Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Can insoluble polysaccharide concentration in dental plaque, sugar exposure and cariogenic microorganisms predict early childhood caries? A follow-up study

T.M. Parisotto [a], R. Stipp [b], L.K.A. Rodrigues [c], R.O. Mattos-Graner [b], L.S. Costa [d], M. Nobre-dos-Santos [b,*]

[a] Laboratory of Microbiology and Molecular Biology, Sao Francisco University Dental School, Bragança Paulista, SP, Brazil

[b] Department of Pediatric Dentistry, Piracicaba Dental School, University of Campinas, Brazil

[c] Department of Operative Dentistry, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, CE, Brazil

[d] Federal University of Rio de Janeiro State, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Accepted 16 April 2015

Keywords:
Caries prediction
Microorganism
Dental plaque
Sugar exposure

ABSTRACT

Background: Insoluble polysaccharide (IP) has been associated with caries prevalence in young children. However, the power of IP to predict ECC needs to be demonstrated.

Aims: To assess the relationships between early childhood caries (ECC) and extracellular insoluble polysaccharides (IP) in dental plaque, sugar exposure and cariogenic microorganisms.

Design: Visible plaque on maxillary incisors was recorded, followed by caries diagnosis in 65 preschoolers (3–4 years) at baseline and after 1 year. Plaque was collected for mutans streptococci (MS), total microorganism (TM) and lactobacilli (LB) enumerations in selective media, as well as for IP analysis, which was later assessed by colorimetry. Sugar/sucrose exposure was assessed by a diet chart.

Results: Positive correlations were found among the prevalence of caries and MS, TM, LB, solid sucrose and visible dental plaque. Additionally, children with IP concentrations in dental plaque higher than 2.36 μg/mg (odds ratio-OR = 6.8), with visible plaque on maxillary incisors (OR = 4.3), harbouring LB (OR = 13) and exposed to solid sugar more than twice/day (OR = 5) showed higher risk of developing caries ($p < 0.05$).

Conclusion: Extracellular insoluble polysaccharides, solid sugar/sucrose, visible dental plaque and cariogenic microorganisms could predict caries development, partially explaining the ECC pattern.

* *Corresponding author at*: Av. Limeira 901, Piracicaba, SP 13414-903, Brazil. Tel.: +55 19 21065290; fax: +55 19 21065218.
E-mail address: nobre@fop.unicamp.br (M. Nobre-dos-Santos).

http://dx.doi.org/10.1016/j.archoralbio.2015.04.003
0003–9969/

1. Introduction

Early childhood caries (ECC) is a multifactorial disease characterized by a process involving mineral transfer from the tooth to the surrounding environment in children younger than 6 years.[1] The dynamic process of dental caries is intimately related to dental plaque if left undisturbed, which covers tooth surfaces as a tightly adherent layer consisting of bacterial, inorganic and organic components; the so-called biofilm.[2]

With respect to the organic components/matrix, the role played by glucans should be highlighted. Glucosyltransferases, enzymes of bacteria such as *Streptococcus mutans* (*S. mutans*), synthesize glucans, which are extracellular polysaccharides formed from sucrose.[3] Depending on the predominant linkage type alpha 1–3 or alpha 1–6, the glucan or extracellular polysaccharide can be water-insoluble (termed mutan) or soluble (termed dextrans), respectively. The matrix formed mostly by the insoluble glucans, contributes to *S. mutans* tenacious adhesion and accumulation. The ability of *S. mutans* to adhere and accumulate on dental surfaces via glucan production is a very significant virulence factor, which could lead to an increased number of infected tooth sites.[4]

Mutans streptococci are the most common pathogens behind ECC, as demonstrated by many studies involving these bacteria.[5–7] However, recent evidences support that there is a wide diversity of species in ECC, including *Slackia exigua* and *Scardovia wiggsiae*, the latter being a candidate as a newly recognized caries pathogen.[8,9] Young children with ECC are usually colonized by mutans streptococci and often have inappropriate feeding practices, such as frequent consumption of carbohydrates and sweetened fluids.[5,10] Inappropriate feeding habits with a high frequency of sugar consumption provide sucrose, the specific substrate for glucan production. IP has been associated with ECC prevalence in young children. However, the power of IP to predict ECC needs to be demonstrated. Moreover, studying caries development after an identification of IP concentration in dental plaque, enable us to consider the child's response to this factor during the disease process. This way we are not assuming that a certain factor preceded caries development. Thus, the purpose of this study was to assess the relationships between ECC and dental plaque IP, sugar exposure and cariogenic microorganisms.

2. Material and methods

2.1. Ethical considerations

This study was independently reviewed and approved by the Ethical Committee in Research of Piracicaba Dental School/UNICAMP (Protocols #015/2006 and #017/2008) and has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki. The preschools granted permission for the study and the children's parents signed a written positive consent form.

2.2. Sample

We based the sample power calculation (85%; $\alpha = 5\%$) on the study previously performed by our group[6] which used similar methodology and found 8.2 μg/mg of difference and a standard deviation of 19% in the extracellular insoluble polysaccharides concentration in dental plaque when caries free and pit and fissure caries children were compared. Since this was a 1-year longitudinal study, the calculated number (52) was increased by 27% to compensate for subject drop-out rate.

A comfort sample of 65 children, from both genders (girls – 51%, boys – 49%) and aged 3–4 year olds, took part in this study. These children were from low socioeconomic backgrounds and attended public preschools in the urban and fluoridated (0.5–0.8 ppm) area of Itatiba, state of São Paulo, Brazil. Children were excluded from the study if they refused to cooperate with the clinical examinations or if they had systemic diseases, severe fluorosis or dental hypoplasia. In the preschools, the children ate the same meals, stayed a minimum of 4 h/day and had their teeth brushed at least once a day with fluoride-containing dentifrice.

The children were submitted to clinical examinations for caries diagnosis; plaque collection for IP and microbiological analyses; dental plaque assessments of the maxillary incisors; and sugar exposure investigation. In order to determine changes in the prevalence of caries of the studied population, only the clinical examinations for caries diagnosis were repeated after 1 year. Thus, according to the changes (follow up scores – baseline scores) in the prevalence of caries, the children were assigned into 3 groups:

1. Caries arrestment (AR): children who had early carious lesions that arrested (decayed, missing, or filled surfaces-dmfs at baseline = 6.18, dmfs at follow up = 4.27, dmfs change = −1.9) ($n = 11$).
2. Caries free (CF): children who were always free of caries and never showed early carious lesions (ECL), cavitations or fillings (dmfs at baseline = 0, dmfs at follow up = 0, dmfs change = 0) ($n = 19$).
3. Caries active (CA): children with caries who continued to develop carious lesions, being it cavitations or ECL (dmfs at baseline = 10.26, dmfs at follow up = 14.86, dmfs increment = 4.6) ($n = 35$).

2.3. Clinically visible dental plaque recording

The presence or absence of clinically visible plaque on the maxillary incisors was recorded under artificial light, with the child lying on a table. This was performed before teeth cleaning for the clinical examinations.

2.4. Clinical examination

- Early childhood caries diagnosis was made according to the World Health Organization's criteria, with an additional measurement of early carious lesions. For further details on how active early carious lesions were diagnosed please refer to Parisotto et al.[7] Thus, in the study, active white chalky spot lesions were also considered caries.

- Before the clinical examinations, a pedodontist was calibrated by replicate examinations on a random sample of 12 children from the population studied, with different clinical situations, including ECL. Clinical photographic slides combined with theoretical discussions were performed to provide visual examples for the examiner of the examination criteria. The calculated Kappa values, according to the surface levels, were 0.78 (before the first examinations at baseline) and 0.82 (before the second examination after 1 year).
- Dental examinations were performed at the preschools, following the cross-infection control measures and using a focusable flashlight, a ball-ended dental probe, a mouth mirror and gauze to clean and dry the teeth. During the examinations, the child lied on a table while the examiner sat behind the child. The units of evaluation were decayed, missing, or filled surfaces.

2.5. *Insoluble extracellular polysaccharide assay*

Pooled supragingival plaque samples were collected from smooth surfaces with a wooden stick (tooth-pick) in the afternoon period, at least 1 h after food intake and tooth brushing. The dental plaque was placed into microcentrifuge tubes and transported in refrigerated boxes (4 °C) to the Pediatric Dentistry Laboratory at Piracicaba Dental School, where they were kept/stored deep-frozen (−20 °C) until analysis. The dental plaque was dried for 24 h in vacuum over P_2O_5 and its dry weight was obtained using an analytical balance (BelEngineering, Via Venezia Giulia, Monza). For extracellular polysaccharide analysis, 0.1 mL of 0.5 M HCl per 1 mg of plaque was added to the tube and after 3 h of constant agitation at room temperature, an equal volume of TISAB II, pH 5.0 (containing 20 g NaOH/L), was added to the tube as a buffer.[6] The samples were centrifuged (12,000 × *g*) for 3 min, the supernatants were discharged and 1 N NaOH (0.1 mL/mg plaque dry weight) was added to the precipitate. The samples were homogenized for 1 min and agitated for 3 h at room temperature. To the supernatant, 3 volumes of 75% ethanol (0.3 mL/mg plaque dry weight) were added, as ethanol precipitates macromolecules such as insoluble extracellular polysaccharide,[11] and the samples were then incubated at −20 °C overnight and subsequently centrifuged (12,000 × *g*) for 3 min. The precipitate was resuspended in 1 N NaOH (0.1 mL/mg plaque dry weight) and the concentration of the insoluble extracellular polysaccharide was determined by colorimetry.[6,12] The readings expressed in absorbance units were transformed to μg IP/mL through linear regression of the calibration curve (3.12–21.25 μg of glucose). The results are expressed as $\log_{10}$ of micrograms of IP per milligram of plaque dry weight.

2.6. *Cariogenic microorganism enumeration*

Pooled supragingival plaque was collected with a sterilized plastic disposable handle (Greiner, Frickenhausen, Germany) from all smooth surfaces, except for the interior of the cavities. The collection was performed in the afternoon period, at least 1 h after food intake and tooth brushing. A disposable handle was used to standardize the amount of plaque collected,[7] as the collection stopped when the handle opening was full. Dental plaque samples were immediately placed in reduced transport fluid[13] and transported in refrigerated boxes (4 °C) to the Pediatric Dentistry Laboratory at Piracicaba Dental School. Within 6 h, microbiological analysis was performed using serial dilutions (10^1–10^7) with 0.9% saline solution. Each dilution was placed in triplicate in three different media: (1) Mitis salivarius agar (Difco, Sparks, MD) with 0.2 units/mL of bacitracin (Sigma, Poole, UK) for mutans streptococci (MS); (2) Rogosa agar (Difco, Sparks, MD) supplemented with 0.13% glacial acetic acid for lactobacilli (LB); and (3) Brain Heart Infusion agar (Difco, Sparks, MD) with 5% defibrinated sheep blood to assess total microorganisms (TM). The plates were incubated for 24 h at 37 °C in a candle-extinguishing jar with 5–10% CO_2 atmosphere, except for the Rogosa agar plates, which were incubated for 48 h.[7] The colony-forming units (CFU) were enumerated using a stereomicroscope and the results are expressed as $\log_{10}$ of CFU/mL.

2.7. *Sugar exposure evaluation*

During the workweek, a diet chart was filled for 3 consecutive days.[7] On this chart, mothers and health professionals included the content of all meals and snacks, as well as the time of the day when the children ate and drank. This chart looks like a table: in the first column, it was written the time of the day of food ingestion, in the second column it was written what was eaten/drunk, for example: 8 a.m. – a couple of milk with chocolate and vanilla cookies, 9 a.m. – orange juice, 12 p.m. – rice, bone, chicken and soda, 3 p.m. – papaya with sugar, 6 p.m. – sandwich of cheese and tomato and soda, 8 p.m. – baby bottle with milk and chocolate. The daily frequency means for the total (solid + liquid) sugar and total (solid + liquid) sucrose exposures, as well as solid sucrose, solid sugar and liquid sucrose exposures, were calculated using this chart, by dividing the total number of sugar/sucrose exposure during the 3 days by the number of days (3). The liquids consumed in the baby bottle were also reported in this chart and considered as liquid sugar exposure.

2.8. *Statistical analysis*

The statistical analyses were performed using the Statistical Analysis System-SAS version 9.1.3 with an alpha level of 0.05 and a 95% confidence interval The correlation between the prevalence of caries (dmfs) and each of the tested variables (concentration of IP in dental plaque, MS counts, TM counts, presence of LB in dental plaque, clinically visible dental plaque on the maxillary incisors and the mean frequencies of solid sugar, solid sucrose, liquid sucrose, total sugar and total sucrose consumption) was assessed using Spearman correlation, as the data showed a non normal distribution. Additionally, chi-square tests, followed by multiple logistic regressions, were performed in order to identify the variables that could explain the development of caries after 1 year. In the multivariate modelling analyses, the dependent variables were children who developed caries after 1 year (CA group) and children who did not (CF and AR groups). The independent variables were: concentration of IP in dental plaque, microbiological components of dental plaque (MS, TM and LB), clinically visible dental plaque and the mean frequencies of

sugar/sucrose consumption. All independent variables were dichotomized based on their median values. The chi-square test was used to select the variables with p values ≤ 0.2, which would enter the stepwise models. In stepwise models, the variables were included one by one, according to the p-value rank in the chi-square test. Also in this process, it was verified the possibility of associations between the independent variables. The models fitting were assessed by the Hosmer and Lemeshow test.

3. Results

As part of a larger clinical study, 188 preschool children (3–4 years old at baseline) were evaluated for dental caries at baseline and after a 1-year follow-up period. After excluding the ones who did not show up/did not cooperate with the dental plaque collections at baseline, children were selected for the present study based on the following criteria: children who had early carious lesions that arrested ($n = 11$), children who were always free caries ($n = 19$) and children with caries who continued to develop carious lesions ($n = 35$). Thus, the final sample size was 65 children. The groups were formed according to changes in caries activity as described above because we wanted to investigate if the evaluated parameters at baseline could be able to explain the changes in caries activity after a 1 year period.

The results shown in Table 3 and bellow demonstrates that several parameters evaluated on baseline could explain the changes in caries activity on follow-up.

The correlations between caries prevalence and sugar/sucrose consumption frequency, cariogenic microorganisms and plaque IP concentrations are shown in Table 1. This table reveals positive significant correlations between caries prevalence in preschool children and MS, TM, LB levels, daily solid sucrose intake and dental plaque presence on the maxillary incisors.

Table 2 displays the variables that reached p values ≤ 0.2, comparing CF versus the CA group as well as CA versus the AR group. These variables were selected for the models shown in Table 3, to determine which investigated parameters at baseline, could explain the changes in caries activity of children of different groups.

Table 1 – Spearman correlation coefficients (r) and probabilities of statistical significance (p) between caries and the variables analyzed – baseline conditions.

Variables	dmf-s	
	r	p
Polysaccharide	−0.013	0.918
Mutans streptococci	0.304	0.014*
Total microorganisms	0.339	0.006*
Lactobacilli	0.435	<0.001*
Liquid sucrose	−0.113	0.372
Solid sucrose	0.254	0.044*
Solid sugar	0.174	0.166
Total sucrose	−0.014	0.912
Total sugar	−0.003	0.979
Presence of dental plaque	0.258	0.038*

dmfs, decayed, missing or filled surfaces. The mean and standard error of dmfs was 6.6 ± 1.26.
* Significant results evaluated by Spearman correlation.

According to Model 1 (AR × CA group), children with dental plaque IP concentrations higher than 2.36 μg/mg were 6.8 times more likely to develop caries than children with lower concentrations. Moreover, preschool children harbouring LB were 13 times more likely to develop caries than children harbouring undetectable levels of these bacteria ($p < 0.05$) (Table 3). According to Model 2 (CA × CF group), children who had clinically visible dental plaque on the maxillary incisors were 4.3 times more likely to develop caries in 1 year than children who did not. In addition, we found that children exposed to solid sugar more than twice a day were 5 times more likely to develop carious lesions than those with lower sugar exposure (Table 3).

4. Discussion

This study of preschool children with a high prevalence of caries (67.7%) and low socioeconomic backgrounds showed for the first time, at least to our knowledge, that dental plaque IP is able to predict ECC development. Dental plaque IP concentration was associated with a higher risk to develop caries, which is in line with Nobre dos Santos et al.[6] who demonstrated that plaque from preschoolers with caries had also significantly higher IP. Similarly, Bayrak et al.[14] also found that IP concentration was significantly higher in children with caries. However, both studies did not evaluate caries development. It is important to highlight that dental plaque rich in IP has an increased porosity, facilitating the transport of bacterial substrates, such as sugar, acids and ions.[2,15] Thus, the higher the IP plaque content, the more tenacious adhesion to the tooth surface and the larger the volume of the matrix diffusion pathways, which increases the extent of acidification within the biofilm[2,15] favouring caries development.[16]

One should keep in mind that sucrose is a specific substrate for IP production[17] and children with caries are exposed to sucrose more frequently and consequently have higher levels of IP.[6] In this respect, our research found a positive correlation between solid sucrose and caries prevalence, which is in line with previous studies.[7,18,19] One possible explanation for this finding is that the solid physical form of sucrose/sugar needs to be triturated by teeth, and this way it can be retained on the tooth surfaces for a prolonged period of time[20,21] in comparison with the liquid form, for example, contributing to the caries process.

Regarding the presence of dental plaque on the maxillary incisors, our data displayed a positive correlation between caries prevalence and this clinical parameter. These results corroborate the findings of Alaluusua and Malmivirta[22] and Leroy et al.[23]. Notably, the accumulation of clinically visible dental plaque is not only related to oral hygiene status[7] but more importantly reflects the sucrose consumption, which is related to IP synthesis by GTF.[24] These enzymes are very important for the virulence of *S. mutans*.[25]

Considering the cariogenic microorganisms, the roles played by the acidogenic and aciduric bacteria are also relevant. In this respect, the present study found a positive significant correlation among caries prevalence, MS counts

Table 2 – Bivariate analysis of the relationships between caries development and the related variables at baseline.

Variables	AR × CA		CF × CA	
	n (%)		*n* (%)	
Polysaccharide[a] (μg/mg of plaque)	$p = 0.082$		$p = 0.933$	
>2.36	2 (10)	18 (90)	10 (36)	18 (64)
≤2.36	9 (35)	17 (65)	9 (35)	17 (65)
Mutans streptococci[a]	$p = 0.749$		$p = 0.315$	
>6.70	5 (26)	14 (74)	5 (26)	14 (74)
≤6.70	6 (22)	21 (78)	14 (40)	21 (60)
Total microorganisms[1]	$p = 0.460$		$p = 0.346$	
>9.11	9 (28)	23 (72)	10 (30)	23 (70)
≤9.11	2 (14)	12 (86)	9 (43)	12 (57)
Lactobacilli	$p = 0.032$*		NA	
Absent	10 (36)	18 (64)	19 (51)	18 (49)
Present	1 (6)	17 (94)	NA	17 (100)
Solid sugar consumption frequency	$p = 0.307$		$p = 0.073$	
>2	4 (17)	20 (83)	6 (23)	20 (77)
≤2	7 (32)	15 (68)	13 (46)	15 (54)
Solid sucrose consumption frequency	$p = 1.000$		$p = 0.229$	
>1	4 (21)	15 (79)	5 (25)	15 (75)
≤1	7 (26)	20 (74)	14 (41)	20 (59)
Liquid sucrose consumption frequency	$p = 0.396$		$p = 0.208$	
>4	6 (30)	14 (70)	11 (44)	14 (56)
≤4	5 (19)	21 (81)	8 (28)	21 (72)
Total sugar consumption frequency	$p = 0.857$		$p = 0.776$	
>6	5 (23)	17 (77)	10 (18)	17 (82)
≤6	6 (25)	18 (75)	9 (33)	18 (67)
Total sucrose consumption frequency	$p = 0.988$		$p = 0.907$	
>5	5 (18)	16 (82)	9 (18)	16 (82)
≤5	6 (24)	19 (76)	10 (34)	19 (66)
Dental biofilm	$p = 1.000$		$p = 0.048$*	
Absent	2 (18)	9 (82)	10 (53)	9 (47)
Present	9 (26)	26 (74)	9 (26)	26 (74)

CF, caries-free group; AR, caries arrestment group; CA, caries development group; NA, not available

* Significant results were evaluated using the chi-square test or Fisher's exact test ($\alpha = 0.05$). Fisher's exact test was applied when the frequencies were smaller than 5.

[a] Values expressed by $\log_{10}$.

Table 3 – Risk factors for early childhood caries development.

Variables	Caries development		OR (95%CI)	*p*-Value
	No (%)	Yes (%)		
Model 1: CA × AR[a]				
Lactobacilli				
Present	10 (36)	18 (64)	13.0 (1.39–121.99)	0.003
Absent	1 (6)	17 (94)	1.00	
Polysaccharide (μg/mg of plaque)				
>2.36	2 (10)	18 (90)	6.8 (1.15–40.30)	
≤2.36	9 (35)	17 (65)	1.00	
Model 2: CA × CF[b]				
Dental plaque				
Present	9 (26)	26 (74)	4.3 (1.12–16.37)	0.007
Absent	10 (53)	9 (47)	1.00	
Solid sugar consumption frequency				
>2	6 (23)	20 (77)	5.0 (1.19–21.02)	
≤2	13 (46)	15 (54)	1.00	

NA, not available; OR, odds ratio; CI, confidence interval.

[a] Likelihood ratio test = 11.81 (2 degrees of freedom); Hosmer and Lemeshow: $p = 0.95$.

[b] Adjusted by liquid sugar exposure frequency. Likelihood ratio test = 12.05 (3 degrees of freedom). Hosmer and Lemeshow: $p = 0.72$.

and the presence of LB. This is in accordance with previous studies.[26,27] It is worth noting that the levels of not only MS and LB but also TM reached statistical significance in the correlation tests. This may suggest that bacteria other than MS and LB, such as *Actinomyces*,[28,29] *S. exigua* and *S. wiggsiae*,[8,9] could also influence the carious process in young children, but this subject was not studied here. In addition, *Actinomyces* species and non-mutans streptococci microorganisms could be bound by Gtf.[17] Glycosyltransferases promote the binding of *S. mutans* to other organisms and concomitantly provide additional structural support for microcolony development.[30]

The strength point of the present research is that it has examined the in vivo caries development in a 1-year follow-up period based on baseline parameters: MS, LB, TM and IP levels, as well as daily sugar/sucrose exposure. Thus, it has considered the child's response to these factors during the disease process. Moreover, this kind of study is of great clinical relevance to dentists, as additional information about caries development in primary teeth contributes to the understanding of the disease process in young children and to target specific preventive measures. However, a limitation of the study is that we could not collect dental plaque at follow-up in order to determine the variables behaviour. Thus, further studies are encouraged.

In conclusion, extracellular insoluble polysaccharides, solid sugar/sucrose, visible dental plaque and cariogenic microorganisms could predict caries development, partially explaining the ECC process.

Why this paper is important to pediatric dentists?

- Additional information about caries development in primary teeth contributes to the understanding of the disease process in young children, which is very helpful for the guidance of preventive measures.

Funding

This study was supported by FAPESP (2008/09510-3) and CNPq (480401/2008-0) grants.

Competing interests

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Ethical approval

The protocol was approved by the local Bioethics Committee of Piracicaba Dental School, University of Campinas, Piracicaba, SP, Brazil (Protocols #015/2006 and #017/2008).

Acknowledgements

We specially thank the volunteers for participating in this research. We also thank the Secretary of Education and Health of the city of Itatiba – SP/Brazil for collaborating with this research. We acknowledge funds by FAPESP (2008/09510-3) and CNPq (480401/2008-0) grants awarded to Prof. Marinês Nobre dos Santos.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2015.04.003.

REFERENCES

1. American Academy of Pediatric Dentistry. *Policy on early childhood caries (ECC): classifications, consequences, and preventive strategies. Reference manual*. 2011.
2. Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. The role of sucrose in cariogenic dental biofilm formation-new insight. *J Dent Res* 2006;**85**(10):878–87.
3. Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res* 2011;**45**(1):69–86.
4. Mattos-Graner RO, Smith DJ, King WF, Mayer MP. Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children. *J Dent Res* 2000;**79**(6):1371–7.
5. Mattos-Graner RO, Zelante F, Line RC, Mayer MP. Association between caries prevalence and clinical, microbiological and dietary variables in 1.0 to 2.5-year-old Brazilian children. *Caries Res* 1998;**32**(5):319–23.
6. Nobre dos Santos M, Melo dos Santos L, Francisco SB, Cury JA. Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. *Caries Res* 2002;**36**(5):347–52.
7. Parisotto TM, Steiner-Oliveira C, Duque C, Peres RCR, Rodrigues LKA, Nobre-dos-Santos M. Relationship among microbiological composition and presence of dental plaque, sugar exposure, social factors and different stages of early childhood caries. *Arch Oral Biol* 2010;**55**(5):365–73.
8. Tanner AC, Kent Jr RL, Holgerson PL, Hughes CV, Loo CY, Kanasi E, et al. Microbiota of severe early childhood caries before and after therapy. *J Dent Res* 2011;**90**(11):1298–305.
9. Tanner AC, Mathney JM, Kent RL, Chalmers NI, Hughes CV, Loo CY, et al. Cultivable anaerobic microbiota of severe early childhood caries. *J Clin Microbiol* 2011;**49**(4):1464–74.
10. Hallett KB, O'Rourke PK. Pattern and severity of early childhood caries. *Community Dent Oral Epidemiol* 2006;**34**(1):25–35.
11. Ccahuana-Vásquez RA, Tabchoury CP, Tenuta LM, Del Bel Cury AA, Vale GC, Cury JA. Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride. *Caries Res* 2007;**41**(1):9–15.
12. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;**28**:350–6.
13. Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. *Appl Microbiol* 1972;**24**(4):638–44.
14. Bayrak S, Okte Z, Fidanci UR. Relationship between caries and dental plaque composition. *Am J Dent* 2011;**24**(1):45–8.

15. van Houte J. Role of microorganisms in caries etiology. *J Dent Res* 1994;**73**(3):672–81.
16. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986;**50**(4):353–80.
17. Vacca-Smith AM, Bowen WH. Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. *Arch Oral Biol* 1998;**43**(2):103–10.
18. Tsai AI, Chen CY, Li LA, Hsiang CL, Hsu KH. Risk indicators for early childhood caries in Taiwan. *Community Dent Oral Epidemiol* 2006;**34**(6):437–45.
19. Johansson I, Holgerson PL, Kressin NR, Nunn ME, Tanner AC. Snacking habits and caries in young children. *Caries Res* 2010;**44**(5):421–30.
20. Gustafsson BE. The Vipeholm dental caries study: survey of the literature on carbohydrates and dental caries. *Acta Odontol Scand* 1954;**11**(3–4):207–31.
21. Touger-Decker R, van Loveren C. Sugars and dental caries. *Am J Clin Nutr* 2003;**78**(4):881S–92S.
22. Alaluusua S, Malmivirta R. Early plaque accumulation – a sign for caries risk in young children. *Community Dent Oral Epidemiol* 1994;**22**(5 Pt 1):273–6.
23. Leroy R, Bogaerts K, Martens L, Declerck D. Risk factors for caries incidence in a cohort of Flemish preschool children. *Clin Oral Investig* 2012;**16**(3):805–12.
24. Smith DJ, Shoushtari B, Heschel RL, King WF, Taubman MA. Immunogenicity and protective immunity induced by synthetic peptides associated with a catalytic subdomain of mutans group streptococcal glucosyltransferase. *Infect Immun* 1997;**65**(11):4424–30.
25. Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK. Role of the *Streptococcus mutans* gtf genes in caries induction in the specific-pathogen-free rat model. *Infect Immun* 1993;**61**(9):3811–7.
26. Ramos-Gomez FJ, Weintraub JA, Gansky SA, Hoover CI, Featherstone JD. Bacterial, behavioral and environmental factors associated with early childhood caries. *J Clin Pediatr Dent* 2002;**26**(2):165–73.
27. Parisotto TM, King WF, Duque C, Mattos-Graner RO, Steiner-Oliveira C, Nobre-dos-Santos M. Immunological and microbiologic changes during caries development in young children. *Caries Res* 2011;**45**(4):377–85.
28. Milnes AR, Bowden GH. The microflora associated with developing lesions of nursing caries. *Caries Res* 1985;**19**(4):289–97.
29. Marchant S, Brailsford SR, Twomey AC, Roberts GJ, Beighton D. The predominant microflora of nursing caries lesions. *Caries Res* 2001;**35**(6):397–406.
30. Koo H, Xiao J, Klein MI, Jeon JG. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *J Bacteriol* 2010;**192**(12):3024–32.