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Associations between Species Mutans *Streptococci* and *Candida Albicans* in Oral Cavity of Children and Adolescents with Intellectual Disabilities

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ABSTRACT

Background: *Oral microorganisms produce damage through the transfer to blood stream, colonising other tissues or direct damage in the oral cavity. Aim is to compare the association between mutans streptococci and the C. albicans in the saliva of the oral cavity of patients with Intellectual disabilities (ID). This study determined the quantitative interaction between mutans streptococci (MS) and the C.albicans (CA) and mutans streptococci species in saliva especially. Scanning electron microscopy identified morphological types of microorganisms in saliva and dental calculus of Chonoan (Chonos) ancestral human remains.*

Patient and Methods: *Our study included 120 patients of both genders: 60 patients with Intellectual disabilities (ID) and 60 patients as a control group (CG). Samples of saliva were taken and bacteria and fungi were grown on TYCSB and Sabouraud agar. Microbiological, serological and quantitative analyses were performed to determine the type of isolation of microorganisms corresponding to the streptococci mutans(SM) c, e, f and k for species S. mutans and d, g for S. sobrinus (SS) and C. albicans (CA). Scanning microscopy was employed to visualize and confirm the morphotype under study. Statistical analysis included t-test proofs for matched data test, Mann-Whitney and Kruskal-Wallis.*

Results: *Forming units CFU per-ml of saliva of C. albicans presented a significant difference observed among ID>CG groups. A correlation of the MS quantity and the C. albicans count was found by age intervals. However, tendencies were different in ID and CG. Also, the CFU of C. albicans was independent from the serotypes of MS (c, e, f, k, d, g and no type), and morphotype of microorganisms present in calculus dental human remains of human Chonoan.*

Conclusion: *These results show a significant non-random association between these two commensal microorganisms in different patient groups, morphotypes of microorganisms present in saliva and dental calculus of de Chonoan ancestral human remains.*

Keywords
Intellectual disabilities, mutans *streptococci*, *Candida albicans,* morphotypes.

Introduction

Oral hygiene is important in oral health, since it implies major control of plaque bacterial, a risk factor in the production of oral infectious diseases, either due to the conditions and the requirement of assistance. Due to this we have patient with intellectual disability (ID) have poor oral hygiene, which brings with it a high prevalence (cross-sectional) of oral pathologies such as Dental caries (caries), Gingivitis and Periodontal diseases, all of which are produced locally by microorganisms present in the bacterial plaque of the teeth [1-4]. The oral cavity is a space made up of soft tissues, hard tissues and saliva colonised by microorganisms such as bacteria, fungi, viruses, mycoplasmas, protozoa and others that are part of the bacterial plaque when calcified and forming dental calculus or tartar. Previous studies made a schematic representation of the adhesion of bacteria in dental plaque according to Kolenbrander PE, London J. [5], being the second largest microbial community in humans after the intestine. The study of the ancestral microbiome in human oral tartar has revealed the importance of the association between humans and microorganisms based on life function, such as the role of acute and chronic diseases and their bioanthropological evolution over time [6]. Ancestral, the presence of *S. mutans* and *P. gingivalis* has been demonstrated in ancient civilizations, specifically Chonoan from the Guaitecas Archipelago in southern Chile [7,8]. Through the remains of human study, compatible infectious diseases such as caries, periodontal and bone lesions in both jaws were visualised, accompanied by poor oral hygiene, as is the case in the ID patient [1]. They may be associated with cultural and physiological factors conforming to they did not have oral hygiene habits [7,8]. Subsequent molecular biology studies such as the indirect immunofluorescence technique and PCR amplification demonstrated the presence of microorganisms in the dental tartar of the Chonoan (Chonos) [9,10]. The PCR technique summit species with two or more variants in some of them present in ancient civilizations, specifically P.gingivalis in Chonoan, with a high frequency of periodontal disease due to microorganisms present in 5 complexes of subgingival microorganisms, one of them is part of a complex network, (*P. gingivalis*, *T. forsythia*, *T. denticola*) are found most frequently in deep periodontal pockets according [13].

Probably, changes in the subsistence patterns produced specifically in diet are a change in the oral microbiome, which favors the development of the mutans streptococci group, as an agent in the production of caries in humans [11,12].

We may think of random mutations of the dextranase gene, whose substrate is sucrose, yielding a big diversity of nucleotides and haplotype maintained by selective or neutral evolution. This gene breaks α 1,6 bonds of glucans and participates in the metabolism of sucrose leading to production of acids that over time would yield to differences between ancient and modern populations [14].

The association between mutans streptococci (mutans-group) and *Candida albicans* happens because they are commensal microorganisms that coexist as a homeostatic mutualism in dental plaques and saliva [15,16]. The MS in humans presents serotype c,e,f, and k for SM and serotypes d and g in SS [17,18]. Usually serotype c is the most frequent in Western society. Caries dental is a multifactorial injury so much in vitro and in vivo, both produced for fermentation, due to the consumption of sucrose in the diet. *C. albicans* participates in an additional form in the acidogenesis process depending on both host and *C. albicans* characteristics [19,20].

At the systemic level *mutans streptococc*i with the presence of serotype k for *S. mutans* possibly corresponds to the no-type or another, in our works [21,22] the one that has been isolated in patients with infective endocarditis [23-25].

C. albicans is a species that is acquired on the first day of birth, in the absence of lesion, *C. albicans* has been identified in saliva or within the oral cavities of 45% of healthy newborns, 45 to 65% healthy children and 30% to 45% healthy adults. The presence of *C. albicans* in the oral cavity may vary between 30% and 50% of the people with varying carriage, depending on the population group study. The oral carriage of *C. albicans* ranges 20% to 75%, owing to factors such as age, smoking, sex, oral hygiene and its association with systemic diseases. It is also found in immunocompromised patients, 95% of patients with *C. albicans* being the causal agent of a wide variety of mucosal infections [26-29].

In recent years a variety the novel mechanisms regulatory as *Candida albicans* undergoes a reversible transition in its morphology (oval and filamentous), increasing its variability according to the environmental conditions of the host, being responsible for the pathogenesis and virulence of the disease [30].

The objective is to study the association between *mutans streptococci* and *C. albicans* in the saliva of patients with ID and their serotypes of MS, morphotypes of microorganisms present in the saliva, and ancestral calculus dental of the remains of human Chonoan.

Patients and Methods

Patients

This study was carried out in 120 males and females' children and adolescents, aged 5 to 19 years, who were studied during 2000 and 2001. They belonged to the public schools of the southern part of the Metropolitan Region of Santiago. Among them 60 presented Intellectual Disabilities (an intelligence Quotient ≤ 70). The information on the grade of intellectual disability (ID) and the presence or absence of cerebral palsy was supplied by the special education school, and 60 were in the control group (CG). These data were obtained by observing ethical protocols at the time of collection. Patients who had received antibiotics less than 21 days before examination were excluded.

Saliva Samples

These were collected according to the following protocol: Two hours after breakfast a dentist brushed the patient's teeth for thirty seconds. Salivary flow was stimulated by applying a 1% solution of citric acid on the dorsal side of the tongue. After one minute, each patient's samples were collected using a glass funnel and kept at 0ºC for microbiological analysis. The volume of saliva collected was at least 0.5 ml [15].

Microbiological samples

The saliva was homogenised in a Vortex mixer (Max Mix typo 37600 Mixer) for 60 seg then, 100 µL of saliva were added to 900 µL of a Na2HPO4 0.2M (pH 7.4) buffer solution (Sigma, St Louis, MI, EEUU). This solution was again homogenised by sonication for 2 min at 37ºC and a volume of 100 µL were streaked in an agar plate with TYCSB [22]. The plates were incubated using an anaerobic system (Gas Pack jars) with a mixture of 95% and N 5% CO_2 for 48 hours at 37ºC. As for *C. albicans*, they were obtained in agar Sabouraud, which were incubated in aerobic conditions for 48 hours to 37ºC [15,16].

The colonies were counted according to the method described by Westergreen and Krasse. The adherent colonies of ms were observed by transillumination in a Spencer magnification lens (10 x). The total number of mutants streptococci colonies present in the Petri plate were obtained using the dilution coefficient and were called colony-forming units per ml of saliva (CFU/ml).

Biochemical study

The biochemical identification of S. mutans or S. sobrinus was done by inoculating 2 colonies in the Todd Hewitt broth [3.1 g Brain Heart Infusion, 20g Peptone, 2g Glucose, 2g Na Cl, 0.4g Na2HPO4, 2.5g Na2CO3] during 18 hours. Bacteria were collected by centrifugation at 5,000 rpm. for 5 min. The pellet was resuspended in 0.2 M Na2HPO4 [pH 7.4] buffer at Nº 5 Mac Farland (1.5 x 10^9 CFU). This suspension was used to identify biotypes of MS (mutans streptococci) through the following micro method: in a sterile plastic box was placed a rectangle glass of 11.5 x 8 cm, divided in 48 pieces of 0.8 cm in diameter each one, and the 30 µL suspension for carbohydrate and Arginine, Esculin hydrolysis, was incubated in a cell culture chamber for 18 hours [15]. The yeasts were identified as *C. albicans* based on formation of a germinal tube (Reynolds-Brude effect), that appears in human plasma when the cell incubated for 2 hours.

Scanning Electron Microscopy

To reveal the adhesion of ms to yeast cells of *C. albicans,* the method described by Holmes et al. was used. Cellular aggregations, previously centrifuged (6000 rpm × 5 min) were set Glutaraldehyde to 2.5% (vol/vol) in a buffer of sodium cacodylate at 0.1 M at 4ºC (pH 7.4) for 90 min. Cells were harvested by centrifugation for 1 min at 12000 rpm and washed 4 times in 0.1 M sodium cacodylate. After this, the cells were fixed with 1% osmium tetroxide at 20ºC (1 hour). Samples were dehydrated in different serial concentrations of ethanol (30, 50, 70, 95 and 100%) and dried with CO_2 using a critical point apparatus. (Polaron England) Saliva and ancestral dental calculus, whose observations were made using Siemens Autoscan SEM [9,16].

Serological Study

This was performed using the double immunodiffusion technique described by Ouchterlony. The antiserum was prepared in female rabbits immunized with *S. mutans* (Ingbritt, serotype c) and *S. sobrinus* (OMZ 176 serotype d). The antigen was extracted by heat for 30 min at 60ºC [21]. The strains were kindly provided by Professor Bratthall (RIP) of Sweden and Professor Loesche of the United States. For *C. albicans* strain ATCC 10231 was used as reference.

Statistical Analysis

The results were analysed with Mann-Whiney test, comparing control and ID (Intellectual Disabilities) groups for the log ms concentration and log o*f C. albicans.* Since samples did not present normal distribution, we used Kruskal-Wallis test for the age (5 to 9, 10 to 14, and 15 to 19 years) comparison, in the control and ID groups. We used Stata® 16.1 and was considered statistically significant if $p < 0.05$.

Results

The analysis of colonies of MS (log/ml) and *C. albicans* (log / ml) of saliva between the control groups CG and intellectual disabilities (ID), test Mann-Whiney showed that for the first group in ms there was no significant difference between the study groups. On the other hand, for the log *C. albicans* there was significant differences with $p = 0.005$ (Table 1).

Table 1: Comparison of the log count in saliva (colonies/ml) in control group and Intellectual disabilities between *mutans streptococci* and *Candida albicans*.

Group	n	MS	Candida. albicans
Control group	60	4,2 ± 1,2	3,2±0,6
Intellectual Disabilities	60	4,8 ± 1,7	3,7±1,2

$p = 0.005$ *C. albicans*.

When we are performing Kruskal-Wallis between age groups for presence of MS (log/ml) we found between age groups significant differences for MS (GC) groups aged between 5 to 9 and 15 to 19 years. ($P<0,005$) and between age 10 to 14 and 15 to 19 years old in ID ($P<0, 04$) (Table 2).

Table 2: Comparison of ms in the log count in saliva (colonies/ml) in Intellectual disabilities and control group.

Age (years)	n	Intellectual Disabilities MS $\bar{x} \pm ds$	Control Group MS $\bar{x} \pm ds$
5 to 9	20	5,18 ± 1,45 µ	5,06 ± 1,04 ≠ ∞
10 to 14	20	5,80 ± 0,84 α	3,92 ± 1,35 ∞
15 to 19	20	3,39 ± 1,66 µ α	3,52 ± 0,54 ≠

Test: Kruskal-Wallis

There were no significant differences for *C. albicans* in the control group. In group ID we found no significance between 5 to 9 and

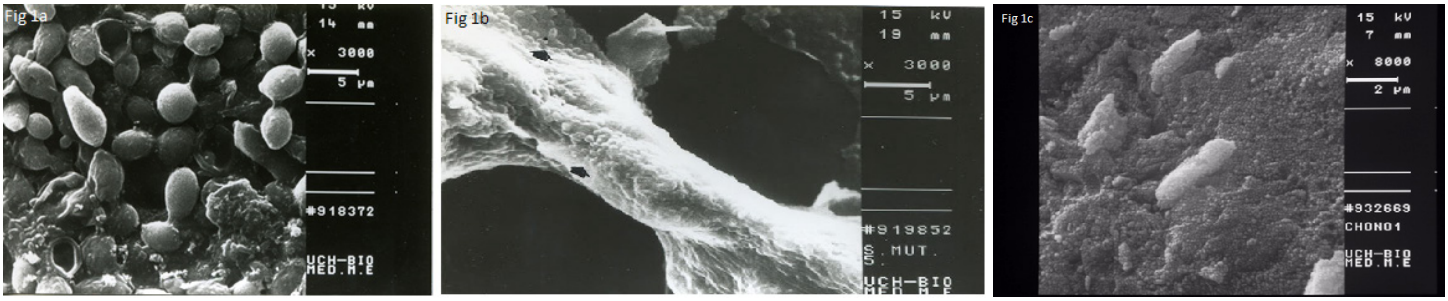

Figure 1a: When Scanning electron microscopy of solid TYCSB culture for MS of saliva, its observe unions through *C.albicans* with yeast filaments. **b**. It vizualize la union de yeast filaments *de C.albicans* colonization by mutans streptococci. **c**. Can be observe one conglomerate of cocaceas morphotype possibly and genus *Candida* in dental tartar ancestral.

15 to 19 years old in *C. albicans.* when compared with 10 to 14 years old; it was also significant between 10 to 14 y 15 to 19 years old (*P*<0,0001) (Table 3). When comparing the Kruskal-Wallis test for *C. albicans* both patient groups for serotypes, no significant difference was found. No-type was only observed in ID (Table 4).

Table 3: Comparison of *Candida albicans* in saliva log count (colonies/ml) in Intellectual disabilities and control group.

Age (years)	n	Intellectual Disabilities Candida albicans $\bar{x} \pm ds$	Control Group Candida albicans $\bar{x} \pm ds$
5 to 9	20	3,37 ± 0,97 ©	3,26 ± 0,55
10 to 14	20	4,49 ± 1,17 © α	3,22 ± 0,82
15 to 19	20	3,16 ± 0,97 α	3,11 ± 0,18

P< 0.005 ©
P<0.001 α

Table 4: Mean scores of *C. albicans* in control group, and Intellectual disabilities children. *S. mutans* serotypes *c,e,f* , *S .sobrinus d,,g* and no-type.

Group			Serotypes	
		c,e,f	d,g	No-Type
	n	60	60	60
Control Group	$\bar{x} \pm ds$	3.15 ± 0.54	3.67 ± 0.78	-
ID	$\bar{x} \pm ds$	3.62 ± 1.21	4.22 ± 1.3	3.94 ± 0.90

C. albicans

We found it through scanning electron microscopy (SEM) colonies in samples of saliva of patients using as culture medium TYCSB for the MS. *C. albicans* present bands between them, probably due to yeast filaments known as germinal tubes present with MS, with its culture medium TYCSB (Figure 1a). We have shown the joint of yeast filaments colonized by ms (Figure 2b). A morphotypes of microorganisms was observed in ancestral dental tartar assigned to the Chonoan (500 ± 70 BP) (Figure 1c).

Discussion

Previous study in Chile of oral health of children and adolescents with ID demonstrated poor oral hygiene, which favors the accumulation of microorganisms in bacterial plaque, activating caries, gingivitis and periodontitis as the most prevalent infectious diseases in the oral cavity [1]. Thus, the intellectually disabled have a high prevalence of caries, gingivitis and periodontitis according to their ages, which differ by the countries studied and the oral health promotion programs [1,4]. In relation to caries, they are produced by an ecological disturbance in the bacterial plaque due to the frequency of ingestion of carbohydrates with the production of acids (low pH) produced by *Lactobacilli, mutans streptococci, C. albicans, Mitis grou*p and others. Previous prevalence studies carried out in patients demonstrated the presence of mutans streptococci and *C. albicans* in saliva, with Down syndrome, which increases the probability of producing caries. A high association is observed in SD between 5 to 9 years and 15 to 19 years in ms colonies/ml and C. albicans colonies/ml in saliva [22]. Our studies in relation to the feasibility of dental caries in patients with ID, in the presence of MS and *C. albicans* in saliva was between 10 and 14 years (Tables 2, 3). These studies are supported by in vitro and in vivo studies on the production of caries by these microorganisms [19,20].

A longitudinal study showed that in Japanese patients with intellectual disabilities, there was a high incidence of caries as a result of the association between *S. mutans and S. sobrinus* [31]. It is inferred that when *S. mutans* are alone after a year, the damage is less, when compared to normal individuals that have both bacteria. Perhaps, it is due to metabolic factors from the host and the microorganisms involved. We identified *S. sobrinus* acidophilus, which appears to be important primarily in surface caries and may be a cariogenic determinant when it occurs with numerous caries. In ours the groups with Down syndrome and ID have average count de d, g serotype, it could be related possibly with the type of caries rampant present in them [17].

Prevalence studies average count of *C. albicans i*n relation to the MS serotypes in patients with Down syndrome showed significant differences when comparing the means of MS in the control group (p<0.05) versus Down syndrome (p<0.02) and acceptable no Type [22]. In ID patients there were no significant differences in the serotypes studied, except in the presence of no type not identified as serotype k or another, which can develop k, infective endocarditis patients [23].

On the other hand, it, has been shown that in calcified dental plaque called dental calculus or dental tartar was found el *S. mutans, A. naeslundii, S. gordonii, F. nucleatum and P. gingivalis (PCR* amplification). Possibly *as the most prevalent microbial agent of caries and periodontal disease until day.* These species presented

two or more *variants, some of them pre*sent in ancient civilizations, specifically the Chonoan of the Guaitecas archipelago in southern Chile (500 ± 70 BP) [9,10].

It should be noted that examining remains human (Chonoan) we see that they had Caries and Periodontal disease are very prevalent, whose diet came from marine diet come from de marine hunters and gatherers of coastal adaptation, consuming hard abrasive foods with low carbohydrate content [8].

According to the above, the bacterial species could vary in relation to quality and quantity according to the constituents of diet. (indigenous wheat, potatoes and some barley), as well as their subsistence pattern and the task they carried out in the environment day, which could recognize that different dental conditions could include a variety of injuries and possible oral defects [8].

The genetic analysis of bacteria from dental calculus brings with it a source of palaeopathological evidence and evolutionary studies, focused on microorganisms and hosts [10]. We think that the microbiome is quite stable over time and that changes can occur at a qualitative or quantitative level, depending on the geographical, environmental and cultural conditions where the human being develops. Distinguish morphotypes could be ser *C. albicans by side conglomerate of cocaceas shape* of ancient dental calculus (Chonoan) forming part of oral microbiome (Figure 1c).

It should be kept in mind that oral health in ID has multiple variables that influence it, such as physical training in hygiene, due to poor oral health index, malocclusion, dental trauma, and complex medication or systemic diseases. The caries index is low in relation to young people with different degrees of disability but is high in disabled adults, therefore it should be continuously monitored in patients with intellectual disabilities [32].

Monitored in Ireland patients, preschool children with intellectual disabilities have a low prevalence of caries when compared to the general population, which may be due to the practice of oral health prevention [33]. On the other hand, a program carried out in India [34]. Where the tooth brushing was assisted in children without having received a previous brushing technique in the patient, 6 to 18 years old for 15 days in children with different disabilities, was met with both success and failure in controlling caries and gingivitis. On the other hand, Dentists and General Practitioners should be aware that oral disease can influence systemic health through clusters that share common characteristics [24].

From the point of view of microbiological control, we have the use of Probiotics as preventives of dental caries with the decrease of *S. mutans* in saliva and dental plaque [35]. This could be in accordance with the strategy in medicine of this association that may alter the relationship of its virulence and resistance to antibiotics promoted by biofilm through horizontal gene transfer HGT [36]. On the other hand, according it points out that the effect of brushing the teeth can be considered a distributive change on the species in the dental plaque where a contest is established between the species that compose it and the host; therefore, the individual should brush their entire mouth. Thus, the species would take some time to recover their habitat and their effect on the host would decrease [37].

On the other hand, the ecological imbalance is the result of the interrelation between microorganisms and the microorganisms that accompany the host, which is essential to maintain oral health. We must keep in mind that molecular biology studies may reveal changes in the interpretation of the results of this association with possible metabolic modifications in time.

In summary, the present study shows in saliva culture, commensalism between mutans streptococci and *C. albicans* in the oral cavity among patients ID. This commensalism would be an interaction between mutans streptococci and its serotypes with *C. albicans.* The ancestral presence or absence of the morphotype Streptococcus and *Candida* in Chonoan, these probably can vary with diet and oral hygiene.

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All the authors of this study declare that they have no conflicts of interest in any way.

Ethical approval

This meta-study was done with data already collected, which were obtained by observing ethical protocols at the time chosen. Parents gave their consent in all cases. The competent children gave their consent in oral form and those who refused to undergo the examination were excluded from the sample.

With respect to publication dealing with the used bone rests in this work, they were not evaluated by an ethics committee when they were published because at that time there were no committees in the field of Social Sciences, particularly Physical or Biological Anthropology. The bone rests corresponding to the Chonos were collected between 1984 and 1987 according to the Law of National Monuments in force at that time and were deposited in the Department of Anthropology of University of Chile until today. As an originating people, the Chonoan have been considered extinct since the first half of the nineteenth century.

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