Detection of Some Biomarkers, Minerals Deficiency and Microorganisms Related to Early Childhood Caries

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Abstract:

In this study, the mean differences of D_3, calcium level, zinc level, iron level and Mn level between study groups including (children with early dental caries and control group) were significant decrease ($P \leq 0.05$) in of D_3, calcium level, zinc level, iron level and Mn level in children with early dental caries compared with the control group. In addition, There were statistically significant increases in total protein intake across groups ($P<0.01$) in total protein in children with early dental caries compared with control group. A total 50 saliva samples of children suffering from early dental caries, the findings indicated that, out of 50 samples, 42(84 percent) provide positive culture, whereas 8(16 percent) samples were negative culture. Out of 42 positive culture on different types of growth media, and the bacterial was identified according to gram stain, biochemical test and Vitek system, the results showed that, Streptococcus sobrinus was considered the most commonly bacterial isolates from children suffering from early dental caries in rate (42.8 percent), followed by Streptococcus mutans in rate (30.9 percent), (14.3 percent) were related to Lactobacillus fermentum and Prevottella oralis in rate (11.9 percent) (11.9 percent) DNA was taken from all probable isolates that previously recognized as Streptococcus sobrinus by vitek2 system, These DNA samples were used in conventional polymerase chain reaction (PCR) to amplify primers specific to the 16s ribosomal RNA gene of Streptococcus sobrinus. After generating a 546 bp DNA fragment, gel electrophoresis showed that all 18 samples of Streptococcus sobrinus matched the allelic ladder. Molecular identification of 16srRNA gene was done for isolates that previously identified as Streptococcus mutans by vitek2 method. The findings indicated all of 13(100 percent) provided good outcomes. Positive findings were identified by the presence of (282bp) bands when compared with allelic ladder. In addition, molecular detection of 16srRNA gene was done for (5) isolates Prevottella oralis isolates and the findings revealed that all 5(100 percent) isolates had this gene. The positive findings were identified by the presence of (530 bp) band compared with allelic ladder.

Objective:

The aim of this study to detection of some biomarkers, minerals deficiency and microorganisms related to early childhood caries.

Keywords:
Early childhood caries, minerals deficiency, microorganisms, early childhood caries, biomarkers.

Introduction:

When a preschool-age child between birth and seventy-one month's old has one or more decaying (non-cavitated or cavitated lesions), missing (as a result of caries) or packed teeth surfaces on their veryny milk teeth, they are said to have early childhood tooth decay (Ballantine et al., 2018). As the name implies, "severe babyhood caries" refers to patterns of cavity development that are either unusual, progressive, acute, or epidemic. (Anil & Anand, 2017). Babyhood caries may be a vital public unhealthiness in elite populations and is additionally found throughout the final population (Pierce *et al*., 2019). Child' teeth are vulnerable to decay as presently as they begin to erupt. Babyhood caries is a communicable disease (Alazmah, 2017). There are many aspects of early childhood caries; baby bottle caries is recognized united of the additional severe manifestations of this syndrome (Gurunathan *et al*., 2019). Babyhood caries are cavities that almost all usually show au courant a child's higher front teeth however may also have an effect on alternative teeth (Duangthip *et al*., 2017). It first appears as little white patches around the gum line on the teeth. It occurs when sugary drinks and meals like milk and juice are left in your child's mouth for an extended period of time (Igic et al., 2018). Bacteria love sugar, which they turn into acids that corrode your child's teeth over time. All of them, as well as other variables including the frequency with which your kid eats, his or her oral hygiene habits, medicines, and other medical and dental problems, can determine whether or not your child is at risk of developing dental caries (Coppes & Fisher-Owens, 2018). The most likely etiological agents of dental caries in children are oral streptococci, such as Eubacteria mutans and Eubacteria sobrinus (Cherukuri et al., 2020). Candida albicans, a fungus, and alternative bacteria including Prevottella and Eubacterium spp. are linked to the occurrence and development of error correcting coding (Hemadi et al., 2017). Many different innate defense mechanisms affect oral microbe survival when biomolecules, particularly macromolecules, are present in spit. This modulates the oral microbiota (Cross & Ruhl, 2018). This means that the amount of protein in saliva may be an accurate predictor of one's oral health. oral bacteria and saliva proteins may be useful indicators for predicting tooth decay risk and prognosis in individuals with certain health conditions (Buzalaf *et al*., 2020). Microorganisms and secretory proteins have important roles to play in ECC prevalence and interference, according to recent research (Pappa et al., 2020). Biomarkers for error correction code risk assessment that may be used to identify tooth decay-causing bacteria and protecting secretions from healthy teeth. Identification of biomarkers for ECC in children is critical not only for early detection, but also for the prevention and treatment of the condition (Devarajan & Somasundaram, 2019). Spit may be a complex mixture of organic and inorganic components necessary to the oral cavity's well-being. There are three main salivary glands that produce spittle, namely the salivary glands, as well as smaller glands situated in the submandibular area and the organs of the mouth, as well as a variety of minor salivary glands (Rosa et al., 2021). The spit also includes desquamated cells of the oral epithelium, bacteria, cartilaginous tube expectoration remnants and food waste as well as its inevitability with animal tissue crevicular fluid (Amado et al., 2019). Dental decay is caused by a variety of causes, including bacteria, nutrition, oral hygiene, medical disorders, and a deficiency in essential nutrients (Chhonkar et al., 2018). For every person at risk of vitamin D insufficiency, one is at risk of missing out on a significant amount of phosphorus absorption (Charoenngam et al., 2019).

Materials and Methods:

Observational case control research is the approach used in the current investigation. From March through June of 2021, researchers gathered study data. The research was carried out at a private dental facility in the Iraqi city of Hilla. A total of 100 children between the ages of 2 and 6 were included in the research (50 with early childhood dental caries and 50 healthy controls). All of the children in the study, as well as the controls, belonged to the same ethnicity (Arabic).

Research and sampling ethics:

Before any samples are collected, the parents of all children participating in the project are notified and verbally consent.

Collection of the blood samples:
To obtain plasma, two milliliters of blood were drawn from each subject and placed in EDTA tubes. The remaining three milliliters of blood were slowly injected into disposable tubes containing separating gel and allowed to clot at room temperature for thirty minutes before being centrifuged at 3000 g for approximately three minutes. The results were analyzed. As a result, serum was procured and kept refrigerated (-20 C) until used in the analysis.

Saliva collection:
In each instance, we used disposable cotton swabs to collect samples, and we followed normal protocol for microscopic analysis and bacterial isolation. To prevent contamination, specimens were meticulously gathered. Rest of sample sent to Department of Microbiology for further analysis. There were no contamination issues because of the contamination-free technique used by Abd AL-Khuder et al. to collect the saliva samples (2021). For the elimination of any remaining material, the mouths of all participants were rinsed for 30–60 seconds with pure water (10mL). It was necessary to collect non-stimulated clean saliva and keep it in an ice bag-lined cold box until it could be examined in the laboratory. After that, each sample was centrifuged at 3000 rpm for 10 minutes to remove unwanted free salivary particles. Afterwards, 1l micropipette tips were used to aspirate the clear salivary solution, which was stored in a 1ml sterile Eppendorf tube for later use at (-20°C) (Mohammed & Hussein, 2021).

Estimation Vitamin D level:
Estimation the D_3 level by use VIDAS® 25 OH Vitamin D Total kit.

Estimation calcium level:
Estimation calcium level by use Calcium kit (arsenazo III method).

Estimation of zinc level:
The estimation of zinc level according to study of Hussain *et al.*, (2016).

Estimation of iron level:
The estimation of iron level according to study of Gmyrek *et al.*, (2009).

Estimation of manganese level:
The estimation of manganese level according to study of Li *et al*., (2011).

Estimation of total protein level:
The estimation of total protein level in saliva according to study of Pandey *et al*., (2015).

Identification of bacterial isolates by gram stain, biochemical tests:
The saliva samples were collected from each case by disposable cotton swabs, and following standard procedure for microscopic examination and isolation of bacteria. At the bedside, an aliquot of the material was put into Blood agar medium for rapid aerobic cultivation. The remainder of the sample was sent to the Department of Microbiology for analysis, where it was inoculated into Blood agar, Mannitol agar, MacConKey agar, and Nutrient agar medium, among other ordinary media, and incubated aerobically and anaerobically at (37oC) for (24) hours. Gram stain, colony morphology, and a biochemical test were used to determine if the bacteria were aerobic or anaerobic. Each isolate was subjected to identification tests that looked at cultural, morphological, and biochemical features (Baron et al., 1994 and MacFadden, 2000).

Identification of bacterial isolates in Compact VITEK-2 System:
Using the Compact VITEK-2 System (BioMerieux), all bacterial isolates were screened and identified according to manufacturer's instructions.

DNA extraction form bacterial culture:
A Genomic DNA purification kit supplemented by a Genomic DNA extraction kit was used to extract genomic DNA from each isolate and direct sample of throat infection (Geneaid, USA). UV-trans- illuminator used to observe.

Identification of some bacterial isolates by *16SrRNA gene*:

Table 1 displays the study's primer sequence and PCR settings .

Table (1): Primers for 16S ribosomal RNA genes, including the size and condition of the resulting amplicon in base pairs (bp).

16Sr RNA Genes	Primer sequence (5′-3′)	Size (bp)	PCR condition	Reference
16SrRNA gene of St. mutans	F: TCGCGAAAAAGATAAACAAACA R: GCCCCTTCACAGTTGGTTAG	282	Initial denaturation 2 min. At 95ºC, annealing at (58 °C) final extension at 72 °C for 7 minutes hold at 4ºC	Gross *et al*., (2012)
16SrRNA gene of St. sobrinus	F: GATAACTACCTGACAGCTGACT R: AAGCTGCCTTAAGGTAATCACT	546	Initial denaturation 2 min. At 95ºC, annealing at (54.5 °C) final extension at 72 °C for 7 minutes hold at 4ºC	Nurelhuda *et al*., (2010)
16S rRNA Gene of Prevottella oralis	F: CACRGTAAACGATGGATGCC R GGTCGGGTTGCAGACC	530	Initial denaturation 2 min. At 95ºC, annealing at (54.1 °C) final extension at 72 °C for 7 minutes hold at 4ºC	Siqueira et al., (2007)

Statistical analysis:

SPSS version 23 was used for the statistical analysis. (Means SD) was used to show continuous variables. The means of the two groups were compared using a student t-test.

Results and discussion:

In this study, the mean differences of D_3, calcium level, zinc level, iron level and Mn level between study groups including (children with early dental caries and control group) was shown in Table (2). The results showed that, there were significant decrease ($P \leq 0.05$) in of D_3, calcium level, zinc level, iron level and Mn level in children with early dental caries compared with control group.

Table (2): Mean difference of some biomarkers between study groups

Biomarkers	Study groups		P-value
	Children with early dental caries (N=50)	Healthy control (N=50)	
D_3 nmol/L	9.45 ± 2.60	24.19 ± 1.42	**P≤ 0.05**
Calcium mg/dl	4.11 ± 1.18	8.96 ± 3.58	**P≤ 0.05**
Zinc µg/dl	72.57 ± 14.70	80.15 ± 4.26	**P≤ 0.05**
Iron mcg/dl	55.01 ± 6.34	81.33 ± 12.07	**P≤ 0.05**
Mn mcg/dl	6.54 ± 3.02	11.81 ± 9.55	**P≤ 0.05**

This research included children with early dental caries as well as a control group (3). According to the findings, children with early dental caries had significantly higher levels of total protein than children in the control group ($P < 0.01$).

Table (3): Mean difference of some biomarkers between study groups

Biomarkers	Study groups		P-value
	Children with early dental caries (N=50)	Healthy control (N=50)	
Protein gm/ml	4.22 ± 0.33	0.89 ± 0.37	**P< 0.01**

As shown in Figure 1, 42 (84 percent) of 50 saliva samples from children with early dental caries gave positive cultures, while 8 (16 percent) of 50 samples were negative.

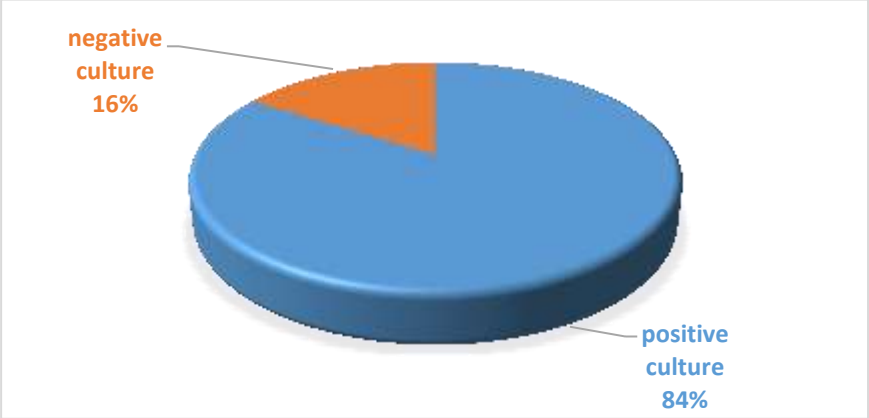

Figure (1): Positive and negative culture of all saliva samples were collected from children suffering from early dental caries

In 42 positive cultures on various growth media, and the bacteria were identified using gram stain, biochemical test and Vitek system, the results showed that Streptococcus sobrinus was the most commonly isolated bacterium from children with early dental caries (42.8 percent), followed by Streptococcus mutans (30.9 percent), (14.3 percent) were related to Lactobacillus fermentum and Prevottella oralis (42.8 percent)) (Table 3)

Table (3): Identification of bacterial isolated from children suffering from early dental caries

No.	Types of bacteria	Rate
1.	*Streptococcus sobrinus*	18(42.8%)
2.	*Streptococcus mutans*	13(30.9%)
3.	*Lactobacillus fermentum*	6(14.5%)
4.	*Prevottella oralis*	5(11.8 %)
Total		**(100%)**

The 16srRNA gene for Streptococcus sobrinus was amplified using DNA from all suspected isolates previously identified as Streptococcus sobrinus using the vitek2 system. Conventional PCR was performed using these DNA samples according to the sequences and program listed in Table (1). When compared to an allelic ladder, all 18 (100%) samples of Streptococcus sobrinus generated the same (546) bp DNA fragment. This is illustrated in Figure (2). Isolates previously identified as Streptococcus mutans by the vitek2 method underwent molecular detection of the 16srRNA gene. As a consequence, every single one of the 13 tests was positive. The existence of (282bp) bands when compared to the allelic ladder revealed positive findings, as shown in Figure (3). Prevottella oralis was tested for the presence of the 16srRNA gene, and the findings revealed that all 5 (100 percent) isolates had it. When compared to an allelic ladder, the positive findings were found by looking for a (530 bp) band

(see Figure) (4).

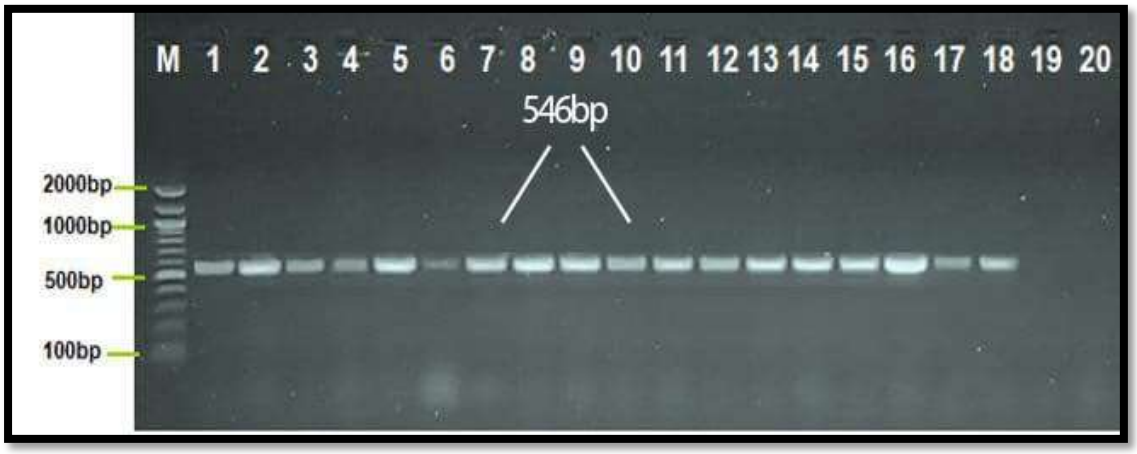

Figure (2): Agarose gel electrophoresis image of PCR product analysis for *16srRNA* gene in *Streptococcus sobrinus* isolates. M (Marker ladder 2000-100bp). Lane (1-18) *16srRNA* gene in *Streptococcus sobrinus* isolates at (546 bp) product size.

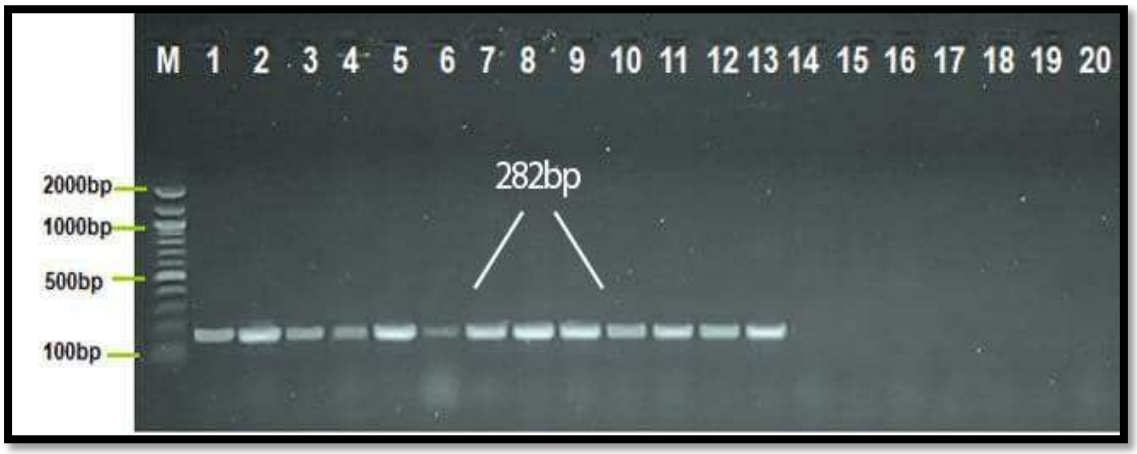

Figure (3): Agarose gel electrophoresis image of PCR product analysis for *16srRNA* gene in *Streptococcus mutans* isolates. M (Marker ladder 2000-100bp). Lane (1-13) *16srRNA* gene in *Streptococcus mutans* isolates at (282 bp) product size.

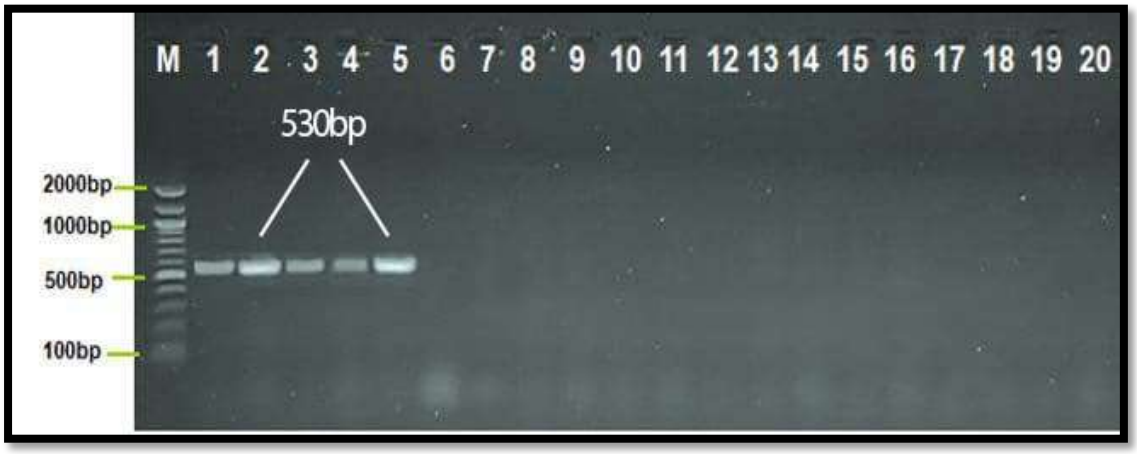

Figure (4): Agarose gel electrophoresis image of PCR product analysis for *16srRNA* gene in *Prevottella oralis* isolates. M (Marker ladder 2000-100bp). Lane (1-5) 16*srRNA* gene in *Prevottella oralis* isolates at (530 bp) product size.

Discussion:

A kid aged seventy-one months or younger is said to have early childhood caries if they have one or more decaying (noncavitated or cavitated lesions), missing (as a result of caries), or crowded tooth surfaces. It is possible that baby tooth decay is a significant public health problem (Childers, 2019). Chronic infectious disease in children is the most prevalent, and it may be difficult to treat. It can begin as soon as the baby's teeth start to emerge (Mathur & Dhillon, 2018). Decay is a severe infectious illness caused by a multifactorial pathophysiology of microorganisms (Ahirwar et al., 2019). Cariogenic bacteria, potential carbohydrates, an inclined tooth, the host, and consequently time are major actors in the genesis of this disease. It's still one of the most widespread diseases in the world (Tahir & Nazir, 2018). Cariogenic bacteria, a kind of bacteria found in the mouth, are responsible for the start and development of tooth decay (Conrads, 2018). *Eubacterium mutans* and eubacteria are the most harmful bacteria. The second most cariogenic bacterium found in mouth flora is Lactobacillus (Krasniqi et al., 2020). However, it's not the caries leader that's critical to the development of caries (Zhang et al., 2020). Most prior studies were quantitative, thus until now, researchers have been unable to pinpoint exactly which eubacteria species are responsible for deterioration (Ahirwar et al., 2019). In a few species-level identification investigations, the most frequent eubacteria species found in saliva, tongue, unhealthily lesioned teeth, dental plaque, etc. were identified as *L. gasseeri*, *L. fermentum*, *L. vaginalis*, and *L. casei* which may play the most important role in caries development (Duar et al., 2017). Vitamin D deficiency is defined as 25(OH) D levels in body fluids less than 20 ng/dL. As far as biological process deficiencies go, it's considered the most prevalent (Lee et al., 2018). However, there is a severe dietary deficit across all age groups and genders in the nation. many things like low dietary intake, seasonal changes, concern about cancer in the sun, keeping the child indoors, and dark pigmentation of the skin cause vitamin D deficiency, such as low dietary intake; weakened body covering synthesis; high rate of exclusive breastfeeding; and vitamin D deficiency in the mother all contribute (Park et al., 2018). The study by Chhonkar et al. (2018) on the relationship between blood vitamin D concentration and tooth loss and dental caries standing found that blood vitamin D concentration was linked to tooth loss. Despite the fact that vitamin D deficiency has been linked to an earlier development of dental caries, Heyden & Wimalawansa (2018) found no evidence to support this theory. Deficiency disease has serious wide-ranging health consequences and is believed to be one among the foremost prevailing matter deficiencies within the world (Lockyer & Buttriss, 2018). However, reliable indicators or biomarkers to assess atomic number 30 status aren't on the market at present. Indirect indicators cherish the prevalence of stunting or anemia, iron deficiency, furthermore as more direct indicators cherish plasma atomic number 30 concentrations are getting used at the present to estimate the prevalence of deficiency disease in populations (Hennigar *et al*., 2021). Youngsters with general zinc deficiency have the next dental caries prevalence and poorer animal tissue health compared to their zinc-sufficient counterparts (Freitas *et al*., 2017). In the poor world, where 2.5 billion children are zinc deficient, deficiency illness is more severe and poses a greater health risk than zinc overdose (Liu et al., 2021). Zinc may be found in a variety of places in the mouth, including saliva, bacterial plaque, and the tissues that hold the teeth together. Oral disorders such as gingivitis, periodontitis, and bad breath may all be treated with atomic number 30 supplementation. Conversely, deficient disease has been linked to poor oral and periodontal health (Nandlal et al., 2021). Atomic number 20 plays a crucial role inside the body, not simply in control overall health however conjointly serving to stay the bones, teeth and jaw strong. If it deficient in calcium, it should expertise a bunch of problems as well as irregular heartbeat, nerve and muscle problems, secretion imbalance and problem engrossing vitamin B12 to call many (Zhang & Yelick, 2021). Atomic number 20 can be found in several of the foods and drinks we tend to consume daily, however people who don’t get enough from these sources ought to bear in mind of the intense consequences (Rouf *et al*., 2019). If an absence of atomic number 20 is observed, the body will begin to require calcium from bones and teeth instead. This can weaken the bones and teeth, inserting it at an elevated risk for damage, decay and pathology (Day *et al*., 2019). Biological fluid macromolecule biomarkers, in particular those that can be quantified precisely and reproducibly, may provide useful information on the body's reaction to a therapy for a sickness or condition, as well as on the semi-permanent observation of oral illnesses (Tonry et al., 2020). Biomarkers will also serve as an early warning system for illness, offering a potential alternative to current oral

diagnostic methods (Miglis et al., 2021). The combination of secretory protein analysis and plaque microbiota analysis may speed up the search for more accurate methods to forecast deterioration (Chattopadhyay et al., 2019). In humans, the most common cariogenic bacteria are Streptococcus mutans, Streptococcus viridans, and Spongia sobrinus. Caries-active (CA) and caries-free (CF) individuals have both been discovered to have S. mutans strains, but it's not clear whether or not they are the initial etiological determinant (Salman et al., 2017).

Conclusion:

Vitamin D_3 and Minerals deficiency were considered the important factors that causes early childhood caries. Increase of total serum protein was also caused this disease. *St. sobrinus*, *St. mutans*, and Lactobacillus were regarded the most cariogenic bacteria in oral flora, with *St. mutans* being the most prevalent. It isn't the cause of caries, but it does play a part in how quickly it spreads.

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