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Mode of Birth Delivery Affects Oral Microbiota in Infants

ABSTRACT

Establishment of the microbiota of the gut has been shown to differ between infants delivered by Caesarian section (C-section) and those delivered vaginally. The aim of the present study was to compare the oral microbiota in infants delivered by these different routes. The oral biofilm was assayed by the Human Oral Microbe Identification Microarray (HOMIM) in healthy three-month-old infants, 38 infants born by C-section, and 25 infants delivered vaginally. Among over 300 bacterial taxa targeted by the HOMIM microarray, *Slackia exigua* was detected only in infants delivered by C-section. Further, significantly more bacterial taxa were detected in the infants delivered vaginally (79 species/species clusters) compared with infants delivered by C-section (54 species/species clusters). Multivariate modeling revealed a strong model that separated the microbiota of C-section and vaginally delivered infants into two distinct colonization patterns. In conclusion, our study indicated differences in the oral microbiota in infants due to mode of delivery, with vaginally delivered infants having a higher number of taxa detected by the HOMIM microarray.

KEY WORDS: newborn, Caesarian section, vaginal delivery, bacterial taxa, HOMIM, *Slackia exigua*.

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INTRODUCTION

The first exposure to micro-organisms in vaginally delivered infants occurs during passage through the birth canal, whereas the first exposure to bacteria in infants born by Caesarian section (C-section) is from the skin of parents and health providers, and medical equipment. Different modes of delivery lead to differences in the intestinal microbiota in infants (Penders *et al*., 2006; Dominguez-Bello *et al*., 2010). Vaginally born children have been reported to have a more diverse gut microbiota, whereas children born by C-section had higher numbers of *Clostridium difficile* and delayed acquisition of bifidobacteria and *Escherichia coli* (Ahrné *et al*., 2005; Penders *et al*., 2006). In the oral cavity, mutans streptococci were detected more frequently and at a younger age in children delivered by C-section than in those delivered vaginally (Li *et al*., 2005). These authors hypothesized that C-section, compared with vaginal birth, lowered the exposure to commensal, protective bacteria from the mother during birth, reducing the natural barrier to colonization by oral pathogens.

Acquisition of oral bacteria in early childhood results mainly from transmission from the mother (Könönen, 2000; Tanner *et al*., 2002), but there is less information about other factors influencing establishment of the microbiota in the oral cavity than reported for the gut. Establishment of the gut microbiota was found not to be a predetermined species-by-species succession, but rather a coordinated interplay between external and internal factors (Fanaro *et al*., 2003; Penders *et al*., 2006). External factors for the gut microbiota included the environment during birth, the mother's microbiota, and infant feeding method (Fallani *et al*., 2010). Internal factors included the developmental stage of the gastrointestinal tract and host factors (Benson *et al*., 2010).

The aim of the present study was to compare oral microbiota, seeking differences in colonization patterns in infants delivered vaginally or by C-section. The human Oral Microbe Identification Microarray was used to detect bacterial taxa.

STUDY POPULATION & METHODS

Study Population

All mothers living in a small inland town and a coastal university city in Northern Sweden who had delivered a healthy baby in the previous 3 mos were invited to consent for their infant to participate in the study. From 300 invited women, 207 accepted (69%), and all infants delivered by C-section (n = 41) and 26 randomly selected vaginally delivered infants were selected for microbial analyses. Phone interviews were conducted with the non-participants, and the

only reason given for non-participation was lack of time. The study was approved by The Regional Ethical Review Board, Umeå, Sweden, and participating mothers signed informed consent at recruitment.

Mode of delivery (C-section or vaginal), intravenous treatment with antibiotics during delivery, and body weight and length were checked against medical records. The mothers completed a questionnaire on other possible confounders, such as health issues (allergy, infections, stomach problems), the infant's use of antibiotics, feeding mode (breast- or bottle-fed), use of a pacifier, and the presence of teeth.

Microbiota by 16S rRNA Probes in HOMIM Microarray

We collected oral biofilm samples by carefully swabbing the cheeks, tongue, and alveolar ridges. DNA was purified from samples with the use of the Gen Elute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA) to obtain 60-1220 ng DNA, which exceeded the amount required for the microarray assay. Four samples were excluded because of low yield after DNA extraction, leaving 38 and 25 of the samples from C-section and vaginal delivery groups, respectively.

The purified DNA of samples was assayed with 422 oligonucleotide probes to the 16S rRNA gene targeting more than 300 bacterial taxa in the HOMIM microarray (http://mim.forsyth.org/homim.html). Samples were analyzed at the HOMIM microarray facility at The Forsyth Institute, Cambridge, MA, USA (Colombo *et al*., 2009). Hybridization signals were read on a six-level scale (0-5), with a lower limit of detection of 10^4 cells (Colombo *et al*., 2009).

Statistical Procedures

Body length and weight at birth and at 3 mos were averaged among infants delivered by the two birth delivery modes. Differences between means were tested by two-sided, independent *t* tests. Dichotomized scores from the HOMIM microarray analyses were used. Lack of signal was set to 0, and all signal levels ≥ 1 to 1. Differences in prevalence distribution between groups were tested with a Chi^2 test. The False Discovery Rate method was used to identify a p-value with less than one false rejection of H0 when true ($p < 0.005$). Thus, a p-value < 0.005 was considered statistically significant to account for multiple comparisons.

Multivariate partial least-squares discriminant analysis (PLS-DA) modeling was performed (SIMCA P+, version 12.0, Umetrics AB, Umeå, Sweden) as described (Sjöström *et al*., 1986; Bylesjö *et al*., 2006). In contrast to traditional regression models, the PLS-DA technique, which defines the maximum separation between class members (here mode of delivery) in the data, is suitable for data where the number of observations is smaller than the number of variables, and where the independent variables co-vary. Dichotomous HOMIM signals, and the selected individual characteristics, gender, weight and length at birth and 3 mos, gestational wks at delivery, treatment with antibiotics during delivery, feeding mode (bottle- or breast-fed), use of pacifier, presence and number of teeth, and town of residence built the X-block, and mode of delivery the Y-block (outcome). An identical model including breast-fed infants born in or later than gestational wk 37 only was also run. Variables were autoscaled to unit variance, and cross-validated prediction of Y calculated (Wold, 1978). Cross-validation was done by a systematic prediction of $1/7^{th}$ of the data by the remaining $6/7^{th}$ of the data. The importance of each x-variable in the model is given by a variable importance in projection (VIP) value. VIP > 1.0 was considered influential and VIP ≥ 1.5 highly influential (Sjöström *et al*., 1986). The R^2- and Q^2-values give the capacity of the x-variables to explain (R^2) and predict (Q^2) the outcome.

RESULTS

Cohort Description

There were no differences by gender or by other characteristics, including breast-feeding, between infants born vaginally and those born by C-section (Table 1). Two infants were born in gestational wk 35 (one delivered by C-section and one by the vaginal route), whereas all other infants were born in gestational wk 37 or later. All infants were healthy at birth and at 3 mos of age. None of the infants had ever received antibiotic treatment, and none was ever given supplements containing probiotic bacteria. With the exception of 15 mothers who received intravenous antibiotics in association with a C-section because of an acute clinical complication, none had antibiotics at delivery. There were no significant differences between participating and non-participating infants in length and weight at birth and at 3 mos of age, or in the socioeconomic variables of their families (data not shown).

Bacteria Detected by HOMIM Microarray

There was reactivity to 85 of the 300 taxa in the HOMIM microarray in oral biofilms of three-month-old infants (Appendix Table). Bacteria detected belonged to 6 phyla or divisions, and approximately half of the taxa detected belonged in *Firmicutes*, particularly *Streptococcus* species, which were detected in all infants (Table 2). Other genera detected in 80 to 99% of all children were *Actinomyces*, *Gemella*, and *Veillonella*. A smaller proportion of the infants (< 15% of the combined groups) had species in the genera *Bacteroides, Selenomonas, Aggregatibacter, Kingella, Neisseria*, and the TM7 division.

Species or species clusters detected in all infants were *Streptococcus* Cluster II, *Streptococcus* Cluster III, *Streptococcus anginosus/intermedius*, and *Streptococcus oralis* (Appendix Table). Species detected in $\geq$ 80% of all children were *Streptococcus* Cluster I, *Streptococcus mitis* biovar 2, *Streptococcus australis, Streptococcus parasanguinis* I and II, *Actinomyces gerenscerіae*, *Gemella hemolysans*, *Veillonella atypical*, and *Veillonella parvula*. Species detected in only a few (< 15%) infants included *Streptococcus sanguinis* and *Streptococcus mutans*, species of *Neisseria*, *Aggregatibacter*, and *Kingella*, and *Actinomyces naeslundii* genospecies1 and 2 (*Actinomyces* clusters I and II, respectively) (Appendix Table).

Species Distribution by Mode of Delivery

There were higher numbers of taxa detected by the microarray in swabs from infants delivered vaginally (79 species/clusters)

Table 1. Gender Proportions, Body Weight and Length, and Feeding Method for Infants Delivered Vaginally or by Caesarian Section

	Vaginal Delivery n = 25[1]	Caesarian Section n = 38[1]	p-value
Boys/Girls (numbers)	16/10	19/21	0.264
Length (cm)[2]			
at birth	51.1 (50.0–52.2)	49.2 (48.4–50.0)	0.849
at 3 mos of age	61.3 (60.4–62.4)	60.2 (59.2–60.8)	0.100
Weight (g)[2]			
at birth	3603 (3394–3813)	3425 (3190–3660)	0.202
at 3 mos of age	6364 (5986–6742)	6069 (5807–6331)	0.221
Breast-fed at 3 mos of age (%)[3]			
Exclusively or partially	27	32	0.836
Not at all	73	68	

[1]Numbers vary slightly for various analyses due to single missing values. Oral samples from four infants, three born by C-section and one vaginally, were not analyzed by microarray, because of low DNA yield.
[2]Mean (95% CI limits). Differences of means were tested with Student's independent *t* test after confirmation of a normal distribution.
[3]Differences in proportions were tested with the Chi[2]-test.

Table 2. Proportions (%) of Three-month-old Infants with a Positive HOMIM Signal by Genus

Phylogenetic Group and Genus	Vaginal Delivery(%)	Caesarian Section(%)	p-value
Actinobacteria			
Actinomyces	92.0	81.6	0.247
Rothia	68.0	65.8	0.856
Bacteroidetes			
Bacteroides	8.0	0.0	0.076
Capnocytophaga	16.0	0.0	0.011
Porphyromonas	4.0	0.0	0.214
Prevotella	40.0	7.9	0.002
Firmicutes			
Eubacterium	16.0	13.2	0.752
Gemella	96.0	100.0	0.214
Granulicatella	36.0	60.5	0.057
Lactobacillus	16.0	63.2	0.000
Parvimonas	4.0	18.4	0.093
Selenomonas	12.0	10.5	0.856
Solobacterium	20.0	10.5	0.293
Streptococcus	100	100	1.000
Veillonella	84.0	97.4	0.055
Fusobacteria			
Fusobacterium	44.0	34.2	0.434
Leptotrichia	44.0	13.2	0.006
Proteobacteria			
Aggregatibacter	4.0	0.0	0.214
Campylobacter	40.0	28.9	0.363
Haemophilus	28.0	7.9	0.033
Kingella	8.0	0.0	0.076
Neisseria	8.0	5.3	0.663
TM7 Division	12.0	0.0	0.029

than in infants delivered by C-section (54 species/clusters) ($p = 0.001$). Of the species or clusters detected, 31 were detected only in infants born vaginally, compared with 6 species or clusters that were detected only in C-section infants (Appendix Fig. 1).

The detection frequencies of 22 species and 2 clusters differed between modes of infant delivery. Species or clusters detected more frequently from C-section compared with vaginally born infants were *Slackia exigua* ($p < 0.001$) and *Lactobacillus* Cluster I ($p < 0.001$) (Fig.). *Haemophilus parainfluenzae* ($p = 0.005$) was detected more frequently from vaginally delivered compared with C-section infants (Fig.). Additional species/clusters displayed marginal significance levels (p-values between 0.02 and 0.006; Fig.).

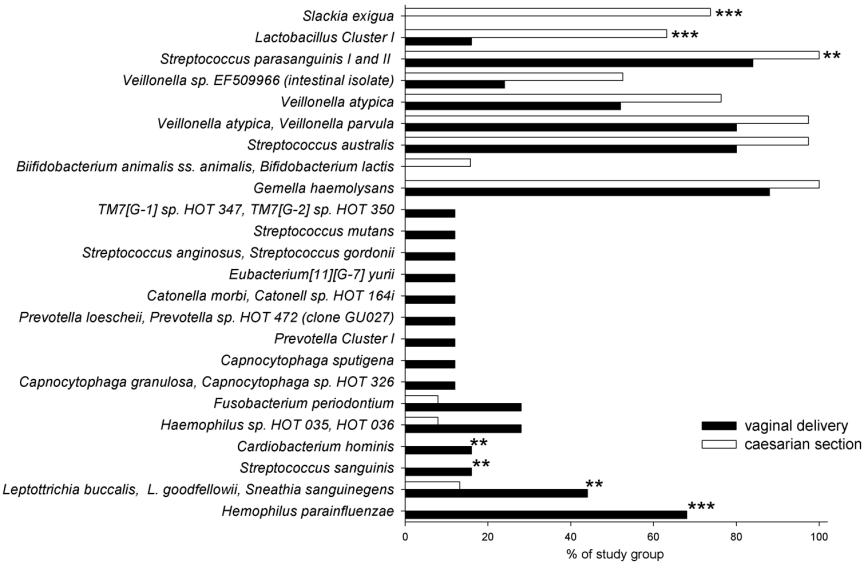

Figure. Reactivity to 24 probes (out of 85 probe reactions) that differed significantly ($p < 0.005$) or marginally ($p < 0.01$) in three-month-old infants born vaginally or by Caesarian section. ***$p \leq 0.005$, **$p < 0.01$ tested by Chi-square. No indication for p-values between 0.02 and 0.01.

PLS-DA Multivariate Modeling of HOMIM 16S rRNA-based Microarray Data

A model with two significant components was obtained by PLS-DA modeling with mode of delivery as outcome (y-variable) and dichotomized HOMIM microarray signals as the x-block, including selected individual characteristics (see statistics) as potential confounders. This model virtually clustered infants delivered by C-section separately from those delivered vaginally (Appendix Fig. 2). The multivariate model, which had an explanatory and predictive capacity of 62% ($R^2 = 0.618$) and 44% ($Q^2 = 0.0.457$), respectively, confirmed associations found in the univariate analyses. Species strongly associated (VIP $\geq$ 1.5) with being born by C-section were *Slackia exigua, Lactobacillus* Cluster I, *Veillonella* sp. EF509966, *Veillonella atypical*, and *V. parvula*, and those associated with being vaginally delivered were *S. sanguinis, Streptococcus* sp. HOT 058, and *Cardiobacterium hominis* (Table 3). The model remained strong ($R^2 = 0.693$, $Q^2 = 0.496$), and the same taxa remained strongly influential when the two pre-term (gestational wk 35) and all formula-fed infants were excluded.

DISCUSSION

The present study investigated the oral microbiota in infants delivered vaginally or by C-section to evaluate if there were differences associated by birth delivery mode. Higher numbers of taxa were detected among infants delivered vaginally, compared with those delivered by C-section, with probes to the 16S rRNA gene of cultivated and uncultivated oral bacteria in a microarray format (HOMIM; Paster and Dewhirst, 2009). Further, the results indicated differences in the microbiota depending on delivery method, including a novel finding that *Slackia exigua* was detected exclusively, and in high prevalence, in infants delivered by C-section. These findings indicate that there were differences in the microbiota of the oral cavity depending on birth delivery method, as has been reported for the microbiota of the lower gastrointestinal tract (Penders *et al*., 2006; Dominguez-Bello *et al*., 2010).

In the current study, 85 species or species clusters out of the approximately 300 taxa evaluated by the HOMIM microarray were detected in the three-month-old infants. This is fewer bacterial taxa than reported for adults in whom approximately 65 to 70% of the microarray species were detected by the same assay (Colombo *et al*., 2009; Preza *et al*., 2009). While there are no direct comparisons between the numbers of taxa detected by HOMIM in infants and those in adults, a lower species diversity of infants compared with individuals in later ages is consistent with separate reports for infants and adults (Könönen, 2000; Hao and Lee, 2004; Morelli, 2008). The lower number of taxa detected in oral biofilms from C-section compared with vaginally delivered infants is in accord with the lower diversity reported for the gut in samples taken immediately after C-section birth (Dominguez-Bello *et al*., 2010).

After birth, bacterial colonization of the gastrointestinal tract, including the mouth, is influenced by the transmission of bacteria from the environment and by genetic factors (Mandar and Mikelsaar, 1996; Dominguez-Bello *et al*., 2010). In the first few months of life, the major influences on the oral microbial succession are person-to-person transmission, composition of the infant's saliva, mode of feeding, and microbial cross-talk. In the neonate, oral bacterial colonization starts with streptococci from the viridans group (Pearce *et al*., 1995; Könönen, 2000), whereas significant colonization of anaerobes was not detected in infants before 2 mos of age (Könönen, 2000). While there are no comparable data with HOMIM in infants, the present frequent detection of species in *Firmicutes*, and particularly within the genus *Streptococcus*, is consistent with previous reports of oral colonization by streptococci in infants (Pearce *et al*., 1995; Könönen, 2005). It is unlikely that these species are transients, considering the detection threshold of about 10^4 cells for the HOMIM microarray, indicating that species detection in this assay likely reflects colonization and growth (Paster and Dewhirst, 2009; Olson *et al*., 2011). Notably, treatment of the mothers with antibiotics during delivery was not influential on the oral microbiota in three-month-old infants.

The present dataset was characterized by a larger number of variables than study participants, and by the presence of species that might be interdependent based on shared environmental needs or inter-species co-aggregation. Under these conditions, the multivariate PLS-DA method is suitable to search for subject

Table 3. Variable Importance (VIP) for Bacteria Associated with Mode of Delivery from PLS-DA Multivariate Modeling

Associated with Vaginal Delivery		Associated with Caesarian Section Delivery	
Bacterial Group[1]	VIP	Bacterial Group[1]	VIP
Streptococcus sanguinis	1.50	***Slackia exigua***	3.26
Cardiobacterium hominis	1.50	***Lactobacillus* Cluster I[3]**	1.85
Streptococcus anginosus, Streptococcus gordonii	1.44	***Veillonella* sp. EF509966 (intestinal isolate)**	1.54
***Prevotella* Cluster I[2]**	1.43	***Veillonella atypica, Veillonella parvula***	1.51
***Prevotella loescheii, Prevotella* sp. HOT 472**	1.43	***Streptococcus parasanguinis* I and II**	1.34
Eubacterium* [11] [G-7] *yurii	1.43	***Veillonella parvula***	1.23
***Catonella morbi, Catonella* sp. HOT 164**	1.43	***Gemella haemolysans***	1.32
***TM7* [G-1] sp. HOT 347, *TM7* [G-2] sp. HOT 350**	1.43	***Gemella morbillorum***	1.26
Haemophilus parainfluenzae	1.41	***Streptococcus australis***	1.20
Leptotrichia buccalis, Leptotrichia goodfellowii, Sneathia sanguinegens	1.31	***Bifidobacterium animalis ss. animalis, Bifidobacterium lactis***	1.13
Bacteroidetes phylum	1.28	***Streptococcus cristatus***	1.08
Campylobacter gracilis	1.27	*Kingella oralis, Eikenella* sp. HOT 009	1.03
***Capnocytophaga granulose, Capnocytophaga* sp. HOT 326**	1.26	*Selenomonas noxia*	1.03
Haemophilus sp. HOT 035, HOT 036	1.25	*Selenomonas sputigena, Selemonas* sp. HOT 143	1.03
Kingella oralis	1.18	*Aggregatibacter segnis, Aggregatibacter* sp. HOT 512	1.03
Prevotella nigrescens	1.05	*Campylobacter concisus*	1.03
Corynebacterium matruchotii	1.05	*Streptococcus* sp. HOT 070, 071	1.02
Neisseria elongata	1.05	***Granulicatella elegans***	1.02
Fusobacterium periodontium	1.05		
Capnocytophaga sputigena	1.05		
Streptococcus mutans	1.04		
Prevotella Cluster IV	1.01		
Prevotella melaninogenica, Prevotella histicola	1.01		

Results are shown for a basic model including all infants with HOMIM microarray data (n = 63) and (in bold) a second model restricted to breast-fed infants and those born in gestational week 37 or later (n = 42). In the PLS-DA model including all 63 infants (model R^2 = 0.620, Q^2 = 0435), length at birth, gestational weeks, and town of residence were associated with mode of birth (VIP ≥ 1.0), whereas in the PLS-DA model restricted to breast-fed infants and infants born in or after gestational week 3 (n = 42) (model R^2 = 0.693, Q^2 = 0.496), only length at 3 mos was influential in addition to bacteria.

[1]Unnamed taxa are identified by their Human Oral Taxon (HOT) number from HOMD (Dewhirst *et al.*, 2010).

[2]*Prevotella* cluster I (probe Y65) targets *P. loescheii*, *Prevotella* spp clone GU027, *Prevotella* spp strain C3MKM081, and *Prevotella* spp strain TFI B31FD (HOT numbers 317, 472, 658).

[3]*Lactobacillus* cluster I (probe W94) targets *L. casei, L. paracasei,* and *L. rhamnosus.*

clustering and for identifying the variables characterizing clusters. With x-variables that were scaled to unit variance, and cross-validation of the explanatory capacity to account for over-interpretation, there was good power to discriminate between modes of delivery. The results were also stable after the model was restricted to infants born in wk 37 or later and those being breast-fed. With PLS-DA, several bacterial species differed between infants based on their delivery method. The biological significance of the species evaluated, and detection of fewer species in the mouth in early infancy of infants delivered by C-section, compared with those delivered vaginally, however, is unknown and will require longitudinal evaluation. It is nevertheless notable that a greater microbial diversity in the intestine and in the mouth has been reported to be associated with health (Marsh, 2006; Preza *et al*., 2008; Sjögren *et al*., 2009; Turnbaugh *et al*., 2009; Luoto *et al*., 2011).

A difference based on mode of delivery was detection of *Slackia exigua* in over 76% of the C-section infants (all with HOMIM scores ≥ 2) compared with non-detection in the vaginal delivery group. *S. exigua* is a Gram-positive, strictly anaerobic, asaccharolytic species that has been isolated in root canal infections, periodontitis, extra-oral surgical wounds, and intestinal abscesses (Abiko *et al*., 2010; Kim *et al*., 2010). While it is not known why *S. exigua* was detected in the C-section but not in the vaginal delivery group, it seems possible that the more diverse microbial biofilm of vaginally delivered infants could suppress establishment of this periodontitis-associated species. The association between *S. exigua* and mode of delivery requires further investigation.

In conclusion, the present study indicates a different colonization pattern in the oral cavity between three-month-old infants delivered vaginally and those delivered by Caesarian section. The reasons for the differences are unknown, as is whether these differences have long-term impact on the oral or general health of the child. Possible reasons for differences will likely include the relative influence of host receptor and mucosal and saliva immune phenotypes, and interactions with environmental exposures.

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