Volume 15, Issue 2, 152415 (1-9)

International Journal of Industrial Chemistry (IJIC)

https://dx.doi.org/10.57647/j.ijic.2024.1502.15

Pre industrialization assessment of polyclonal IgY antibodies to whole-cell *S. mutans*

Samaneh Sadat Rasi Varaei[1], Aliasghar Rahimian[1], Hooman Askari[1], Abbas Bahador[2,3], Maryam Pourhajibagher[4*], Mahdi Aminian[1‡]

[1]*Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.*
[2]*Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.*
[3]*Fellowship in Clinical Laboratory Sciences, BioHealth Lab, Tehran, Iran.*
[4]*Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran.*

***Corresponding author:** *m-pourhajibagher@sina.tums.ac.ir; ‡ amminian@tums.ac.ir

Original Research

Received:
5 June 2024
Revised:
21 June 2024
Accepted:
26 June 2024
Published online:
30 June 2024

Abstract:

Dental caries is a multifactorial disease with high prevalence around the world. Various strategies have been developed and proved beneficial in managing the disease; however, novel techniques have also been investigated. One of such strategies involves the administration of polyclonal antibodies (passive immunization), usually in the form of an oral mouthwash. While many studies have taken place over the years, to our knowledge, the effectiveness of these antibodies in prevention of the biofilm formed by different *S. mutans* isolates of the same serotype has not been investigated. Nine clinical isolates of *S. mutans* were obtained, all serotype c. Gtf-B was purified and together with one of the clinical isolate (SM4), were used as antigen for hen immunization. IgY polyclonal antibodies were purified, and applied to wells containing a distinct *S. mutans* isolate. Bacteria growth and biofilm formation was investigated by measuring absorbance at 600 nm and performing a biofilm assay based on crystal violet. Polyclonal antibodies to Gtf-B reduced bacterial growth between 9 and 35% compared to controls in four clinical isolates ($P<0.05$). As for biofilm formation, the polyclonal antibodies to Gtf-B inhibited biofilms by 17-54%, which was significant for all clinical isolates ($p<0.05$). On the other hand, polyclonal antibodies to whole-cell *S. mutans* reduced bacterial growth from 43 to 61%, which was meaningful for all isolates as well as the control strain (SM25175). As for biofilm formation, the anti-whole-cell antibodies reduced biofilm formation by 6-51%, which was significant for only four strains.

Keywords: IgY polyclonal antibodies; Dental caries; *Streptococcus mutans*

1. Introduction

Dental caries is a multifactorial disease characterized by the destruction of tooth hard tissue through biofilm formation, mainly by *Streptococcus mutans* [1–4]. The acid produced from the fermentation of sugars by the bacteria is thought to be primarily responsible for demineralizing tooth hard tissue [5]. While the disease has been considered both infectious and non-communicable by authorities, there is a consensus that its progression highly depends on the factors that affect biofilm formation or influence the growth and metabolism of *S. mutans* [3, 6]. These factors include dietary intake of sugars, level of salivary flow, oral hygiene, and use of fluoride and antibacterial agents [6–8]. Although some of the traditional strategies for managing dental caries have proved to be effective, due to the high prevalence of the disease, many novel techniques have also been investigated over the years [9]. Active and passive immunization against *S. mutans* has been investigated as such techniques for decades [7, 10]. Many whole-cell vaccines have been developed to provide active immunity against *S. mutans*; however, the cross-reactivity of generated antibodies with heart and skeletal muscle tissue has limited their applicability. As a result, researchers have tried to identify and target specific antigens that provide both effective and safe

immunity against human dental caries [11]. Since biofilm formation is essential for disease pathogenesis, enzymes involved in its generation have been investigated as potential targets in numerous studies [12, 13].

Glucosyltransferases (GTFs) are critical enzymes for producing the extracellular polysaccharides of biofilm and the colonization of bacteria. These enzymes employ the glucosyl residue from sucrose to synthesize glucose polymers, known as glucans and release the fructose moiety [14]. The polymers are either soluble in water with a high number of α(1–6) glycosidic linkages (dextrans) or have a low solubility and a high number of α(1–3)-linkages (mutans) [15]. There are at least three glucosyltransferases in *S. mutans*, all of which are encoded by separate genes and synthesis structurally different glucans: GTFB (previously known as GTF-I) synthesizes insoluble glucans with a high number of α(1–3)-linkages (mutan). GTFC (also known as GTF-SI) produces both soluble and insoluble polymers. GTFD (also known as GTF-S) synthesizes water-soluble glucans with a lot of α(1–6)-linkages (dextran) [12, 15, 16]. The function of each of these enzymes seems to be crucial for the formation of biofilm [17]. GTFC has the highest affinity for the enamel and seemingly produces a glucan surface to provide a binding site for other organisms [16, 17]. GTFB, on the other hand, is readily adsorbed into the surfaces of *S. mutans* bacteria, is responsible for forming cell clusters, and promotes the formation of microcolonies with the assistance of GTFC [16]. GTFB is mainly referred to as cell-associated in older studies [18]. GTFD produces dextran polymers that can serve as both a source of energy for the biofilm and a primer for GTFB enzyme [16]. The enzyme was called cell-free GTF due to its spontaneous release into culture supernatant in past studies [18].

Workers have investigated the effectiveness of passive immunization using a wide range of antibodies in preventing dental caries [12]. From both economic and moral perspectives, egg yolk antibodies present several advantages over other traditional sources of polyclonal antibodies. These include the reduced cost of animal husbandry, a large amount of antibody obtained from hens (17-35 g total antibody per chicken each year), and the far less amount of stress and pain caused to animals for the production of antibodies [19, 20]. A few studies have investigated the effectiveness of IgY antibodies against dental caries [21–27]. Serotype c seems to be the most prevalent serotype among them all [28, 29]. While the antibodies generated against one serotype might have different affinities towards the other isolates of the same serotype, to our knowledge, no one has investigated the matter. Here, we examined the efficacy of the generated antibodies against a single serotype c isolates in preventing biofilm formation by various isolates of serotype c from different patients with dental caries. IgY polyclonal antibodies were generated against both GTFB and whole-cell of an *S. mutans* isolate from a patient with dental caries. Subsequently, the efficacy of antibodies in preventing bacterial growth and biofilm formation by other serotypes c *S. mutans* isolates, as well as a standard strain, ATCC25175, was investigated *in vitro*.

2. Materials and methods

Bacterial strains and isolation of S. mutans from patients

An *S. mutans* standard strain (ATCC25175, serotype c) was obtained. Plaque samples were collected from nine healthy volunteers (without any chronic conditions) at Tehran University of Medical Sciences, department of microbiology with the approval of ethical committee (Ref no.). Formal consent was taken from each participant and they were asked specifically not to brush their teeth 24 hours before collecting the sample. Isolation of bacteria was done by applying a swap on dental plaques, and then dipping it into 1 mL of sterile PBS. Afterwards, the BHI agar plates were streaked with isolated bacteria and incubation was done at 37 °C under anaerobic conditions for two days. Identity of colonies was investigated by performing polymerase chain reaction using exclusive primers targeting a preserved sequence on 16 rRNA genes of *S. mutans* (GenBank ref). Positive colonies (in PCR testing) were cultured in Todd Hewitt Broth media (THB) at 37 °C under anaerobic condition and a number of stocks were made.

Preparation of antigens

The cell-associated Gtf (GtfB) from *S. mutans* and inactivated *S. mutans* bacteria (whole-cell) were used for immunization of hens. A clinical isolate of *S. mutans* (SM4), was used as the antigen source. The isolate was cultured in Todd Hewitt Broth media (THB) at 37 °C under anaerobic condition and was grown to an optical density (OD) of 0.6 at 600 nm. In order to neutralize the bacteria, formaldehyde was added to a final concentration of 0.5% v/v and incubated for 24 hours at 4 °C. The bacteria was centrifuged, and washed three times using 0.9% w/v NaCl solution and then resuspended in the same solution again to reach an OD of 0.6 at 600 nm. Bacterial suspension was streaked on a BHI agar plate to ensure complete inactivation.

Extraction of Cell-associated GtfB

The GtfB extract was prepared as described before by Hamada et al. with slight modifications [30]. Briefly, *S. mutans* was grown to a density of 1×10^9 CFU/mL and the bacteria pellet was isolated by centrifugation. The pellet was then washed with 10 mM phosphate buffer (pH 6.0) through centrifugation and resuspension. The cell pellet was finally suspended in 0.5% formaldehyde and incubated at 4°C for 24 hours to neutralize the infectivity of the bacteria. Afterwards, the pellet was washed again 3 times with PBS to remove residual formaldehyde and then was resuspended in PBS at 1×10^9 CFU/mL cell density. The inactivated *S. mutans* pellet was dissolved in 8 M urea and was incubated at RT for 4 h. Afterwards, Centrifugation was done at 16,000 g for 20 minutes. The resulting supernatant was considered as crude extract for purification of GtfB enzyme.

Purification of GtfB

DEAE-Sepharose anion exchange chromatography was carried out for purification of GtfB enzyme from crude extract. Bond proteins were eluted using phosphate buffer containing different NaCl concentration (0-1.0 M). Fractions were collected and analyzed through Gtf enzyme assay as de-

scribed in the following section. The fraction exhibiting maximum enzyme activity was considered as the optimum elution condition. A rough estimation on GtfB amount of the preparation was made through analytical 10% SDS-Poly acrylamide gel electrophoresis (SDS-PAGE) and consequent densitometry of 130 kDa GtfB against BSA standard concentrations. To obtain purer GtfB enzyme for injection, the GtfB preparation was further purified through a preparative SDS-PAGE. Antigen preparation was mixed with non-reducing loading buffer and was loaded on a single 10% SDS-PAGE mega-well at a concentration of 2-3 mg total protein per milliliter of sample (DEAE-anion exchange chromatography elution). Electrophoresis was carried out using a vertical electrophoresis system (ENDURU, Labnet). Zinc-imidazole negative staining method was used for detection of the protein band of interest (130 KDa) on SDS-PAGE gel. After it was cut from the gel, the 130 kDa GtfB protein was extracted from gel slice through passive elution. Briefly, the gel slice was soaked in elution buffer containing 0.1% SDS and was crashed by a Teflon pestle. The crashed gel fragments were incubated for 24 hours in elution buffer on a rotator. Gel fragments were then separated through centrifugation and the supernatant was subjected to acetone precipitation for SDS removal and to concentrate the sample. The purity of this final sample was evaluated through SDS-PAGE analysis. The total protein content of antigen preparations was estimated through Bradford protein assay.

Gtf activity assay

The activity of GtfB enzyme in antigen preparation was investigated. Different dilutions of antigen solution were prepared in phosphate buffer (0.1 M with pH=6) and sodium acetate (0.1 M with pH 6.2) containing 5% sucrose (3 mL total volume). The resulting mixtures alongside with a negative control (without antigen solution) were incubated at 37 °C for 4 hours. Each mixture was added with 1.5 mL of absolute ethanol and further was incubated at 4 °C overnight. The mixtures were centrifuged at 20,000 g for 30 minutes and the resulting water-insoluble glucan was quantified through phenol sulfuric acid method. Briefly, precipitates were washed 3 times with 60% ethanol. After addition of 50 µL of potassium phosphate buffer, 25 µL of 80% phenol and 2.5 mL of concentrated sulfuric acid, the mixtures were incubated at 25 °C in a water bath until development of orange color. The absorbance of the samples was read at 490 nm. The experiment was performed in duplicate.

Immunization of chickens

Six white Leghorn chickens were included in the present study. Chickens were either immunized with 500 µL inactivated bacterial whole-cell (2.7×10^9 CFU/mL) combined with 500 µL of complete Freund adjuvant or 500 µL of GtfB combined with 500 µL PBS and 1 mL complete Freund adjuvant in groups of two. Also, two chickens were considered as the control group receiving the immunization adjuvants alone (1 mL complete Freund adjuvant combined with 500 µL PBS). The injections were delivered to the right and left pectoral muscles. To increase the antibody titer, animals received booster shots containing Freund in-complete adjuvant on day 14 and 25.

Antibody titer assay using ELISA

The antibody activity of collected egg yolk and purified IgY against *S. mutans* (serotype c) and GtfB was determined using an indirect ELISA. Microplate wells (96-well Nun. Polysorb) were coated with 100 µL of either *S. mutans* (OD_{600}= 1.0) or GtfB (1 µg/well) preparations in carbonate buffer. For analysis of non-immune IgY samples, both *S. mutans* and GtfB preparations were coated as described. 5% skimmed milk in PBS containing 0.1% tween 20 (PBS-T) was used as the blocking agent. Wells were blocked for 2 h in RT and were washed 3 times with PBS-T. IgY preparations were diluted in PBS-T and were added to the wells in triplicate as the primary antibody. After 2 h incubation at 37 °C, wells were washed 3 times with PBS-T. Rabbit anti-IgY HRP conjugate was diluted 1:16000 in 2% skimmed milk and was added to the wells as the detecting reagent. Plate was incubated for 1h at 37 °C and then was washed 3 times with PBS-T. Wells were incubated with 100 µL of HRP substrate (TMB) in presence of H_2O_2 for 10 min and the reaction was stopped by addition of 100 µL of 1 M HCl. Using an ELISA plate reader, the absorbance of the wells in 450 nm was measured.

IgY purification

Eggs were collected daily and were labeled with an identification number. Egg yolks were separated from the white and were washed with water to remove albumin. Approximately 10-15 mL yolk was obtained from an average-sized egg. For purification of IgY fraction, the yolks were diluted 1:7 in distilled water and after adjusting the pH to 0.5, the samples were kept frozen at -20 °C for 24 hours. The next day, frozen samples were thawed at room temperature and centrifuged at 12,000 g for 15 minutes. The resulting supernatants were added with 8.8% NaCl and the pH of the samples was adjusted to 4. After a 2 h incubation at RT the samples were centrifuged at 5000 rpm for 10 min. The precipitates were dissolved in 15 mL of PBS and were stored at -20 °C.

ELISA mapping of purified IgY against GtfB and *S. mutans*

To evaluate the binding specificity after the isolation of IgY, indirect ELISA was performed to detect the specific interaction between the IgY and target antigens. Purified GtfB was coated in 1, 0.5, 0.25, 0.12 and 0.06 µg/well and *S. mutans* was coated at OD600 of 1 1, 0.5, 0.25, 0.12. control wells were coated with Bovine serum albumin (BSA) ELISA was performed as described before with different antigen amounts. Purified IgY were diluted 1:10 in blocking buffer (2% skim milk in PBS-T) and were added to coated and control wells. The signal ratio for antigen coated and control well was then evaluated for each antigen concentration.

Inhibitory effect of anti-GtfB IgY against GtfB enzymatic activity

The enzymatic activity of GtfB in presence of different dilutions of anti-GtfB IgY was evaluated as described before. The purified Enzyme was diluted 1:10 in reaction buffer and

was further added with different dilutions of IgY starting from 1:2 to 1:100.

Effect of IgY preparations on biofilm formation and growth of *S. mutans*:

Formation of bacterial biofilm from 16 clinical isolates and a standard isolate of *S. mutans* (serotype c) was tested in presence of obtained anti-*S. mutans* and anti-GtfB IgY preparations or lysozyme and non-immune egg-yolk extract. In a sterile 96-well plate, 100 µL of each bacterial isolate suspension with OD_{600} = 0.025 was added with 100 µL of IgY preparations (anti-GtfB or Anti-whole-cell *S. mutans*) or negative IgY control solution obtained from egg yolk or PBS. The plate was incubated for 30h at 37 °C in a candle jar.

In order to assess the inhibition of bacterial growth, the absorbance of wells was measured at 600 nm. To assess the inhibition of biofilm formation, the culture media was discarded and then the plate was washed 3 times with PBS. The plate was then dried by 10 minutes of incubation at 65 °C. In order to fix the biofilm, 100 µL of 96% ethanol was added to each well and the plate was incubated for 15 minutes at RT. The plate was then washed once with PBS and placed at RT for 15 minutes. 200 µL of 0.5% crystal violet was added to each well and the plate was incubated for 20 minutes at RT. Subsequently, the crystal violet content was removed and the plate was rinsed 3 times with PBS. Finally, 150 µL of 33% acetic acid was added into the wells and absorbance was immediately read at 570 nm using an Eon microplate reader device (BioTek, U.S.).

3. Results

Characteristics of antigens:

Activity of GtfB extraction was evaluated through a colorimetric assay. The obtained optical densities showed correlation with the dilution of enzyme being tested Fig. 1A, the protein content of chromatography elution was 1.1 mg/mL as analyzed by Bradford protein assay. Fig. 2 shows the electrophoresis analysis GtfB purification.

Immunization of chickens

Immunization was performed on days zero, 14 and 25. Fig. 3 shows the patterns of produced antibody levels against each of the injected antigens. The immune response of the both immunized groups showed a significant difference compared to the control groups. As shown in the figure, after the third injection, the antibody level increased signifi-

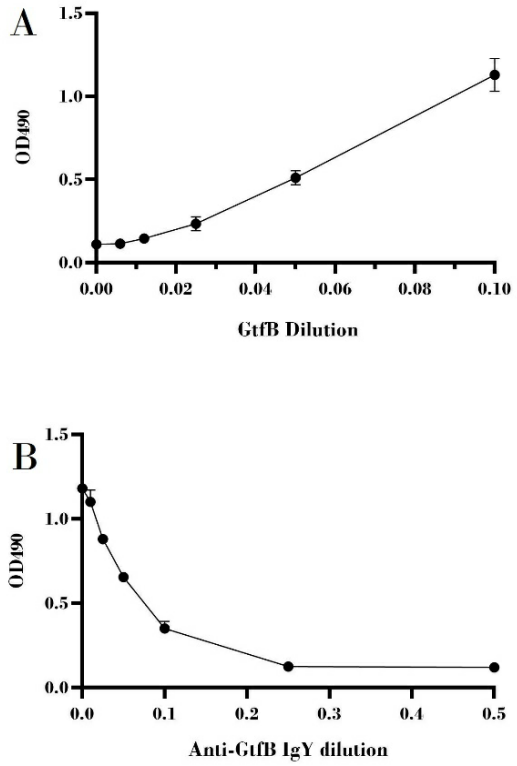

Figure 1. Enzymatic activity of purified GtfB enzyme. In colorimetric enzyme assay, increase in amount of purified GtfB fraction, resulted in proportional signal enhancement (A). in presence of anti-GtfB IgY, signal was decreased proportionally with increased antibody concentration (B).

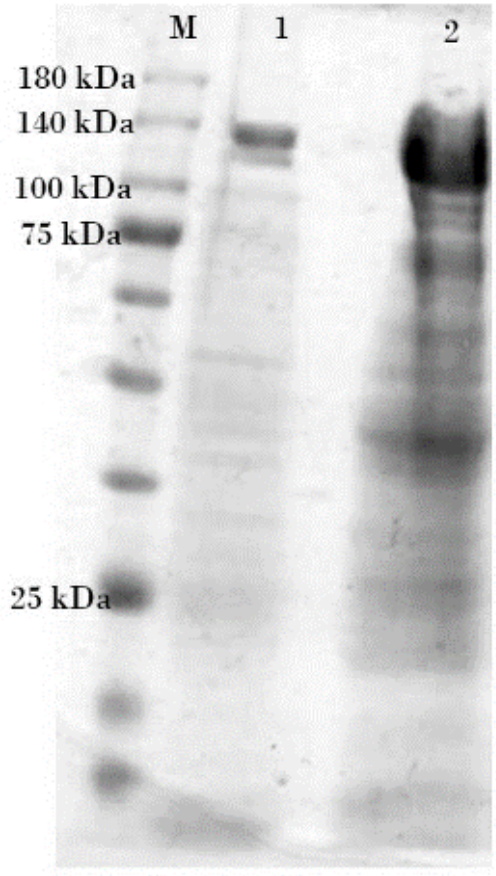

Figure 2. SDS-PAGE analysis of GtfB purification. Crude urea extraction (lane 2) and chromatography-purified Gtf (130 kDa) (lane 1). Molecular weight marker SMO bio pm2500 (M).

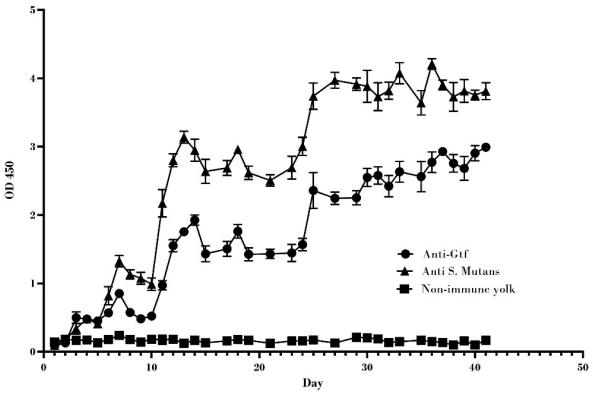

Figure 3. Relative antibody rise against injected antigens in egg yolk of study animals. Immune yolks show an antibody rising pattern against injected antigens which is significantly different from non-immune yolks. Relative active IgY amount is shown as ELISA signal (OD_{450}). For OD values more than 2, the reaction mix was diluted and the resulting OD was then normalized to dilution factor.

cantly and remained high until the end of the period.

Characteristics of purified IgY

Purity of IgY preparations was evaluated thorough SDS-PAGE. As seen in Fig. 4 antibody preparations showed acceptable purity. As analyzed by densitometry, the purity of IgY preparations were more than 80%. Elisa mapping signal showed proportional increase with coated antigen amount. Anti-*S. mutans* IgY showed up to 10-fold more signal against *S. mutans* coated on plate surface compared to control BSA-coated well. This ratio was more than 7-fold for anti-GtfB IgY (Fig. 5). Purified GtfB activity was suppressed in presence of anti-GtfB IgY with a concentration-dependent manner. 1:4 dilution of the IgY completely suppressed the enzymatic activity of the GtfB (Fig. 1B).

Effect of IgY preparations on growth and biofilm formation of *S. mutans* isolates

We studied the inhibitory effect of two types of IgY (Anti-GtfB and Anti-*S. mutans*) on the growth and biofilm formation of a reference isolate (SM25175) and different clinical isolates Anti-GtfB was able to reduce bacterial growth by 9-35% in different isolates and standard strain which was statistically significant ($p<0.05$) for SM3, SM5, SM6 and SM9 isolates (Fig. 6). Anti-GtfB also reduced the bacterial biofilm formation by 17-54% in different isolates and the reference strain. This inhibition in biofilm formation was significant for all isolates except for the standard strain (Fig. 7). According to our results exposure to Anti-SM4 IgY resulted in inhibition of bacterial growth by 43-61% in various isolates, which was statistically significant for all isolates. AntiSM4 was also able to reduce the formation of bacterial biofilms by 6-51% in various isolates. This decrease in biofilm formation was statistically significant for SM3, SM4, SM5 and SM6 isolates (Fig. 8). Overall, the anti-GtfB preparation against SM4 showed a significant cross-reactive inhibitory effect on biofilm formation

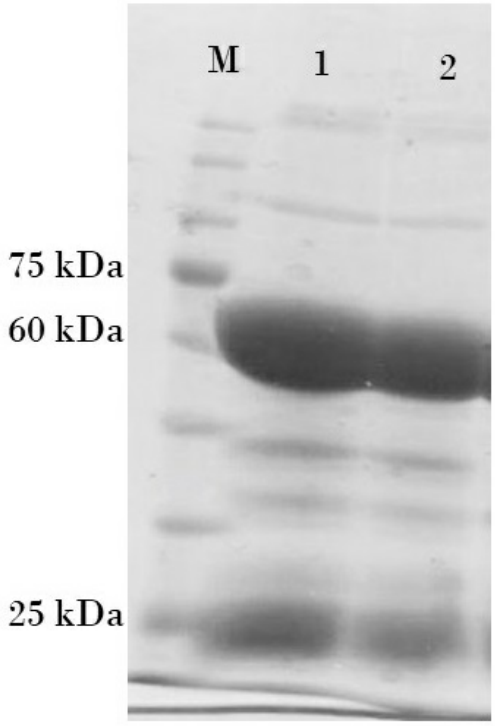

Figure 4. Reducing SDS-PAGE analysis of IgY preparations. The IgY molecules consist of two Heavy and two light chains. The molecular weight of heavy chains is 65-70 kDa and the light chains are 25 kDa. In reducing condition, the SDS-PAGE analysis of purified IgY shows two major bands in mentioned molecular weights. Lane 1) Purified Anti-GtfB IgY. Lane 2) Purified Anti-*S. mutans* IgY. M) Molecular weight marker SMOBIO PM2500.

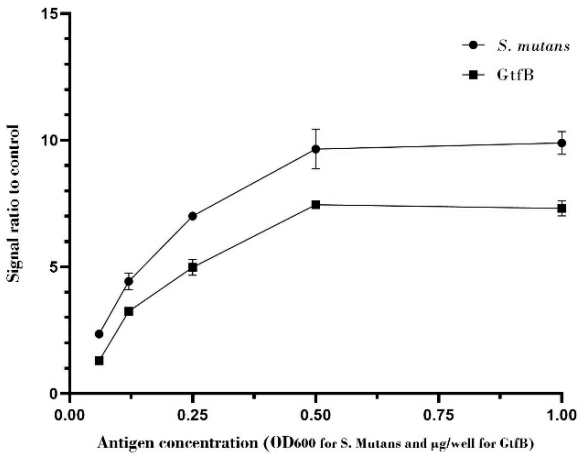

Figure 5. ELISA mapping of purified anti-GtfB and anti-*S. mutans* IgY. The Elisa signal of IgY against antigen-coated wells in comparison to BSA-coated control wells is presented. The ELISA showed proportional signal correlative to the coated antigen amount.

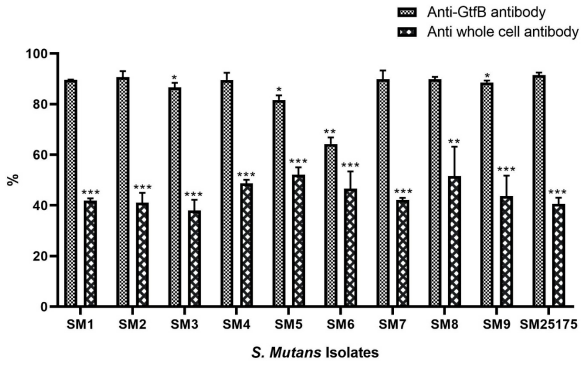

Figure 6. Effect of anti GtfB and anti *S. mutans* IgY preparations on growth of different clinical isolates and a reference strain of *S. mutans*.The anti-GtfB IgY does not show significant growth inhibition activity against most of the clinical isolates. Contrarily, the anti-GtfB IgY preparation against whole cell SM4 is able to significantly affect the growth capability of the reference strain and all clinical isolates. For each isolate, values are shown in percentage compared to the same untreated isolate. *) p-value<0.05. **) p-value<0.01. ***) p-value<0.001.

against different clinical isolates as well as the reference strain. Inhibition of biofilm formation occurred significantly more effective in presence of anti-GtfB IgY rather than IgY preparation against whole cell *S. mutans*. Bovine serum albumen (BSA) and Non-immune IgY treatments did not show any significant effect neither on growth nor on biofilm formation of reference strain SM25175. Though, Lysozyme treatment showed a significant inhibitory effect on growth and biofilm formation of reference strain.

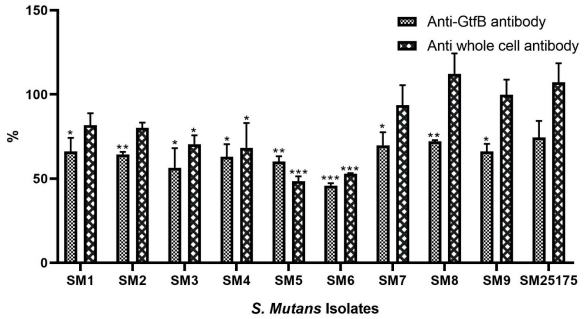

Figure 7. Effect of anti GtfB and anti *S. mutans* IgY preparations on biofilm formation of different clinical isolates and a reference strain of *S. mutans*. The anti-GtfB IgY treatment significantly decreased biofilm formation in all clinical isolates but reference strain. Treatment with the anti-*S. mutans* IgY preparation significantly affected biofilm formation in some of clinical isolates (SM3, SM4, SM5 and SM6). For each isolate, percentages are shown compared to the same untreated isolate. *) p-value<0.05. **) p-value<0.01. ***) p-value<0.001.

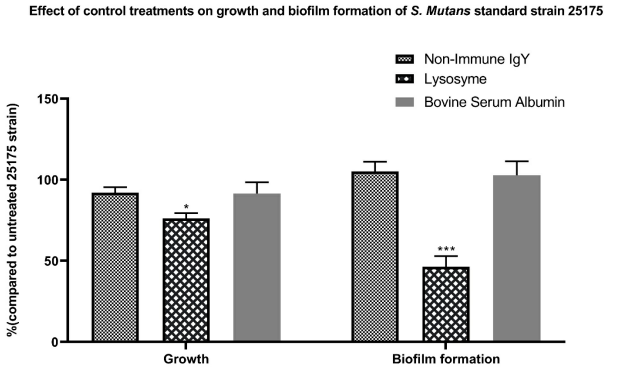

Figure 8. Effect of control treatments on biofilm formation and growth of *S. mutans* reference strain 25175. Exposure to non-immune IgY preparation or bovine serum albumin does not result in significant decrease in growth or formation of biofilm in reference strain 25175. While lysozyme treatment is able to affect both growth and biofilm formation. Percent are shown compared to the untreated reference strain 25175. *) p-value<0.05. ***) p-value<0.001.

4. Discussion:

Passive immunization has long been suggested as a method for the prevention of dental caries. Oral streptococci species, especially *S. mutans*, are thought to play an essential role in the etiology of the disease [30, 31]. GTF enzymes are critical for the formation of biofilm and colonization of bacteria and have long been considered potential targets in therapeutic approaches for managing dental caries [10]. Here, IgY polyclonal antibodies were generated against whole-cell *S. mutans* serotype c and GtfB enzyme, and their ability to inhibit bacterial growth and biofilm formation was investigated in vitro. It was found that polyclonal antibodies generated against GtfB were able to significantly reduce bacterial growth only in four isolates (P<0.05), namely SM3, SM5, SM6, and SM9. The extent of inhibition ranged between 9 and 35% compared to controls in Todd Hewitt Broth media (THB) without further addition of sucrose. Anti-GtfB was far more effective in preventing the formation of biofilm, We observed that treatment with IgY polyclonal antibody generated against GtfB reduced the formation of biofilm by 17-54%, which was significant for all of the clinical isolates (p<0.05). IgY polyclonal antibody generated against whole-cell *S. mutans*, on the other hand, was able to significantly reduce bacterial growth in all isolates as well as the control strain (SM25175). The extent of growth inhibition much higher as compared to anti-GtfB and varied between 43-61%. As for biofilm formation, anti whole-cell *S. mutans* was able to reduce its formation by 6-51% in different isolates. The effect of anti-whole cell was meaningful in four isolates-that is, SM3, SM4, SM5 and SM6. The effectiveness of anti-GtfB polyclonal antibody in prevention of biofilm formation can be attributed to its ability to inhibit the activity of GtfB enzyme. As it is shown in Fig. 1, the treatment with IgY polyclonal antibody against GtfB reduced the activity by half at a titer of 1:10, which might explain the effectiveness of anti-GtfB against biofilm

formation.

Oral passive immunization has long been investigated as a strategy for preventing dental carries. Otake et al. carried out one of the first studies on the effectiveness of IgY antibodies against *S. mutans* serotypes C in pathogen-free rats supplemented with a cariogenic diet containing egg yolk antibodies. The study found that diets containing at least 2% immune yolk powder or 0.5% partially-purified IgY (water-soluble fraction) significantly hindered the development of dental caries [21]. We observed that the antibodies generated against GtfB are far more effective in preventing biofilm formation than the antibody generated against whole-cell *S. mutans*. The efficacy of these two strategies (generating antibody against GtfB or whole-cell bacteria) in passive immunization has been investigated in vivo before. Hamada et al. generated IgY antibodies against both whole *S. mutans* (serotype c) and purified GtfB (cell-associated GTF) and GTFD (cell-free GTF) and investigated the efficacy of antibodies both in pathogen-free rats and in vitro. They observed that either of these two antibodies can inhibit the adherence of bacteria to a glass surface and that polyclonal antibodies generated against GTFs inhibited enzymatic activity. In addition, antibodies generated against GtfB were reported to be effective in preventing the development of dental caries in pathogen-free rats; however, antibodies against GTFD and whole-cell *S. mutans* failed to provide such protection [22]. Here, we showed that the antibodies generated against GtfB are more effective in preventing the formation of biofilm. Thus, a rational cause for the higher potency of anti-GtfB polyclonal antibody in prevention of dental caries might reside in its relative effectiveness in prevention of biofilm.

In another study, passive immunization with IgY antibodies (against whole-cell) was investigated in a study on 11 human subjects (1). The study suggested that the ratio of *S. mutans* to total streptococci was significantly reduced in dental plaques of the subjects who received immune IgY mouth rinse treatment for 7 seven days. In addition, it was shown that the IgY antibodies, which were generated against serotype c, could react with all of the serotypes except for b when the serotypes were grown in BHI media containing 5% sucrose. However, a high reactivity was only observed for serotypes c, e, and f when the serotypes were grown in BHI media without sucrose. The antibody's overall affinity for all serotypes was much lower compared to that for the serotypes grown sucrose-rich media. The authors hypothesized that this was because the *S. mutans* species are covered with insoluble glycans, which are synthesized in the presence of sucrose. As the bacteria used for antigen was also grown in a sucrose-supplemented media, the antibodies must have been generated against these glycans to a greater extent than the serotypes-specific antigens located at the surface of the bacteria. This conclusion was supported by their finding that *S. mutans* serotype b, the serotype with least reactivity toward generated antibody, showed no GTase activity and, therefore, could not have produced insoluble glycans in the sucrose-rich media. In our study, the bacteria were grown in BHI media without further supplementation of sucrose, so glycans ought to have a minimal presence on the surface of bacteria. Therefore, the antibody reactivity might depend more on the antigenicity of the specific antigens located on each isolate rather than the glycan coating. In addition, the antibodies were generated against serotype c of S. mutans due to the fact that among all *S. mutans* serotypes, serotype c is the most prevalent one and has long been considered to be primarily responsible for the development of dental caries [28, 29]. However, there were also limitations associated with this study. To begin with, the number of isolates used is relatively low and they are all belong to the most prevalent serotype of *S. mutans*. In addition, many studies have suggested that other cariogenic bacteria, like Streptococcus mitis, are also responsible for development of dental caries [8, 32].

5. Conclusion

Our study aimed to investigate the effectivity of IgY polyclonal antibodies in reducing the growth and the formation of biofilm within the same serotype of *S. mutans* in vitro. We showed that antibodies generated against GftB enzyme are far more effective in prevention of biofilm formation as compared to antibodies against whole-cell *S. mutans* in vitro, as it significantly inhibited its formation in all isolates. However, anti-whole-cell showed a higher capability in reducing the bacterial growth in vitro. As a final comment, we believe that before conducting any clinical trials or in vivo studies, the effectivity of passive immunization against any microorganism partly responsibly should be investigated in vitro. For instance, antibodies generated against *Streptococcus mitis*, *Streptococcus mutans* or any other organism responsible for development of dental caries can be first shown to have an acceptable effect in vitro and then be used separately or together to formulate a mouth rinse wash and finally be assessed in a clinical trial.

Funding
The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Authors Contributions
Authors had equal role in designing and performing the experiments and preparing the paper.

Availability of Data and Materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors have no relevant financial or non-financial interests to disclose.

References

[1] L. H. He and M. V. Swain. "3.306 - Microindentation. In: Ducheyne P, editor. Comprehensive Biomaterials.". *Oxford: Elsevier*, :75–90, 2011.

[2] V. Machiulskiene, G. Campus, J. C. Carvalho, I. Dige, K. R. Ekstrand, A. Jablonski-Momeni, and et al. "Terminology of dental caries and dental caries management: consensus report of a workshop organized by ORCA and Cariology Research Group of IADR.". *Caries research*, **54**(1):7–14, 2020.

[3] M. Rathee and A. Sapra. "Dental Caries. StatPearls. Treasure Island (FL): StatPearls Publishing". *Copyright © 2021, StatPearls Publishing LLC*, , 2021.

[4] S. Garcia, M. Blackledge, S. Michalek, L. Su, T. Ptacek, P. Eipers, and et al. "Targeting of Streptococcus mutans biofilms by a novel small molecule prevents dental caries and preserves the oral microbiome.". *Journal of dental research*, **96**(7):807–14, 2017.

[5] S. D. Forssten, M. Björklund, and A. C. Ouwehand. "Streptococcus mutans, caries and simulation models". *Nutrients*, **2**(3):290–8, 2010.

[6] S. Twetman. "Prevention of dental caries as a non-communicable disease". *European journal of oral sciences*, **126**:19–25, 2018.

[7] I. D. Mandel. "Caries prevention: Current Strategies, New Directions". *The Journal of the American Dental Association*, **127**(10):1477–88, 1996.

[8] X. Chen, E. B. Daliri, N. Kim, J. R. Kim, D. Yoo, and D. H. Oh. "Microbial Etiology and Prevention of Dental Caries: Exploiting Natural Products to Inhibit Cariogenic Biofilms". *Pathogens (Basel, Switzerland)*, **9**(7), 2020.

[9] C. Sicca, E. Bobbio, N. Quartuccio, G. Nicolò, and A. Cistaro. "Prevention of dental caries: A review of effective treatments". *Journal of clinical and experimental dentistry*, **8**(5):e604, 2016.

[10] Q. Zhang, Q. Ma, Y. Wang, H. Wu, and J. Zou. "Molecular mechanisms of inhibiting glucosyltransferases for biofilm formation in Streptococcus mutans". *International Journal of Oral Science*, **13**(1): 30, 2021.

[11] G. Cherukuri, C. Veeramachaneni, G. V. Rao, V. B. Pacha, and S. B. Balla. "Insight into status of dental caries vaccination: A review". *Journal of conservative dentistry : JCD*, **23**(6):544–9, 2020.

[12] T. Koga, T. Oho, Y. Shimazaki, and Y. Nakano. "Immunization against dental caries". *Vaccine*, **20**(16): 2027–44, 2002.

[13] S. A. Rather, S. C. Sharma, and A. Mahmood. "Antibodies generated against dextransucrase exhibit potential anticariostatic properties in Streptococcus mutans". *Applied microbiology and biotechnology*, **104** (4):1761–72, 2020.

[14] J. A. Lemos, S. R. Palmer, L. Zeng, Z. T. Wen, J. K. Kajfasz, I. A. Freires, and et al. "The Biology of Streptococcus mutans". *Microbiology spectrum*, **7**(1), 2019.

[15] J. Kreth, L. Zhu, J. Merritt, W. Shi, and F. Qi. "Role of sucrose in the fitness of Streptococcus mutans". *Oral microbiology and immunology*, **23**(3):213–9, 2008.

[16] W. H. Bowen and H. Koo. "Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms". *Caries Res*, **45**(1):69–86, 2011.

[17] W. Krzyściak, A. Jurczak, D. Kościelniak, B. Bystrowska, and A. Skalniak. "The virulence of Streptococcus mutans and the ability to form biofilms". *European Journal of Clinical Microbiology & Infectious Diseases*, **33**(4):499–515, 2014.

[18] T. Fujiwara, Y. Terao, T. Hoshino, S. Kawabata, T. Ooshima, S. Sobue, and et al. "Molecular analyses of glucosyltransferase genes among strains of Streptococcus mutans". *FEMS microbiology letters*, **161**(2):331–6, 1998.

[19] E. P. V. Pereira, M. F. van Tilburg, E. Florean, and M. I. F. Guedes. "Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review". *International immunopharmacology*, **73**:293–303, 2019.

[20] R. Schade, X. Zhang, and H. Terzolo. " Use of IgY Antibodies in Human and Veterinary Medicine". *Bioactive Egg Compounds. lst ed. Springer: Berlin*, , 2007.

[21] S. Otake, Y. Nishihara, M. Makimura, H. Hatta, M. Kim, T. Yamamoto, and et al. "Protection of rats against dental caries by passive immunization with hen-egg-yolk antibody (IgY)". *J Dent Res*, **70**(3): 162–6, 1991.

[22] S. Hamada, T. Horikoshi, T. Minami, S. Kawabata, J. Hiraoka, T. Fujiwara, and et al. "Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of Streptococcus mutans". *Infection and immunity*, **59**(11):4161–7, 1991.

[23] H. Hatta, K. Tsuda, M. Ozeki, M. Kim, T. Yamamoto, S. Otake, and et al. "Passive immunization against dental plaque formation in humans: effect of a mouth rinse containing egg yolk antibodies (IgY) specific to Streptococcus mutans". *Caries Res*, **31**(4):268–74, 1997.

[24] H. M. Chang, R. F. Ou-Yang, Y. T. Chen, and C. C. Chen. "Productivity and some properties of immunoglobulin specific against Streptococcus mutans serotype c in chicken egg yolk (IgY)". *Journal of agricultural and food chemistry*, **47**(1):61–6, 1999.

[25] C. Krüger, S. K. Pearson, Y. Kodama, A. Vacca Smith, W. H. Bowen, and L. Hammarström. "The effects of egg-derived antibodies to glucosyltransferases on dental caries in rats". *Caries Res*, **38**(1):9–14, 2004.

[26] S. V. Nguyen, F. C. Icatlo, J.r, T. Nakano, E. Isogai, K. Hirose, H. Mizugai, and et al. "Anti-cell-associated glucosyltransferase immunoglobulin Y suppression of salivary mutans streptococci in healthy young adults". *Journal of the American Dental Association*, **142**(8): 943–9, 2011.

[27] E. W. Bachtiar, A. Afdhal, R. Meidyawati, R. D. Soejoedono, and E. Poerwaningsih. "Effect of topical anti-Streptococcus mutans IgY gel on quantity of S. mutans on rats' tooth surface". *Acta microbiologica et immunologica Hungarica*, **63**(2):159–69, 2016.

[28] S. Hamada, N. Masuda, and S. Kotani. " Isolation and serotyping of Streptococcus mutans from teeth and feces of children". *Journal of clinical microbiology*, **11**(4):314–8, 1980.

[29] M. Matsumoto-Nakano. "Dental Caries". *Reference Module in Biomedical Sciences: Elsevier*, , 2014.

[30] S. Hamada, T. Horikoshi, T. Minami, N. Okahashi, and T. Koga. "Purification and Characterization of Cell-associated Glucosyltransferase Synthesizing Water-insoluble Glucan from Serotype c Streptococcus mutans". *Microbiology*, **135**(2):335–44, 1989.

[31] Y. Abiko. "Passive immunization against dental caries and periodontal disease: development of recombinant and human monoclonal antibodies". *Critical Reviews in Oral Biology & Medicine*, **11**(2):140–58, 2000.

[32] J. A. Aas, A. L. Griffen, S. R. Dardis, A. M. Lee, I. Olsen, F. E. Dewhirst, and et al. "Bacteria of dental caries in primary and permanent teeth in children and young adults". *Journal of clinical microbiology*, **46** (4):1407–17, 2008.