The antibacterial effects of vitamin D3 against mutans streptococci: an in vitro study

Purpose
This study aims to evaluate the antimicrobial effects of the cholecalciferol vitamin D3 against *Streptococcus sobrinus* (*Strep. sobrinus*) and *Streptococcus mutans* (*Strep. mutans*) bacteria in vitro that is considered the main causative bacteria in dental caries development.

Materials and Methods
The antimicrobial effects of vitamin D3 were evaluated against *Strep. sobrinus* and *Strep. mutans* using the agar disc diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of vitamin D3 were determined using a microdilution method following the guidelines by the Clinical Laboratory Standards Institute (CLSI). Scanning electron microscope (SEM) was used to evaluate the morphological changes of bacterial cells following exposure to vitamin D3.

Results
*Strep. sobrinus* was more sensitive to vitamin D3 compared to *Strep. mutans* bacteria. The MIC values of vitamin D3 against *Strep. sobrinus* and *Strep. mutans* were 60 µg/mL and 250 µg/mL respectively whereas the MBC values were 120 µg/mL and 500 µg/mL, respectively. Moreover, significant changes in the bacterial morphology were observed in treated bacterial cells with vitamin D3 as compared to the untreated control bacteria using SEM.

Conclusion
These findings suggested that vitamin D3 has excellent antimicrobial effects against *Strep. sobrinus* and *Strep. mutans* and may be considered as a promising compound in the prevention of dental caries in the future. Further research is recommended to elucidate the mechanism of vitamin D3 on these bacteria.

Keywords: Vitamin D3, Cholecalciferol, Streptococcus sobrinus, Streptococcus mutans, antibacterial effect
have indicated a significant association between vitamin D deficiency and higher dental caries prevalence among children and adults (3, 6).

The potential role of vitamin D in inducing the innate immunity and improving the body’s resistance against different pathogens is well documented (7). Theoretically, the role of vitamin D in combating diseases is conceptualized by modulating the immune response of the infected host by production of antimicrobial peptides and inducing cell-specific receptors related to pathogen clearance (8). Almost all human cells have a specific vitamin D receptor (VDR), including B and T lymphocytes, macrophages, dendritic cells, and monocytes (7). Vitamin D boosts the expression of powerful antimicrobial peptides, such as cathelicidin and β defensin as well as cytokines response that exist in neutrophils, monocytes, and natural killer cells through its effects on the VDRs. Additionally, the level of vitamin D has a direct influence on macrophages, enhances oxidative burst of macrophages including maturation, production of cytokines and releases hydrogen peroxide. In addition, vitamin D assists neutrophil motility and phagocytic function (7).

Moreover, the efficacy of vitamin D against diseases is not only via modulation of the immune system but also via direct antimicrobial activities against different bacteria although little is known about the direct effects of vitamin D on bacteria such as Mycobacteria (8). The mechanism by which vitamin D inhibits Mycobacterial growth remains to be studied further. Vitamin D inhibits Helicobacter pylori growth (9) via the collapse and destabilization of the cell membrane structures and ultimately lysis of the bacterial cells (9). Vitamin D inhibits the growth of Porphyromonas gingivalis by decreasing the virulence factors of associated genes contributing in bacterial colonization, inactivation of host defence mechanisms, tissue destruction and nutrient acquisition (10). Besides that, it was indicated that vitamin D derivatives are bactericidal and possess lytic activity against Strep. mutans and target the bacitracin-associated efflux system (11).

Mutans streptococci mainly Streptococcus mutans (Strep. mutans) and Streptococcus sobrinus (Strep. sobrinus) are Gram positive and facultative anaerobic bacteria and are mainly found in the oral cavity. They are the main causative bacteria responsible for initiating dental caries (12); these bacteria can easily produce extracellular polysaccharides in large quantities from fermented carbohydrates and are strongly bound to teeth surfaces. They are able to survive in an acidic environment (13, 14). Therefore, eradicating such cariogenic bacteria would be considered a basic and essential step in preventing dental caries.

Recently, searching for novel antimicrobial agents is of great interest where overuse or misuse of antibiotics and antibacterial agents have led to antimicrobial resistance (15). Several antimicrobial agents such as chlorhexidine, triclosan and cetylpyridinium chloride are widely used as effective antibacterial agents against oral pathogens to reduce dental plaque and oral diseases including dental caries (16). However, side effects such as tooth discoloration and bacterial resistance still hinder their use (17, 18). Antibiotics is still an expensive option and misuse of them results in significant antibiotic resistance and contributes to increased health care costs (18). Using other alternative therapeutic products such as vitamin D3 which is considered an inexpensive prophylactic option could be an essential step to discover a novel antimicrobial agent since the search for novel antimicrobial agents has been of great interest in the last few decades.

To the best of our knowledge, two previous studies by Grenier et al. (10) and Saputo et al. (11) have determined the antibacterial activities of vitamin D against Strep. mutans. However, in these studies (10, 11), different study methods and different vitamin D compounds (alfacalcidol, doxercalciferol, and calcitriol) were used. The antimicrobial activity of vitamin D3 against Strep. sobrinus was very much lacking in the literature. Hence, this study may extend our knowledge about the antibacterial activity of another vitamin D compound which is cholecalciferol vitamin D3 against the two most cariogenic bacteria that causes dental caries, namely Strep. sobrinus and Strep. mutans bacteria. Therefore, we hypothesized that vitamin D3 might inhibit the growth of these bacteria which in turn may help in preventing dental caries. The objective of this study is to assess the antibacterial effects of cholecalciferol vitamin D3 against Strep. sobrinus and Strep. mutans in vitro.

Materials and Methods

Preparation of vitamin D3

100 mg of analytical standard vitamin D3 (Cholecalciferol) was obtained from Sigma Chemical (Sigma-Aldrich, Germany, Cat. No.: 47763) and was dissolved in 4mL of 95% ethanol to obtain 25mg/mL stock solution. This stock solution was then diluted in distilled water to obtain the working stocks and to reduce ethanol toxicity. The working stocks were aliquoted and kept at −80°C until used; once the working stocks were used, they were discarded.

Bacterial strains and growth conditions

Bacterial strain from the glycerol stock under −80°C was sub-cultured. The Strep. sobrinus DSM 20742 obtained from the German Collection of Microorganisms and Cell Cultures (Germany) and Strep. mutans (ATCC 25175 American Type Culture Collection, USA) were cultured on Brain heart infusion broth (BHI) and Brain heart infusion agar at 37°C under aerobic conditions for 18–24 hours. Microbiological media was obtained from Sigma-Aldrich (St. Louis, MO, USA and Oxoid Ltd, Basingstoke, UK) and prepared according to the manufacturer’s instructions.

Antibacterial susceptibility assay

The antibacterial susceptibility of vitamin D3 was investigated using the disc diffusion method on Mueller-Hinton agar plates (Sigma-Aldrich, St. Louis, MO, USA). Agar plates were inoculated with bacterial suspensions at a concentration of 1×10⁶ CFU/mL. Then sterile blank discs (6-mm diameter) which were impregnated with 20 µL of (500, 1000, 2000, and 4000 µg/mL) cholecalciferol vitamin D3 solutions were applied to give a final concentration of 10, 20, 40 and 80 μg/disc respectively, together with a positive (0.12% chlorhexidine) and negative control (2% ethanol). Preliminary experiments were carried out to test the effects of the sol-
vent (ethanol) on the tested bacteria which showed that at the dilution used, ethanol had no effect on bacterial growth. After 24 hours incubation at 37°C, the inhibition zones were observed and measured in millimetres.

**Minimum inhibitory concentration and minimum bactericidal concentration**

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method following the National Committee for Clinical Laboratory Standards (19). In this study, MIC and MBC experiments of vitamin D3 against Strep. sobrinus were carried out. Strep. sobrinus showed high sensitivity to vitamin D3 at lower concentrations; however, these low concentrations did not work on Strep. mutans, hence higher concentrations of vitamin D3 were used. The vitamin D3 stock used for Strep. sobrinus was 240 µg/mL whereas 2000 µg/mL was used for Strep. mutans.

Serial dilutions of vitamin D3 stocks were carried out in BHI broth in a sterile 96-well plate. Then, 100 µL of bacterial inoculum (a final concentration of 1×10^6 CFU/mL) was added to each well. These assays were tested in triplicates along with positive and negative controls. The positive controls contained bacterial cells in BHI broth to determine the bacteria growth throughout the experiment. The negative controls contained two-fold serial dilutions of the tested vitamin D3 in BHI broth without any bacteria and served as primary negative control to determine the changes in absorbance due to the different vitamin D3 concentrations. In addition, another negative control contained uninoculated BHI broth without vitamin D3 to evaluate the sterility of the BHI broth (20). Then the plates were incubated aerobically at 37°C for 24 hours. The growth of bacteria was determined at OD 600 nm using a microplate Spectrophotometer (Infinite M200 Pro, Tecan). The MIC was assessed by subtracting the mean OD 600 values of the incubated test medium from the inoculated primary negative control. The MIC was considered as the lowest concentration of tested vitamin D3 at which the OD 600 absorbance falls below 0.05 with respect to the primary negative control (21). Three triplicate experiments were completed at different time intervals.

The MBC was determined by taking 10 µL aliquot from the clear wells and were plated on BHI plates and incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of tested vitamin D3 that did not show any bacterial growth on BHI plates. The MBC of vitamin D3 against Strep. sobrinus at MIC concentrations was 60 µg/mL and 250 µg/mL, respectively.

Scanning electron microscope (SEM)

In this experiment, the morphological changes were assessed for the untreated and treated Strep. sobrinus and Strep. mutans with vitamin D3 application using SEM.

Briefly, overnight cultures of Strep. sobrinus and Strep. mutans were treated with cholecalciferol vitamin D3 at MIC values and incubated for 18–24 hours at 37°C along with untreated bacteria cultures that serve as growth controls. The treated bacteria were fixed in 2.5% glutaraldehyde for 4–6 hours then washed with 0.1 M sodium phosphate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide for 2 hours at 4°C. After washing again with 0.1 M sodium phosphate buffer, the samples were dehydrated using a series of alcohols. The specimens were coated with a thin layer of platinum and were observed under SEM.

**Statistical analysis**

The data was entered and analysed using Statistical Package for Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA). No data corrections were applied before the analysis. Bacterial measurement data under SEM were presented as the mean± standard deviations. The distribution of the data did not meet the requirements for normality and homogeneity of variance assumptions and therefore the length and width measurements between untreated and treated bacteria were determined by the nonparametric Mann–Whitney U test. The confidence interval was set to 95% and p < 0.05 was considered statistically significant.

**Results**

**Antibacterial activity of vitamin D3**

In this experiment, vitamin D3 was investigated to evaluate its antibacterial activity against Strep. sobrinus and Strep. mutans using the disc diffusion method. The results revealed no inhibition zones for both bacteria against all tested concentrations of vitamin D3.

**Minimum inhibitory concentration and minimum bactericidal concentration**

The MIC is considered the lowest vitamin D3 concentration that inhibited bacterial growth, as measured at OD 600. The MBC is defined as the lowest concentration of tested vitamin D3 that did not show any bacterial growth on BHI plates.

The MIC values of vitamin D3 against Strep. sobrinus and Strep. mutans were 60 µg/mL and 250 µg/mL, respectively, as shown in Figure 1 and 2. The MBC of vitamin D3 against Strep. sobrinus and Strep. mutans were 120 µg/mL and 500 µg/mL, respectively.

**Scanning electron microscope**

SEM examination was conducted to investigate the possible changes in the morphology of Strep. sobrinus and Strep. mutans treated with vitamin D3.

![Figure 1. MIC value of vitamin D3 against Strep. sobrinus.](image-url)
Vitamin D effect on mutans streptococci

mutans bacteria in response to cholecalciferol vitamin D3 application. The morphology of the tested bacteria was observed for the untreated and vitamin D3 treated bacterial cells. The untreated Strep. sobrinus and Strep. mutans exhibited the typical streptococcal appearance as ovoidal (elongated) cells with smooth uniform shape and intact cell membranes (Fig.3a, c and Fig.4a, c, e). However, the treated Strep. sobrinus significantly appeared shorter and swollen compared to untreated Strep. sobrinus bacteria (Fig.3b, d) with mean length of 0.96±1.95 µm, 0.78±0.11 µm p=0.021 and mean width of 0.47±0.04 µm, 0.51±0.06 µm p=0.048 for non-treated and treated bacteria, respectively. On the other hand, the treated Strep. mutans cells did not exhibit any clear changes in their size compared to the untreated cells. Additionally, both treated Strep. sobrinus and Strep. mutans bacterial cells showed distinct surface alternations of formation of cell membrane blebs (Fig.3b, d) and (Fig.4b), membrane damage/rupture (Fig.3f) and (Fig.4f), cell membrane clumping (Fig.3f), intracellular material leakage (Fig.4b), wrinkled and rough cell membrane (Fig.3f). Furthermore, the bacterium-to-bacteria contact area appeared flattened and wider in the treated Strep. mutans (Fig.4b). Thus, the observed morphological alternations in both bacteria appear to be related to the damage in cell wall and cell membrane.

Discussion

Vitamin D deficiency has been linked to the etiology of many chronic diseases such as respiratory infections (22), asthma, allergic diseases (23), rheumatoid arthritis (24). Vitamin D supplements in asthmatic patients is associated with reduction of bacterial respiratory infections including H. influenzae, S. pneumoniae, beta-haemolytic Streptococcus spp., S. aureus, and Chlamydia pneumoniae (22).

Earlier studies have shown that young children and adults who had low serum vitamin D had higher dental caries occurrence compared to individuals with adequate serum vitamin D levels (3, 6). Vitamin D supplementation was associated with a 47% reduced risk of caries (25). In addition, serum vitamin D levels above 30–40 ng/mL may significantly reduce the risk of dental caries (26). It is unclear whether the circulating hormone vitamin D has exerted a direct antibacterial activity against oral bacteria that causes dental caries, or this is based on the findings that vitamin D regulates calcium and phosphate homeostasis that is essential for calcification, mineralization and maintenance of hard tissue, oral bone and teeth (2), or the fact that vitamin D regulates the expression of endogenous antimicrobial peptides which are human cathelicidin (LL-37) and defensins that have broad spectrum antimicrobial activities against many bacteria (8, 26). The results of this study showed that cholecalciferol vitamin D3 was able to inhibit the normal growth of Strep. sobrinus and Strep. mutans and altered their normal cell morphology. Therefore, it suggests that cholecalciferol vitamin D3 has a direct antibacterial action against these bacteria, which is totally different from its hormonal effects.

The antibacterial susceptibility of vitamin D3 was investigated using the disc agar diffusion method. This method is one of the popular methods used to determine the antimicrobial effects of an agent (27). However, this test can be considered for materials which are soluble and capable of diffusing into the surrounding environment (28). This may explain why there was no zone of inhibition (ZOI) in the present study. It appears that the insolubility of the cholecalciferol vitamin D3 may have hindered its diffusion to the surrounding agar surface and the inhibition zone.

In recent years, there was increasing attention towards the sunshine vitamin. Few studies had reported the antibacterial activities of vitamin D analogues including vitamin D3 products against different bacteria including Mycobacteria (8), Helicobacter pylori (9) and Streptococcus mutans (10, 11). Varied MIC values were reported from previous studies depending on the applied methods, vitamin D compounds used and bacteria species. Hosoda and colleagues (9) have found that vitamin D3 species (vitamin D3: 25-hydroxyvitamin D3; 1α,25-Dihydroxyvitamin D3) at 5 µM concentration reduced the colony forming unit (CFU) count and exhibited the antibacterial action against H. pylori. A recent study found that the MIC for 1,25(OH)2D3 ranging from 3.125 to 6.25 µg/mL inhibited the growth of oral Porphyromonas gingivalis (10). Another study indicated that 1,25(OH)2D3 showed inhibition activities against S. mutans ATCC 35668 at MIC of 200 µg/mL, while MBC was > 400 µg/mL. In addition, a study by Saputo et al. (11) has determined the antibacterial activities of three vitamin D compounds, namely alfalcacidol, doxercalciferol, and calcitriol against Strep. mutans. They have concluded that vitamin D derivatives possess lytic activity against Strep. mutans at MIC of 16 µg/mL (11). In addition, the minimum biofilm inhibitory concentration of doxercalciferol and alfalcacidol was 64 µg/mL and 128 µg/mL, respectively; however, no biofilm formation inhibition was detected using calcitriol at any of these concentrations (11).

Both Strep. sobrinus and Strep. mutans are considered the most cariogenic bacteria causing dental caries; they are equally virulent in causing dental caries (12). Currently, chlorhexidine is considered the most effective oral antimicrobial agent due to its broad-spectrum action against Gram positive and Gram negative bacteria (29). Research has found that Strep. sobrinus has a higher resistance to chlorhexidine compared to Strep. mutans, and it may reappear earlier in saliva and plaque at higher levels than Strep. mutans after the application of chlorhexidine (30). However, in this study, we found that Strep. sobrinus is more sensitive to vitamin D3 compared to Strep. mutans, as the MIC and MBC values of vitamin D3 against Strep. sobrinus were lower than Strep.
Figure 3. Scanning electron microscope of untreated Strep. sobrinus (3a,c,e). Strep. sobrinus treated with vitamin D$_3$ at MIC (3b,d,f) showing the formation of cell membrane blebs (red arrows) (3b,d), cell membrane damage/ruptured (green arrow) (3f), membrane clumping (blue arrows) (3f), wrinkled and rough cell membrane (yellow arrows) (3f).
Figure 4. Scanning electron microscope of untreated Strep. mutans (4a,c,e). Strep. mutans treated with vitamin D$_3$ at MIC (4b,d,f) showing the formation of cell membrane blebs (red arrow) (4b), intracellular materials leakage (blue arrows) (4b), and the bacterium-to-bacterium contact area appeared flattened and wider (orange arrow) (4b). Bacterial cell distortion (white arrows) (4d) and cell membrane damage/ruptured (green arrows) (4f).
interactions with the peptidoglycan of this Gram positive bacteria, mainly Strep. sobrinus.

Moreover, due to the absence of studies that evaluated the antibacterial activities of vitamin D3 against oral bacteria, we were unable to compare our MIC and MBC values against the tested bacteria.

The microbial cell wall serves as a selective environmental barrier and contains determinants required for bacterial colonization and survival (31). The first barrier that an antimicrobial agent must overcome when interacting with its target is the bacterial cell wall (32). It was indicated that Gram positive bacteria were less sensitive to antibacterial agents compared to Gram negative bacteria because of the presence of a thicker peptidoglycan layer which acts as an additional barrier for the entry of antimicrobial agents inside the bacterial cells (33). From SEM results, it was demonstrated that treatment of Strep. sobrinus and Strep. mutans with cholecalciferol vitamin D3 exhibited considerable morphological changes. Treated Strep. sobrinus cells appeared shorter compared to untreated cells (Fig.3b, d). It appeared that vitamin D3 may impede the growth of Strep. sobrinus. Bacteria that grow in the presence of a compound which has antibacterial properties may experience environmental stress that could influence its ability to use nutrients efficiently and thereby slow down its normal growth (34). Morphological changes such as formation of blebs, wrinkled surfaces and cellular membrane damages were observed in the present study and the membrane damages are considered a key factor in the inactivation of bacteria (35). Such morphological changes in the surfaces of bacterial cells following the treatment with antimicrobial agent have been previously reported (35, 36, 37) and the results of the present study were consistent with them.

The SEM analysis in the present study proposed a possible mechanism for the antibacterial action of vitamin D3. Vitamin D3 attaches to the treated bacterial cell wall through interactions with the peptidoglycan of this Gram positive strain. The adherence of cholecalciferol vitamin D3 to the cell wall caused disruption to the bacterial cell wall and membrane, making them shrink, become rough and increase the internal cellular pressure causing bleb-like formation and eventually causing cell membrane rupture and bacteria damage. Based on SEM findings, it is evident that vitamin D3 is considered a membrane-active agent and is toxic to these oral bacteria and therefore affecting its normal growth.

To our knowledge this is the first study assessing the antibacterial activity of cholecalciferol vitamin D3 against Strep. sobrinus and Strep. mutans bacteria in vitro. Cholecalciferol vitamin D3 exhibited MIC and MBC as well as clear morphological alternations on both bacteria even though the exact mechanism by which vitamin D3 inhibited Strep. sobrinus and Strep. mutans growth remains to be discovered. More studies to evaluate its effects on the bacterial membrane ultrastructure need to be considered.

Conclusion

The findings of this study suggest that vitamin D3 has a direct antimicrobial effect against mutants streptococci bacteria in vitro. It appears that vitamin D3 is a membrane-active agent that affects bacterial cell wall and causes membrane disruption. It significantly altered the cellular structure of both the Strep. sobrinus and Strep. mutans cell walls and obviously hindered the normal growth of these bacteria. Therefore, vitamin D3 could be considered as a promising compound that may be used in caries prevention. Further research is recommended to explicate the mechanism of antibacterial activity of vitamin D3 on cariogenic oral bacteria.

Türkçe Özet: Vitamin D3’ün Mutans Streptokokklara Karşı Antibakteri etkisini in vitro araştırıp, özellikle Strep. sobrinus ve Strep. mutans’a karşı antibakteriyel etkiyi önlerken, bakterinin hücre yüzeyi ve hücre zarında meydana gelen değişiklikleri belirlemek için, vitamin D3’ün bakterilerin hücre Entwicklungindeki önemi. Vitamin D3’ün disc ottuğunun yontemi kullanılarak değerlendirdi. Vitamin D3’in minimum inhibitory konsantrasyonu (MIC) ve minimum bakterisit konsantrasyonu (MBC), Klinik Laboratuar Standartları Enstitüsü (CLSI) yönergelerine göre mikrodilüsyon yöntemi kullanılarak belirlendi. Vitamin D3’ünün potansiyel antibakteriyel etkisini araştırması, bu bakteriyel etkisini daha fazla araştırılması gerektiği düşünüldü. Vitamin D3’in bakterilerle MIC değerleri, Strep. sobrinus ve Strep. mutans için sırasıyla 60 µg/mL ve 250 µg / mL iken MBC değerleri ise sırasıyla 120 µg/mL ve 500 µg/mL idi. Ayrıca vitamin D3, bakterilerin hücre yüzeyindeki etkisini artırdı ve bakterilerin hücre yüzeyindeki etkisini arttırdı. Vitamin D3’in bakteri hücrelerinin hücrenin hücre merkezindeki etkisini arttırdı. Vitamin D3’in bakteri hücrelerinin hücrenin hücre merkezindeki etkisini arttırdı. Vitamin D3’in bakteri hücrelerinin hücrenin hücre merkezindeki etkisini arttırdı. Vitamin D3’in bakteri hücrelerinin hücrenin hücre merkezindeki etkisini arttırdı.

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Author contributions: MMMA, ASH and HAT designed the study. MMMA and ASH participated in generating the data for the study. MMMA, SABN and NAEBE participated in gathering the data for the study. MMMA, HAT, SABN and NAEBE participated in the analysis of the data. MMMA and ASH wrote the majority of the original draft of the paper. MIAH, HAT and HBSGK participated in writing the paper.

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