Effects of soybean milk, chitosan, and anti-\textit{Streptococcus mutans} IgY in malnourished rats’ dental biofilm and the IgY persistency in saliva

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Abstract: Objective: This study aims to evaluate the effect of soybean milk containing a combination of anti-\textit{Streptococcus mutans} IgY and chitosan to the colonization of \textit{S. mutans} in the saliva and to the IgY persistency in the saliva. Materials and Methods: Experimental malnourished Sprague-Dawley rats were fed with soybean milk that is enriched with anti-\textit{S. mutans} IgY and chitosan. After 15 days of feeding, we evaluated the \textit{S. mutans} in dental biofilm, in addition to the persistency level of anti-\textit{S. mutans} IgY. Results: The rats that received soybean milk supplemented with anti-\textit{S. mutans} IgY had the lowest number of \textit{S. mutans} colonies (\(p < 0.05\)). Anti-\textit{S. mutans} IgY was detected in saliva after 15 days of feeding. Conclusions: Soybean milk supplemented with anti-\textit{S. mutans} IgY and chitosan could significantly reduce \textit{S. mutans} biofilm, and the supplemented anti-\textit{S. mutans} IgY persisted in these rats’ saliva following the feeding period.

Keywords: \textit{S. mutans}, IgY, biofilm, chitosan, soy milk

Introduction

Low birth weight (LBW) is still one of the most frequent health problems in babies born in Indonesia. The incidence rate for LBW in Indonesia is 9%, which is above the average rate of 6.6% in Thailand and 5.3% in Vietnam [1]. LBW is also one of the factors that cause malnutrition, inhibiting baby growth and development [2], in addition to disrupting oral cavity such as dental caries [3]. Soy milk is a processed product that is widely used in Indonesia as a substitution for meat product. In addition to its low cost and easiness to obtain, soy milk contains proteins, fats, vitamins, and minerals that are useful for growth [4]. Therefore, soy milk can be used as a supplement to help restore the condition of malnourished children [4, 5]. Moreover, Fontela et al. reported that soy milk can increase the production of SlgA [6], which can inhibit the activity of \textit{S. mutans}, the cause of dental caries in oral cavity. The caries process begins with the formation of bacterial biofilm, particularly associated with the \textit{S. mutans}. Moreover, the frequency of caries in Indonesia is still relatively high. RISKESDAS stated that, in 2007, caries prevalence in Indonesia is 46.5% [7]. There have been various efforts that were attempted in preventing caries, including through active and passive immunization [8–13]. Recently, passive immunization and anti-microbial substances are the two things that are mostly utilized for the development of caries vaccines [14–17]. One method is by utilizing Immunoglobulin Y derived from chickens.

Moreover, a number of studies in the dental field have reported that the anti-\textit{S. mutans} IgY could inhibit the attachment of \textit{S. mutans}, the bacteria that causes dental caries, into the teeth pellicle [9, 16, 18–24]. Immunoglobulin IgY can be found in chicken eggs, which functions to protect the chicken embryo from bacterial infections. As a protein, IgY is more resistant towards changes in environmental pH and temperature.
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compared to human immunoglobulin. In addition, the IgY has no cross reaction with the complement system of mammals. Therefore, IgY has a potential to be used to prevent diseases. Smith et al. [9] and Otake et al. [18] in their study reported that the effectiveness of IgY could inhibit the attachment of *S. mutans*, the bacteria that causes dental caries, into the teeth pellicle. Addition of anti-*S. mutans* IgY in soy milk is intended to provide protection against caries in children who are given soy milk as the natural source for sucrose.

However, previous studies have reported that anti-*S. mutans* IgY is susceptible to changes in temperature [25] and pH level [25, 26]. Hence, to overcome the possible damage of IgY prior to its administration as passive immunization, chitosan might be a good biomaterial as preservative. Chitosan is a linear polysaccharide substance that is extracted from chitin of crustaceans shell waste. Several studies have shown that chitosan also has antibacterial effects [27]. A number of players in the health industry also utilize chitosan for the purpose of lowering cholesterol levels and as a substance to inhibit fat absorption [28–30]. Chitosan and anti-*S. mutans* IgY added to soy milk is expected to reduce the level of fat as well as prevent *S. mutans* biofilm and cariogenic bacteria formation.

Furthermore, human saliva contains specific immunity that is mediated predominantly by secretory immunoglobulin A (IgA) antibodies [31], in addition to an array of antimicrobial molecules whose presence does not depend on previous exposure to microbial antigens [32, 33]. These non-immunoglobulin defense factors contribute to the protection of dental and mucosal surfaces in the oral cavity by modulating microbial colonization and metabolism. Furthermore, submandibular–sublingual mucins and other salivary glycoproteins, such as the parotid salivary agglutinin, are capable of aggregating oral microorganisms in the fluid phase, which results in the clearance of microorganisms from the mouth by swallowing [34, 35].

Hence, based on the results of these previous studies and considering the role of IgY in preventing the attachment of *S. mutans* as well as the content in soy milk that have the potential to improve nutrition, this current study was conducted to determine whether the addition of anti-*S. mutans* IgY and chitosan into soy milk can decrease the number of colonies of *S. mutans* in dental biofilm.

Materials and Methods

**Animal model**

Induction of malnourished rats was conducted during the prenatal period of Sprague-Dawley rats in the 8th week of pregnancy, by reducing the protein intake in their food. The postnatal induction was conducted after 3 weeks of lactation and 3 weeks post-lactation stages. Each rat was then infected with 50 µL of 5 × 106 CFU/mL cultured *S. mutans*.

Twenty Sprague-Dawley rats were divided into 5 groups, each consisted of 4 rats. Group (1) was the control, group (2) was fed only with soy milk, group (3) was fed with soy milk that is enriched with 0.01% chitosan, group (4) was fed with soy milk enriched with 0.1% anti-*S. mutans* IgY, and group (5) was fed with soy milk enriched with both 0.01% chitosan and 0.1% anti-*S. mutans* IgY. The chitosan used was in powder form and was produced by the Indonesian Nuclear Agency (BATAN), with 70% degree of deacetylation and dissolved in acetic acid.

**Dental biofilm sampling**

The rats were anesthetized with ether, and the sampling of dental biofilm was done by swabbing the surface of the teeth using cotton bud. The sample was then stored in Eppendorf tube containing 200 µL sterile phosphate-buffered saline (PBS) solution, inside an ice box. The cotton bud that was used for taking samples was squeezed in the Eppendorf tube, which was later centrifuged for 10 min at 1000 rpm. Subsequently, 50 µL of sample was obtained using micropipette and dripped on to TYS-A solid media, before being spread for further incubation in anaerobic jar. It was also supplied with 10% of CO₂ gas, 5% of N₂, and 85% of O₂ for 1 min, prior to 72 h of incubation at 37 °C. After 72 h of incubation, the number of *S. mutans* colonies was counted [36].

**ELISA**

To detect the persistency of anti-*S. mutans* IgY, we used a modified version of the ELISA that was described by Akitake and Nakai [37]. Briefly, the 96-well plates were coated with sodium carbonate buffer (pH 9.6). After blocking with blotto (skim milk 5% diluted in TBS), we collected the rat’s saliva that was diluted in a tenfold serial dilution in blotto, and 100 µL of each dilution was added to the plates. Plates were then washed with PBS-T (0.05% (v/v) Tween 20 in PBS, pH 7.2) and incubated for 1 h after adding horseradish peroxide anti-rabbit chicken IgY, which was diluted 1:1500 in blotto. The plates were also washed with PBS-T, and the TMB (Sigma) was added as a substrate to each well. After 10-minute incubation, the reaction was stopped by adding 1 N of HCl. The 450 nm wavelength was measured using an ELISA reader.

**Western blot analysis**

Electrophoretically transferred protein (2.5 mg protein/saliva per well) from unstained 10% sodium dodecyl sul-
fate (SDS)–polyacrylamide gels was used for Western blot. The proteins were transferred onto nitrocellulose membranes (Bio-Rad). After transferring, the membranes were blocked with blocking buffer (5% (w/v) nonfat milk powder or blotto, 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween 20) and rinsed with wash buffer (blocking buffer without milk powder). The membranes were then incubated with the IgY diluted (1:100) in blocking buffer, washed with the washing buffer and incubated with HRP-anti-rabbit chicken IgY that was diluted (1:2000) in blocking buffer, before being subjected to a subsequent wash. We then detected the dot that corresponds to the anti- S. mutans IgY.

Results

To determine the effect of soy milk–IgY–chitosan, we measured the number of colonization in the experimental groups as compared to the control group. According to the Kruskal–Wallis test result (Table I), there are at least two groups with significant differences (df = 4, σ = 11,847).

We observed that there was more number of bacteria in the group of rats that were being fed with only soy milk, compared to the control group. Moreover, this observation was the greatest among the experimental groups. Provision of soy milk tends to increase the number of bacteria in the biofilm, despite the Mann–Whitney test result that suggested no significant statistical difference (p-value = 0.149).

The group of rats that received soy milk enriched with anti- S. mutans IgY and chitosan has the lowest S. mutans count in the dental biofilm (p-value < 0.05). On the other hand, rats that have received soy milk in addition to the IgY had resulted in nonsignificant difference of S. mutans count compared to the group that has received soy milk plus chitosan as well as in comparison to the experimental group receiving only soy milk.

Figure 1 shows the persistency of anti-S. mutans in the rats group receiving IgY either with or without chitosan. This was indicated by the high outside diameter (OD) of ~0.4 whereas, in the other groups, they were shown to have an OD below 0.35. The average OD values were also subjected to a Kruskal–Wallis test, yielding a significant p-value of less than 0.05. Furthermore, the

Table I  The average amount of S. mutans each group after 15 days of feeding

| Group                              | S. mutans count (CFU/mL) |
|------------------------------------|--------------------------|
| The control group                  | 495.00075                |
| Soy milk only                      | 1540                     |
| Soy milk + Chitosan                | 126.6333                 |
| Soy milk + IgY anti-S. mutans      | 1525                     |
| Soy milk + IgY anti-S. mutans + Chitosan | 39.985                  |

Fig. 1. The persistency of IgY anti-S. mutans in rat’s saliva. The IgY was detected in group of rats that received a combination of soy milk and IgY with or without chitosan (OD > 0.3)
Western blot analysis result also confirmed the IgY protein band at 41 kDa (Fig. 2).

Discussion

Sheikh et al. previously reported that consumption of soy milk can decrease the pH of saliva till 5.87 [36], an acidity level that is close to the critical pH for dissolving hydroxyapatite in the tooth enamel [39]. Meanwhile, carbohydrate in soybean milk is a form of polysaccharide and oligosaccharide, such as sucrose. Sucrose is the most cariogenic carbohydrate because it can be easily fermented by acidogenic bacteria such as the S. mutans, in addition to producing a polysaccharide that facilitates the attachment of bacteria to the teeth surface. Furthermore, Fontenla et al. reported that soy milk can increase the production of SIgA [6], which is one of the components for the body’s defense system against oral bacteria. It can be concluded that although soybean milk can increase the SIgA level, it is more likely to increase the risk of caries either by lowering the pH of the saliva or by inducing greater level of bacterial activity in the biofilm.

In this study, the group of rats that received soy milk enriched with anti-S. mutans IgY and chitosan has the lowest S. mutans count in the dental biofilm (p-value < 0.05). In contrast, the rats group that had received both soy milk and IgY resulted in a nonsignificant difference of S. mutans count in comparison to not only the group receiving soy milk and chitosan but also the experimental group receiving only soy milk. Nonetheless, we have discovered that soy milk feeding could indeed prevent S. mutans colonization in all of four rat groups. This result supports previous studies that were reported by Tarsi et al. [39], who concluded on the antibacterial effects of chitosan, including that of anti-S. mutans.

Furthermore, according to a study conducted by Otake et al. [18], anti-S. mutans IgY can inhibit S. mutans colonization. However, in this study, no significant difference was reported between rats only receiving soy milk compared to those receiving both soy milk and IgY. This may occur because the anti-S. mutans IgY could become unstable and damaged during the feeding process; hence, the effect could not be optimal.

Moreover, the number of bacteria in rats that received a combination of soy milk with anti-S. mutans IgY and chitosan is the lowest compared with the other groups. This result suggests that soy milk that is enriched with anti-S. mutans IgY and chitosan can provide the best anti-S. mutans effect. In this supplement, the existence of chitosan, which also functions as a natural preservative, can optimize the stability or persistence of the anticaries effect of the anti-S. mutans IgY.

The supplementation of soy milk enriched with anti-S. mutans IgY and chitosan is the most effective in reducing the number of S. mutans in mice dental biofilm. Even though the statistical result shows no significant difference (p-value = 0.146), the number of S. mutans in the rats group receiving soy milk, anti-S. mutans IgY plus chitosan is twelve times lower than the control group, indicating that the soya milk enriched with anti-S. mutans IgY and chitosan has a tendency to reduce the number of S. mutans in the dental biofilm.

Moreover, it should be noted that giving soy milk alone can increase the amount of S. mutans in dental biofilm. This is supported by the theory that soy milk contains sucrose which facilitates S. mutans attachment to the surface of the teeth [40]. The result also suggested that the number of S. mutans in the rats that received soy milk was three times higher than the control group, albeit the nonstatistical significance (p-value = 0.149). It can be concluded that feeding of soy milk that was enriched with anti-S. mutans IgY and chitosan can effectively reduce the number of S. mutans in rat dental biofilm.

Another important issue regarding passive immunization to prevent colonization in the tooth hard surface is the stability or persistence of IgY in the saliva. As shown in Table 1 and Fig. 2, this study shows the persistency of anti-S. mutans IgY in the rats group that have received IgY either with or without chitosan. This was indicated by the high OD value of ~0.4 in comparison to the other groups, which are associated with OD value below 0.35. This average OD values were also subjected to Kruskal–Wallis test, yielding a significant p-value of less than 0.05.

Furthermore, based on the experimental result in the groups that were given anti-S. mutans IgY after 15 days, it can be suggested that the anti-S. mutans IgY can still persist in the saliva (Fig. 1). The stability of IgY to acid
and alkali has been previously studied under various conditions. It was found that the IgY activity was reduced at pH 3.5 or lower and almost completely lost with an irreversible change at pH 3. Under alkaline conditions, the activity of IgY did not change until the pH was increased to 11. However, it was markedly reduced at pH 12 or higher [26, 41, 42]. Previously, IgY has also been thermally treated at various temperatures at different periods of time. The antigen binding activity of the IgY decreased with increasing temperature and heating time. IgY is stable at temperature ranging between 60° and 70 °C. The activity of IgY decreased by heating for 15 min at 70 °C or higher, and IgY can be denatured when thermally treated at temperatures higher than 75 °C [43].

From the Western blot analysis, there was a band spotted in lane 6 with a molecular weight of 41 kDa, which indicated the IgY protein [44]. This result can prove that the band that appeared on the membrane was anti- S. mutans IgY. This supports the ELISA result that shows how persistently the anti- S. mutans IgY can still be detected after 15 days in oral cavity.

Conclusions

Soybean milk supplemented with anti- S. mutans IgY and chitosan could significantly reduce S. mutans biofilm, and the supplemented anti- S. mutans IgY persisted in these rat’s saliva following the feeding period.

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Authors’ contribution: EWB: Study design, wrote the manuscript. manuscript drafting and critical discussion; RDS: supervised the research study; BMB: revised the manuscript; AH: animal experiment; FN: Immunology assay; YM: FA performed the statistical analysis and wrote the methodology.

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