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Salivary IgA antibody responses to *Streptococcus mitis* and *Streptococcus mutans* in preterm and fullterm newborn children

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ABSTRACT

Objectives: The intensities and specificities of salivary IgA antibody responses to antigens of *Streptococcus mutans*, the main pathogen of dental caries, may influence colonization by these organisms during the first 1.5 year of life. Thus, the ontogeny of salivary IgA responses to oral colonizers continues to warrant investigation, especially with regard to the influence of birth conditions, e.g. prematurity, on the ability of children to efficiently respond to oral microorganisms. In this study, we characterised the salivary antibody responses to two bacterial species which are prototypes of pioneer and pathogenic microorganisms of the oral cavity (*Streptococcus mitis* and *Streptococcus mutans*, respectively) in fullterm (FT) and preterm (PT) newborn children.

Methods: Salivas from 123 infants (70 FT and 53 PT) were collected during the first 10 h after birth and levels of IgA and IgM antibodies and the presence of *S. mutans* and *S. mitis* were analysed respectively by ELISA and by checkerboard DNA–DNA hybridization. Two subgroups of 24 FT and 24 PT children were compared with respect to patterns of antibody specificities against *S. mutans* and *S. mitis* antigens, using Western blot assays. Cross-adsorption of 10 infant's saliva was tested to *S. mitis*, *S. mutans* and *Enterococcus faecalis* antigens.

Results: Salivary levels of IgA at birth were 2.5-fold higher in FT than in PT children (Mann–Whitney, $P < 0.05$). Salivary IgA antibodies reactive with several antigens of *S. mitis* and *S. mutans* were detected at birth in children with undetectable levels of those bacteria. Adsorption of infant saliva with cells of *S. mutans* produced a reduction of antibodies recognizing *S. mitis* antigens in half of the neonates. The diversity and intensity of IgA responses were lower in PT compared to FT children, although those differences were not significant.

Conclusion: These data provide evidence that children have salivary IgA antibodies shortly after birth, which might influence the establishment of the oral microbiota, and that the levels of salivary antibody might be related to prematurity.

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1. Introduction

The mucosal immune system represents the first line of defence in the adaptive immune response to mucosal infection. Secretory IgA (SIgA) present in saliva¹ may control the oral microbiota by reducing the adherence of bacteria to the oral mucosa and teeth.² The total levels of SIgA in saliva have been considered as an indicator of maturation of the mucosal immune system in children.^{3–6} Transient reductions in the levels of IgA detected in saliva were associated with increased susceptibility to infections of the gastrointestinal tract.^{4,6} Several factors might influence the development of an effective mucosal immune response, including nutritional status, breastfeeding, gestational age, exposition to antigens and genetic factors.⁷ Newborn infants are known to have a higher frequency of microbial infections than older children and adults soon after birth, due to immaturity of the immune system.⁸ Babies born prematurely (less than 37 weeks gestation) have 5 times higher susceptibility to bacterial infections.⁹ Streptococci such as *S. mitis* represent the majority of bacteria that initially colonize the oral cavity.^{10–12} After tooth eruption new species colonize such as *S. mutans*,^{5,13} although such species can be also detected in children before tooth eruption.¹⁴ Prospective study of 5- to 24-month-old children heavily exposed to *S. mutans* showed a complex pattern of salivary IgA antibody reactivity to antigens from *S. mutans* and *S. mitis*,^{15,16} suggesting that responses to virulence-associated antigens early in life may influence the ability of *S. mutans* to colonize the oral cavity. Several recent studies showed that SIgA is present in saliva and other secretions at birth.^{6,7} However, the influence of these antibodies in the establishment of the oral microbiota is unknown.

In this study, we characterised the levels and specificities of salivary IgA antibodies to *S. mitis* and *S. mutans* antigens in newborn children, and compared intensities and complexities of antibody responses between fullterm (FT) and preterm (PT) children.

2. Materials and methods

2.1. Study design

A total of 123 (70 FT and 53 PT) newborn children in the Hospital of the University of Ribeirao Preto, Brazil were enrolled in this study, under mothers consent for their participation. This study was approved by the Ethical Committee of the Medical School of Ribeirao Preto, SP, Brazil, 2963/2007. To be included in the study population, only healthy newborns less than 10 h old were included in this study. Children with congenital malformations, perinatal hypoxia, intracranial haemorrhage, with length or weight incompatible with gestational ages, or under antibiotic therapy were excluded from this study. Gestational age was estimated from the reported date of last menstruation period and somatic evaluation. Information on maternal and gestational background was obtained by interviewing the mother. A total of 123 children, 70 FT and 53 PT were born at mean gestational ages of 39.2 (SD: 1.23, range: 37–41) and 34.5

(SD: 2.21, range: 30–36.5) weeks respectively. Forty-one (18 PT and 23 FT) and 82 (35 PT and 47 FT) infants were delivered by caesarean and vaginally section respectively. Amongst those children ($n = 123$) from whom volumes of saliva collected were suitable for laboratory analysis, two subsets of children were selected for immunological analysis: 24 PT (<37 weeks of gestation) and 24 FT children. For the purposes of comparison, these PT and FT children were paired based on total salivary levels of IgA, gender, racial background and breastfeeding.

2.2. Collection of samples

Samples of whole saliva unstimulated were collected using sterile polypropylene transfer pipettes. Collections were performed in all children at approximately 4–10 h after birth in order to standardize the collection and the oral microbial exposure, and at least 3 h after breastfeeding to avoid contamination with non-salivary components, but in four children (3 FT and 1 PT) saliva samples were collected before the first breastfeeding. Solution of 250 mM EDTA, pH 5.2 (Sigma, St. Louis, MO, USA) was added to each sample prior to transport on ice to the laboratory where they were stored at -80°C until analysis. Samples of colostrum and maternal milk were collected by manual expression into empty sterile containers on the 1st day of lactation from 20 puerperal mothers of the some children in the study. After collection, the maternal samples also were transported on ice to the laboratory, centrifuged to remove lipids components and stored at -80°C until use.

2.3. Detection of Streptococcal species in the oral cavity

The presence of *S. mutans* and *S. mitis* in the samples of saliva of newborn children was investigated by chequerboard DNA–DNA hybridization with species-specific probes as described by Socransky et al.¹⁷ Briefly, 0.5 M NaOH, pH 13.4 (Sigma) was added to saliva samples. After boiling, samples were applied and fixed by exposure to ultra-violet light (Hybrilinker, UVP, Upland, CA, USA) in individual lanes on a nylon membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using the chequerboard slot blot device (Minislot 30, Immunetics, Cambridge, MA, USA). Digoxigenin-labelled whole genomic DNA probes were prepared for each one of the reference strains (*S. mutans* [UA159] and *S. mitis* [ATCC506]) using a random primer technique. These two DNA probes were hybridized perpendicularly to the lanes of the bacterial samples using the Miniblotter 45 (Immunetics Cambridge, MA, USA) at 70°C . Bound probes were detected using phosphatase-conjugated antibody to digoxigenin (Roche Applied Science, Mannheim, Germany) and revealed with CDP-Star[®] (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. The signals developed on X-ray films were scanned in a densitometer (Bio-Rad GS-700 Imaging Densitometer, Hercules, CA, USA) and evaluated using the ImageQuant Software (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). Signals were converted to absolute counts by comparison

with the standards on the same membrane. Failure to detect a signal was recorded as zero.

2.4. Concentration of protein, IgA and IgM

Total concentration of protein in saliva was determined by the method of Bradford (Sigma) to check for variations in salivary flow. Total levels of IgA and IgM were determined in capture ELISA assays as previously described.¹⁵

2.5. Salivary and maternal milk IgA reactivity against bacterial antigens

Patterns of reactivity of salivary IgA and breast milk antibody against *S. mitis* (ATCC 906) and *S. mutans* (UA 159) Ags were determined in Western blot assays. Sixteen micrograms of antigen extracts prepared as previously described¹⁵ were loaded per lane, separated by sodium dodecyl sulphate–6% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After being stained with Red Ponceau (Sigma), membranes were washed and blocked overnight at 4 °C (in Tris-buffered saline–Tween, pH 7.5, 5% nonfat milk). Incubations with samples diluted 1:100 were performed at room temperature for 2 h. As negative controls, membranes were incubated only with blocking buffer, and as positive controls, membranes were incubated with a standard saliva sample obtained from an adult whose pattern of reaction with *S. mutans* and *S. mitis* antigen extracts had been previously measured. The secondary antibody was goat IgG anti-human IgA conjugated with horseradish peroxidase (1:4000 dilution). Antibody reactions were developed using an ECL system (Amersham Biosciences). For this purpose, immunoblots were incubated with ECL detection solution and then exposed to the

same X-ray film for 5 min. The developed X-ray films were scanned in a scanning densitometer (Bio-Rad GS-700 Imaging Densitometer) to analyse patterns of antigen recognition, including the number and intensity of reactive bands. A film blank value was subtracted from the value of the reactive band.

In order to determine whether any of the antibodies detected were uniquely specific for a single species, ten saliva samples (3 PT and 7 FT) were adsorbed sequentially with antigens of cells of *S. mitis*, *S. mutans* and *Enterococcus faecalis* as described by Cole et al.¹⁸ Antibody activities remaining after adsorption (percent) were determined by dividing the optical density at 450 nm of each adsorbed saliva by that of the corresponding unadsorbed saliva at the same dilution and multiplying by 100.

2.6. Statistical analysis

Associations between concentrations of IgA, IgM and total protein, and patterns of antibody reactions were tested by Spearman correlation analysis. Differences in densitometry values of reactive Ags between the subsets of PT and FT children were analysed by a Mann–Whitney *U* test. Comparisons of the frequencies of children with distinct IgA antibody specificities were tested by a chi-square test. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Levels of IgA and IgM

Immunoglobulin A and IgM were detected in all saliva samples tested ($n = 123$). There were statistically significant differences

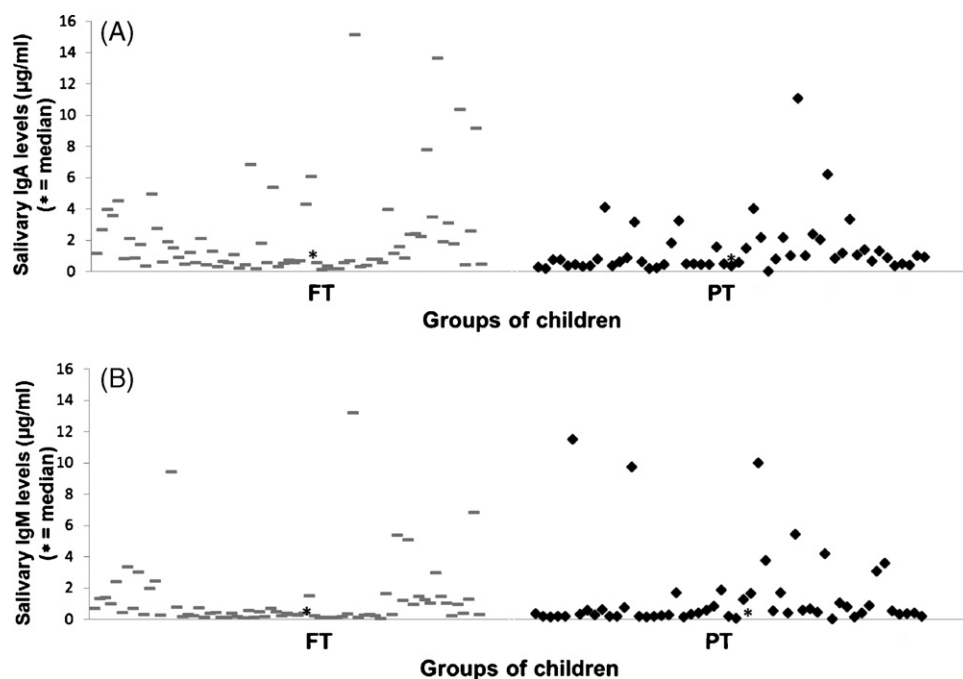


Fig. 1 – Levels of salivary IgA (A) and IgM (B) observed amongst 123 children, Preterm (PT) and Fullterm (FT) measured in the same samples. Squares represent absolute amounts of immunoglobulins in salivas ($\mu\text{g/ml}$). Asterisks represent the median levels of Ig in each group of children.

in levels of salivary IgA between PT (median: 0.78, interquartile range [IQR]: 0.43–1.49) and FT (median: 1.09, IQR: 0.55–2.75) (Mann–Whitney *U* test, $P < 0.05$). A positive correlation was observed between salivary levels of IgA and IgM in each group (Spearman's, $r > 0.75$, $P < 0.01$). Fluctuation of absolute levels of IgA (A) and IgM (B) are shown in Fig. 1. The median concentration of total protein in saliva was 834.3 $\mu\text{g/ml}$ (IQR: 613.9–1219.4), with similar levels in FT and PT infants (Mann–Whitney, $P > 0.05$). The median ratios of values of IgA normalized by protein concentration (median ratio, 0.10, IQR: 0.05–0.20) determined for PT was significantly lower than that observed in FT infants (median ratio, 0.22, IQR: 0.06–0.40; Mann–Whitney; $P < 0.05$). No significant differences were detected in median ratios of values of IgM normalized by protein concentration between groups (PT = median ratio, 0.08, IQR: 0.02–0.15 vs FT = median ratio, 0.10, IQR: 0.02–0.20, Mann–Whitney; $P > 0.05$). The median concentration of total IgA in maternal milk was 2567.8 $\mu\text{g/ml}$ (IQR: 834.0–3986.3) not differing between mothers of preterm and full-term infants (Mann–Whitney, $P > 0.05$). Also, the levels of immunoglobulins and proteins were similar in infants delivered by caesarean section or vaginally (Mann–Whitney; $P > 0.05$).

3.2. Complexity and intensity of IgA response to *S. mutans* and *S. mitis* Ags

Detection of streptococci in oral samples using chequerboard DNA–DNA hybridization assays showed that no children have *S. mitis* or *S. mutans* in saliva samples at the levels tested. Fifty and 37.5% of PT ($n = 12$) and FT ($n = 9$) respectively did not show IgA-reactive bands to the antigen extracts tested. However, amongst the IgA-reactive children, several bands of IgA reactivity with *S. mutans* and *S. mitis* antigens were identified, especially in FT children. Examples of immunoblots incubated with salivas from three representative pairs of PT and FT children against Ags from *S. mutans* and *S. mitis* are shown in Fig. 2A. Maternal and child patterns of IgA-reactivity with *S. mitis* and *S. mutans* antigens were compared. Interestingly, few coincident bands were noted between mother and child. Median percentage values of coincident bands to total number of bands identified were 5 and 8% for *S. mitis* and *S. mutans*, respectively. Three pairs of examples of immunoblots comparing the mother milk and her baby's saliva are shown in Fig. 2B. In addition, the immunoblots from two children (1 PT and 1 FT) who were not yet breast fed presented IgA response to antigens from *S. mutans* and *S. mitis* (Fig. 2A, pair 10).

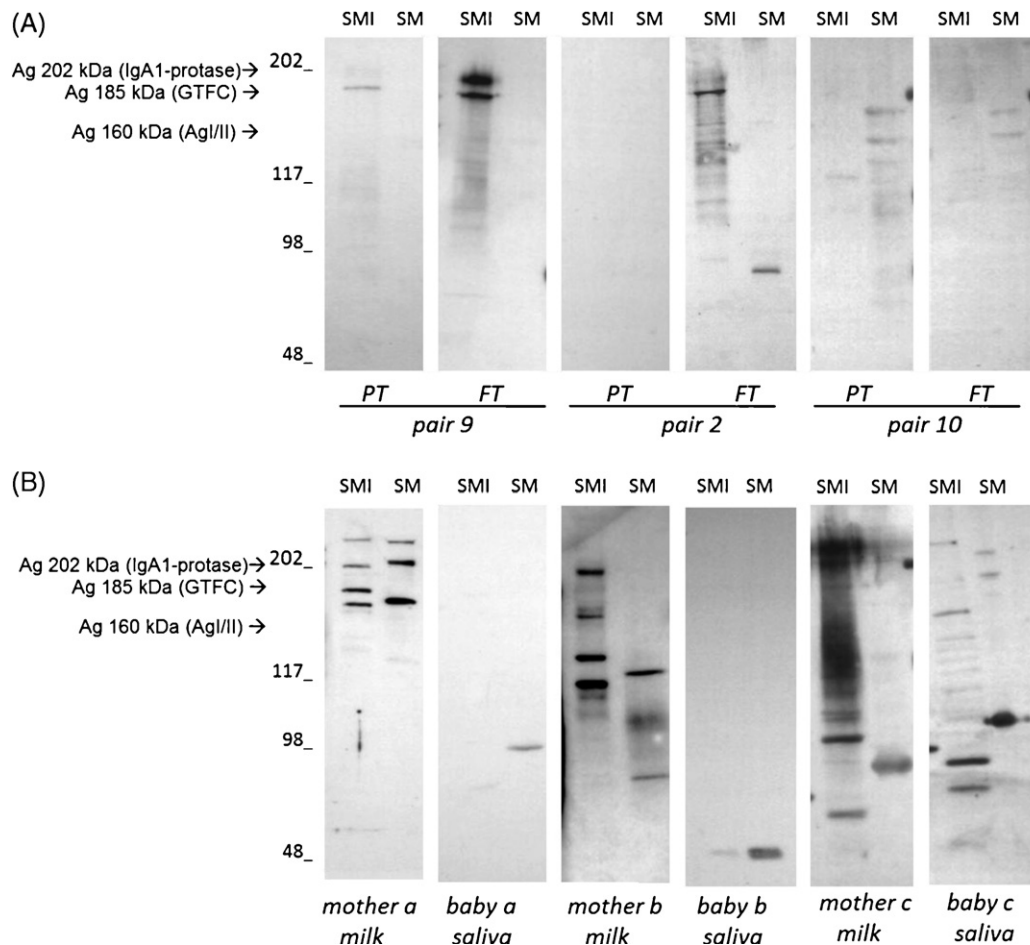


Fig. 2 – (A–B) Patterns of IgA specificities against antigens from *S. mitis* (SMI) and *S. mutans* (SM) in 3 pairs (A) of preterm (PT) and fullterm (FT) children's salivas. Standard molecular sizes (kDa) and specific antigens (202, 185 and 160 kDa) are indicated on the left side of the immunoblots. (B) Comparison of complexity of IgA antibody reactivities between 2 samples of milk's mother and their respective baby's salivas.

Table 1 – Comparisons of the frequencies of children with positive salivary IgA responses and sum of mean intensities to total and specific Ags from *Streptococcus mitis* (SMI) and *Streptococcus mutans* (SM) between preterm (PT) and fullterm (FT) groups.

Antigens	Number (%) of children with salivary IgA reactive		Sum of mean Intensity of salivary IgA reactive \pm SD	
	PT n = 24	FT n = 24	PT n = 24	FT n = 24
<i>Streptococcus mitis</i>				
Total SMI Ags	11(46)	12(50)	296 \pm 575	10201 \pm 29482
202 kDa Ags (IgA1-protease)	3(13)	7(29)	85 \pm 244	107 \pm 226
Mean number of reactive Ags \pm SD	1.0 \pm 1.5 ^a	2.7 \pm 4.1 ^a		
<i>Streptococcus mutans</i>				
Total SM Ags	7(29)	11(46)	215 \pm 567	466 \pm 896
160 kDa Ags (GTF C)	2(8)	6(25)	16 \pm 53	47 \pm 125
185 kDa Ags (Ag I/II)	4(17)	4(17)	21 \pm 88	46 \pm 104
Mean number of reactive Ags \pm SD	0.9 \pm 1.6	1.5 \pm 2.0		

^a Significant differences between PT and FT groups; Mann–Whitney U test: $P \leq 0.05$.

Antigens in both species are shown to react with salivary IgA in both pairs. Taken together, these data suggest that the IgA reactivities seen in the children's salivas are derived, at least in part, from the child.

The numbers of children that presented detectable IgA antibodies to antigens of each *Streptococcal* species and mean numbers of reactive bands detected are shown in Table 1. Although IgA antibody responses were detected more frequently to *S. mitis* Ags ($n = 23$, [11 PT and 12 FT]) when compared to *S. mutans* antigens ($n = 18$, [7 PT and 11 FT]) those differences were not significant (Mann–Whitney, $P > 0.05$). Additionally, the number of IgA-reactive bands to *S. mitis* antigens was significantly higher in FT than in PT children (Mann–Whitney U test, $P \leq 0.05$). Six percent of the SDS–PAGE gels analysed allowed the visualization of important antigens from *S. mutans*: Ag I/II (185 kDa), GTF C (160 kDa) and GbpB (56 kDa) and of *S. mitis*: IgA-protease (202 kDa). Twenty-one percent of children ($n = 10$, [3PT and 7FT]) had IgA reactive to Ag 202 kDa–*S. mitis* and 16.5 ($n = 8$, [2PT and 6FT]) and 17 ($n = 8$ [4FT and 4 PT]) % of children presented IgA reactive to 185 and 160 kDa–*S. mutans* Ags respectively (Table 1). We did not find children with IgA reactive with bands in the 56 kDa region of *S. mutans* blots. There were no significant differences in the number of PT and FT children with IgA responses to these antigens (Qui Square test, $q < 2.01$; $P > 0.27$).

There were variations in the intensities and numbers of IgA antibody reactions with the recognized bands amongst children in both groups. Table 1 shows the sums of intensities of IgA reactions with all bands detected for each species (total intensities) observed in children of the FT and PT groups. In general, FT children presented the highest intensity of IgA to all antigens tested but those differences were not statistically significant (Mann–Whitney U test, $P > 0.2$), likely due to the high variability in intensities of response amongst children of each group.

The results showed that SIgA antibody from 10 samples (3 PT and 7 FT) tested did not react with *E. faecalis* antigens, as SIgA responses to *S. mutans* and *S. mitis* were not reduced by *E. faecalis* cross-adsorption. On the other hand, when samples ($n = 10$) were adsorbed with cells of *S. mitis*, there were mean reductions of 22% of SIgA to *S. mutans* in 5 children (4 FT and 1

PT). In the same children ($n = 5$), there was also a mean reduction of 45% of SIgA to *S. mitis* when samples were adsorbed previously with cells of *S. mutans*.

4. Discussion

Salivary IgA antibodies play several roles in the modulation of the establishment of the microbiota compatible with health homeostasis¹⁹ and form a first line of defence against specific pathogens.¹⁹ Salivary IgA antibodies neutralize antigenic components involved in microbial virulence and might block surface adhesins important for colonization of the mucosa.²⁰ In the saliva, secretory IgA predominates, but early in life, IgM is also normally detected.⁶ Previously, it was described that IgA can be detected in saliva at birth.^{6,7} Here we show that detectable levels of salivary IgA antibodies reactive with common bacterial species of the oral cavity can be detected in the first hours of life.

With respect to gestational age, not only were the total levels of salivary IgA higher in FT (up to 2.5-fold) but also the complexity of IgA against bacterial species (Fig. 2A, Table 1), suggesting that prematurity can lead to a delay in IgA responses at initial stages of antigenic challenge. Longitudinal comparisons of levels of IgA in PT and FT infants could be helpful to clarify the extent to which this difference is maintained over time.

Previously, we suggested that patterns of specificity of IgA antibody responses to *S. mutans* antigens might be more important than total levels of reactive IgA antibodies.¹⁵ In this study, we observed that patterns of protein bands reactive with salivary IgA were variable amongst newborn (Fig. 2A). We reasoned that mucosal responses, most frequently detected in newborns to antigens of *S. mitis*, a pioneer colonizer of oral mucosa, might develop earlier than to *S. mutans*, which colonize children at a later age.^{5,13,14} By separating proteins in 6% SDS–PAGE gels it is possible to visualize the three main cell-associated antigens of *S. mutans*, Ag I/II,²¹ GTF C²² and GbpB⁵ with molecular masses of 185, 160 and 56 kDa respectively. These antigens are involved in the capacity of *S. mutans* to adhere and accumulate in the dental biofilm. A previous study

showed that some five-month-old children presented with salivary IgA reactive to all this antigens, especially to GbpB and may have a role in modulating the level of colonization by *S. mutans*.¹⁵ In the present study, approximately 30% of the children evaluated ($n = 16/48$) presented IgA against AgI/II and GTFC, but not against GbpB (Table 1). Also, 20% of saliva samples from newborn children were reactive with a *S. mitis* 202 kDa component (Table 1), suggesting the presence of IgA reactive to IgA1-protease, an antigen important for *S. mitis* establishment in the oral cavity.^{23,24}

In the present study we analysed the specificity of salivary SIgA antibodies reactive with *S. mutans*, *S. mitis* and *E. faecalis*, to test whether SIgA antibodies reactive with commensal oral bacteria were induced by these bacteria and were, therefore, specific to them or whether they were induced by cross-reactions with other bacteria. The results of cross-adsorption showed that in half of the saliva tested ($n = 5$ of 10), there was a reduction of the salivary IgA to *S. mutans* when the plate was previously adsorbed with *S. mitis* antigens. A similar result of levels of salivary IgA to *S. mitis* occurred when the plate was covered with *S. mutans*. The elimination of salivary IgA antibodies reactive with the test species following sequential adsorption of saliva samples with each streptococcal species supports partially the conclusion that the antibodies were cross-reactive rather than species specific, as described previously.^{18,25}

Although in our previous study we detected SIgA antibodies reactive with *S. mutans* in saliva of preterm infants who did not harbour *S. mutans*,¹⁵ here, the presence of specific antibody at birth is unlikely to have been induced within 10 h, since it takes at least a week for the uptake, processing of antigen, B cell selection and migration to local sites, differentiation into plasma cells leading to antibody secretion and endosomal transfer into the gland lumen. Thus, some hypotheses can be raised to address this early response of SIgA to *S. mutans* and *S. mitis* antigens. Firstly, the presence of residual of IgA from maternal milk in the oral cavity of children cannot be excluded, even though the samples have been collected at least 3 h after breastfeeding. For this reason, we compared immunoblotting of infant salivary samples with their respective maternal milk samples (Fig. 2B). The majority of antigens that were more frequently reactive in the infant salivary samples were not recognized by maternal milk (Fig. 2B). Additionally, immunoblots from children who did not receive maternal milk (Fig. 1A, pair 10) presented with IgA antibody reactivity with *S. mutans* and *S. mitis* antigens. The persistence of secretory antibodies in the oral cavity (e.g., following breast feeding) strongly depend on their adhesion to salivary pellicle on tooth surfaces.²⁶ Since newborns are edentulous, this condition for persistence of maternal IgA is absent.

An alternative hypothesis could be associated with the plurispecific protection at mucosal surfaces, proposed by Quan and coworkers,²⁷ who found that SIgA antibodies from human saliva reacted with actin, myosin and tubulin but also with antigens from *Streptococcus pyogenes*. Also, those antibodies could result from stimulation without antigenic exposure, as the result of anti-idiotypic induction²⁸ or intra-uterine stimulation. Thus, several bacteria have been isolated from umbilical cord blood, amniotic fluid and foetal membranes without clinical or histological evidence of infection or inflammation in pairs of mothers and children.²⁹

In summary, the results show that detectable levels of salivary IgA antibodies reactive to oral bacterial species can be detected within the first hours after birth. Furthermore, the salivary IgA concentrations and IgA antibody specificities appear to be influenced by the gestational age, which might reflect the level of immunological maturity of the mucosal immune system. These findings support further study about the investigation of antibody and microbial sources from mother in order to clarify the role and development of mucosal immune response in neonates.

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Conflict of interest: The authors declare no conflict of interest.

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Ethical approval: This study was approved by the Ethical Committee of the Medical School of Ribeirão Preto, SP, Brazil, reference number: 2963/2007.

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