Comparison between Clinical Aspects and Salivary Microbial Profile of Children with and without Early Childhood Caries: A Preliminary Study

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***Purpose**: To compare the clinical aspects and microbial profile of children with and without early childhood caries (ECC). **Study design:** 14 patients (7 without caries and 7 with ECC) were submitted to anamnesis, clinical exam and saliva collection for microbiological analyses. Counts of Streptococcus mutans, Lactobacillus spp. Candida spp., and total microorganisms were performed by culture methods. Microbial diversity was characterized by PCR-DGGE. Demographic/clinical data and salivary microbial counts were compared between groups. **Results**: Habits of hygiene and breastfeeding presented no association with ECC. Use of pacifiers was associated with absence of caries (p=0.035). Counts of total microorganisms and Candida spp. did not differ between the groups. The ECC group presented larger quantity of S. mutans (p=0.026) and Lactobacillus spp. (p=0.002). There was no correlation between microorganisms and breastfeeding and pacifier use. The dmf-t of ECC Group was 10.5±1.9 and the modified dmf-t was 11.3±3.6. The DGGE demonstrated difference in the pattern of bands between the groups. **Conclusion**: Pacifiers usage was associated with the absence of ECC and microorganism number was higher in the caries group. The PCR-DGGE revealed a characteristic microbial diversity in the ECC Group, being an excellent tool for observing the dynamics of the salivary microbial community in these patients.*

Key words: *Early childhood caries, Preschool children, Salivary microbes, Polymerase Chain Reaction.*

INTRODUCTION

According to the American Academy of Pediatric Dentistry[1], children under the age of six years, presenting one or more caries lesions, are categorized in a group of individuals with early childhood caries (ECC). This is an aggressive form of caries disease which, when found in children under the age of three years, is denominated severe early childhood caries, and may present as the atypical, progressive, acute and rampant type[2].

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In spite of the advancement in the area of cariology and reduction in prevalence and levels of caries in the population over the last 30 years, in many countries ECC continues to be a public health problem[3]; which has attracted the attention of the scientific community with regard to the factors involved in the etiology of the disease[4]. Risk factors such as the presence of cariogenic biofilm, prolonged and frequent consumption of sugary foods, and systemic, environmental, behavioral and lifestyle factors are associated with the development of ECC[5,6]. Moreover, the diversity of the microbial community involved has an influence on the clinical signs and progression of this pathology[7].

Caries is a biofilm-dependent disease[4] and is strongly associated with the presence of *Streptococcus mutans*, considered one of the main cariogenic bacteria. In addition to this microorganism, other microbial species are usually found in association with ECC, such as *Lactobacillus ssp., Candida ssp., Bifidobacteria ssp., Actinomyces ssp.* and *Veillonella ssp.*[4,8-11]. As the oral cavity represents a diverse ecosystem, with over 600 microbial species in multiple *habitats*[12], studies that investigate the composition of oral microbiota in patients with ECC may help elucidate the shifts in the microbial population necessary for the establishment of this pathology and hence advance the understanding of the factors that trigger the disease.

Molecular analyses[7,12] have demonstrated that there are differences in the microbial composition of biofilm between individuals and even within the individual, considering the different areas of the oral cavity. Among the methods of molecular analyses available, denaturing gel gradient electrophoresis (DGGE) arise as a powerful technique for qualitative analyses of polymicrobial ecosystems. The microbial profiles obtained by DGGE from both saliva and biofilm samples may be compared with other microbiological analyses[2]

and with the patient's clinical data; thereby contributing to better understanding of the nature of ECC. Thus, the aim of the present study was to compare the clinical and microbiological aspects of children with and without early childhood caries and to characterize the microbial diversity of these children by means of PCR-DGGE.

MATERIALS AND METHOD

The sample of the present study was composed of 14 patients aged between 2 and 4 years, from the Pediatric Dental Clinic at the Federal University of Rio de Janeiro, accompanied by their guardians, for initial clinical exam, without any previous dental treatment. The patients were included in two different groups: 1- Patients with ECC; 2- Control Group of caries-free patients. In order to be included in the study, the patients had to present good general health, in accordance with the criteria of Zero *et al*.[13], and could not be making use of antimicrobial agents for a period of 6 months prior to the exam. This study was conducted after obtaining approval from the Research Ethics Committee of the "Hospital Universitário Clementino Fraga Filho" – Federal University of Rio de Janeiro (HCFUF/UFRJ) and the signatures of the guardians were obtained with reference to the Term of Free and Informed Consent.

During the first consultation, careful anamnesis was performed with the guardians, in which information about gender, age, deleterious habits, diet and oral hygiene were collected. The clinical exam was also performed during the initial consultation, in which the entire oral cavity was inspected, and after professional prophylaxis, all the teeth present were evaluated in accordance with the modified dmf-t index[14]. Thus, in addition to the data with reference to dental caries, commonly evaluated in epidemiological surveys (teeth with open cavities, with extraction indicated, or restored), the teeth affected by active white spot lesions were also noted. These had to present a whitened, opaque and porous appearance[15]. The healthy teeth were also noted, adding that a single, previously trained examiner performed the clinical exam.

For the examiner calibration process, five children were randomly selected from a Pediatric Dental Clinic at UFRJ. A total of 100 teeth were examined in two distinct time intervals, by the post-graduate professor responsible for the clinic, and after this, by the examiner of the study. The inter-examiner agreement was evaluated (Kappa=0.838), as well as the intra-examiner (Kappa=0.836) agreement. The children who participated in the calibration process were not included in the main study.

Saliva Collection

With the patient comfortably seated in the dental chair, saliva collection was performed at a time prior to the clinical exam. This collection was done by means of using a 1000µL pipette with sterilized tips. It should be pointed out that this procedure was performed without the tip coming into contact with the peribuccal tissues. The saliva collected was stored in a sterile 1,5ml micro centrifuge tube for each patient. The saliva samples were immediately taken to the Interdisciplinary Dental Research Laboratory of the Dental School of UFRJ for seeding in cultures and DNA extraction.

With the purpose of investigating the salivary levels of total microorganisms, *Streptococcus mutans*, *Lactobacillus spp*. and *Candida spp*., in both study groups, 100 µL of saliva (dilutions between 10^{-1} and 10^{-5}) were plated in duplicate in Petri dishes, with the aid of a Drigalsky loop. The following culture media were used: (1) BHI agar (Difco, USA) for total microorganism colony counts; agar Mitis salivarius (Difco, USA) with addition of bacitracin and potassium telurite to identify *S. mutans* colonies; (2) agar Rogosa (Difco, USA) for *Lactobacillus spp*., growth; and (3) CHROMagar Agar (Chromagar, France) for analyzing colonies of *Candida spp.* The plates were incubated under microaerophilic conditions at 37ºC for 48h, except for those used for identification of *Candida* species, which were incubated in a microbiological incubator at 37ºC for 24h. The results of microbial counts were expressed by mean number of colony forming units (CFU/mL) of *S. mutans*, *Lactobacillus spp*., *Candida spp*., and total microorganisms. The experiment was conducted in duplicate.

DNA Extraction

DNA extraction was performed as previously described[5]. The saliva samples were thawed and vortexed for 30 seconds. A 500 µL sample was used for bacterial DNA extraction. Samples were centrifuged for 2 minutes and 180 µL of enzymatic solution (20mg/ml lysozyme; 20mM Tris-HCL, [pH 8,0]; 2 mM EDTA; 1.2% Triton) were added to the pellet, resuspended and incubated for 30 minutes at 37ºC to guarantee adequate lysis of the gram-positive bacteria. The bacterial DNA was then purified with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. The extracted DNA were stored at -20ºC until further use.

PCR Amplification

For the DGGE technique, a Polymerase Chain Reaction (PCR) was initially performed with universal primers PRBA338f GC and PRUN518r, specific for the V3 region of the bacterial 16S rRNA genes. The PCR mix contained 10 mM Tris-HCl pH 8.3, 2,5 mM MgCl2, 200 µM of each nucleotide (Promega), 2,5 U of *Taq* DNA polimerase (Life Technologies), 10 µM of each primer and 40ng of DNA. PCR reaction was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) with the following conditions: initial denaturation of 92°C for 2 minutes, 30 cycles of [92°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute] and a final extension of 72°C for 6 minutes, The presence of the PCR products was confirmed by electrophoresis in a 1.2% agarose gel in a Tris-borate-EDTA (TBE) *buffer* run at 80V. The gel was stained for 15 minutes with 0.5µgml of 1% ethidium bromide and visualized under short-wave ultraviolet light.

DGGE Analysis

The DGGE of the PCR products was attained using a Dcode System (universal system for the detection of mutations; Bio-Rad, USA). The gel contained 6% (w/v) polyacrylamide with a denaturing gradient of 45-65% (urea and formamide). All the gels were loaded with DNA markers in the first and last lanes to allow standardization of the samples, as indicated by the manufacturer. Electrophoresis was performed in a 1X Tris-acetate-EDTA *buffer*, at a temperature of 60 ºC at a constant voltage of 75V, for 16 hours. The DGGE gel was stained with Sybr Gold (Invitrogen, São Paulo, Brazil) and visualized with the aid of the software program Storm 860 Imaging System (GE Healthcare, Munich, Germany).

Microbial profile analyses by DGGE

The DGGE gels were aligned with the aid of the software GelCompar (GelCompar II Software, version 5.10, Applied Maths, Belgium) and dendrograms were generated using the software BioNumerics version 6.0 (Applied Maths, Ghent, Belgium). Banding pattern was compared using the Pearson correlation coefficient and analyses of the groups was performed by the *weighted pair group* method with mean bonds (UPGMA),

For this analysis, each patient in the group with caries and those from the group without caries were identified as caries and healthy, respectively.

The data were analyzed with the statistical software program SPSS version 20.0 (SPSS Inc, Chicago. USA). The Kolmogorov-Smirnov test was used to verify the normal distribution of the microbial culture results. In view of the normal distribution of data, the Student's-*t* test was used to observe differences between the groups of children with and without caries, in relation to the microbial counts.

With regard to the demographic and clinical data, such as gender, age, habits and diet, the chi-square test was used for comparison between the groups. Furthermore, in order to verify a possible correlation between the microbial count data and the breast-feeding and pacifier-use data, both the Pearson correlation coefficient and chi-square test were used. For all analyses, a level of significance of 5% was considered.

RESULTS

Of the 14 patients examined, 7 were diagnosed with ECC and 7 had no caries lesions. The patients' mean was age 2.7 years ± 0.9, without difference between the groups (p=0.278) (Table 1). As regards gender, 64.3% of the participants were male, and no difference in prevalence of caries among boys and girls was observed (p=0.133) (Table 1). When considering the oral hygiene habit, 100% of the guardians responded that they brushed the child's teeth after meals and between them, 7 were still breast/bottle fed. Considering these children who are still breast/bottle fed, only one performed hygiene after feeding, however, this hygiene habit presented no association with the presence of caries (p=0.607). In addition, the breast/bottle feeding also showed no association with the presence or absence of caries (p=0.543) (Table 1). With regard to the factors investigated, only the use of pacifiers presented association with the absence of caries, bearing in mind that the entire group of children who had EEC (n=7) did not suck pacifiers (p=0.035) (Table 1).

Table 1: Characterization of sample studied as regards gender, age, diet, pacifier-sucking habit and hygiene.

Variable*	ECC		*p*-Value
	No (n=7)	Yes (n=7)	
Gender			
Boy (n)	6	3	0.133
Girl (n)	1	4	
Age/ mean ± (SD)	2.42 ±0.78	3.00 ±1.00	0.278
Still breast/bottle-fed			
Yes (n)	3	4	0.543
No (n)	4	3	
Use of Pacifiers			
Yes (n)	4	0	0.035
No (n)	3	7	
Hygiene after feeding**			
Yes (n)	1	0	0.607
No (n)	3	3	

Note: The chi-square test was used for statistical analyses. ECC = early childhood caries; SD = standard deviation.**Only patients who were breast/bottle-fed were considered.

When analyzing the data of microbial culture (Figure 1), the quantity of total microorganisms did not differ between the studied groups (p=0.583). However, when some of the microorganisms specifically involved in ECC were investigated, the group of patients with caries presented a higher number of *S. mutans* (p=0.026) and *Lactobacillus spp.* (p=0.002) colonies. Whereas the number of *Candida spp.* colonies did not differ between the groups (p=0.479), as the mean values of this microorganism were 0.92 ± 1.2 and 0.55 ± 0.7 for children with and without caries, respectively.

The absence of correlation could be verified between the microbial culture data for each type of microorganism evaluated, and breast/bottle feeding and pacifier-use; that is to say, between children who are breast/bottle fed and those who are not, the total microorganism count (p=0.956), *S. mutans* (p=0.102), *Candida spp*. (p=0.184) e *Lactobacillus spp*. (p=0.143) was similar. In the same way, children who sucked pacifiers also did not present a higher number of total microorganisms (p=0.949), *S. mutans* (p=0.245), *Candida spp*. (p=0.286) and *Lactobacillus spp*. (p=0.283).

The mean dmf-t of the group with ECC was 10.5 ± 1.9, while the modified dmf-t was 11.3 ± 3.6. Within this group, caries patient 5 (Table 2 and Figure 1) presented the lowest dmf-t. The modified dmf-t data and microorganism counts of each volunteer/patient in the study may be observed in Table 2.

The DNA samples of only six patient in each group were submitted to the DGGE analyses, because the quantity of saliva expectorated by two patients (caries 1 and healthy 1) was insufficient to enable both microbiological analyses to be performed. It was observed that the mean number of bands of each group, indicated by the position of the bands in the gel, was similar (p=0.502): Group without caries (22.67 ±4.46), and with caries (23.5 ±5.32).

The result of the DGGE analysis demonstrated a difference in the pattern of bands of the studied sample, in which two large clusters were formed, one of patients with caries and the other of healthy patients (Figure 1). One of the patients (healthy 4) remained between the two clusters, however closer to the group of caries-free patients. Two caries-free patients (healthy 2 and 3) were agglomerated into the *cluster* of the group with caries, in the same way as two children with caries disease (caries 6 and 7) formed part of the cluster of the group without caries.

DISCUSSION

Studies using culture based techniques for assessing microbial diversity are useful in understanding the impact of the composition of the oral microbiota in diverse pathogenic processes. However, PCR-DGGE has been shown to be an important, culture-independent tool for observing changes in the pattern of microbial communities in various diseases as well; particularly when this pattern is compared with various environmental conditions[5,7], as it helps in the investigation of possible trigger factors for a certain condition. Thus, the aim of the present study to compare the clinical aspects

and microbial profile of children with and without early childhood caries (ECC), monitored by PCR-DGGE, is justified.

ECC is clinically characterized as the presence of one or more teeth with caries lesions[1,2], with progression of the lesion being extremely rapid, normally presenting in a destructive form, mainly when it affects children under 3 years of age[16]. The clinical data of the present study demonstrated this pattern of tooth destruction, bearing in mind the mean value of the modified dmf-t of the group of children with ECC (11.3 ± 3.6).

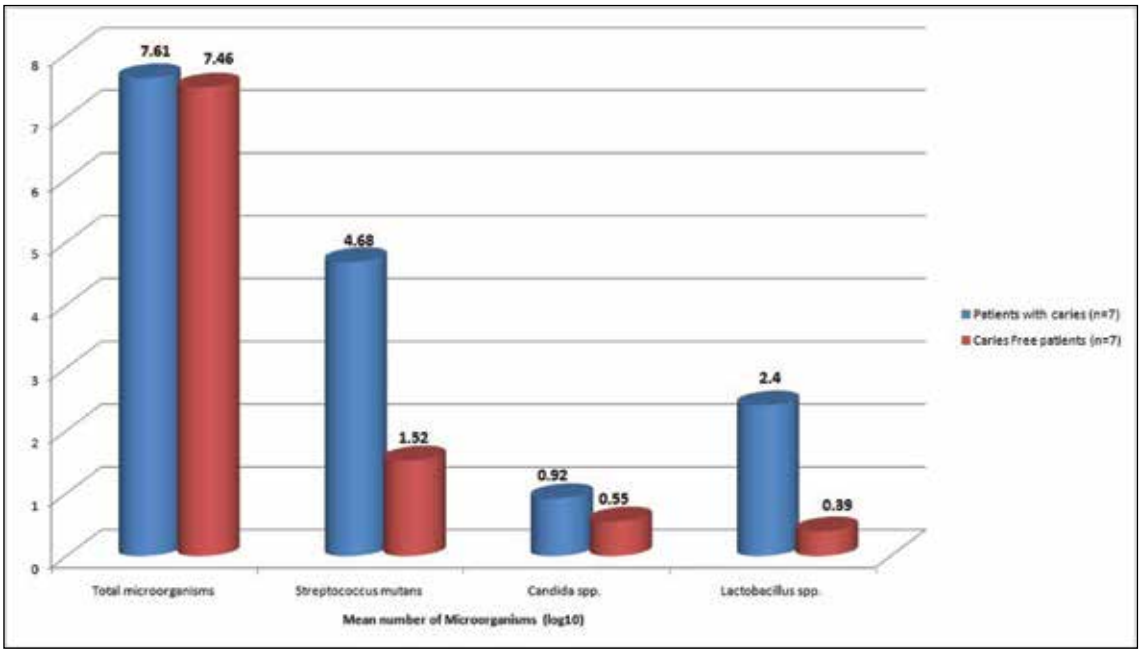
The association between ECC and breast/bottle feeding is still controversial in the literature[17,18]. According to Mohebbi *et al.*[19], breastfeeding for over one year, and particularly at night, is strongly associated with ECC. However, in a systematic review[20] the authors affirmed that there is no consistent evidence that demonstrates the association between ECC and breastfeeding, specifically. In addition, an *in vitro* study conducted in 2009[21] demonstrated that mother's milk, in particular, is not cariogenic, but indeed it is a protective food against caries. This study affirmed that the milk was capable of preventing *S. mutans* colonization on hydroxyapatite discs. In the present research we also observed no association between

Table 2: Characteristics of patients with and without early childhood caries, considering mean number of microorganisms (UFC/mL) and modified dmf-t index.

Volunteers	Total Micro-organisms (CFU/mL)	*S. mutans* (CFUmL)	*Candida spp.*(CFU/m)	*Lactobacillus spp.*(CFU/m)	*Dmf-t*
Caries1	7.41	5.51	2.13	3.74	13
Caries2	7.41	4.83	ND	4.28	9
Caries3	7.41	5.74	ND	3.61	15
Caries4	8.00	5.72	1.48	ND	13
Caries5	7.30	5.43	ND	ND	6
Caries6	8.04	5.51	2.81	5.16	15
Caries7	7.71	ND	ND	ND	8
Caries free1	6.48	ND	ND	ND	0
Caries free2	7.38	5.24	1.65	2.76	0
Caries free3	6.77	5.26	ND	ND	0
Caries free4	7.98	ND	1.00	ND	0
Caries free5	8.13	ND	ND	ND	0
Caries free6	8.00	ND	ND	ND	0
Caries free7	7.48	ND	1.18	ND	0

Note: CFU- Colony Forming Units; ND - Not detected; dmf-t -Decayed, missing, filled primary teeth. For better comparison, the values of microorganism counts are expressed in logarithmic scale ($\log_{10}$).

Figure 1: Mean number (CFU/mL) of total microorganisms, *Streptococcus mutans*, *Candida spp.* and *Lactobacillus spp.*, of groups of children with and without caries.



breast/bottle feeding in general, to the above-mentioned condition. Moreover, the children who were breast/bottle-fed did not present higher quantities of any of the microorganisms evaluated, when compared with children who were not breast/bottle-fed. In any event, it is worth pointing out that this is a preliminary study with a small number of children involved. Therefore, future researches are necessary to elucidate this question.

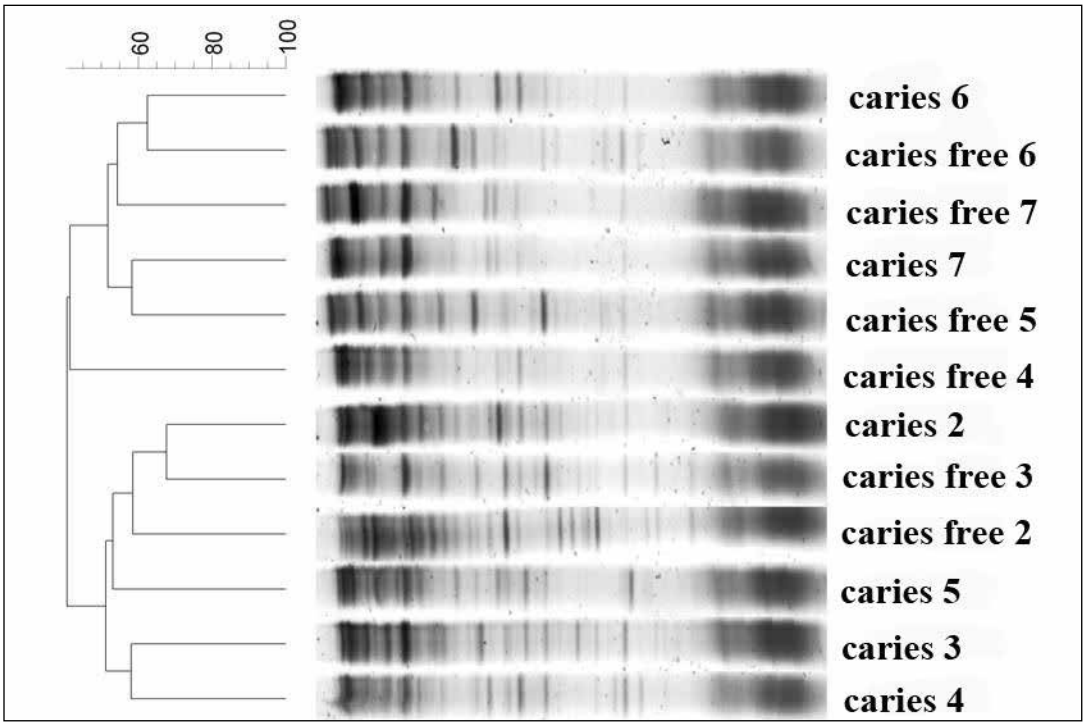
In addition, no difference was observed between the quantity of total microorganisms, *S. mutans*, *Lactobacillus spp*. and *Candida spp*. in children who did or did not suck pacifiers. However, the use of pacifiers was associated with the absence of caries, as all the children in the caries group did not suck pacifiers, and among those of the group without caries, four sucked pacifiers. It is known that one of the determinant factors of caries disease is saliva, which participates directly in the process of de/remineralization of the dental structure, due to its supersaturation with calcium and phosphate ions in relation to tooth enamel. Saliva also has other important functions in caries prevention, such as buffering the acids produced in oral biofilm and antimicrobial properties[22]. Thus, the authors of the present study suggest that the use of pacifiers may have increased the salivary flow of these children, thereby contributing to a lower prevalence of caries lesions. In addition, it is suggested that children who suck pacifiers ingest foods in more prolonged time intervals, thus favoring the increase in the pH of both saliva and biofilm. However, how this pacifier was offered to these children was not investigated; for example, whether it was sweetened or no, which would make it relevant to prepare further studies, particularly with a larger number of children, with the purpose of seeking stronger evidence with respect to this topic. With further respect to these results, it is important to point out that the use of pacifiers may lead to the development of a future malocclusion, which restricts its indication.

Studies have demonstrated the association of ECC with the presence of certain microorganisms such as *S. mutans*, *Lactobacillus spp.* and *Candida albicans*[4,6,23]. *S. mutans* is considered a classical cariogenic microorganism, present in initial carious lesions[7,23]. The presence of this bacterium in high numbers is generally associated with caries, and the American Academy of Pediatric Dentistry[1] has recommended its reduction. In the present study, the patients in the group with caries presented the highest counts of this microorganism, when compared with the group without caries. Whereas, with regard to *Candida spp.*, although there was no statistical difference between the groups studied, the patient who did not have caries lesions presented slightly lower counts. Klinke *et al*.[24] have stated that the *Candida spp.* counts were shown to be lower, when compared with the colony counts of *S. mutans* and *Lactobacillus spp*.

The presence of high numbers of *Lactobacillus spp*. is associated with the presence of retentive areas, as these bacteria are described as being related to the progression of carious lesions[11]. Therefore, the result obtained in this study is in agreement with the literature, with the difference between the group with and without caries being significant and the highest values of counts of this microorganism being present in the group with caries lesions.

In spite of the difference found in the cariogenic bacterial counts between the groups of the study, we observed no difference with regard to the values of total microorganisms, corroborating the results of the number of bands observed in the DGGE analysis, that were similar between these groups. It is known that the oral cavity is colonized by diverse microbial species, with this number being higher than 600 different species[12]; therefore, equivalence in the total microorganism counts between the two groups was to be expected. Mainly because the microbial population is in constant competition; that is to say, under a certain environmental pressure

Figure 2: DGGE profiles of bacterial communities of saliva samples of 12 children: Six with early childhood caries (ECC), identified as caries, and six without ECC (identified as caries free).



such as regular conditions of sugar and low pH, this microbiota only becomes altered in order to become an inducer of disease [25]. In other words, there is an increase in some species to the detriment of others, which probably does not alter the final count when the general number of microorganisms is counted.

In terms of microbial ecology, the PCR-DGGE technique represents an important tool for detecting alterations in microbial patterns[26]. When observing the data of the dendrogram, one perceives that two large main groups were formed: one in patients with caries and the other in patients without caries. These results corroborate the findings of Li *et al*.[16], in which these authors, by means of the PCR-DGGE technique, also observed the same pattern among their volunteers, and affirmed that the bacterial philotypes present were shown to be associated with disease (patients with caries) or healthy (caries-free). These authors added that these results, as in the case of those of the present study, demonstrated that caries disease may be presumed by means of the pattern of the bands of patients, obtained by the DGGE technique.

When each cluster/group (children with and without caries) was analyzed separately, one observed that there were patients with inverse conditions of health or disease within each of them. Jiang *et al*.[23], when analyzing the microbiota of patients with and without ECC, observed 3% of dissimilarity of the microbial population found in each group evaluated, which justifies the presence of these discrepancies in our study. Furthermore, by means of an individualized analysis, it could be verified that the healthy patients 2 and 3 agglomerated into the caries cluster, presented high levels of *S. mutans* (Table 2), in the same way as did the patients themselves of this group, which could explain a greater proximity in the pattern of bands of these caries-free individuals to those who have the disease. In the same way, the caries patient 7 presented microbial count characteristics (Table 2) very similar to patients without caries, which may also have influenced his/her position in the cluster of patients without the disease. Whereas caries patient 6, in spite of having been agglomerated into the cluster of caries-free patients, did not present microbial counts that were similar to any of the patients in this group. According to Tao *et al*.[5] each individual presents his/her own microbial identity, which may justify the appearance of distinct cases in studies that involve the PCR-DGGE technique. Moreover, the type and/or quality of diet may also represent confounding factors in these cases, as they may influence the individual's microbiota. Therefore researches with a large number of patients and a strict study designs must be conducted.

CONCLUSION

Our results demonstrated association between the use of pacifiers and absence of caries lesion, when a group of children with and without ECC was considered. Moreover, a high number of cariogenic bacteria was found in the oral cavity of children with the disease, in addition to a characteristic pattern of bands. PCR-DGGE was shown to be an excellent analytical tool for observing the dynamics of the microbial community of these patients, helping one to gain a better understanding of this disease. However, as this was a preliminary study, the authors suggest that further researches should be conducted with a larger number of children, in order to provide increasing evidence about the findings of the present study.

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