-455; Bisignano *et al.*, 2012, p. 57-63; Charteris, Collins, Kelly, & Morelli, 1998, p. 759-768).

*Lactobacillus acidophilus* is a strain in the group of *Lactobacillus* spp. (Batt, Robinson, & Patel, 2000, p. 1134- 1157; Nair & Takeda, 2011, p. 87-109). It is a Gram-positive, non-spore forming bacilli which normally isolated from gastrointestinal tract of human. The optimal growth temperature is between 30 - 40 °C enhanced by 5% CO<sub>2</sub> at pH 5.5 - 5.8. These strain produces several kinds of bacteriocin such as Lactacin B, Lactacin F, Brevicin 37 and Gassericin A which affect specific strains in complex microbial biofilm. Furthermore, it also produces several antimicrobial substances such as lactic acid, hydrogen peroxide and various bacteriocins (Batt, Robinson, & Patel, 2000, p. 1134- 1157). *In vitro* studies show that *Lactobacillus casei* strain GG produces different antimicrobial components such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins, and adhesion inhibitors against *Streptococcus* spp. (Deneke, Gorbach, Jacobus, & Silva, 1987, p. 1231-1233; Hasslöf, Hedberg, Twetman, & Stecksén-Blicks, 2010, p. 18). According to Kojima Y. *et al.* (2016), *Lactobacillus* spp. can inhibit insoluble glucan formation of *S. mutans* (Kojima, Maeda, Ohshima, & Seneviratne, 2016, p.27-32). Thus, *Lactobacillus* spp. have been considered as a potential probiotic (Ahmed, Aly, Ghareeb, & Mohamed, 2008, p. 128-136).

Prebiotics have been defined as non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the human colon, which have the potential to improve the host's health. The most common prebiotics are non-digestible polysaccharides (NDO) for instance, lactosucrose, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and isomalto-oligosaccharides (Larque, Sabater-Molina, Toorella, & Zamora, 2009, p. 315-328). However, only FOS, GOS and inulin have been tested *in vivo* to meet all the requirements for current criteria of prebiotics (Marcel, 2007, p. 830S-837S).

Galacto-oligosaccharides are polymers consisting of 2-8 saccharides with glucose terminus (Boler & Fahey, 2011, p. 13-26). They provide benefits to hosts by stimulating growth of selected members of the gut microbiota (Boler & Fahey, 2011, p. 13-26; Goncalves, Rodrigues, Teixeira, & Torres, 2010, p. 257-259).



GOS have more efficiency to enhance growth of intestinal *Bifidobacterium* spp. than FOS (Li *et al.*, 2015, p. 158-168).

Since prebiotics and probiotics have negative effect on cariogenic bacteria, so several studies attempted to improve spectrum of oral microbial modulation by combine them together. These combination is called synbiotics (Gibson, Loo, Probert, & Rastall, 2004, p. 257-259) which are a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and enhancing microbial nutrients in the gastrointestinal tract (Kojima Y., Ohshima T., Seneviratne C. J. J., & N., 2016, p. 27-32). The recent *in vitro* study showed that *L. acidophilus* cultured with conjac glucomanan could inhibit *S. mutans* growth (Al-Ghazzewi & Tester, 2011, p. 234-237). According to Kondepudi *et al* (2012) (Ambalam, Kondepudi, Nilsson, Ljungh, & Wadström, 2012, p. 489-497), GOS increased growth rates of *B. breve*, *B. longum* and *B. pseudocatenulatum*. However, there are few studies of probiotics and prebiotics in dental caries modulation (Ambalam, Kondepudi, Nilsson, Ljungh, & Wadström, 2012, p. 489-497). Thus, this study aim to investigate the inhibitory effect of *Lactobacillus acidophilus* (TISTR 2365<sup>T</sup> = DSMZ 20079<sup>T</sup>) on *Streptococcus mutans* (DSMZ 20523<sup>T</sup>) after treatment with galacto-oligosaccharides (GOS).

## METHODS

### 1. Media, prebiotic and microorganism preparation

#### 1.1 Media preparation

Brain heart infusion broth, BHI (Difco, USA), de Man Rogosa and Sharpe broth, MRS (Difco, USA) and Tryptic Soy Broth (TSB plus tryptone 10 g, yeast extract 5 g, KH<sub>2</sub>PO<sub>4</sub> 1.33 g, K<sub>2</sub>HPO<sub>4</sub> 2.66 g, MgSO<sub>4</sub>.7H<sub>2</sub>O 10 mg, FeCl<sub>2</sub> 10 mg, MnSO<sub>4</sub>. 4H<sub>2</sub>O 10 mg, NaCl 10 mg and glucose 2mg/ml) (Hamilton & Bowden, 1982, p. 255-262) were used for both strains cultivation. For agar media preparation, 1.5 % of agar powder was added to culture broth. All prepared media were autoclaved at 121<sup>0</sup>C, pressure 15 lb/inch<sup>2</sup>, for 15 minutes.

#### 1.2 Prebiotic preparation

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

#### 1.3 Microorganisms preparation

The microorganisms in this experiment were cariogenic bacteria and probiotic as in Table (1). From the lyophilized stock, they were cultured in MRS broth at 37<sup>0</sup>C in 5% CO<sub>2</sub> for 18-24 hours. The overnight cultures were then inoculated on MRS agar by using streak plate technique. A single colony was transferred to fresh media allow them to growth effectively.



**Table 1.** Microorganism, culture media and conditions for cultivation

Item	Detail
<b>Cariogenic microorganism</b>	<i>S. mutans</i> DSMZ* 20523 <sup>T</sup>
<b>Probiotics</b>	<i>L. acidophilus</i> TISTR** 2365 <sup>T</sup> = DSMZ 20079 <sup>T</sup>
<b>Prebiotic</b>	Galactoto-oligosaccharides (GOS)
<b>Culture media</b>	de Man, Rogosa and Sharpe (MRS), BHI and TYE
<b>Cultivation condition</b>	5% CO <sub>2</sub> , 37 °C

\*DSMZ : Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany

\*\*TISTR : Thailand Institute of Scientific and Technological Research, Thailand

## 2. Determination the efficacy of prebiotic-treated probiotic on cariogenic bacteria

The growth inhibition activity of treated-probiotic on cariogenic bacteria was examined by using dilution method (Ferraro & Jorgensen, 2009).

### 2.1 Determination the number of microorganism from growth curve

500 µl of each strain from section 1.3 was inoculated into 20 ml MRS broth. Both cultures were investigated by the optical density (OD) 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 Optimization the proportion of *S. mutans* and *L. acidophilus*

*S. mutans* and *L. acidophilus* at mid log phase were varied to 1:1, 1:2, 1:10, 1:15, 1:20, 1:30, 1:50, 1:100 and 1:150 ratios. The number of both strains at 0, 1, 3, 6, 8, 10, 12, 14, 16, 18, 24, 36 and 48 hrs were enumerated by colony counting method as mentioned in section 2.1.

### 2.3 Effect of prebiotic on probiotic to inhibit *S. mutans*

The optimized proportion between *S. mutans* and *L. acidophilus* from section 2.2 were cultured in MRS supplemented with different concentration of GOS at 1%, 2% and 3% (v/v). The viable count of both strains were tested as described in section 2.1.

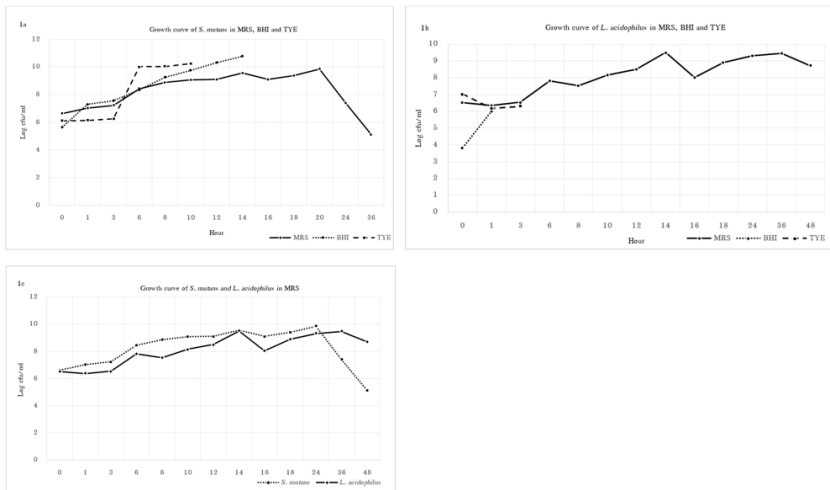
## 3. Data Collection and data analysis

All experiments were performed triplicate to investigate the suitable concentration of GOS which showed the highest ability to enhance growth of *L. acidophilus* against *S. mutans*. The viable count and growth rate of *S. mutans* and *L. acidophilus* were analyzed by using statistical analysis ( $p < 0.05$ ) (Kruskal Wallis test).

## RESULTS

### 1. Growth curve of microorganisms

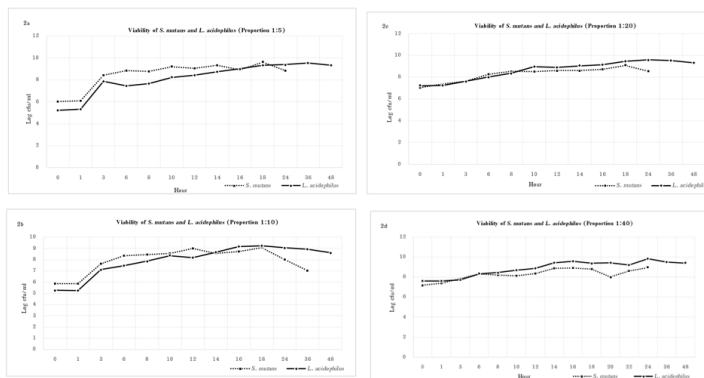
*L. acidophilus* and *S. mutans* were grown in three media to find the most suitable culture media for both strains. The results have shown that *L. acidophilus* and *S. mutans* grew well on MRS medium at similar growth rate 0.481 hr<sup>-1</sup> and 0.491 hr<sup>-1</sup>, respectively (Figure 1c). Thus, MRS medium was selected for next experiment.



**Figure 1** The growth curve of *S. mutans* and *L. acidophilus* in MRS, BHI and TYE media are shown in 1a and 1b, respectively. The growth curve of both strains in MRS is represented in 1c.

### 2. Optimization the proportion of *S. mutans* and *L. acidophilus*

The growth rate of *S. mutans* was greater than *L. acidophilus* at the 1:5 and 1:10 ratio in MRS medium whereas the growth rate of *S. mutans* was less than *L. acidophilus* at 1:40. However, the viable count of *L. acidophilus* was not significant difference with *S. mutans* at 1:20 ratios (Figure 2). Thus, the ratio of 1:20 was the most suitable proportion of both strains for further experiment.



**Figure 2** The growth of *S. mutans* and *L. acidophilus* in various proportions

### 3. Effect of prebiotic on probiotic to inhibit *S. mutans*

From previous result, *S. mutans* and *L. acidophilus* at proportion 1:20 were then co-cultured in MRS broth supplemented with 1%, 2% and 3% (v/v) of GOS. The viable count of them were determined by colony counting method on MRS agar. Without GOS, the growth rate of *S. mutans* and *L. acidophilus* were similar at 0.5502 and 0.5220 hr<sup>-1</sup>, respectively. The maximum growth rate of *S. mutans* and *L. acidophilus* were 0.6885 hr<sup>-1</sup> and 0.6288 hr<sup>-1</sup> at 1% (v/v) of GOS. Then the growth rate of *S. mutans* significantly decreased at 2% and 3% (v/v) of GOS (0.0109 hr<sup>-1</sup>). While *L. acidophilus* slightly decreased when compared with control at 2% and 3% (v/v) of GOS. Hence, the concentration of GOS at 3% (v/v) showed most capable for the reduction growth rate of *S. mutans* while growth rate of *L. acidophilus* was stable (Figure 3). This seemed to provide the promised capability for the clinical application.

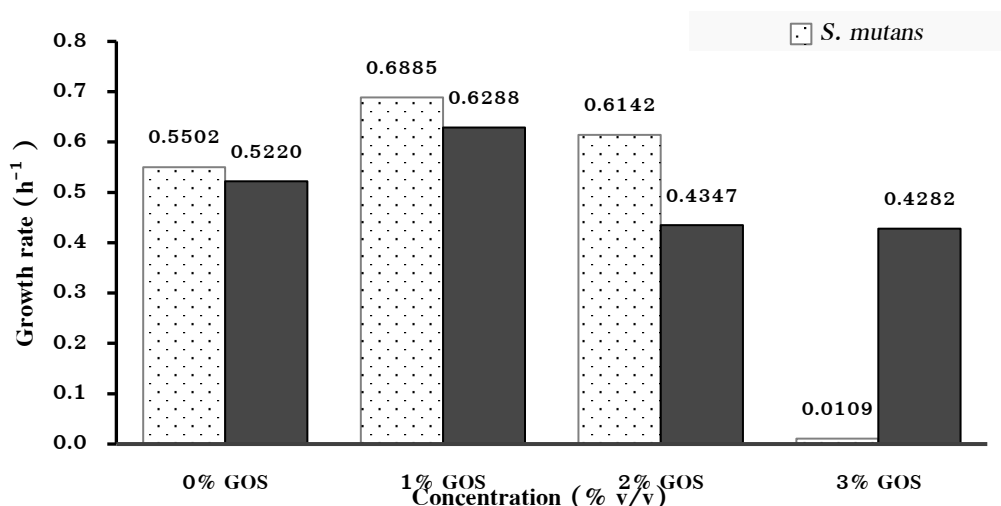


Figure 3 The growth rate of *S. mutans* and *L. acidophilus* in various concentrations of GOS

### Discussion

Prebiotics are oligosaccharides which are used to promote the functions of natural probiotics in a 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 are no many studies 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. In this experiment, *L. acidophilus* preferred MRS for growth because it required more trace elements which were found only in MRS. While, *S. mutans* grew in all type of media and gave maximum growth rate in BHI medium. Thus, MRS medium was the most suitable to use for co-cultures of *S. mutans* and *L. acidophilus*.

According to growth curve at log phase of *S. mutans* and *L. acidophilus* were range from 6–8 hrs and the average number of *S. mutans* has shown about  $2.7 \times 10^8$  CFU/ml which was greater than *L. acidophilus* at  $8.2 \times 10^7$  CFU/ml. To determine the similar initial number of them, *S. mutans* and *L. acidophilus* were co-cultures in various ratios of 1:5, 1:10, 1:20 and 1:40. *L. acidophilus* decreased the number of *S. mutans* which correspondent to the study of Singh *et al.* (2011) (Chawla, Damle, & Singh, 2011, p. 389–394) and Aimi *et al.* (2004) (Aimi *et al.*, 2004, p. 219–223), After 6 hrs., the number of *S. mutans* gradually decreased while that of *L. acidophilus* increased along with the pH of media apparently drop to 4.5. This might be the environment was suitable for *L. acidophilus*. From previous research found that *L. acidophilus* produced variety of antimicrobial substances such as Lactacin F and Lactacin B to compete growth of *S. mutans* (Batt, Robinson, & Patel, 2000, p. 1134–1157; Bermudez-Brito, Gil, Gómez-Llorente, Muñoz-Quezada, & Plaza-Díaz, 2012, p. 160–174).

The GOS seems to provide a safely synbiotic effect when applied along with *L. acidophilus* to inhibit growth of *S. mutans*. The number of *S. mutans* increased in the addition of GOS at 1 and 2% (v/v). The viable count of *S. mutans* significantly decreased while there was no change for *L. acidophilus* at 3% (v/v). The GOS is derived from milk. It is commercially available as the ingredient in food for both infants and adults. Therefore, it is not toxic when utilized for the clinical application.



### Conclusion

The GOS with the appropriate concentration has an efficiency to enhance *L. acidophilus* to inhibit the growth of *S. mutans*.

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