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Activity of polyclonal IgY from Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis to inhibit colonization of Fusobacterium nucleatum and Streptococcus sanguinis ABSTRACT Background: Fusobacterium nucleatum (F. nucleatum) and Streptococcus sanguinis (S. sanguinis) play a role in the formation of dental plaque which is the cause of periodontitis. Immunoglobulin Y (IgY) is present in serum and egg yolk. IgY can bind to the surface components of bacteria. F. nucleatum and S. sanguinis have the same type IV pili as Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans). Saliva binding protein (SsaB) on S. sanguinis is a FimA homolog, FimA is a surface element of Porphyromonas gingivalis (P. gingivalis). F. nucleatum and P. gingivalis have the same outer membran protein (OMP) molecular mass. Purpose: The study aimed to determine the activity of A. actinomycetemcomitans and P. gingivalis polyclonal IgY in serum and egg yolk that can inhibit colonization of F. nucleatum and S.

sanguinis. Methods: IgY samples were diluted with (phosphate buffer saline) PBS. Several holes were made in nutrient medium, add 10µl antigen (F. nucleatum/S. sanguinis) into the center hole, then add 10 µl PBS, 1:1, 1:2, 1:4, 1:8, 1:16 A. actinomycetemcomitans or P. gingivalis polyclonal IgY into the surrounding holes. Incubation at 37? C, results are observed after 24-48 hours. We used Kruskal Wallis dan Man Whitney test to analysed that data. Results: A. actinomycetemcomitans and P. gingivalis polyclonal IgY groups in serum against F. nucleatum and S. sanguinis showed precipitation line at 1:1 and 1:2 dilutions, whereas in egg yolk at 1:1 dilutions. There was no significant differences in each dilution ($p>0.05$) and there was no differences between serum and egg yolk ($p>0.05$). Conclusion: IgY polyclonal of A. actinomycetemcomitans and P. gingivalis in serum also in egg yolk have activities that can inhibit colonization of F. nucleatum and S. sanguinis. Keywords: Aggregatibacter actinomycetemcomitans; Fusobacterium nucleatum; IgY; Porphyromonas gingivalis; Streptococcus sanguinis INTRODUCTION Gingivitis generally occurs within 10 to 21 days, after the formation of dental plaque with poor oral hygiene and untreated conditions, can progress to periodontitis.1 Periodontitis is the result of ecological imbalances of microbial communities in dental biofilms that support the growth of Streptococcus pathogenic bacteria.2 Streptococcus sanguinis (S. sanguinis) is a pioneer bacterium on the surface of the tooth, this bacterium plays an important role in the process of plaque maturation because of its ability to aggregate with other bacteria and cause periodontal disease.3 The initiation and development of periodontitis is caused by Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Porphyromonas gingivalis (P. gingivalis), Tannerella forsythia (T. forsythia), Treponema denticola (T. denticola), Prevotella intermedia (P. intermedia) and Fusobacterium nucleatum (F. nucleatum) bacterium.4 Inhibition of plaque matrix formation and inhibition of initiation of bacterial aggregation can prevent colonization the beginning of the bacteria so that the final colonization is also not formed.5 F. nucleatum and S. sanguinis bacteria have a role in the formation of dental plaque which is can caused of periodontitis. Over the past two decades, dentists have used antibiotic therapy for periodontal disease treatment.6 In addition, mouthwash can prevent plaque formation. Chlorhexidine is the main component of mouthwash.7 To reduced antibiotic resistance and side effects of chlorhexidine gluconate mouthwash such as taste disorders, oral irritation, local allergy symptoms, etc., the new therapeutic approach is needed, and one possible is by exploring passive oral immunotherapy using IgY as a control method in inhibiting dental plaque.7,8 Immunoglobulin Y (IgY) is one of the antibodies classes that contained in the blood serum and yolk of amphibian, reptile and poultry groups.9 IgY is a polyclonal antibody that has been shown to be an efficient for prevention and treatment of several diseases. 10 Low cost of using IgY and it can be produced through an easy production process that makes it an attractive antibody for research and diagnosis.11 IgY can inhibit the attachment of bacteria to host cells.12 IgY antibacterium binds to certain components on the bacteria target surface such as outer membrane protein (OMP), lipopolysaccharide (LPS), flagella and fimbriae. 13 It has been reported that A. actinomycetemcomitans has type IV pili.14 Type IV pili are also expressed by other Gram- positive pathogens such as Clostridium perfringens (C. perfringens) and S. sanguinis.15 Phenotyping screening also shows type IV pili present on cell surface of F. nucleatum.16 P. gingivalis pili contains FimA.17 S. sanguinis bacteria contain SsaB on their surface, which is a FimA homolog. The homology is related to bacterial adhesion molecule, allowing have the same role to adhesion activity in host.18 P. gingivalis and F. nucleatum have an outer membrane protein with a 40-kDa molecular mass.19 Based on this background, the aims of this study is to determine if the IgY anti A. actinomycetemcomitans and P. gingivalis could be used to inhibit colonization of F. nucleatum and S. sanguinis bacteria. MATERIALS AND METHODS This type of research is an experimental laboratory. The samples of this study were polyclonal IgY anti A. actinomycetemcomitans and polyclonal IgY anti P. gingivalis in serum and egg yolk. The number of replications is determined by the Federer formula and the number of replications, more than 3 was obtained. IgY produced from laying hens that have been injected with A. actinomycetemcomitans serotype b strain Y4 ATCC 4371 and P. gingivalis ATCC 3327 until four times (booster) and then analyzed that serum and egg yolk with Elisa. Polyclonal IgY anti A. actinomycetemcomitans and P. gingivalis used is IgY in serum and egg yolk. 11 The research method has gone through the ethical clearence test procedure carried out

in Faculty of Dental Medicine, Universitas Airlangga with the ethical clearance serificate number: 294/HRECC.FODM/XI/2018. Serum is diluted to 1:16 dilution with an phosphate buffer saline (PBS). Each vial/bottle contains 20 µl of assay buffer. The first vial is mixed with 20µl serum samples. Dilution of the sample is 1:1. After that, 1:1 sample dilution was transferred as much as 20 µl into the second vial. Dilution in the vial is 1:2. Dilution of 1:2 samples was transferred as much as 20 µl into the third vial. Dilution in the vial is 1:4. 1:4 sample dilutions were transferred as much as 20 µl into the fourth vial. Dilution in the vial is 1:8. 1:8 sample dilutions were transferred as much as 20 µl into the fifth vial. Dilution in the vial is 1:16. The procedures was repeated for IgY samples in egg yolk.20 The nutrient medium solution to be cooled to 50-60℃ and put into 15ml petri dishes on a horizontal surface. Medium is left alone for 30 minutes. To be punched with gel punch in the marked section. PBS and serum sample dilutions 1:1, 1:2, 1:4, 1:8, 1:16 were put in each of the holes at the outer rim of the medium, each hole contains 10µl of the samples. 10µl of the antigen (F.nucleatum/S.sanguinis) are inserted into the center hole of each petri dish. Petri dishes were incubated at 37℃ for 24 hours. After incubation, the line of precipitation between the antigen hole and the IgY serum holes was observed. The perocedures was repeated for IgY samples in egg yolk.20,21 RESULTS This study shows that there were inhibition to the colonization of F. nucleatum and S. sanguinis bacteria in the A. actinomycetemcomitans polyclonal IgY group in serum and egg yolk (Figure 1). Based on these results, the IgY anti A. actinomycetemcomitan group in serum showed a precipitation line against F. nucleatum and S. sanguinis at 1:1 and 1:2 dilutions of treatment groups. Whereas, in the IgY anti A. actinomycetemcomitan group in the egg yolk showed the presence of precipitation lines against F. nucleatum and S. sanguinis at 1:1 dilution treatment. Data analysis was performed using the Kruskal Wallis test shown in Table 1 and 2, and the Man Whitney test in Table 3. Based on Table 1 and 2, it is known that all F/S, F/T, S/S and S/T groups did not show significant value because the p value equals 0.051 which means that is greater than 0.05 (p> 0.05). This indicates that concentration has no effect on the results. Based on Table 3, it shows that the value of F/S with F/T is 0.514 and S/S with S/T is 0.514. These results, according to statistical calculation, showed no difference between the polyclonal IgY group of A. actinomycetemcomitans in serum and egg yolk against F. nucleatum and S. sanguinis due to insignificant results i.e p value >0.05. This study shows that there is colonization inhibition of F. nucleatum and S. sanguinis bacteria of the IgY anti P. gingivalis group in serum and egg yolk. This inhibition can be characterized by the precipitation line shown in Figure 3. Based on these results of IgY anti P. gingivalis group in serum against F. nucleatum and S. sanguinis showed a precipitation line against F. nucleatum and S. sanguinis at 1:1 and 1:2 dilutions treatment groups. Whereas in the P. gingivalis polyclonal IgY group in egg yolk showed the presence of precipitation lines against F. nucleatum and S. sanguinis at 1:1 dilution treatment group. Data analysis was performed using the Kruskal Wallis test shown in Table 4 and 5 and the Man Whitney test in Table 6. Based on Table 4 and 5, it is known that all F/S, F/T, S/S and S/T group did not show significant values because the p value equals 0.051 which means that it greater than 0.05 (p>0.05). This indicates that concentration has no effect on the results. Based on Table 6, it shows that the values of F/S with F/T is 0.514 and S/S with S/T is 0.514. These results, according to statistical calculation, showed no difference between the polyclonal IgY group of P. gingivalis in serum and egg yolk against F. nucleatum and S.sanguinis due to insignificant results i.e p value >0.05. DISCUSSION This study used IgY anti A. actinomycetemcomitans and IgY anti P. gingivalis in serum and egg yolk to be tested against two other bacteria i.e F. nucleatum and S. sanguinis, using a double immunodiffusion method. A. actinomycetemcomitans polyclonal IgY and P. gingivalis polyclonal IgY are obtained from chickens previously immunized with specific antigens i.e A. actinomycetemcomitans or P. gingivalis after that, the serum was taken. To tested the two polyclonal IgY we used a double immunodifusion methods. The results of the data from the IgY anti A. actinomycetemcomitans group in serum against the F. nucleatum and S. sanguinis bacteria, found a formation precipitation line at 1:1 and 1:2 dilutions. Whereas, for the IgY anti A. actinomycetemcomitans group in egg yolk against F. nucleatum and S. sanguinis, there was a formation of precipitation line at 1:1 dilution. Similar results were also found in the IgY anti P. gingivalis group. The results of the IgY anti P. gingivalis group in serum against the F. nucleatum and S. sanguinis bacteria, found the

precipitation line at 1:1 and 1:2 dilutions. Whereas, for the IgY anti P. gingivalis group in egg yolk against F. nucleatum and S. sanguinis, there was a formation of precipitation line at 1:1 dilution. This shows that precipitation lines are formed at low dilutions, the concentration of antibodies in dilution is higher than other higher dilution groups, which means that the group with low levels of dilution has the appropriate concentration to be able to interact with the antigen. If the antibody concentration is too low, it will give the appearance of negative results.22 These results are the same as the previous research conducted by Sharon et al. (2016),20 the results obtained are precipitation line seen in 1:2 dilution. The precipitation lines formed between antigens and antibodies, both in the IgY anti A. actinomycetemcomitans group in serum and egg yolk against F. nucleatum and S. sanguinis, as well as IgY anti P. gingivalis group in serum and egg yolk against F. nucleatum and S. sanguinis showed some form activity between the IgY anti A. actinomycetemcomitans with F. nucleatum and S. sanguinis, as well as IgY anti P. gingivalis with F. nucleatum and S. sanguinis. The activity is in the form of binding between antibodies and antigens. This can be because IgY is a polyclonal antibody that can bind to various epitope antigens. IgY anti A. actinomycetemcomitans can bind to pili from bacteria, such as F. nucleatum and S. sanguis because all of them have type IV pili. Polyclonal IgY P. gingivalis can also bind to the homologous element found on the surface of S. sanguinis namely SsaB, and can bind to OMP F. nucleatum because it has the same molecular mass. This binding can inhibit the colonization of F. nucleatum and S. sanguinis bacteria due to the disruption of the function of the surface components of these bacteria which can be useful for their adhesion. Previous research conducted by Lee et al. (2002),23 showed that Salmonella specific IgY binds to Salmonella surface molecules so that it can inhibit homologous Salmonella growth. IgY can affect colonization of Salmonella enteriditis and Salmonella typhimurium by binding to OMP. OMP Salmonella is useful for adhesion and mucosal invasion. The binding causes disruption of OMP's biological function, so that invasive Salmonella is reduced due to the loss of the ability to colonize the digestive tract.24 Based on the results of statistical analysis there were no differences in the effect of each group of polyclonal IgY A. actinomycetemcomitans dilution, nor the polyclonal IgY P. gingivalis groups. This is consistent with the theory that this test less sensitive because the formation of precipitation lines depends on the equivalent concentration of antigen antibodies and specific antibodies.25 It can be concluded that A. actinomycetemcomitans and P. gingivalis polyclonal IgY in serum and egg yolk have activities that can inhibit colonization of F. nucleatum and S. sanguinis. Table 1. Data analysis of A. actinomycetemcomitans polyclonal IgY group against F. nucleatum. Antigen AA1 (1:1) AA2 (1:2) AA3 (1:4) AA4 (1:8) AA5 AA6 (1:16) (PBS) p value F/S + + - - - + + - - - F/T + - - - - + - - - - - - - - 0.051 0.051 Table 2. Data analysis of A. actinomycetemcomitans polyclonal IgY group against S. sanguinis. AA1 AA2 AA3 AA4 AA5 AA6 Antigen (1:1) (1:2) (1:4) (1:8) (1:16) (PBS) p value S/S + + - - - - + + - - - - 0.051 S/T + - - - - - + - - - - - 0.051 Note: +: there is a precipitation line; -: there is no precipitation line; *: significant (p value <0.05). Table 3. Comparison of the results of A.actinomycetemcomitans polyclonal IgY group in the serum and egg yolk against F.nucleatum and S.sanguinis. Antigen p value F/S F/T 0.514 S/S S/T 0.514 Note: *significant (p value <0.05) Table 4. Data analysis of P. gingivalis polyclonal IgY against F. nucleatum. Antigen PG1 PG2 PG3 PG4 PG5 PG6 (1:1) (1:2) (1:4) (1:8) (1:16) (PBS) p value F/S + + F/T + + + + - - - - - - - - - - - - - - - - - - 0.051 0.051 Table 5. Data analysis of P. gingivalis polyclonal IgY against S. sanguinis Antigen PG1 PG2 PG3 PG4 PG5 PG6 (1:1) (1:2) (1:4) (1:8) (1:16) (PBS) p value S/S + + S/T + + + + - - - - - - - - - - - - - - - - - - 0.051 0.051 Note: +: there is a precipitation line; -: there is no precipitation line; *: significant (p value <0.05) Table 6. Comparison of the result of IgY P.gingivalis group in the serum and egg yolk against F.nucleatum and S.sanguinis Antigen p value F/S F/T 0.514 S/S S/T 0.514 Note: *: signifikan (p value <0.05) Figure 1. The precipitation line of A. actinomycetemcomitans polyclonal IgY groups in serum and egg yolk against F. nucleatum and S. sanguinis. (S/S: S. sanguinis/IgY in Serum; S/T: S. sanguinis/IgY in egg yolk; F/S: F. nucletum/IgY in serum; F/T : F.nucleatum/IgY in egg yolk). Figure 2. Result diagrams of the A. actinomycetemcomitans polyclonal IgY group against bacteria: a. F. nucleatum; b. S. sanguinis; a. Serum preparation; b. Egg yolk preparation. Figure 3. The precipitation line of IgY anti P. gingivalis group in the serum and egg yolk against F. nucleatum and S. sanguinis. (S/S: S. sanguinis / IgY in serum; S/T : S.sanguinis/IgY in egg yolk; F/S: F. nucletum / IgY

in serum : F.nucleatum/IgY in egg yolk). Figure 4. Result diagrams of IgY anti P. gingivalis group against F. nucleatum and S. sanguinis; a) serum preparation; b) Egg yolks preparation.