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Oral microflora in preschool children attending a fluoride varnish program: a cross-sectional study

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Abstract

Background: To compare the oral microflora in preschool children attending a fluoride varnish program with a reference group receiving a standard oral health program without fluoride varnish applications. A second aim was to relate the microbial composition to the caries prevalence.

Methods: Five hundred seven 3-year-old children were enrolled from a cohort of 3403 preschool children taking part in a community based oral health project. Two hundred sixty-three of them had attended caries-preventive program with semi-annual applications of a fluoride varnish since the age of 1 year (test group) while 237 had received standard preventive care (reference group). Oral samples were collected with a sterile swab and analysed with checkerboard DNA-DNA hybridization using 12 pre-determined bacterial probes. Caries and background data were collected from clinical examinations and questionnaires.

Results: Gram-positive streptococci (*S. intermedius*, *S. salivarius*, *S. oralis*) were most frequently detected and displayed the highest counts in both groups. There were no significant differences between the groups concerning prevalence of any of the selected bacterial strains except for *S. oralis* that occurred less frequently in the reference group. In children with caries, *V. parvula* were significantly more common ($p < 0.05$) while strains of Lactobacillus, Bifidobacterium and Neisseria were more prevalent among the caries-free children ($p < 0.05$).

Conclusions: A 2-year community program with semi-annual fluoride varnish applications did not seem to significantly influence the oral microflora in preschool children.

Trial registration: www.controlled-trials.com (ISRCTN35086887) 20131216 'retrospectively registered'.

Keywords: Bacteria, Caries prevention, Community health program, Fluoride varnish, Preschool children

Background

Early childhood caries (ECC) is a public health problem associated with impaired oral health-related quality of life and high costs for families as well as for society. There are numerous biological, medical, behavioural, psychological, cultural, and life-style factors associated with the disease but in spite of extensive research over the years, there are knowledge gaps concerning its prevention and management [1]. Fluoridated toothpaste and fluoride varnish are currently the most effective

strategies to prevent ECC [2]. Therefore, such community oral health programs have been launched for vulnerable children with increased caries risk [3–6]. Little is however known on the long-term impact of such fluoride-based programs on oral bacteria. It is generally thought that a low-fluoride exposure can interact with the consecutive cycles of de- and remineralization while higher fluoride concentrations may hamper bacterial acid production [7, 8]. A reduced pH-drop would favour the growth of health-associated bacteria that prefer pH values around neutrality rather than the acid-producing and acid-tolerating species associated with caries [9]. Fluoride varnish contains typically 5% sodium fluoride (22,600 ppm F) and after application, fluoride is slowly released to the oral biofilm over a period of several

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weeks. Since it is not fully clear whether or not this sustained fluoride release has an influence on the bacterial composition of biofilm, it was thought of interest to apply this research question in a current community oral health project [10]. The primary aim of the present study was to compare the oral microflora in preschool children participating in an extended preventive program with semi-annual fluoride varnish applications with children receiving a standard program with no varnish applications. The null hypothesis was that no differences would be obtained between the groups. A secondary aim was to investigate the microbial composition in children who had developed early childhood caries at the age of 3 years versus those that remained caries free.

Methods

This cross-sectional study was carried out as a part of a cluster-randomized controlled field trial aiming to evaluate the effectiveness of fluoride varnish applications on caries development in toddlers living in multicultural areas in greater Stockholm, Sweden [10]. The original trial was designed with two parallel arms that compared children receiving a standard oral health program (reference group) with children who had the same standard oral health program supplemented with semi-annual fluoride varnish applications (test group). The study comprised 23 public dental clinics involving 3403 toddlers and the intervention was carried out between 1- and 3-years of age. From the main study, 7 public dental clinics representing both programs were selected by convenience and invited to the present investigation. 507 children (263 from the test group and 244 from the reference group) were consecutively enrolled in connection with the scheduled 36-month examination. All parents gave their written informed consent after verbal and written information and an interpreter was used when necessary. The project was approved by the Regional Ethic Committee (EPN; no 2013/143–32).

Intervention

The children in the test group received topical applications of fluoride varnish (Duraphat®, 22.5 mg of fluoride per ml, Colgate-Palmolive) every 6th month from 1 year of age (totally 5 applications) as a supplement to a standard oral health program. The standard program (reference group) consisted of yearly dental examinations with tooth-brushing instructions, fluoride toothpaste enforcement and dietary counselling. The children of the reference group did not receive any fluoride varnish applications. At the end of each dental visit, all participating children were given a free tube of fluoride toothpaste (1100 ppm) and a soft toothbrush.

Sample collection

The samples were collected with aid of a sterile swab that was rotated inside the lips to capture saliva and supra-gingival plaque. The swab was then transferred to a test tube coded with a unique number and immediately sent by surface mail to the laboratory. The samples were stored frozen at -20°C until further processing. The samplings were performed between March and November 2013 and the samples were analysed within 3–15 months (median 12 months) after collection.

Microbial analyses

The samples were processed with the checkerboard DNA-DNA hybridization technology as described by Wall-Manning et al. (2002) [11]. DNA was extracted with mutanolysin and lysozyme as previously described [12] and the DNA quality was evaluated from the UV extinction at 260 nm using NanoDrop 2000 (Thermo Scientific, Fisher Scientific, Gothenburg, Sweden). Whole genomic DNA probes were prepared from a panel of 12 bacterial species with strain designation according to the Culture Collection, University of Gothenburg (CCUG), or Oral Microbiology, Gothenburg, Sweden (OMGS): *Actinomyces odontolyticus* OMGS G67; *Bifidobacterium dentium* OMGS G174; *Capnocytophaga ochracea* OMGS 1233; *Haemophilus parainfluenzae* CCUG 12836; *Lactobacillus casei* OMGS 3184; *Lactobacillus salivarius* OMGS 3830; *Neisseria subflava* CCUG 23930; *Streptococcus intermedius* CCUG 17827; *Streptococcus oralis* OMGS 2470; *Streptococcus mutans* OMGS 2482; *Streptococcus salivarius* OMGS 2473; *Veillonella parvula* OMGS G186. The detection level was $>10^4$ cells per mL sample.

Caries data

Caries data was extracted from the clinical examination at 3 years of age performed by the child's regular dental team as previously described [10]. The International Caries Detection and Assessment System (ICDAS II) was used according to Ismail and co-workers [13]. Furthermore, data from a structured parental interview concerning socioeconomic conditions, the child's general health and oral health related habits were registered.

Dropouts

Data from seven children were lost due to technical errors and these children were excluded from the final material.

Statistical analysis

All data was processed with the IBM SPSS software (version 22.0, Chicago, IL USA). Differences in percentage distribution of bacterial growth between the groups were calculated with chi-square tests. We considered a *p*-value less than 0.05 as statistically significant. A power

estimation based on the prevalence of *S. mutans* was made since systematic reviews have highlighted this bacterium as a strong biomarker for caries development in early childhood [14, 15]. We anticipated that a 50% difference in the prevalence of high counts ($\geq 10^5$ cells) would be of “clinical importance”. With α and β set at 0.05 and 0.2 respectively, it was calculated that 170 participants in each group should be recruited to get sufficient power to limit the risk of Type I and Type II errors.

Results

The background characteristics and caries data of the participating children is summarized in Table 1. The proportion of parents with immigrant background (other language than Swedish at home) was significantly higher in the test group ($p < 0.05$). Likewise, one or both parents were more often smoking on a daily basis in the test group. The children of the test group displayed also a higher prevalence of caries than the reference group.

The microbial data is presented in Table 2. Gram positive streptococci dominated the samples in both groups; there were no significant differences in the prevalence of *S. intermedius*, *S. salivarius* and *S. mutans* between the groups while *S. oralis* seemed to occur less frequently in the reference group ($p < 0.05$). Among the non-streptococci, *V. parvula*, *L. salivarius*, *B. dentium* and *H. parainfluenzae* were frequently harboured in both groups albeit in low counts. High counts were commonly displayed for *S. salivarius* and *N. subflava*. The results from the checkerboard hybridisation in relation to caries prevalence are shown in Table 3. The prevalence (ICDAS 1–6) for the entire study group ($n = 500$) was 23.4% and we found a more frequent occurrence of

Table 1 Study group characteristics and caries prevalence at 3 years of age

Variable	Fluoride (Test) <i>n</i> = 263		<i>P</i>
	Standard (Reference) <i>n</i> = 237		
Girls/boys	55/45%	53/47%	NS
Chronic disease (yes)	11%	10%	NS
Immigrant background ^a (yes)	86%	76%	$p < 0.05$
Mother's education (<9 years)	22%	21%	NS
Family income (<20,000 SEK/month)	46%	37%	NS
Mother and/or father smoking (yes)	42%	34%	$p < 0.05$
Candy (>1 week)	37%	32%	NS
Sweet drinks (>2 times per day)	6%	2%	$p < 0.05$
Daily tooth brushing (yes)	91%	94%	NS
ICDAS 1–6 (all lesions)	29%	17%	$p < 0.05$
ICDAS 3–6 (moderate/extensive lesions)	17%	8%	$p < 0.05$

^aOther language than Swedish at home, NS not statistically significant

Table 2 Prevalence ($\geq 10^4$ cells) and high counts ($> 10^5$ cells) of selected oral bacteria in the two intervention groups

Strain	Fluoride (Test) <i>n</i> = 263		Standard (Reference) <i>n</i> = 237	
	$\geq 10^4$	$> 10^5$	$\geq 10^4$	$> 10^5$
<i>Actinomyces odontolyticus</i>	21	0	26	3
<i>Bifidobacterium dentium</i>	39	0	34	0
<i>Capnocytophaga ochracea</i>	2	0	4	0
<i>Haemophilus parainfluenzae</i>	38	0	33	0
<i>Lactobacillus casei</i>	3	0	4	0
<i>Lactobacillus salivarius</i>	42	2	39	3
<i>Neisseria subflava</i>	21	7	28	8
<i>Streptococcus intermedius</i>	83	7	83	5
<i>Streptococcus mutans</i>	66	4	66	4
<i>Streptococcus oralis</i>	89	3	72 ^a	5
<i>Streptococcus salivarius</i>	79	15	75	11
<i>Veillonella parvula</i>	66	5	61	7

^aSignificantly lower than the test group ($p < 0.05$; chi-square test)
Values in table denote percentage

V. parvula among children with caries compared with those that were caries free (ICDAS 0). On the other hand, the *B. dentium*, *L. casei*, *L. salivarius* and *N. subflava* strains were more frequently detected from the caries-free children ($p < 0.05$). No differences were obtained concerning *A. odontolyticus* or any of the streptococci strains.

Discussion

The main finding of this study was that the oral microflora appeared basically unchanged following a supplementary

Table 3 Prevalence ($\geq 10^4$ cells) and high counts ($> 10^5$ cells) of selected oral bacteria in relation to caries

Strain	Caries (ICDAS 1–6) <i>n</i> = 117		Caries-free (ICDAS 0) <i>n</i> = 383	
	$\geq 10^4$	$> 10^5$	$\geq 10^4$	$> 10^5$
<i>Actinomyces odontolyticus</i>	17	2	26	2
<i>Bifidobacterium dentium</i>	23	3	40 ^a	2
<i>Capnocytophaga ochracea</i>	2	0	2	0
<i>Haemophilus parainfluenzae</i>	28	2	38	1
<i>Lactobacillus casei</i>	0	0	4 ^a	0
<i>Lactobacillus salivarius</i>	31	2	44 ^a	3
<i>Neisseria subflava</i>	11	2	30 ^a	9 ^a
<i>Streptococcus intermedius</i>	80	6	84	6
<i>Streptococcus mutans</i>	63	3	70	5
<i>Streptococcus oralis</i>	75	4	82	4
<i>Streptococcus salivarius</i>	71	11	79	14
<i>Veillonella parvula</i>	56 ^b	5	27	6

^aSignificantly higher compared with children with caries ($p < 0.05$; chi-square test)

^bSignificantly higher compared with caries-free children ($p < 0.05$; chi-square test)
Values in table denote percentage

semi-annual varnish program in a group of vulnerable preschool children. Thus, the null hypothesis could not be rejected. It is likely that the negative results were a consequence of, and mirrored the absent benefits of the fluoride varnish program on caries increment as reported from the main project [10] and from similar studies in young children [16, 17]. A second explanation could be that the semi-annual schedule of varnish applications was too infrequent to make a difference. It is suggested that fluoride in concentrations found in dental plaque may act as a metabolic inhibitor and lower the bacterial acid production in the oral biofilm but the clinical implications are still unclear [7]. Many bacteria are highly susceptible to fluoride in planktonic stages or simple biofilm models but this may not be the case in complex biofilms communities in vivo [9, 18]. Chau and co-workers [19] have recently shown that fluoride varnish applications can affect biofilm formation and acidogenicity but these effects were strongly reduced by time and biofilm age. Notably, the present samplings were performed approximately 6 months after the latest varnish application. A third but less plausible explanation could be that the daily exposure of fluoride from tooth paste in both groups may have obscured the results. Therefore, further clinical research to elucidate the impact of fluoride on bacterial physiology and adaptation seems warranted.

Recent findings from molecular-based studies have confirmed the importance of mutans streptococci, Actinomyces and Veillonella for the development of early and severe childhood caries [20–22]. We were able to verify a frequent recovery of *V. parvula* but found no differences in the *S. mutans* or *A. odontolyticus* counts between caries-free children and those with initial, moderate and extensive lesions. Interestingly, we noted a higher prevalence of Lactobacillus, Neisseria and Bifidobacterium species among children free from caries. These findings support the concept that caries is more due to absence or under-abundance of beneficial bacteria rather than linked to specific pathogens [23, 24]. Thus, future research should focus on functional rather than phylogenetic diversity in order to fully understand host-microbiome interactions. An illustration of this complexity is *L. rhamnosus* that can be linked to both mineral loss [25] and caries prevention [26]. The relatively frequent detection of Neisseria and Haemophilus in our samples was likely a reflection of the low age of the present subjects and the oral sampling technique representing more oral structures than just dental plaque.

The obtained results must however be regarded with caution. First of all, the study groups constituted a convenience sample from a major project and the groups were unfortunately not fully balanced concerning socio-economy and caries. A previous study has established a relationship between social deprivation and the isolation

frequencies of caries-associated microorganisms, such as lactobacilli and mutans streptococci, in 3- and 4-year-old-children [27]. However, when the data was adjusted for the imbalance between the groups concerning immigrant status, parental smoking, sweet drinks and caries, the bacterial outcome remained unchanged. Secondly, the checkerboard DNA-DNA hybridization technique has its strengths and shortcomings; the main advantage is that the method permits enumeration of large numbers of species in a large numbers of samples and hence considered as a useful tool for the enumeration of bacterial species in complex microbial systems [28]. Among the limitations, possible cross-reactions and varying reproducibility for different strains has been discussed [29]. In fact, the probes used were not particularly specific and did not differentiate between genotypes of the same species. For example, among some Gram-positive genera (e.g. Actinomyces, Lactobacillus and Streptococcus) there was a risk for cross-reactions between closely related species. Such cross-reactions between the probes were checked prior to the study and the selection of the present twelve DNA probes was done in order to get as few cross-reactions as possible. Furthermore, the selected species represented early colonizers of the oral cavity (mucosal membranes and teeth) and therefore considered suitable for this low age group. The methodological limitations made us however to present data according as proportions over the thresholds 10^4 and 10^5 cells respectively [29]. We used the swab-technique to collect oral samples rather than dental samples for practical reasons; because of the low age of the subjects, it was not possible to collect enough plaque and saliva for separate analysis. One should also keep in mind that the present assay only mirrored 12 selected species out of the over 600 prevalent taxa at species level that are reported from the oral cavity [30].

Conclusions

Within the limitations of the present study, the findings suggested that the composition of the oral microflora did not differ in preschool children involved in a 2-year community fluoride varnish program when compared with those attending a standard oral health program without fluoride varnish applications.

Abbreviations

CCUG: Culture Collection University of Gothenburg; ECC: Early childhood caries; ICDAS: International Caries Detection and Assessment System; OMGS: Oral microbiology Gothenburg Sweden; Ppm: Parts per million; SEK: Swedish Crowns

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Availability of data and materials

The whole dataset generated and analyzed during the current study are available in the Dryad Digital Repository, <http://dx.doi.org/10.5061/dryad.7v387>.

Authors' contributions

MA - contributed to design, acquisition and coordination, managed database, performed statistical analyses and drafted the manuscript. MG - contributed to conception and design, acquisition and critically revised manuscript. GD - contributed to conception and design and critically revised manuscript. GD - contributed to conception and design, microbial analyses and critically revised manuscript. ST - contributed to conception and design, performed statistical analyses drafted manuscript and critically revised manuscript. All read and approved the final manuscript.

Competing interests

The authors declare that they have no proprietary, financial, professional or other personal interests of any nature or kind in any product, service, or company that could be construed as influencing the position presented in this paper.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by Regional Ethic Committee (EPN; no 2013/143–32), Stockholm, Sweden. Each parent was provided with a written informed consent. All the information including personal details and clinical information that have been used for this study were de-identified.

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References

- García R, Borrelli B, Dhar V, Douglass J, Gomez FR, Hieftje K, Horowitz A, Li Y, Ng MW, Twetman S, Tinanoff N. Progress in early childhood caries and opportunities in research, policy, and clinical Management. *Pediatr Dent*. 2015;37:294–9.
- Twetman S, Dhar V. Evidence of effectiveness of current therapies to prevent and treat early childhood caries. *Pediatr Dent*. 2015;37:246–53.
- Slade GD, Baillie RS, Roberts-Thomson K, Leach AJ, Raye I, Endean C, Simmons B, Morris P. Effect of health promotion and fluoride varnish on dental caries among Australian Aboriginal children: results from a community-randomized controlled trial. *Community Dent Oral Epidemiol*. 2011;39:29–43.
- Ramos-Gomez FJ, Gansky SA, Featherstone JD, Jue B, Gonzalez-Beristain R, Santo W, Martinez E, Weintraub JA. Mother and youth access (MAYA) maternal chlorhexidine, counselling and paediatric fluoride varnish randomized clinical trial to prevent early childhood caries. *Int J Paediatr Dent*. 2012;22:169–79.
- Divaris K, Preisser JS, Slade GD. Surface-specific efficacy of fluoride varnish in caries prevention in the primary dentition: results of a community randomized clinical trial. *Car Res*. 2013;47:78–87.
- Oliveira BH, Salazar M, Carvalho DM, Falcão A, Campos K, Nadanovsky P. Biannual fluoride varnish applications and caries incidence in preschoolers: a 24-month follow-up randomized placebo-controlled clinical trial. *Car Res*. 2014;48:228–36.
- Buzalaf MA, Pessan JP, Honório HM, Ten Cate JM. Mechanisms of action of fluoride for caries control. *Monogr Oral Sci*. 2011;22:97–114.
- Mannaa A, Carlén A, Zaura E, Buijs MJ, Bukhary S, Lingström P. Effects of high-fluoride dentifrice (5,000-ppm) on caries-related plaque and salivary variables. *Clin Oral Invest*. 2014;18:1419–26.
- Marsh PD, Head DA, Devine DA. Ecological approaches to oral biofilms: control without killing. *Car Res*. 2015;49:46–54.
- Anderson M, Dahllöf G, Twetman S, Jansson L, Bergenlid AC, Grindefjord M. Effectiveness of early preventive intervention with semi-annual fluoride varnish applications in toddlers living in high-risk areas: A stratified cluster randomized controlled trial. *Car Res*. 2016;50:17–23.
- Wall-Manning GM, Sissons CH, Anderson SA, Lee M. Checkerboard DNA–DNA hybridisation technology focused on the analysis of Gram-positive cariogenic bacteria. *J Microbiol Methods*. 2002;51:301–11.
- Teanpaisan R, Dahlen G. Use of polymerase chain reaction techniques and sodium dodecyl sulfatepolyacrylamide gel electrophoresis for differentiation of oral *Lactobacillus* species. *Oral Microbiol Immunol*. 2006;21:79–83.
- Ismail AI, Sohn W, Tellez M, Amaya A, Sen A, Hasson H, Pitts NB. The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. *Community Dent Oral Epidemiol*. 2007;35:170–8.
- Thenisch NL, Bachmann LM, Imfeld T, Leisebach Minder T, Steurer J. Are mutans streptococci detected in preschool children a reliable predictive factor for dental caries risk? A systematic review. *Car Res*. 2006;40:366–74.
- Parisotto TM, Steiner-Oliveira C, Silva CM, Rodrigues LK, Nobre-dos-Santos M. Early childhood caries and mutans streptococci: a systematic review. *Oral Health Prev Dent*. 2010;8:59–70.
- Hardman MC, Davies GM, Duxbury JT, Davies RM. A cluster randomised controlled trial to evaluate the effectiveness of fluoride varnish as a public health measure to reduce caries in children. *Car Res*. 2007;41:371–6.
- Agouropoulos A, Twetman S, Pandis N, Kavvadia K, Papagiannoulis L. Caries-preventive effectiveness of fluoride varnish as adjunct to oral health promotion and supervised tooth brushing in preschool children: a double-blind randomized controlled trial. *J Dent*. 2014;42:1277–83.
- Marquis RE, Clock SA, Mota-Meira M. Fluoride and organic weak acids as modulators of microbial physiology. *FEMS Microbiol Rev*. 2003;26:493–510.
- Chau NP, Pandit S, Jung JE, Jeon JG. Evaluation of *Streptococcus mutans* adhesion to fluoride varnishes and subsequent change in biofilm accumulation and acidogenicity. *J Dent*. 2014;42:726–34.
- Tanner AC, Mathney JM, Kent RL, Chalmers NI, Hughes CV, Loo CY, Pradhan N, Kanasi E, Hwang J, Dahlan MA, Papadopoulou E, Dewhirst FE. Cultivable anaerobic microbiota of severe early childhood caries. *J Clin Microbiol*. 2011;49:1464–74.
- Jiang W, Zhang J, Chen H. Pyrosequencing analysis of oral microbiota in children with severe early childhood dental caries. *Current Microbiol*. 2013; 67:537–42.
- Chalmers NI, Oh K, Hughes CV, Pradhan N, Kanasi E, Ehrlich Y, Dewhirst FE, Tanner AC. Pulp and plaque microbiotas of children with severe early childhood caries. *J Oral Microbiol*. 2015;7:25951.
- Corby PM, Lyons-Weiler J, Bretz WA, Hart TC, Aas JA, Boumenna T, Goss J, Corby AL, Junior HM, Weyant RJ, Paster BJ. Microbial risk indicators of early childhood caries. *J Clin Microbiol*. 2005;43:5753–9.
- Wade WG. The oral microbiome in health and disease. *Pharmacol Res*. 2013; 69:137–43.
- Schwendicke F, Dörfer C, Kneist S, Meyer-Lueckel H, Paris S. Cariogenic effects of probiotic *Lactobacillus rhamnosus* GG in a dental biofilm model. *Car Res*. 2014;48:186–92.
- Stecksén-Blicks C, Sjöström I, Twetman S. Effect of long-term consumption of milk supplemented with probiotic lactobacilli and fluoride on dental caries and general health in preschool children: a cluster-randomized study. *Car Res*. 2009;43:374–81.
- Radford JR, Ballantyne HM, Nugent ZJ, Robertson M, Longbottom C, Pitts NB, Beighton D, Brailsford SR. Does social deprivation in 1, 2, 3 and 4-year-old Scottish infants influence the frequency isolation of caries-associated microorganisms? *J Dent*. 2001;29:325–32.
- Socransky SS, Haffajee AD, Smith C, Martin L, Haffajee JA, Uzel NG, Goodson JM. Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems. *Oral Microbiol Immunol*. 2004;19:352–62.
- Dahlén G, Preus HR, Baelum V. Methodological issues in the quantification of subgingival microorganisms using the checkerboard technique. *J Microbiol Methods*. 2015;110:68–77.
- Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A, Wade WG. The human oral microbiome. *J Bacteriol*. 2010;192:5002–17.