Protein profiling of *Streptococcus mutans* isolated from the surface of the tongue in children with early childhood caries

H Aldilavita¹, F P Gultom¹* and E W Bachtiar¹

¹Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta 10430, Indonesia

*E-mail: gultom_ferry@yahoo.com

Abstract. The pathogenesis of early childhood caries (ECC) is influenced by the virulence factors of *Streptococcus mutans*, a major etiological factor of dental caries protein profile. To analyze the differences in the protein profiles of *S. mutans* isolated from the surface of the tongue in ECC and caries-free children. Saliva samples were collected from 32 children (16 with ECC and 16 caries-free). Protein profiling was performed using the SDS-PAGE method. Examination of protein bands on the polyacrylamide gel revealed differences in the frequencies of several *S. mutans* proteins. The molecular weights of the proteins observed in the ECC and free children were as follows: 13, 29, 39, 41.3, 74, and 94.5 kDa. The proteins virulent to ECC (41.3 kDa and 74 kDa) were more frequently expressed in children with ECC when compared to the caries-free children. Differences in the protein profiles of *S. mutans* isolated from the tongue were observed between the ECC and caries-free children in this study.

1. Introduction
According to the American Academy of Pediatric Dentistry (AAPD), early childhood caries (ECC) is defined as the presence of one or more decayed, missing, or filled tooth surface in children below 71 months of age [1]. The prevalence and severity of caries in children remains high in several countries, including Indonesia. According to the Basic Health Research (RISKESDAS) in 2013, the national DMFT index was 4.6. In 1988, the prevalence of ECC in preschool-aged children in Jakarta and in the surrounding areas was 85.17% [2,3]. ECC reduces chewing ability and interferes with growth and development, thus, decreasing the quality of life in children [4].

ECC can occur due to host, environmental, and microorganism factors. *Streptococcus mutans* is one of the main causes of ECC, and is usually present in the dental plaque and on the tongue [5]. The tongue is located at the floor of the mouth, and is important for various functions such as swallowing, taste, and speech [6]. The papillary structures of the tongue facilitate the accumulation of dental plaque and microorganisms, including *S. mutans*, on the dorsal surface, especially when oral hygiene is not maintained [5].

The main virulent factors of *S. mutans* are the ability to produce large amounts of organic acids and to withstand a low pH; in addition, they can produce large amounts of glucans by metabolizing sucrose to glucan with the aid of a *S. mutans* protein, glucosyltransferase (Gtf) [7]. Glucan can help in the attaching and colonizing of *S. mutans* to the tooth surface [8] thus, initiating the pathogenesis of caries.
S. mutans also produce glucan binding proteins, which can increase the attachment of these microorganisms to the tooth surface. Furthermore, the proteins produced by S. mutans are correlated to the virulence of this microorganism with regard to the development of caries [9].

The present study aimed to evaluate the protein profile of S. mutans isolated from the tongue surface of children with ECC.

2. Methods
Included in this study were 32 children (16 with ECC and 16 non-ECC) aged below 71 months from the Al-Mutazam Kindergarten, Depok, West Java. Samples were obtained from dental plaque on both surfaces (ventral and dorsum) of the tongue using dental floss, and transferred into microcentrifuge tubes containing PBS and PMSF. Saliva was collected using a transfer pipette; the child was instructed to keep the mouth open for one min. The saliva was transferred into a 1.5 mL microcentrifuge tube and its viscosity was noted (high or low) by tilting the microcentrifuge tube.

The samples were cultured using 5 μL of TYS agar (10 g of Trypticase Peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) and were incubated under anaerobic conditions at 37 °C for 72 h. Then, the S. mutans colonies were transferred from the TYS agar to a TYS Broth by picking up each colony with a toothpick, and incubated under anaerobic conditions at 37 °C for 72 h.

The antigen lysis method was used to prepare S. mutans antigen. To obtain the antigen, the samples in the TYS Broth were placed in a centrifuge for 10 min. The pellet obtained was resuspended in 1 mL of cell resuspension buffer; 0.2 mL lysozyme solution was added to the suspended cells and the solution was allowed to rest for 5 min. Then, it was centrifuged again for 5 min. The pellet was collected, was resuspended in 0.3 mL of TEN buffer and 0.065 SDS (10%), and was reincubated for 10 min.

After obtaining the S. mutans antigen, the Bradford assay was used to calculate the antigen concentration. The assay was performed using 10 μL of each standard protein and the S. mutans antigen in microwell plates; 190 μL of Bradford reagent was added to the standard protein and S. mutans antigen. A microplate reader was used to measure the absorbance values of all samples and controls.

The antigen concentrations were calculated and equated to 100 μL for the SDS-PAGE method. 10 μL of the buffer samples were added and were placed onto the thermoblock at 95°C for 5 min. The resolving and stacking gels were made until well formed. Next, 15 μL of samples and 5 μL of protein marker were placed in the wells. The tank electrophoresis was connected to the power source (150 V, 80 mA) for 70 min. After electrophoresis, the gel was placed in a closed container containing the blue page, and on a shaker at 60 rpm for one night. The gel was washed with distillated water or aquadest every 30 min. The results of the SDS-PAGE were observed on the scanner. The visible protein bands were recorded in a table based on which, a chart was prepared to analyze the S. mutans protein profile.

3. Results
Figure 1 shows the protein profile of S. mutans isolated from the ventral surfaces of the tongues of children with ECC and the caries-free subjects. The profiles in both samples included proteins of various molecular weights (13 kDa, 29 kDa, 39 kDa, 41.3 kDa, 74 kDa, and 95 kDa). The frequencies of the proteins weighing 13 kDa and 29 kDa were similar in the ECC and caries-free groups (16/16 samples; 100%). Proteins weighing 39 kDa were detected in 1 of 16 samples (6.25%) in ECC patients, and in 13 of 16 samples (81.25%) in the caries-free children. Four out of 16 samples (25%) in the ECC group contained proteins weighing 41.3 kDa when compared to 1 out of 16 (6.25%) children in the caries-free group. Protein with 74 kDa molecular weight had a greater frequency (5/16; 31.25%) in the ECC group than compared to the caries-free group (3/16; 18.75%). On the contrary, the
frequency of proteins weighing 94.5 kDa in the ECC group (2/16; 12.5%) was lower than that in the caries-free group (4/16; 25%).

**Figure 1.** Block chart demonstrating the frequency of protein expression in S. mutans. The microorganisms were isolated from the ventral surfaces of the tongues of children belonging to the early childhood caries and caries-free groups (n = 16 in each group).

A marked difference in the frequency of the 39 kDa protein was noted between the ECC (1) and caries-free (13) groups when compared with the other proteins (Figure 1).

**Figure 2.** Bar graph showing the frequency of protein expression of *S. mutans* isolated from the dorsal surfaces of the tongues of children in the ECC and caries-free groups (n = 16 in each group).
Figure 2 showed the protein profile of *S. mutans* isolated from the dorsum of the tongue. The frequencies of the 13 (16/16; 100%) and 74 kDa (3/16; 18.75%) proteins were similar between the two groups. Conversely, the frequencies of the 39 kDa proteins were visibly different between the ECC group (4/16; 81.25%) and the caries-free group (12/16; 75%).

Clear differences in the protein profiles of *S. mutans* from the dorsum and ventral surfaces of the tongue surface were noted between the ECC and caries-free groups (Figure 1 and Figure 2). The frequencies of the proteins virulent to ECC (41.3 kDa and 74 kDa) were higher in the ECC groups when compared with the caries-free group. Therefore, the first and second hypothesis in this study was accepted.

4. Discussion
In the present study, there was an examination of the expression pattern of proteins from *S. mutans* that were isolated from the dorsum and ventral surfaces of the tongue surface. A protein profile is a protein characteristic of bacterial cells that can be qualitatively determined using SDS-PAGE [10], which is a molecular technique that helps to identify protein cells based on the molecular weight of the protein subunit [11]. The frequency of the 13 kDa proteins, called as antigen D in *S. mutans* [12], was same in the ECC and caries-free groups in the samples isolated from the ventral and dorsal surfaces of the tongues. According to Russell R.B (1993), this protein was identified previously as a low molecular mass protein [13]. According to Roman C et al (2013), antigen D can respond to enzymes involved in amino acid synthesis and can interact with other proteins during the process of early caries development [12]. This might explain why these proteins were present in all (100%) of the individuals in both groups.

The 29 kDa molecular weight protein is called antigen A [14]. It is not known to be involved in the forming of dextran bonds that play a role in initial colony formation. Nevertheless, previous studies suggest that although the role of antigen A is still not clear, immunization against it might protect the host against caries. Antigen A induces Ig A antibodies to interfere with bacterial attachment and colonization that could lead to caries [15]. These findings were supported by the results of this study, where the frequency of the 29 kDa proteins in the dorsum of the tongue was higher in the caries-free children when compared to those with ECC, indicating that increased levels of antigen A would induce IgA to protect the host against caries. However, the frequencies of these proteins in samples isolated from the ventral surface of the tongue were similar in both groups. The role of antigen A in patients with ECC might be reduced because of other more dominant factors such as diet, salivary flow rate, salivary viscosity or the host, resulting in the development of caries [16].

Proteins with a molecular weight of 39 kDa are known as antigen III [12]. In the current study, the frequencies of the 39 kDa proteins isolated from the ventral and dorsal surfaces of the tongue were higher in the caries-free children. According to Russel W (1995), this antigen was thought to induce IgA production in caries-free children to protect them against ECC [17]. This might explain the higher levels of antigen III in the caries-free groups in this study.

The frequency of the 41.3 kDa protein, also called GbpB protein [18], in saliva isolated from the dorsum and ventral surfaces of the tongue was high in children with ECC when compared to those without caries. Mattos-Graner et al. (2001) stated that GbpB had a positive relationship with biofilm formation [19]. GbpB is also known to play a role in cell wall synthesis, which is important for bacterial growth; therefore, interruptions in GbpB production might prove lethal for bacterial development. These findings indicate that the presence of GbpB could increase bacterial growth leading to an increase in biofilm formation in the oral cavity. Conversely, the absence of GbpB might inhibit bacterial growth and reduce the formation of biofilm [19].

The 74 kDa proteins, also called GbpA proteins [20], are thought to have hypercariogenic properties because of changes in plaque structure, thereby increasing acid production and forming tight barriers between the tooth surface and saliva [21]. This leads to the demineralization of the enamel, which is further facilitated by the inability of the saliva to act as a buffer. GbpA was also
found to be able to increase bacterial attachment under the influence of sucrose [21]. In the present study, the frequency of 74 kDa proteins in saliva isolated from the ventral surface of the tongue was higher in the ECC group when compared with the caries-free group, suggesting that GbpA was required for caries development in these children. However, on the dorsum of tongue, the frequency of this protein was the same in both groups. GbpA in the caries-free children did not influence caries development probably because of habits such as maintenance of good oral hygiene, fluoride use, healthy diet, and routine visits to the dentist. In addition, the presence of several S. mutans proteins that might activate the immune system and protect the child from antigens A and III cannot be discounted [12,22].

The 94.5 kDa proteins, also called extracellular dextranase (DexA), [23] are found to be more frequently present in saliva isolated from the ventral and dorsal surfaces of the tongue in caries-free children. Several studies have reported that DexA might inhibit the attachment of S. mutans to the tooth surface thereby inhibiting caries development by altering the production of water-insoluble glucan (WIG) in glucan. WIG is a pathogen that plays an important role in biofilm formation and adhesion to tooth surfaces. In the study by Otsuka et al. (2014), extracellular dextranase was found to decrease the amount of WIG in biofilms, indicating that it might play an inhibitory role in the development of caries [24].

5. Conclusion
The protein profile of S. mutans isolated from the dorsum and ventral surface of tongue showed that the frequency of expression of proteins virulent to ECC (molecular weights, 41.3 kDa and 74 kDa) were higher in children with ECC when compared to the caries-free children.

References
[1] AAPD 2008 Definition of early childhood caries (ECC). Am Acad Pediatr Dent. 4 1.
[2] Pengembangan dan Penelitian, 2013 Riset Kesehatan Dasar Kementrian Kesehatan Republik Indonesia
[3] Sugito FS, Djoharnas H and Darwita RR 2008 Breastfeeding and early childhood caries severity of children under three years old in DKI Jakarta Makara, Kesehat. 12 86–91.
[4] Fung MHT, Wong MCM, Lo ECM and Chu CH 2013 Arresting Early Childhood Caries with Silver Diamine Fluoride-A Literature Review Oral. Hyg. Health. 1 1–7.
[5] Sylvania DA, Gultom FP and Bachtiar BM 2014 Korelasi Kuantitas Streptococcus mutans pada Plak Lidah dengan Risiko Karies Tinggi Correlation between Quantity of Streptococcus mutans in Tongue Plaque and Saliva and High Risk Caries. Dept. Oral Biologi, Fakultas Kedokteran Gigi, Universitas Indonesia. 2–8.
[6] Ghom AG and Anil S 2014 Textbook of Oral Medicine. New Delhi, India: Jaypee Brother Med Pub; pp. 501–502.
[7] Daboor SM, Syed F and Masood S 2015 Fly ash based biopesticides: A comprehensive review Indian. J. Microbiol. Res. 2 76–82.
[8] Nishimura J, Saito T, Yoneyama H, Lan Bai L, Okumura K, et al 2012 Biofilm Formation by Streptococcus mutans and Related Bacteria Adv. Microbiol.2 208–15.
[9] Kawada-matsuo M and Komatsuzawa H 2017 Role of Streptococcus mutans two-component systems in antimicrobial peptide resistance in the oral cavity Jpn. Dent. Sci. Rev. 53 86–94.
[10] Anastasya RE 2011 Analisis Keterkaitan Ekspresi Protein Streptococcus mutans yang Diisolasi dari Subjek Karies dan Bebas Karies [Skripsi]. Universitas Indonesia. 38–39.
[11] Tahmourespour A, Nabinejad A, Shirian H and Ghasemi N 2013 The Comparison of Proteins Elaborated by Streptococcus mutans Strains Isolated from Caries Free and Susceptible Subjects Iran. J. Basic. Med. Sci. 16 656–7.
[12] Roman C, Rivera A, Santellan R, Teutle B, Yanez A, et al 2013 Immunogenic antigens from streptococcus mutans which stimulate secretory iga response from parotid saliva in children with caries World Appl. Sci. J. 28 297–303.

[13] Russell RRB, Hogg SD and Sutcliffe IC 1993 Identification of Streptococcus mutans antigen D as the HPr component of the sugar phosphotransferase system JFEMS Microbiol. 107 67–70.

[14] Dao MYL, Chavez C, Hirachi Y and Ferretti JJ 1989 Molecular cloning of the Streptococcus mutans gene specifying antigen A American Society for Microbiology. 57 3372–6.

[15] Gómez SI, Jaramillo LM, Moreno GC, Roa NS and Rodriguez A 2015 Differential reactivity of salivary igA and igG against streptococcus mutans proteins in humans with different caries experience Acta Odontol. 28 3–12.

[16] Warna D, et al. 2011 Hubungan biofilm streptococcus mutans terhadap resiko terjadinya karies gigi J.K.G Unej. 8 127–30.

[17] Russell MW and Harrington DJ 1995 Identity of Streptococcus mutans Surface Protein Antigen III and Wall-Associated Protein Antigen A American Society for Microbiology. 63 733–5.

[18] Irwandi RA, Bachtiar EW, Yuniaututi M 2012 Pengaruh immunoglobulin-y terhadap protein streptococcus mutans yang diisolasi dari subjek karies dan bebas karies IDJ. 1.

[19] Mattos-graner RO, Jin S, King WF, Chen T, Smith DJ, et al 2001 Cloning of the Streptococcus mutans gene encoding glucan binding protein B and analysis of genetic diversity and protein production in clinical isolates American Society for Microbiology. 69 6931–41.

[20] Shah DSH and Russell RRB 2004 A novel glucan binding protein with lipase activity from the oral pathogen streplococcus mutans Microbioloy. 150 1947–56.

[21] Hazlett KRO, Mazurkiewicz JE, Banas JA, Al HET and Mmun INI 1999 Inactivation of the gbpA Gene of Streptococcus mutans Alters Structural and Functional Aspects of Plaque Biofilm Which Are Compensated by Recombination of the gtfB andgtfC Genes American Society for Microbiology. 67 3909–14.

[22] Pay MN, Widiati S and Sriyono NW 2016 Identifikasi faktor yang mempengaruhi perilaku anak dalam pemeliharaan kebersihan gigi dan mulut: Studi pada Pusat Pengembangan Anak Agape Sikumana Kota Kupang, Nusa Tenggara Timur, Indonesia Maj. Ked. Gi. Ind. 2 27–34.

[23] Igarashi T, Yamamoto A and Goto N 1995 Sequence analysis of the Streptococcus mutans Ingbritt dexA gene encoding extracellular dextranase Microbiology O. 39 853–60.

[24] Otsuka R, Imai S, Murata T, Nomura Y and Okamoto M 2015 Application of chimeric glucanase comprising mutanase and dextranase for prevention of dental biofilm formation Microbiol. Immunol. 59 28–36.