Pacifier cleaning practices and risk of allergy development

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Abbreviations: HR=hazard ratio; IgE=immunoglobulin E; OPLS=orthogonal projection to latent structures; OR=odds ratio; PCR=polymerase chain reaction; T-RFLP= terminal restriction fragment length polymorphism

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What is known: Infants with a diverse gut microbial flora are less likely to develop eczema and allergy.

What this study adds: Parental sucking of their infant’s pacifier is associated with a reduced risk of allergy development and an altered oral flora in their child. Transfer of oral microbes from parent to infant via the pacifier might be used in primary prevention.

Contribution statement
Bill Hesselmar (BH) has been involved in the planning and design of the study and interpretation of the data. BH has been responsible for the design of the protocols used, construction and validation of clinical databases and diagnoses. BH has, together with RS and NÅ, examined the children at all follow-ups. As corresponding author, BH is responsible for the writing of the manuscript and for data/statistical analyses, with the exception of the analyses of the saliva samples, which were done by FS.

Fei Sjöberg has been responsible for data analysis, data interpretation and writing concerning the T-RFLP analysis.

Robert Saalman (RS) has been involved in the planning and design of the study, interpretation of the data and writing of the manuscript. RS has examined children regarding allergic manifestations related to food allergy.

Nils Åberg has been involved in the planning and design of the study, in the design of the protocols used. NÅ was responsible for the collection of basic data regarding the children, and has, together with BH and RS examined the children at all follow-ups. He has participated in discussions related to the production of the manuscript.

Ingegerd Adlerberth (IA) has been involved in the planning and in the design of the study, and in the interpretation of the data. IA and AW initiated the ALLERGYFLORA cohort on which the current study is based. IA is chiefly responsible for the microbial analyses and a co-supervisor of Fei Sjöberg.

Senior author Agnes E. Wold (AEW) has been involved in the planning and in the design of the study, and in the interpretation of the data. AEW is the primary investigator of the ALLERGYFLORA cohort, on which the current study is based and is the main supervisor of FS. Agnes Wold has been involved in the writing of the manuscript an in the design of figures.

All authors had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.
Abstract

Objective Immune stimulation through exposure to commensal microbes may protect against allergy development. Oral microbes may be transferred from parents to infants via pacifiers. We investigated whether pacifier-cleaning practices affected the risk of sensitization and allergy development in infants up to the age of 36 months.

Methods A birth-cohort of 184 infants was examined for clinical allergy and sensitisation to airborne and food allergens at 18 and 36 months of age and, in addition, promptly upon occurrence of symptoms. Pacifier use and pacifier-cleaning practices were recorded during interviews with the parents when the children were 6 months old. The oral microbiota of the infants was characterised by analysis of saliva samples collected at 4 months of age.

Results Children whose parents ‘cleaned’ their pacifier by sucking it (n=65) were less likely to have asthma (OR 0.12; 95% CI 0.01-0.99), eczema (OR 0.37; 95% CI 0.15-0.91) and sensitization (OR 0.37; 95% CI 0.10-1.27) at 18 months of age than children whose parents did not use this cleaning technique (n=58). Protection against eczema remained at age 36 months (hazard ratio 0.51; p=0.04). Vaginal delivery and parental pacifier sucking yielded independent and additive protective effects against eczema development. The salivary microbiota differed between children whose parents ‘cleaned’ their pacifier by sucking it and children whose parents did not use this practice.

Conclusions Parental sucking of their infant’s pacifier may reduce the risk of allergy development, possibly via immune stimulation by microbes transferred to the baby via the parent’s saliva.
Introduction

The prevalence of immunoglobulin (Ig) E-mediated (atopic) allergy increased strongly during the 20th century, and today 1 in 3 children in affluent countries are affected.\(^1\) Allergy results from the failure to develop immune tolerance to harmless inhaled or ingested proteins, termed allergens. Instead, an immune response occurs (sensitization), which may lead to symptoms (clinical allergy) upon renewed contact with the allergen(s).

The cause of the increased prevalence of allergy is unknown, although circumstantial evidence points to reduced exposure of infants and young children to microbes as a risk factor, observations that were summarized as ‘the hygiene hypothesis’.\(^2\) Hence, poverty, crowded housing, large families, early contact with pets or farm animals, and early exposure to food-borne microbes are associated with a reduced risk of allergy development.\(^2-^5\) Furthermore, acquisition of commensal gut bacteria is delayed in western infants\(^6\) and a gut microbiota of low complexity during the neonatal period is a risk factor for allergy development.\(^7-^9\) Vaginal delivery, which leads to neonatal exposure to a complex maternal microbiota, is associated with a reduced risk of allergy development compared with delivery by caesarean section.\(^10-^12\)

The oral cavity contains a complex mixture of aerobic and anaerobic bacteria with more than 600 prevalent taxa.\(^13\) Babies may be exposed to parent’s oral microbes, transferred in saliva via kissing. Saliva is also transferred if the parent puts the baby’s feeding spoon or pacifier (dummy/soother) into their own mouth before giving it back to the baby. Whether early exposure to oral microbes is protective against allergy development has, to the best of our knowledge, not yet been studied. The aim of the study was to examine whether the mode by which parents clean their baby’s pacifier affects the risk of allergy development in the baby.
Methods

Inclusion and follow-up of ALLERGYFLORA cohort

Pregnant women were recruited into the ALLERGYFLORA study at Mölndal Hospital, Greater Gothenburg, Sweden, and their infants were included in the birth cohort 1–3 days after delivery. Exclusion criteria were preterm birth (<38 weeks’ gestation) and neonatal intensive care. We mainly approached families with at least one allergic parent so that the birth cohort would include a high proportion of children with allergic disease. All parents provided written informed consent for their children to participate in the study. The study was approved by the Ethics Committee of the University of Gothenburg, Sweden (R 448-97 and Ö 446-00).

Upon inclusion, a structured interview was conducted focusing on the pregnancy, delivery, and family structure and housing conditions. Diaries covering the infant’s first and second 6 months of life were kept by the parents, who were asked to record food introduction, weaning, diseases and medications, and other significant events. This information was reported during structured telephone interviews with the parents when the children were 6 months old. During this interview the parents were also asked: ‘Does the child use a pacifier?’ and ‘Is it cleaned by boiling, rinsing in tap water, or by the parents sucking on it?’ (more than one option could be selected).

Sensitization and clinical allergy diagnoses

A paediatric allergist examined the children, reviewed their medical charts, and performed a structured interview with the parents when the children were 18 and 36 months old, and whenever symptoms suggesting the commencement of allergy occurred. Venous blood was analysed for eosinophilic granulocyte counts, which increase in patients with allergies, and for allergen-specific IgE (“sensitization”, see below).
For clinical diagnosis, the examining paediatrician used the following criteria.

**Eczema:** Diagnosed according to Williams’ criteria for “atopic dermatitis”\(^{14}\). ‘Eczema at 18 months’ denoted diagnosis at any time before or at 18 months; ‘eczema at 36 months’ required symptoms after 24 months of age.

**Asthma:** Persistent wheezing for ≥4 weeks or ≥3 episodes of wheezing combined with ≥1 minor criterion (symptoms between colds, eczema, allergic rhinoconjunctivitis, or food allergy). For ‘asthma at 36 months’, ≥1 wheezing episode should have occurred after 24 months of age, and response to inhaled glucocorticoids or leukotriene antagonists was included among the minor criteria.

**Sensitization:** Presence of specific IgE against inhalant allergens (birch, timothy grass, mugwort, cat, dog, horse, D. pteronyssinus, D. farinae and mould; Phadiatop\(^{®}\), Phadia AB, Uppsala, Sweden) or against food antigens (milk, egg, soy, fish, wheat and peanut; ImmunoCAP\(^{®}\) food-mix test, Phadia AB). A positive reaction was defined as a sum of allergen-specific IgEs ≥0.35 kU/L in either assay.

**Parental history of allergy:** A doctor’s diagnosis of asthma, allergic rhinoconjunctivitis, or eczema, as reported at interviews.

**Analysis of infants’ salivary microbiota**

For infants included after February 2001, saliva samples were collected at 4 months of age. The samples were stored at -20°C until microbiota fingerprinting was performed using terminal restriction fragment length polymorphism (T-RFLP). Bacterial DNA was extracted from 150 μL saliva (QIAlert DNA Stool Mini Kit, Qiagen, Hildens, Germany) according to the manufacturer’s instructions, but including shaking (1200 rpm) with 2 mm glass beads (10–12 beads/sample) for 30 min at 5°C after addition of the ASL buffer. Extracted DNA,
50 ng as measured by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA; version 3.3), was mixed with 0.3 μM of the 16S rRNA universal primers ENV1 (5'-6-FAM-AGA GTT TGA TII TGG CTC AG -3'; *Escherichia coli* 16S rRNA gene sequence nucleotide positions 8–27) and ENV 2 (5'-CGG ITA CCT TGT TAC GAC TT-3'; *E. coli* 16S rRNA gene sequence nucleotide positions 1511–1492), 25 μL Hot Start Taq Master Mix (2.5 units Taq DNA polymerase, 1.5 mM MgCl₂, 200 μM dNTP) (Qiagen), 1 mM MgCl₂, and water to a final volume of 50 μL. The ENV1 primer was labelled with 6-FAM fluorescent dye. The polymerase chain reaction (PCR) program was run as follows: an initial activation of Taq polymerase at 95°C for 15 min; 25 cycles of 94°C for 1 min (DNA denaturation), 50°C for 45 s (primer annealing), and 72°C for 7 min (primer extension), with a final extension at 72°C for 7 min. PCR amplification products were examined by agarose gel electrophoresis. Triplicate samples were pooled and purified (QIAquick PCR purification kit, Qiagen), and 100 ng was digested using 16 U of *Msp*I endonuclease (New England Biolabs, Ipswich, MA, USA) at 37°C for 5 h in a final volume of 5 μL. A mixture of 1 μL of digested DNA, 9 μL of formamide, and 0.5 μL of DNA size standard (LIZ 1200, Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) was denatured at 95°C for 3 min, placed on ice, and separated for 80 min at 15 kV on an ABI PRISM 310 genetic analyser with POP-4 gel matrix and 5 s injection time (Applied Biosystems). Fragment lengths were analysed using GeneMapper (version 4; Applied Biosystems) and the local institution’s Southern blot method. ‘True’ peaks were separated from ‘noise’ by statistical analysis using Perl and R software (www.ibest.edo/tools/trflp_stats/index.php). Peaks with a relative area >3 standard deviations from the mean were identified as true signals and removed. This process was repeated until no more ‘true’ peaks were identified. Saliva samples were analysed in triplicate; only peaks appearing in all runs were included (terminal restriction fragments were considered to be identical if they differed by no more than ±1bp in different runs), and the
peak areas were averaged.

**Statistical analyses**

Statistical analyses were performed using SPSS software (version 15.0.1.1; SPSS, Chicago, IL, USA). Fisher’s exact and $\chi^2$ tests were used to compare proportions; the $\chi^2$ test was used for trend analysis; the Mann–Whitney test and analysis of variance (ANOVA) were used to compare continuous data and binary logistic regression to adjust for confounding variables. Kaplan–Meier analyses were used to monitor allergy development over time, with the use of Cox regression to adjust for confounders. Orthogonal projection to latent structures (OPLS) was used to analyse the salivary microbiota in relation to pacifier cleaning practices (SIMCA-P+ software, version 12.0; Umetrics AB, Umeå, Sweden).
Results

Between 1998 and 2003, 206 pregnant women were recruited into the study; 187 infants met the criteria for inclusion in the ALLERGYFLORA birth cohort. A total of 184 children were followed until the child was 18 months old and 174 until the age of 36 months. Eighty per cent of the children had at least one allergic parent and 74% used a pacifier during the first 6 months of life (table 1). As seen in table 2, almost all parents cleaned the pacifier by rinsing it in tap water, but approximately half of them also boiled it and almost half reported ‘cleaning’ the pacifier by sucking it before giving it back to the infant.

Pacifier cleaning practices and allergy at 18 months of age

By the age of 18 months, 25% of the children had developed eczema and 5% asthma. Sensitization to food antigens occurred in 15%, while sensitization to inhaled antigens was uncommon (table 3).

We examined whether the presence of eczema, asthma or atopy at 18 months was related to pacifier cleaning practices during the child’s first 6 months of life. Figure 1 shows the Odds Ratio of allergy and sensitization in relation to pacifier use and pacifier cleaning practices. As seen in the figure, pacifier use per se was not significantly associated with clinical allergy or sensitization (figure 1). In pacifier-using children, a cleaning method was compared to those not using that specific cleaning method. Boiling the pacifier was associated with an increased prevalence of asthma, but the effect was not significant. However, parental ‘cleaning’ of the pacifier by sucking it was strongly associated with the risk of allergy development. Both eczema (odds ratio [OR] 0.37; 95% CI 0.15-0.91; p=0.02) and asthma (OR 0.12; 95% CI 0.01-0.99; p=0.03) were strongly reduced in children whose parents had this habit. There was also a trend towards lower risk of sensitization in this group (OR 0.37; 95% CI 0.10-1.27;
p=0.08) (figure 1). Furthermore, the blood eosinophil count at 18 months of age was also lower in children whose parents sucked on their pacifier than in other pacifier-using children (figure 2).

**Parental pacifier sucking and respiratory infections in the child**

Respiratory infections in the child’s first 6 months were recorded by parents and reported at the 6-month telephone interview. On average, 1.5 respiratory infections per child were noted by the parents (confidence interval: 1.3-1.7). This frequency did not differ between children whose parents sucked their pacifier (1.4) and other pacifier-using children (1.5; ANOVA, p=0.75).

**Analysis of confounding factors**

We analysed the relationship between parental pacifier sucking and possible confounding factors. Parents of vaginally delivered infants were significantly more likely to suck the child’s pacifier than parents of babies delivered by caesarean section (Fisher’s exact test, p=0.02; table 4). Conversely, mothers with a University level degree were tended to be less likely to suck the child’s pacifier, although this association was not significant (table 4). After logistic regression to adjust for delivery mode and mother’s occupation, the protective effect of parental pacifier sucking on eczema development during the child’s first 18 months of life remained (OR 0.27, 95% confidence interval 0.086–0.819; p=0.02). The protective effect on asthma could not be analysed in this way because of the low number of cases.

We speculated that delivery via the vaginal route, which exposes the infant to the maternal vaginal microbiota, and parent/infant pacifier sharing, which leads to exposure to parental oral microbiota, may confer additive protective effects against allergy development by exposure to a variety of commensal microbes. We thus stratified the cohort into three groups: (1) vaginally delivered infants with pacifier-sucking parents; (2) caesarean-delivered infants
whose parents did not suck their pacifiers; and (3) infants who were either vaginally delivered or exposed to parents’ oral microbiota by pacifier sharing. We then compared the prevalence of eczema in infants at 18 months of age in these three groups (figure 3). The group exposed to both maternal vaginal and parental oral microbiota had the lowest prevalence of eczema (20%), whereas infants exposed to neither maternal vaginal or parental oral microbiota had the highest prevalence (54%). The group of children who were either vaginally delivered or whose parents sucked their pacifiers had an intermediate eczema-prevalence (31%) (figure 3).

Parental pacifier sucking and allergy during the child’s first 36 months of life

The children in the cohort were followed up at 36 months of age. Kaplan–Meier curves were calculated for eczema, asthma, and sensitization (figure 4). Development of eczema up to 36 months of age was significantly less likely in infants whose parents sucked on their pacifiers during their first 6 months of life, as compared to other pacifier-using children (p=0.04). The protective effect of parental pacifier sucking on asthma and sensitization was not statistically significant.

Parental pacifier sucking and child’s salivary microbiota at 4 months old

If parents’ salivary microbes are transferred to the infant via the pacifier, it may affect development of the infant’s salivary microbiota. This hypothesis was examined by fingerprinting bacterial DNA in infants’ saliva, which had been sampled from 64 infants at 4 months of age. We excluded caesarean-delivered infants in this analysis, since their oral microbiota has been shown to differ from that of vaginally delivered infants16, and since mode of delivery was associated with parental sucking of the baby’s pacifier. After exclusion of caesarean-delivered infants and non-pacifier users, 33 infants remained, 21 of them had parents who sucked on their pacifier.

T-RFLP analysis of bacterial DNA from infants’ saliva was used to identify different bacterial
groups and O-PLS (orthogonal projection onto latent structures), a regression variety of principal component analysis, to reveal whether infants whose parents cleaned their pacifier by sucking it could be separated from those whose parents did not have this habit, based on microbiota composition. Figure 5 shows that these two groups could be distinguished based on the pattern of microbes in their saliva. One infant (outlier bottom left) had a microbiota clearly deviant from the others.
Discussion

Early acquisition of a complex intestinal microbiota has been identified to provide protection against allergy development, suggesting that commensal bacteria may provide the developing immune system with crucial signals for its proper maturation. Here, we demonstrate that a common parental practice - sucking on the baby’s pacifier before it is given back to the infant - is associated with protection against early eczema development and asthma symptoms. Furthermore, the blood eosinophil counts were lower at 18 months of age in children whose parents sucked on their pacifiers, compared to other pacifier-using children whose parents did not have this habit.

It is possible that the observed effect of ‘parental pacifier sucking’ is due to the transfer of oral bacteria from the parents, via the pacifier, to their baby. By no doubt, this habit allows for a close oral contact between parents and child, facilitating bacterial transfer at a very young age, before the child starts to use spoons etc. Using T-RFLP as a fingerprinting method to characterise the infant’s salivary bacteria, we have obtained suggestive evidence that this practice influenced the infant’s oral microbiota composition. T-RFLP is a suitable method for depicting complex bacterial communities; cleaving of total bacterial DNA generates a series of peaks each of which represents a bacterial group (usually a genus). As identification of T-RFLP peaks requires a substantial amount of additional work, we do not, at present, know the identity of the microbes that are more or less prevalent in the saliva of the two groups of infants.

We examined whether selection bias could have caused the association. The parental practice of ‘cleaning’ the child’s pacifier by sucking it was neither associated with parental allergy, duration of breast-feeding, smoking, or pet keeping. However, it was significantly positively
associated with the baby having been born vaginally. The basis for this association can only be speculated upon. During a vaginal delivery, the baby is exposed to bodily fluids and sometimes soiled with faeces; after a normal delivery the mother and baby will be speedily discharged from the hospital. In contrast, delivery by caesarean section is a strictly sterile surgical procedure followed by hospital stay and substantial medical attention. Theoretically, the ‘sterility culture’ of the hospital may be transmitted to the parents and influence they’re subsequent care of their baby even when they return home. It cannot be excluded that ‘parental pacifier sucking’ is a marker of a common general behaviour of ‘close oral contact’, allowing for bacterial transfer from parent to child by other oral routes like spoons, feeding bottles, kisses etc. As a consequence, parents who do not suck their baby’s pacifier might be generally more cautious against bacterial transfer.

Importantly, however, the protective effect against eczema of parental pacifier sucking remained after controlling for delivery mode by multiple regression analysis. Furthermore, vaginal delivery and parental pacifier sucking conferred additive and independent protective effects against allergy development in the infant. Hence, the prevalence of eczema was approximately 2.5 times lower at 18 months of age in vaginally delivered children whose parents sucked their pacifiers than in caesarean section-delivered children whose parents did not have this habit (20% vs 54%); an intermediate protective effect was seen in children exposed to either vaginal delivery or parental pacifier sucking. Thus, vaginal delivery, which is a source for transfer of a complex microbiota from mother to infant\textsuperscript{17} and parent and infant sharing of a pacifier might both lead to microbial stimulation, with beneficial effects on allergy development. Infants delivered by caesarean section have an increased risk of asthma\textsuperscript{10-12} and sensitization\textsuperscript{11} and may benefit the most from the increased bacterial exposure linked to parental pacifier sucking. This is in line with the suggestion that ‘children delivered by caesarean section could be targeted for future preventive interventions, such as the use of
probiotics’. Respiratory pathogens might be transferred by parental pacifier sucking, but we found no increased respiratory infection rate in their children. Another concern is that cariogenic bacteria may be transferred. However, caries seems to be unrelated to pacifier use and may even be negatively associated with ‘close’ salivary contact between infant and parent. Transfer of a complex microbiota from parent to infant may help build up resistance to colonization with more pathogenic bacteria.

The relatively small scale of the present study may be a potential weakness. Negative findings could be a result of the small sample size. However, the size of the study is also its strength, as it was possible to employ a very detailed and structured follow-up. Diaries were used by the parents and structured interviews were conducted every 6 months. Children with signs and symptoms suggestive of allergic disease were examined by a study paediatrician within a few days and diagnoses were all based on strict criteria. For eczema, we used the validated criteria of Williams. Asthma in early childhood is notoriously difficult to distinguish from viral wheeze, which is unlikely to progress to true asthma. Our criteria for an asthma diagnosis are based on prior studies demonstrating that atopy and/or symptoms between episodes and eczema are significant indicators of asthma in young infants with wheezing, especially persistent asthma with onset in early childhood. Even though this is far from the strict asthma criteria used in older children and adults, the criteria used are probably the best available without using infant lung function tests and invasive methods. With these limitations in mind, it is important to emphasize the need to re-evaluate the findings from the study in larger studies, and in older children with a better-defined asthma and an established atopic profile. A follow up of the cohort is on-going in which both lung function and airway reactivity are tested.
Oral tolerance is the normal physiological response to harmless proteins, and it has been known for decades that the presence of commensal microbiota is a prerequisite for the development of normal tolerance against harmless protein antigens. Hitherto, attention has almost exclusively focused on the small intestine as the inductive site for oral tolerance and the influence of gut microbiota on handling of dietary antigens. However, the oral cavity is actually the first site of encounter between foreign antigens and the lymphoid system and allows the lymphoid to sample antigens that have not been denatured by acid and cut by digestive enzymes. The oropharynx is surrounded by dense lymphoid tissue, i.e. the adenoids, and palatine and pharyngeal tonsils. Similar to the gut-associated lymphoid tissues, these are covered by M-cells that are specialized in antigen uptake and delivery to antigen-presenting cells and lymphocytes beneath the epithelium. Furthermore, dendritic cells capture antigens in the oral mucosa and migrate to cervico-mandibular lymph nodes, similar to the way dietary antigens are carried to the mesenteric lymph nodes. The tonsils are rich in T cells with a regulatory phenotype, and applications of contact allergens on the oral mucosa leads to active tolerance induction. Thus, there is no reason to believe that active oral tolerance could not be induced already in the oral cavity. Exposure of the infant to parental saliva might accelerate development of a complex oral/pharyngeal microbiota which, similar to a complex gut microbiota, might beneficially affect tolerogenic handling of antigens by the oral/pharyngeal lymphoid tissues. Moreover, oral bacteria are swallowed and hence also affect the composition of the microbiota in the small intestine, which may in turn regulate tolerance development in the gut. Further studies are now required to establish if parental pacifier sucking could be a simple and safe method to reduce allergy development in infants and young children, as our study suggests.

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