The influence of *Lactobacillus rhamnosus* GG on the binding ability of *Streptococcus mutans*

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Abstract

Probiotics has currently attracted for means of preventive treatment measurement instead of using non-specific and broad spectrum antimicrobials. In previous studies, two main probiotics species, Lactobacillus and Bifidobateria, showed the reduction of DMFS and *S. mutans* counts. However, the timing of introducing probiotic species to oral cavity is not clear. The aim of this study is to evaluate the changes of binding ability of *S. mutans* in various concentrations and inoculation time of *L. rhamnosus* GG. Adding the following concentration of *L. rhamnosus* GG, 1×10^6 CFU, 1×10^7 CFU and 1×10^8 CFU, to *S. mutans* medium demonstrates significant reduction of *S. mutans* counts. Additionally, more reduction was observed when *L. rhamnosus* were inoculated prior to *S. mutans* or simultaneously inoculated compared to when *S. mutans* were inoculated prior to *L. rhamnosus* after 3 hours of incubation. Based on this research, the timing of introducing probiotics should be considered when probiotics are utilized as a preventive treatment measurement.

Key words : Probiotics, *Lactobacillus rhamnosus*, *Streptococcus mutans*

Ⅰ. Introduction

The reduction of dental caries has been a long term issue in dentistry. There have been many different approaches to achieve this goal by using mechanical or non-mechanical treatments. As a non-mechanical treatment, using antimicrobials such as fluoride, chlorhexidine, triclosan, and xylitol has been used previously and was somewhat successful[1-6]. However, the concept of the probiotics, which is the substitution of the oral flora from harmful to beneficial species currently, has been an attraction as a non-mechanical treatment modality[7,8]. The success of using probiotics in gastro-intestinal field is well established and supported in literature[9]. The putative probiotic mechanisms of action are the same in the mouth as they are parts of the gastrointestinal track[10]. *L. rhamnosus* GG reduced the risk of caries significantly in the 3 to 4 years old[11]. Probiotics, Bifidobateria in yogurt may reduce the levels of selected caries-associated microorganisms in saliva[12]. However, the timing of introducing probiotics to oral cavities is not clear. The purpose of this study is to evaluate the change on binding ability of S. mutans in various concentrations and inoculation time with *L. rhamnosus* GG.

Ⅱ. Materials and Methods

Bacterial strain and culture

Lactobacillus rhamnosus GG and *Streptococcus mutant* ATCC 25175 were purchased from American Type Culture Collection (ATCC) and cultured MRS broth (BD bioscience, MD, USA) and Todd Hewitt broth (BD bioscience) at 37℃ in anaerobic atmosphere, respectively.

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Bacteria count

L. rhamnosus and *S. mutans* were counted by using Petroff-Hauser counting chamber (Hausser Scientific Co., PA., USA) after staining with safranin O for 5 min at room temperature (RT). After five times counting, the average was obtained.

Preparation of saliva

Saliva was obtained from a 42 years old healthy female after chewing paraffin wax to facilitate saliva flow for 3-4 minutes , then spilt out the saliva into a 50ml sterilized conical tube (while saliva was collected, the conical tube was placed in the ice). The collected saliva was centrifuged at 11,000×g for 10 minutes at 4℃ by high speed refrigerated centrifuge, diluted 1:1 ratio with PBS and filtered with polyvinylidene difluoride filter (pore size: 0.2 ㎛; PALL life science).

Preparationof hydroxyapatite

Hydroxyapatite (HA: size 80 um, Bio-Rad) 2 mg for each group for 3 trials was washed twice with 1 ml of PBS and removed the supernatant (for each rinse, HA in the 15 ml conical tube was centrifuged at 3,000×g for 10 seconds).

Binding assay

The HA was coated with the prepared saliva,and rotated at 60 rpm for 30 minutes at room temperature (RT). The saliva coated-HA was then washed twice with PBS and the supernatant was removed (for each rinse, the tube contained the saliva coated-HA was centrifugedat 3000×g for 10 seconds). The first sets of saliva coated-HA was dispensed into 1.5 ml tubes for 3 trials in various concentrations of *L. rhamnosus* GG, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 CFU/ml respectively in the presence of *S. mutans*,1×10^6 CFU/ml (binding assay 1). The second sets of saliva coated -HA was dispensed into 1.5 ml tubes for 3 trials in different inoculation time as follows: *S. mutans* only (SM), *S. mutans* and *L. rhamnosus* simultaneously (SM+LGG), *S. mutans*-inoculated one hour prior to *L. rhamnosus* GG (SM1h+LGG), *L. rhamnosus*-inoculated one hour prior to *S. mutans* (LGG1h+ SM), *S. mutans*- inoculated two hours prior to *L. rhamnosus* GG (SM2h+LGG), *L. rhamnosus*-inoculated two hours prior to *S. mutans* (LGG2h+ SM), *S. mutans*- inoculated three hours prior to *L. rhamnosus* GG (SM3h+LGG), *L. rhamnosus*-inoculated three hours prior to *S. mutans* (LGG3h+ SM).After the both bacteria were inoculated, they were incubated for 3 hours in an aerobic atmosphere at 37℃. *S. mutans* DNA was extracted with G-spin™ Genomic DNA extracted Kit for bacteria (iNtROn biotechnology, Inc) according to manufacturer's protocol. DNA (2 ㎕) was then mixed with SYBR Premix Ex Taq, ROX reference Dye (Takara Bio, Otsu, Japan) and each primer (0.2 ㎛M). The condition for the real-time PCR reactions was 40 cycles of denaturation at 94℃ for 15 s, annealing at 60℃ for 15 s and extension at 72℃ for 33 s, and performed by using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Dissociation curves, which were verified by melting curve analysis, were obtained to confirm non-specific amplification of DNA. The sequences of the primers for real-time PCR were as follows: 3′- CTA CAC TTT CGG GTG GCT TG-5′ and 3′-GAA GCT TTT CAC CAT TAG AAG CTG-5′ for the *S. mutans* gene, 3′-GCA GTC GTT AGC CAC CCG AA-5′ and 3′-ACT CCT GTA ATT GCC AGC CG-5′ for *L. rhamnosus* gene. The standard curve was generated by using given *S. mutans* count in different concentrations (1×10^3~10^7) and Cycle Threshold (Ct) of the amplificated DNA of *S. mutans*. The count of *S. mutans* and *L. rhamnosus* binding on the HA was then calculated from the standard curve.

Statistical Analysis

The results were initially entered Microsoft Excel 2007,and performed statistical analysis by utilizing T-test. The data was regarded as statistically significant if p-value was less than 0.05.

Ⅲ. Result

Binding assay 1

S. mutans counts in different concentration of *L. rhamnosus* (Fig. 1).

After 3 hours of incubation, compared to the base line of *S. mutans* counts (1×10^6 CFU), adding 1×10^5 CFU of *L. rhamnosus* GG did not affect *S. mutans* count: however, *L. rhamnosus* GG 1×10^6 CFU, 1×10^7 CFU and 1×10^8 CFU were significantlyreduced *S. mutans* counts.

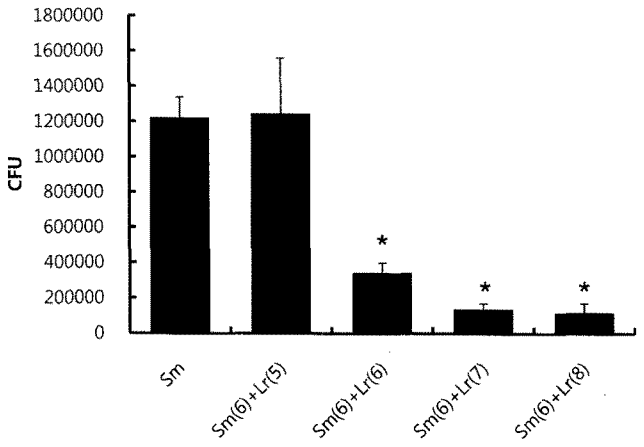

Fig. 1. Inhibitory effect of *L. rhamnosus* on *S. mutans* binding ability to HA in various concentrations. *Streptococcus mutans* (SM) counts after 3 hours of incubation with different concentration of *Lactobacillus rhamnosus* GG (LGG): (5), (6), (7) and (8) indicates 1×10^5 CFU, 1×10^6 CFU, 1×10^7 CFU and 1×10^8 CFU respectively, * represents $p<0.05$, statistically significant by T-test.

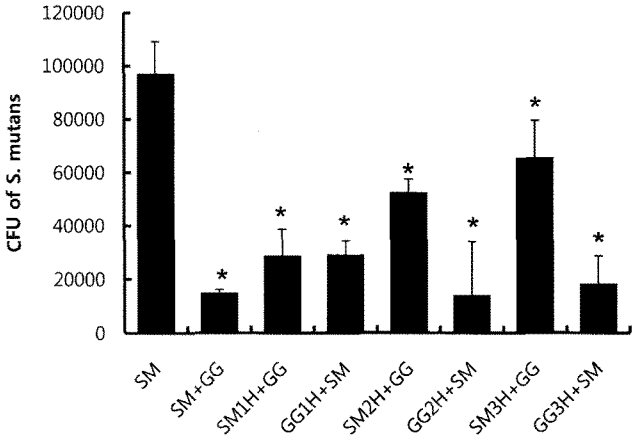

Fig. 2. Inhibitory effect of *L. rhamnosus* on *S. mutans* binding ability to HA in different inoculation time intervals. *Streptococcus mutans* counts in different inoculation time after 3 hours of incubation: *S. mutans* only (SM), *S. mutans* and *L. rhamnosus*-inoculated simultaneously (SM+LGG), *S. mutans*- inoculated 1 hour prior to *L. rhamnosus* GG (SM1h+LGG), *L. rhamnosus*-inoculated 1 hour prior to *S. mutans* (LGG1h+ SM), *S. mutans*- inoculated 2 hours prior to *L. rhamnosus* GG (SM2h+LGG), *L. rhamnosus*-inoculated 2 hours prior to *S. mutans* (LGG2h+ SM), *S. mutans*- inoculated 3 hours prior to *L. rhamnosus* GG (SM3h+LGG), and *L. rhamnosus*-inoculated 3 hours prior to *S. mutans* (LGG3h+ SM).* represents $p<0.05$, statistically significant by T-test.

Binding assay 2

S. mutans counts in different inoculationtime (Fig. 2).

S. mutans counts significantly dropped in all the experimental groups compared to the control. Additionally, more *S. mutans* countreduction was observed when *L. rhamnosus* were inoculated prior to *S. mutans* or simultaneously inoculated after 3 hours of incubation.

Ⅳ. Discussion

Since the initial recognition of possible involvement of oral microbial in dental caries by Dr. W. D. Miller in 1890, numerous studies have been performed to find out the main causative microorganism. As a result, *Streptococcus mutans* was identified as the main pathogens for dental caries even if it is not absolute in all incidents[13]. Based on the previous literature, dental caries are the demineralizing process of the hard tooth structure, which is mainly result of acids produced by bacteria after the consumption of fermentable sugar.The effort to decrease dental caries has been made for decades through the methods of excavating or using antimicrobial agents such as Chlorhexidine, Triclosan, Xylitol, Hexetidine, fluoride and Sanguinaria extracts, to modify dental biofilm composition. However, most antimicrobial agents are non-specific and broad spectrumantimicrobials; therefore, they should not be used routinely except fluoride.

Hence, the probiotictherapy, which alters detrimental to favorable oral microorganism, in contrast to antimicrobial therapy,has attracted as a non-mechanical preventive modality in dentistry. According to various sources, the reduction of the target microorganism, *Streptococcus mutans*, was reported by using different methods to deliver probiotics. The level of caries-associated *mutans streptococci*counts was reduced after daily ingestion of 53 g of the ice-cream, containing *Bifidobacterium lactis* with 1×10^7 CFU/g for 10 days; however,the optimal dose to suppress bacteria still needs to be investigated[14]. The probiotic lozenge, containing *Lactobacillus reuteri* significantly reduced *Streptococcus mutans* counts, especially among high risk carious group, whose *Streptococcus mutans* counts were above 1×10^6 CFU/ml at base line[15]. The yoghurt with live bacteria, *Streptococcus thermophilus* and *Lactobacillus bul-*

garicus, showed selective anti-mutans activity[16].The inhibition of *Streptococcus mutans* adhesion by probiotics, Lactobacillus and Bifidobacterium was dose-dependent as follows; 1×10^7 CFU/ml of the probiotics didn't show inhibition effect, but 1×10^8 CFU/ml of the probiotics showed clearly inhibition effect, and 1×10^9 CFU/ml of probiotics was almost abolished *Streptococcus mutans*[17].

Our study also shows the dose-dependent inhibition from the *Streptococcus mutans* binding assay. The concentration of more than 1×10^6 CFU/ml of *Lactobacillus rhamnosus* GG markedly illustrated the reduction of *Streptococcus mutans* counts. Additionally, when *Lactobacillus rhamnosus* GG was inoculated 2-3 hours prior to *Streptococcus mutans*, the inhibition effect of *Streptococcus mutans* was increased. In a previous study, the effect of inhibition of streptococcus was more evident when probiotic organism added before streptococcus in a model mimicking intestinal[18]. Based on the result, timing of introducing probiotics could be an important factor. Therefore, it must be determined in which age probiotics should be introduced as dental caries prevention regimen, prior to or after the eruption of the dentitions.

As previously stated,there are many literatures, supporting the probiotic therapy as one of non-mechanical optionsof dental treatments. However, other factors such as decreasing pH should be considered, than solely relying on the reduction of *S. mutans* before probiotics are commonly recommended. In addition, more randomized clinical research must be conducted after intra/inter examiner's calibration. Presently, the trend of dentistry is gearing toward the conservation of natural tooth structure as much as possible by the early stage of the prevention and minimally invasive restoration if any type of operative treatments is necessary. Therefore, the probiotic treatment could be beneficial as a preventive measurement. However, there is still some limitation and needs further research to set up guidelines prior to accepting certain probiotic as a common regimen for dental caries prevention.

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국문초록

Lactobacillus rhamnosus GG가 *Streptococcus mutans*의 부착능에 미치는 영향

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최근 들어, 수복 치료 등을 이용한 질병의 직접적인 치료보다는 예방 치료에 대한 중요성이 강조되면서, 그 동안 사용되었던 화학적인 약물을 통한 예방 치료 이외에도 유산균등을 이용한 probiotics에 대한관심이 높아지고 있다. 여러 논문에서 Lactobacillus와 Bifidobateria들이 DMFS와 *S. mutans*를 감소한다고 보고하고 있다. 하지만, 이러한 미생물들의 접종 시기와 접종 농도에 대한 언급은 부족하다. 따라서 본 실험에서는 probiotics의 대표적인 균인, *L. rhamnosus* GG를 이용하여 예방 효과를 보기 위한 적정 농도를 재정립하고, 접종시기를 고찰하였다. *S. mutans* 배지에 1×10^6 CFU이상 농도의 *L. rhamnosus*를 접종하였을 때 *S. mutans* count가 현저하게 감소하는 것을 볼 수 있었다. 접종 순서는 *L. rhamnosus*와 *S. mutans*를 동시에 접종하거나 *L. rhamnosus*를 *S. mutans* 보다 선행하여 접종하였을 때가, *S. mutans*를 *L. rhamnosus* 보다 선행하여 접종하였을 때에 비교하여 *S. mutans* count를 더 감소시켰다. 이 실험의 결과로 보아 probiotics의 접종 시기는 probiotics을 임상에 적용할 때 반드시 고려되어야 하겠다.

주요어: Probiotics, *Lactobacillus rhamnosus*, *Streptococcus mutans*