Review Article

Salivary Proteins, Enzymes and Immune Factors Associated With Early Childhood Caries: A Narrative Review

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ABSTRACT

Background: Early Childhood Caries (ECC) has many etiologies such as families' socioeconomic status, parents' education and awareness, prolonged and improper bottle or breastfeeding, consuming sweet foods and high-sugar diets, brushing techniques, immaturity of children's immune system, family size, and Streptococcus mutans.

Evidence Acquisition: The data used in our review were searched from articles published between 1950 to 2021 and using ECC, children, saliva, salivary biomarkers, salivary enzymes, salivary peptides, salivary proteins, and immunity as keywords, collected from official web pages (Scopus, PubMed, Embase, and Google scholar) and documents published from different international institutions. The search was limited to articles published in the English language. After screening the abstract, the full text of 194 related studies was reviewed. Finally, 78 most related studies were selected.

Results and Conclusions: ECC-related salivary proteins and peptides are Proline-rich proteins, salivary mucins, Lactoferrin, immunoglobulins, Toll-like receptors, Lysozyme, Histatins, Statherin, Defensins, Calprotectin, and Cytokines. ECC-related enzymes are Amylase, Lysozyme, Lactoperoxidase, Alkaline phosphatase, Carbonic anhydrase VI, Lactate dehydrogenase, and Glucosyltransferase B. Immunity factors affecting ECC include IgA (sIgA), IgG, IgM, salivary mucins, Lactoferrin, TLRs, Histatins, Statins, Defensins, Calprotectin, Lysozyme, Lactoperoxidase, Cytokines and interleukins, Cathelicidin (LL-37), Agglutinin, Cysteine, and Neutrophils.

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Introduction

Early Childhood Caries (ECC) [1] is one of the acute and progressive types of tooth decay [2] and in this review article, we go through its etiology, prevalence, and involved salivary factors, e.g., salivary proteins, enzymes, lipids, antioxidants, and other known factors investigated by previous articles on this area. Rampant caries is a clinical syndrome in infants and young children known in the past by various names such as nursing caries, baby bottle tooth decay, and nursing bottle syndrome that is currently known as ECC [2, 3].

This complication's hallmark is the rapid progression of caries on the labial surfaces of the upper anterior deciduous teeth and lingual surfaces of the lower molar teeth [2, 3]. The prevalence of caries in children in different articles varied from 11% to 74% depending on the parents' awareness, the age of the children studied, the study population, and other factors involved [1, 4-6]. The known etiologies for this disease are mostly based on the socioeconomic status of families, parents' education and their level of awareness [1, 4-9], prolonged and improper bottle- or breastfeeding [1, 10-12], and consuming sweet foods and high-sugar diets [1, 4, 8, 10, 13, 14]. Although some articles also attributed other factors such as gender, brushing techniques or lack thereof [1, 8, 15], immaturity of children's immune system [13], and family size [13], the main element acting on top of the previously mentioned factors (e.g., improper diet and parental ignorance) causing various caries in children's mouth is a bacterium that has been widely reported in articles named Streptococcus mutans [1, 4, 8, 10, 12-14, 16-20]. Moreover, some studies mentioned the association of lactobacilli with Streptococcus mutans in pathogenicity [17, 21, 22].

Previous studies have also suggested ways to treat and prevent ECC: Educating parents and raising their level of awareness [1, 4-7], diet modification, and prevention of Streptococcus mutans colonization [4, 12-14], using fluoride in the form of toothpaste or varnish for children [23] to name a few. Given the importance and prevalence of this type of progressive caries in children, we examined some of the salivary biomarkers involved in previous studies more closely.

Literature review

The data used in our review were searched from articles published between 1950 to 2021 and using ECC,

Table 1. ECC-related salivary proteins and peptides and their function

ECC-Related Salivary Proteins and Peptides	Functions	Ref.
Proteins in general	Detection, as biomarkers	[24-27]
Proline-rich protein	Enamel remineralization	[22, 30]
Salivary mucins	Prevention of demineralization by acid-producing microbes	[18, 22]
Lactoferrin	Antimicrobial activity	[17, 18, 22, 31-33]
Immunoglobulins	Bacterial binding, immune activity	[18, 20, 22, 34-38]
Histatin	Antifungal and antimicrobial	[22, 27, 43-46, 54]
Alpha and beta defensin	Antimicrobial and antiviral	[29, 30, 37, 45-50]
Lysozyme	Antimicrobial activity, cell wall lysis	[1, 32, 40, 41]
Statherin	Binding to HAP, inhibiting the spontaneous deposition of calcium and phosphate salts from supersaturated saliva and inhibiting HAP crystalline growth, inhibiting bacterial and fungal colonization	[18, 22, 24, 30]
Calprotectin	Antimicrobial properties and regulation of inflammation	[50]
Interleukins	Involved in cytokine cascade, one of the first host responses to inflammation, a diagnostic biomarker in ECC saliva in children	[51, 52]
CD14 and CD63	CD14: Bacterial detection CD63: Antimicrobial protein quality control	[37, 53]

HAP: Hydroxyapatite; ECC: Early childhood caries.

children, saliva, salivary biomarkers, salivary enzymes, salivary peptides, salivary proteins, and immunity as keywords, collected from official web pages (Scopus, PubMed, Embase and Google scholar) and documents published from different international institutions. The search was limited to articles published in the English language. After the abstract screening, the full text of 194 related studies was reviewed. Finally, 78 most related studies were selected. The full text of all the articles was found. The following types of studies were excluded from this review: Animal studies, studies published in languages other than English, case reports, medical record reviews, meeting abstracts, editorials, letters, and commentaries. The included studies were the ones in which authors published information about ECC and salivary biomarkers in children.

Discussion

Salivary proteins and peptides

The substances in saliva can contain a lot of health information. Therefore, the use of saliva to diagnose various types of oral diseases has been in the spotlight since it is non-invasive, easy to work with, and inexpensive. Salivary proteomics can also lead to the discovery of diagnostic biomarkers [24]. Numerous proteomics studies have been performed on the saliva of children with ECC and control groups without the disease. A study conducted by Mahjoub et al. in 2014 observed that the protein content of saliva in the group of patients with S-ECC has increased compared to the control group [25]. A study by Tian et al. in 2017 found a marked difference in salivary peptides between children with and without ECC relapse after treatment, and salivary proteins in children without ECC relapse appear to be more sensitive to treatment. It seems that two peptides with m/z values of 3162.0 Da and 3290.4 Da can have diagnostic values for ECC return [26]. Comparing the protein peaks in the saliva of children with ECC and the control group, studies have shown higher levels of APO A1, SPTAN1 [24], histatin-rich and PITSLRE protein kinase beta SV1 isoform [27] in the former group. Therefore, it is suggested that these proteins be used as diagnostic biomarkers. Besides all of the same origins of the uncharacterized protein AF_2048 originating from Archaeoglobus fulgidus, three peptide peaks were also found that are lower in people with ECC than in healthy people. These three peptides can also be used as diagnostic markers of ECC [24]:

Proline-rich proteins (PRPs): PRPs are important oral glycoproteins involved in remineralizing tooth enamel and regulating the oral cavity's microbial flora. Acidic PRPs (aPRPs) are involved in the formation of dental pellicles and enhance the adhesion of S. mutans to hydroxyapatite (HAP) surfaces. They also maintain calcium homeostasis in saliva [22]. A study by Bhalla et al. in 2010 using electrophoresis analysis showed that the number of PRP bands in non-caries groups was significantly higher than in ECC groups [28]. Most articles have suggested a direct relationship between increased PRP and increased incidence of ECC [22, 29, 30]. A study by Ribeiro et al. in 2013 found that proline-rich peptides IB-4 could increase the risk of ECC [29].

Salivary mucins: Salivary mucins are large, highly glycosylated proteins secreted by submandibular acinar cells. The mucin family in human saliva is divided into two types: High molecular weight weighing about 1000 kDa and low molecular weight weighing about

Table 2. ECC-related enzymes and their function in saliva

Enzyme	Function in Saliva	References
Amylase (Alpha Amylase)	Helps digest starch, associated with bacterial plaque activity	[17, 31, 55]
Lysozyme	The antimicrobial effect, destruction of bacterial cell wall	[17, 18, 22, 33, 57, 58]
Lactoperoxidase	Antimicrobial effect	[18, 33, 60, 61]
Alkaline phosphatase	Effect on balance control of remineralization and demineralization (control of caries in tooth tissue)	[63]
Carbonic anhydrase VI	Effect on neutralizing bacterial acids	[64]
Lactate dehydrogenase	Indicates tissue damage (necrosis, etc.)	[66]
Glucosyltransferase B	Basic role in pathogenesis and caries (produced by bacteria in the oral environment)	[67, 68]

150-200 kDa. Mucins found in the oral cavity include MUC5B, MUC7, MUC19, and MUC1. These glycoproteins prevent the demineralization of teeth by acid-producing microbes. Mucins exert their protective role by binding to microorganisms, preventing their accumulation, attachment, and influencing tooth enamel. Previous studies have reported no significant association between salivary mucin levels (MUC5B and MUC7) and ECC incidence [18, 22].

Lactoferrin: Lactoferrin is an iron-binding cationic glycoprotein with a molecular weight of about 80 kDa. It is secreted by serous acini in the major and minor salivary glands and acts effectively against S. mutans, fungi, parasites, and viruses. Salivary lactoferrin can also originate from neutrophil granulocytes and gingival crevicular fluid. With its N-terminal's help, this glycoprotein can bind to the bacteria and kill them directly by destroying the bacterial wall. Also, as a cationic glycoprotein, lactoferrin can prevent the accumulation of microbes. Previous studies have shown that salivary lactoferrin levels are associated with ECC, with salivary lactoferrin levels decreasing as the patient recovers from ECC [17, 18, 22, 31]. A study conducted by Moslemi et al. in 2015 observed that the concentration of lysozyme and lactoferrin in the salivary samples of patients with ECC was lower than in people without caries and after completing the treatment, the mean concentration of these two proteins in the subjects did not change. It seems that low concentrations of salivary lysozyme and lactoferrin can be considered risk factors for caries [32]. Also, a study conducted by Gudipaneni et al. in 2014 found that lactoferrin-containing toothpaste effectively reduces the number of salivary bacteria in people with S-ECC [33].

Immunoglobulins: Salivary immunoglobulins make up 5%-15% of total salivary proteins. The most abundant antibody found in saliva is IgA, followed by IgG and IgM. IgA can neutralize bacterial toxins and enzymes, and interfere with the adherence of the bacteria to the tooth surface by physically blocking their adhesion, inhibiting their metabolism, reducing their hydrophobicity, and aggregating them together [18, 22]. Previous studies have shown that salivary immunoglobulin levels are associated with caries and ECC, so the amount of immunoglobulins in people with ECC is higher than in those without caries [20, 34-37]. A 2016 study by Colombo et al. found that children with S-ECC had a decreased salivary IgA immune response to S. mutans GbpB, which reduced their ability to manage MS infection and its cariogenic potential [38]. A 2003 study by De Farias et al. found that salivary IgA and IgG levels were higher in the ECC group than in the healthy group, while the mean amylase activity, total protein concentration, and total IgM were similar between the two groups [36].

Toll-like receptors: TLRs are one non-specific immunity factors and one of the immune system's initial reactions in response to substances produced by various pathogens, such as LPS. TLRs are critical mediators in the defense against gram-positive bacteria (which are important causes of caries). In a study conducted in 2014 by Malekafzali et al., it was observed that the concentration of TLR-2 in the group of patients with ECC increased compared to the control group. This rate decreased again in patients with ECC after treatment, which indicates the immune system's role and especially TLR-2 in the pathogenesis of ECC [39].

Lysozyme: Lysozyme is an antibacterial enzyme found abundantly in body fluids such as saliva, serum, tears, and amniotic fluid. Lysozyme prevents the adhesion and accumulation of bacteria. This enzyme also can kill and inhibit the growth of bacteria [1, 40]. Studies have shown that lysozyme levels in people with ECC are lower than in people without caries. It seems that its concentration can be related to ECC [32]. In a study in 2015, Lertsirivorakul et al. observed an increase in salivary lysozyme expression and activity in children with ECC, suggesting that there may be an association between increased salivary lysozyme and oral immunity in response to ECC [41].

Histatins: Histatins are 12 small histidine-rich cationic peptides identified by their histidine-rich structure in the length of 7 to 38 amino acids [42]. Histatins, and especially histatin 1, can reduce bacterial colonization because of their ability to inhibit glycoproteins on the HAP surface and block the site of bacterial attachment to the tooth surface [43, 44]. Previous studies have shown that the concentration of histatin in the saliva of patients with ECC was higher than that of people without caries [22, 27, 45]. However, a study by Sun et al. in 2016 comparing the protein content of saliva in children with ECC and the same children after four weeks of treatment showed that the concentration of histatin-1 in children with ECC increased after four weeks of treatment [46].

Statherin: Statherin is an acidic peptide with low molecular weight, containing 43 amino acids, that has many functions including binding to HAP, inhibition of spontaneous deposition of calcium and phosphate salts from supersaturated saliva, and inhibition of

HAP crystalline growth. Statherin can also prevent the colonization of bacteria and fungi. Studies show that statins play a protective role in people with ECC. One study also noted the absence of caries when statherin levels were high [18, 22, 24, 30].

Defensins: Defensins are essential antimicrobial peptides in the body that contain 29-35 amino acids. Two types of defensin have been identified so far, known as α- and β-defensin. α-defensins (HNP 1-3) are secreted by neutrophils and are found in GCF. β-defensins (hBD-1, hBD-2, and hBD-3) secreted by oral epithelial cells are found in saliva and superficial gingival fluid and have anti-inflammatory properties [30, 45, 47, 48]. In some studies, the concentration of α-defensin in people with ECC was lower than in the control group [29, 49]. However, a study conducted by Luthfi et al. in 2019 observed that the secretion of HNP 1-3 in the saliva increases in people with ECC compared to the control group [37], and a study by Jurczak et al. in 2015 found that β-defensin-2 concentrations were higher in patients with ECC than in the control group. This increase in concentration was also associated with disease progression [45]. A study conducted in 2011 by Toomarian et al. showed no difference in the concentration of HNP 1-3 in patients with ECC compared to the control group [50]. A 2016 study by Colombo et al. found that the number of antimicrobial peptides (AMPs) in saliva alone was not related to ECC severity. Its data showed a positive correlation between the levels of S. mutans with salivary hBD-2 or HTN-5, and also between hBD-2, -3, LL-37, and HTN-5. The stimulus of caries seems to trigger the response that occurs with these peptides [47].

Calprotectin: Calprotectin is a calcium- and zinc-binding protein that makes up 30%-60% of the proteins in the cytoplasm of neutrophils. This protein has antimicrobial and inflammatory regulatory properties and is used as a marker in diagnosing some diseases. A study conducted in 2011 by Toomarian et al. showed no difference in calprotectin concentration between the saliva samples of the S-ECC group and the control group. This study also stated that we do not know whether S-ECC can be predicted by salivary protein calprotectin or α-defensin [50].

Cytokines: Interleukins are inflammatory cytokines that include different types such as IL-6, IL-8, IL-10, and IL-12. They increase in response to oral bacterial infection. IL-6 has both pro-inflammatory and anti-inflammatory activities and is an important cytokine. A study conducted by George et al. in 2018 on children with ECC and ventricular septal defect observed that the mean concentration of IL-6 decreased after one month of treatment [51]. A study by Ribeiro et al. in 2018 found that the average amount of IL-6 in children with ECC is two times higher than in children without tooth decay and the level of vascular endothelial growth factor (another cytokine) in patients with ECC was 63% higher than in the control group. The study also observed that high obesity and high sugar intake were associated with pro-inflammatory cytokines and tooth decay [52].

CD14 is a 55 kDa cell membrane surface glycoprotein that detects bacterial products such as LPS, endotoxins, and peptidoglycans. Human saliva contains higher concentrations of this glycoprotein than serum. CD14 has been shown to regulate the immune response to stimuli. In a study by Biria et al. in 2010, the mean CD14 concentration was higher in patients with ECC than in controls (57.82 and 31.92 ng/mL, respectively). This concentration also decreased to 11.38 ng/mL with the treatment of caries. It seems that increased CD14 concentration can be considered an inflammatory marker and primary immune response during ECC [53].

CD63 is a membrane protein found abundantly on the azurophilic granules' membranes and plays a role in controlling antimicrobial proteins' quality. HNP 1-3 secretion also occurs as a result of neutrophil activation. This activation is made possible by depleting azurophilic granules, which can be observed by examining the membrane of neutrophil granules. A study conducted by Luthfi et al. in 2019 observed that the secretion of HNP 1-3 into saliva in people with ECC increased compared to the control group and the amount of CD63 in the level of salivary neutrophils decreased [37].

Enzymes

Enzymes of various natures (mostly proteins) are essential components of saliva. Excerpts from enzymes studied in previous articles are given below:

Amylase: Amylase in saliva helps digest starch. Since the consumption of starch in the human diet is so widespread, it is crucial to be aware of its association with salivary amylase and its pathogenicity following this interaction to understand the pathogenic properties of foods [55]. Alpha-amylase is the most abundant saliva enzyme and makes up 40-50% of the salivary glands' total protein. This enzyme has several distinct biological functions that may allow or

inhibit tooth decay [17]. Although there is evidence of amylase-producing bacteria in dental plaque, approximately 25% of plaque alpha-amylase activity is dependent on bacterial plaque activity [55]. In the article by Débora Gonçalves de Farias et al. studying several children aged 12 to 47 months and examining them in two groups without caries and with ECC, they concluded that the level of amylase activity in the two groups was not significantly different. They concluded that the two groups' level of amylase activity was not significantly different [36]. Gabriela N. Borghi and colleagues performed experiments on ECC and caries-free children and found that alpha-amylase activity was considerably higher in caries-free children. In contrast to Débora Gonçalves de Farias's results, they observed a significant negative correlation between alpha-amylase activity and caries. They stated that ECC's risk is much higher at alpha-amylase activity less than 122.8 U/mL and biofilm observation in maxillary incisors [56]. Sharma A et al., who studied children with ECC and applied comprehensive dental treatment, found a significant decrease in amylase levels as treatment progressed [31].

Lysozyme: Lysozyme is an enzyme with antimicrobial properties that can activate autolysis in bacteria and destroy the bacterial cell wall [17, 18, 22, 33, 57, 58]. Lysozyme use in toothpaste has been shown to reduce Lactobacillus acidophilus and S. mutans colonies in children with ECC [33]. Studies have shown that lysozyme activity and levels increase in children with SECC; this increase is thought to be due to an increased immune response to caries [31, 41, 59]. One study studied children with ECC during treatment and found similar results, that lysozyme levels decrease as oral health improved. However, in another study, conflicting results were obtained: In the caries-free (CF) children, the amount of lysozyme enzyme was significantly higher than in children with ECC, and no significant difference was observed in lysozyme levels after treatment in ECC children [32].

Lactoperoxidase: This enzyme is one of the antimicrobial factors in saliva [18, 33, 60, 61]. This enzyme is a significant factor in the nonspecific immune response in saliva. Its primary function is to oxidize thiocyanate ions in the presence of hydrogen peroxide to achieve its antimicrobial activity [61]. The presence of lactoperoxidase in toothpaste consumed by children with ECC has shown a significant reduction of Lactobacillus acidophilus and S. mutans colonies [33]. In proportion to the severity of caries, this enzyme's level in saliva increases to be useful in reducing the density of oral bacteria and clearing them from the oral cavity with bactericidal or bacteriostatic effects [60].

Alkaline phosphatase [62]: The exact balance between remineralization and demineralization, which affects hard tooth tissue, depends on salivary calcium, phosphate, and alkaline phosphatase levels [63]. In one study, this enzyme's level in the CF group was significantly lower than in the ECC group [63]. However, another study did not report a significant difference in alkaline phosphatase concentrations in non-stimulatory saliva between the two groups SECC and CF [59].

Carbonic anhydrase VI (CA VI): Studies have shown that CA VI penetrates saliva into the biofilm to facilitate the neutralization of bacterial acids [64]. A study on the relationship between this enzyme and ECC also found that this enzyme's level in children with ECC is significantly higher [56].

Lactate dehydrogenase [65]: This enzyme is present in the cytoplasm of almost all tissues in the body. Its main action is to catalyze the oxidation reaction of lactate to pyruvate. This enzyme is always inside the cell, and its extracellular presence can be a sign of necrosis or tissue damage. This enzyme is also present in the mouth, and the salivary glands produce it. The primary source of this enzyme is the oral epithelium [66].

Glucosyltransferase B (GtfBkill): Glucosyltransferases are bacterial-derived enzymes that play a crucial role in the formation and pathogenesis of caries by synthesizing glucan polymers from sucrose and starch hydrolysis [67].

Immunity and Saliva

1- Antibodies: In general, they play their role in the immune system by agglutinating and inactivating bacteria [30]:

a) IgA (secretory IgA, sIgA): As the most important antimicrobial protein in saliva and the most salivary immunoglobulin type, it is synthesized from B lymphocytes adjacent to the secretory epithelium. After secretion in the interstitial fluid, it is taken up by the acinar cells and ducts of the salivary gland and subsequently secreted into the saliva [18, 30, 60]. IgA levels in children with ECC, SECC, and rampant caries increase significantly [20, 34-36, 38, 59, 69, 70]. One study suggests that in children with SECC, the IgA response to Streptococcus mutans may be reduced [38]. There is a negative correlation between IgA and dental caries,

and with comprehensive dental treatment for children with caries, IgA levels decrease again [31, 68].

b) IgG: It is usually present in saliva from the leakage of gingival groove fluid into the oral cavity [30]. Studies have shown that its level is higher in ECC children [36, 69].

c) IgM: The level of this antibody is similar in ECC and CF groups with no significant difference between them [36].

2- Salivary mucins[1]

3- Lactoferrin[1]

4- Toll-like receptors (TLRs)[1]

5- Histatins[1]

6- Statins[1]

7- Defensins[1]

8- Calprotectin[1]

9- Lysozyme[1,2]

10-Lactoperoxidase[2]

11- Cytokines and interleukins[3]

12- Cathelicidin (LL-37):

This peptide has broad-spectrum bactericidal properties and is significantly associated with dental caries along with defensins [30, 47].

13- Agglutinin: It is an agglutinating glycoprotein of various microbes (especially Streptococcus mutans) that enters saliva from the parotid, submandibular, and sublingual salivary glands [30, 71-75].

14- Cysteine: It is an amino acid that plays a role in immunity by limiting the protease enzyme activity. It originates from the sublingual gland and mostly from the submandibular gland [30].

15- Neutrophils: In saliva, neutrophils are prominent members of the first line of immune cells against pathogenic microbes [37]. Neutrophils perform chemotaxis and phagocytosis as defenses against inflammatory mediators secreted by microbes and kill germs by releasing human antimicrobial peptides (AMP) (HNP 1-3) as an antibacterial mechanism [76]. In ECC, saliva increases the secretion of HNP 1-3 to remove biofilms and reduce tooth decay but reduces CD63 expression on the surface of salivary neutrophils [37, 77]. However, it has been stated that the neutrophil count was not significantly different between ECC and CF children [78].

1. These headings are explained in detail in the Proteins and Peptides section.
2. These headings are explained in detail in the Enzymes section.
3. These headings are explained in detail in the Cytokines section.

Conclusion

ECC-related salivary proteins and peptides are PRPs, salivary mucins, Lactoferrin, immunoglobulins, TLRs, Lysozyme, Histatins, Statherin, Defensins, Calprotectin, and Cytokines. ECC-related enzymes are Amylase, Lysozyme, Lactoperoxidase, Alkaline phosphatase, Carbonic anhydrase VI, Lactate dehydrogenase, and Glucosyltransferase B. Immunity factors affecting ECC include IgA(sIgA), IgG, IgM, Salivary mucins, Lactoferrin, TLRs, Histatins, Statins, Defensins, Calprotectin, Lysozyme, Lactoperoxidase, Cytokines and interleukins, Cathelicidin (LL-37), Agglutinin, Cysteine, and Neutrophils.

Ethical Considerations

Compliance with ethical guidelines

All methods of this study were carried out in accordance with relevant guidelines and regulations.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflicts of interest

The authors declare no conflict of interest.

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