**Original Research Article**

**Microbiology of Dental Calculus of Prehistoric People from Cabeçuda Shell Mound, Santa Catarina, Brazil**

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ABSTRACT

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| **Background and Aim**: Oral health investigations contribute to the understanding of the culture and biology of past populations. It is possible to go beyond the main diseases, such as caries and tooth loss, addressing detailed information on morphology, pathology, and physiology. A progressing analysis of microfossils in dental calculus confirms the good preservation of biofilm in the matrix. Health models associating synergic relationships of dental & oral pathology with systemic diseases are important to understand modern, as well as past human life and death. The aim of this investigation is to the absence of dental caries in one human past population, that could be associated with a specific oral microbiota and health conditions.  **Methodology**: Dental calculi were extracted from the human teeth of 20 different adult and subadult individuals, both sexes. The morphology of biofilm components was described, especially in the search for Vibrios. Coastal human groups lived exposed to the saltwater environment, and the one studied here, from the shell mound of Cabeçuda, Santa Catarina State, Brazil, was free from caries (estimate date 2,800 to 1,500 BP). The archaeological series belonged to the Museu Nacional of Rio de Janeiro; sample selection occurred in its Biological Anthropology laboratory. Calculi extraction & preparation was in the laboratory of paleoparasitology at Fiocruz, the SEM analysis was at Hertha Mayer Laboratory, Federal University of Rio de Janeiro. After appropriate hygienization, dental calculi were extracted and disaggregated, and parts were scanned. The images were analyzed, considering morphotypes in the biofilm, their density, and spatial distribution.  **Results**: No Vibrio was detected, but 5 different morphotypes were described and associated with possible genders (*Lactobacillus*, *Porphyromonas*, *Actinomyces*, *Leptotrichia*, *Rothya*, *Neisseria*, *Staphylococcus*, *Veillonella*, *Peptostreptococcus*, *Streptococcus*).  **Conclusion**: Morphotypes are a good first approach to the main components of biofilm, results can be improved with better disaggregation techniques. Complementary genomics and proteomics techniques should be more conclusive. |

*Keywords: dental calculus – biofilm – Vibrio – shell mound - prehistory*

1. INTRODUCTION

Dental caries is a dynamic and multifactorial infectious disease [1,2], which results from the interaction between the oral microbiota, the mouth environment, and host susceptibility. Even in the case of increased carbohydrate consumption, there may be no karyogenesis, without expressive activity and multiplication of cariogenic bacteria [3]. The composition of the oral microbiota and the presence of growth of acidity-tolerant bacteria, as well as the enamel demineralization processes, contribute to enamel erosion and, therefore, caries of dental surfaces, starting karyogenesis [4]. At birth, humans have a sterile oral cavity. Exposure to bacteria in the mother's body, the environment, food, and other factors determine the formation of biofilms in the oral cavity. Changes suffered throughout life bring new exposures and colonization by hundreds of species [5, 6, 7, 8] by different routes of introduction [8, 9, 10, 11].

Studying the teeth, mucous membranes, and other components of the mouth environment and their biofilms is important to understanding health and disease, whether of individuals or human groups. In this context, dental calculus is a true reference for paleopathological, and even cultural aspects [12, 13]. Oral versus systemic health has been the subject of paleopathology for decades [14, 15, 16]. The community of microorganisms in the oral cavity constitutes an ecologically active environment impacting health.

In general, human coexistence with the oral microbiota is formed by many saprophytes and commensals, remaining generally healthy and balanced [17, 18, 19]. Increased consumption of starchy foods, processed foods, and ultra-processed foods [1, 10, 15, 20, 21, 22] changes the microecological balance between host and microorganisms causing a dysbiotic state [23]. The impact on oral health causes a progressive increase in the prevalence of dental carie and its systemic consequences [11].

Frequencies of dental caries in different periods and populations [24, 25, 26] establish their determinant factors. In Brazil, the adoption of crops and other forms of food production introduced more cariogenic components to the diet of pre-horticulturists. Brazilian pre-horticultural groups living in coastal environments in the South-Southeast, between 8,000 and 1,500 years ago, as a rule, represented a period before the introduction of starch-rich cultivars [27, 28, 29]. The frequency of caries [30] was low, increasing when carbohydrates began to be more intensively used. Caries occurs in low frequencies among the builders of monumental shell mounds, called sambaquis in Brazil [31].

Different processes of microbial interaction and the growth of the population of cariogenic bacteria can be modulated, with microorganisms capable of interfering with the proliferation of others [33]. The performance of microorganisms [6, 19, 34, 35, 36, 37, 38] and their ability to produce non-physiological events must consider the complexity of their diversity and interactions.

In a review concerning the composition of the oral microbiota and its correlation with oral and systemic diseases [23], the authors call attention to the presence of the genus Vibrio, which acts as a growth modulator of other microorganisms. Santos (1978) [39] studied its ability to induce the composition of the biofilm, through quorum sensing, interfering in the growth of other microorganisms through metabolites eliminated in the environment [40]. Acting on the plaques that form on tooth surfaces, those microorganisms modulate the presence of cariogenic bacteria reducing caries development.

Despite the difficulties in studying dental calculus, pioneers such as Dobney and Brothwell (1988) [41], and more recent authors, such as Charlier et al. (2010) [42] and Power et al. (2014) [43], confirmed the morphological preservation of microfossils from the biofilm. Advances in techniques and methodologies today allow, contrary to what still occurred about two decades ago, to identify significant results and eliminate possible contamination biases, for example, expanding the potential of investigations on oral microbiota [12, 13].

The present work searches for evidence of Vibrio in the biofilms of prehistoric dental calculi, a way to contribute to studies on the health of such groups, especially the absence of dental caries.

**An ancient group without dental caries**

Shell mounds are prehistoric monuments for funerary use, which preserve many remnants of human bodies. These sites are found mainly along the Atlantic coast, in South and Southeastern Brazil [27, 28]. Artifacts, faunal skeletons, and human remains of non-horticultural groups that lived by gathering, fishing, and occasionally hunting, between 8,000 and 1,500 years before the present are found in those sites, together with funerary structures and fireplaces. Testimonies of their lifestyle include economic evidence [44], cultural material [29], physical and genetic characteristics [45], and demographic [46, 47] as well as paleopathological, nutritional, and other health evidence [31, 48] recurrently described and discussed.

In Brazil, one of the largest shell mounds is the Cabeçuda (Figure 1), which is located between the lagoons of Santo Antônio dos Anjos and Imaruí, in the municipality of Laguna, State of Santa Catarina. It was a monumental site, but today it is almost destroyed by economic exploitation. The few meters remaining from its base continue to be studied by the Museu Nacional of the Federal University of Rio de Janeiro, and University of Southern Santa Catarina. By the time of the first excavation in the year 1950, the testimony of the original monument measured about 20m in height and 53,000m² in area [49]. It was a typical funerary site (Figure 2) that may have contained hundreds of burials [46]. The series of human skeletons, which specimens were used in the present study, comes from a single funerary area [50] excavated by Luiz de Castro Faria [49].

![Foto em preto e branco em cima de uma superfície de pedra

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T1CTWFHVlY9N3RWR0JNTU1CQj1bb2Z0W1FCaoNRanR6b3RmVnSQkMzGoLSIjcbZ5/7//v////////////////////7//////////////////////////////v////7///////////////////////////////////////////////////////////////////////////7//////////////////////////////////////////////////////////////v//////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////+OGWlptmfmaIkFFHTVFhW1FWZltCR1tHPUJhUUdqTVFWPVZCQkI9Qj1CPUc9Nz1CPU1CMTE3Nzc3PTc9Qj1HTTc3NzdNTVs3PTdCUU09ak0xPUdqWz1CQkI3Nz1NR0dCUUdCR0JCPVFNQk1HR1tqZnSITVFNm3S9xufw5/T+//7/8P7/+N7++Ovn5/T+//////////////////////////7//v7////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////+/////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////v/Cm41viG9WR2p6jVtWQkdHR0dNR0c9Nzc3Nz1CQlFbPUdNUU1HNz1Cb1Y3MTE3MTdHMTc9PT03Nz09NzE3NzdCYVFCMTc9Rz09PT1CPTdHR1s3NzFWNzdRZkdNQjdCQkc9R0IxNzdCQkdCNzc9amZWUUdCPUJham+Dr5Cqr5vMqpulvevPzH6gxsK0m7n++P7+///////////////////+//////7+//7+/////////////v/////////////////////////////////////////////////////////////////////////////////////////////////+/////////////////////////v////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////i0UWFHVltvTWFWW0dHW0JCPUJCN0dHTTc9PTE3Nzc3Nzc3QltbQjc9PVFHMTcxLDc3MTcxPVZhN0JHPTE3MTc9Rzc9PTdHUUIxUVFHQjc9Qk1CNzc9Nz1HQj1CTUdHN01CUXRHPTc9Nz09Nzc3PUdNUTc9Nz16YUdWiH5mYVFWiGphVmFhamqDpa+9oLTe8PD+/v///////////////////v7+/v/+/v7///////////////////////////////////////////////////////7/////////////////////////////////////////////////////////////////////////////////+P////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////7/////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////uXphRz03QlaIVkdRPT1NQkc3PUI9PTc9Rzc3PTc9NzcxMTdNNz09UTc9PTc9N0I3MT1CNzExMTE3R1E9PTc3PTExQjc9QkJNQkdWUUdhQkI3Nz1WWz1CQls9UVZmW1FCTUdHTVZbak09TUI9PTc9N0JWTU09Nz1RQj09apZvW0dNalFRR0JNVmZhVo1mg4h+g8Le4ef0+Pj///////7+/vTw3sa94ev0tOv+/v7////////////////////////////+////////////////////////////////////////////////////////////////////////////////////////////////////////9P/////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////ZYUdCPTcxTT1RTVFCQj03PTcxNzc9Nzc3QkI9Nz09Nz03MTE3PTc3MTE3PUJNPUdNRzdHQj0xMSwxR1s3PUJNUTE3UT1NTWGbYVFvUU1RZk03R01HYVtbYW+Wb3TGqqB0b4NhiFZ6g35Nb2ZNb01hUU1qW2Fhek1NQj1CVltqemFbW01NUUJCR0JCR1ZNTVtRVqqvoL3U4evw9Ov+6/j44a+QtHp+iH6viLTM9P/////////////////+//////7+///4////////////////////////////////////////////////////////////////////////////////////////////////////////8P////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////7///hWNzc9Nzc3Nzc3Nzc3Rz03PT1HQkJRUX5+lpuDQlZ0eo1vVlFNTVs3NzdNQm9WQkdCR0KQN0I9Qjc3TVZNakdNVkdNdGp6oG90m4iIiHqQjY1qUYhHTWGDTVtmjZu5tJuqUYO0xtSNqq+QYY29vXqQjb1vZmGIb4ONdFtHTXRbZmZqb2ZRUVFCYU1CQkdNR1FHR1abfq/GtNTw5+fwz6Wqr4iDW1t6fm9qdJuIlrnw8P7//v/////////////++LnZ9N7C6/Tn////////////////////////////////////////////////////////////////////////////////////////////////////6/////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////////5hW1ZNRz1CTU1qPT1Ng6Vqb2+bqrmqxtTe2cyvwr2Wr7TCwm+0eqqNelaNUapqb3RhVmF6Qk1bVkJqloiNlq+QenqWiFGIdHqqtIO9xmGbpaVbarRbb5Clfn5qub29z8y5loi95+vMloOIoLSqwnqIZsLMjdSqqqCWjYiDlmpqb1tmY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Figure 1 - View of the sambaqui called Cabeçuda shell mound, located next to the Imaruí Lagoon, in Laguna, State of Santa Catarina, Brazil (Photo: L.C. Faria, 1952) [49].

Foto em preto e branco de criança em pé

O conteúdo gerado por IA pode estar incorreto.

Figure 2 – Human burial in a flexed position recorded during the archaeological field work in the 1950s (Photo: L.C. Faria, 1952) [49].

In Cabeçuda people tooth loss was not very significant, thanks to tooth impaction by hypercementosis [51]. The complete absence of caries was a subject of interest since the first study of this material, even considering that coastal shell mounds usually have a low frequency of caries. A pioneering investigation pointed to the absence of caries in 1329 human teeth of 83 adult/sub-adult individuals. The secondary dentitions were represented by 231 maxilla teeth and 1088 mandibula teeth. The remaining 38 were deciduous [52]. At the time, the diagnostic criterion was the presence of a cavity on the surface of the crown or root, detected with the aid of an exploratory dental probe (0.5 mm). The total absence of dental caries in Cabeçuda was initially attributed to the hyper protein nutrition of the pre-horticultor coastal fishing-gathering groups, and a possible genetic factor, given that similar coastal groups exposed to similar habits and environment, presented low caries frequencies, but not zero. The absence of caries in the Cabeçuda people was confirmed in a later study [51], based on the same criterion, using the same probe size. The second investigation used 33 probably female, 36 probably male, and 7 of undetermined sex [53].

Integrating different paleopathological skeletal signs from the same human remains, Souza (1999) [54] proposed a systemic interpretation. General health conditions associated with their exposure to environmental risks provided a systemic approach to the absence of caries. Physiological stress indicators such as porotic hyperostosis, cribra orbitalia, linear enamel hypoplasia, arrested growth lines in long bones, periosteal reactions and auditory and mandibula torus. A chronic exposure to recurrent infections caused by saltwater *Vibrio* was then proposed by Souza (1999) [54]. Colonizing skin and mucous membranes, including the oral cavity, *Vibrio* would cause cutaneous, gastroenteric, and other infections. Different health problems could be caused by *Vibrio*, such as gastroenteritis, periostitis, otitis, skin and mucous membrane infections; even fatal cases are described in contemporaneous coastal populations [55, 56]. Colonizing the mucous membranes and modifying the ecology of the oral cavity [39], Vibrio would be able to modulate the cariogenic microbiota, interfering in cariogenesis.

The present investigation uses scanning electron microscopy (SEM) to analyze the supragingival dental calculi from individuals buried in Cabeçuda, searching for microfossils of the former microorganisms in the biofilm. The study aims especially the possible evidence of the presence of *Vibrio*. Here the taxa identified are presented, and technical difficulties limiting the identification of microfossils morphologically compatible with Vibrio are discussed. Suggestions on methodological adjustments necessary for the continuity in this line of research are proposed.

2. material and methods

Dental calculi from 20 different adult or sub-adult individuals, exhumed during archaeological research in the 1950s, in the shell mound of Cabeçuda, were analyzed. The skeletons used in the present study had their antiquity estimated by dating to the archaeological layers where they were buried. Such calibrated dates reveal ages between 2,300 and 1,500 years before the present [44]. Among the individuals studied, six had ages estimated by cementum annulation of the dental roots [47], with the following results: two of them were between 40.5 and 40.8 years old, two were between 28.1 and 28.5 years old, one of them was 18.8 years old and the other was 32.7 years old. The others were identified in the catalog of the collection only as adults, nine of them had the sex estimated.[53], from incomplete skeletons, with limitations in their estimation. Four males and five females were identified. Of these, only three male skeletons and one female skeleton were also confirmed by discriminant analysis [57]. The results of the research were used to obtain the Master Degree in Public Health, at the National School of Public Health (Fiocruz) of the first author in 2014. The co-authors were the supervisors of the Graduation Program.

Voluminous calculus justified the choice of specimens for this study. Some calculi came from previously disconnected teeth (n=14), and others from teeth in situ (n=6). Except for one upper left canine, the others were molars: three upper left, eight upper right, five lower left, and three lower right. Molar teeth were chosen because they had larger calculi (Figure 3). After selection, the material was described and documented on cards, macro, and microscopic photos (50x, PROSCOPE digital magnifying glass Avantgarde@).

The calculi and enamel surfaces were previously sanitized with soft brushes and distilled water. A curette was used to extract the calculi without removing the tooth from the socket. The fragments were reserved in Eppendorf and identified. The stage of choosing and selecting the samples, as well as extracting the dental calculus, was carried out at the Biological Anthropology Laboratory of the National Museum of Rio de Janeiro.

Uma imagem contendo mesa, comida, rosquinha, azul

O conteúdo gerado por IA pode estar incorreto.Figure 3 - Teeth from burials of the Cabeçuda shell mound, note the accumulation of dental calculus (Photo: T.P.)

Subsequently, the material was taken to the Paleoparasitology Laboratory of the Sergio Arouca National School of Public Health, of the Oswaldo Cruz Foundation, where the fragments were reexamined under a digital magnifying glass (50x), weighed on a SPLABOR digital scale, and then partitioned and reserved in new 2ml Eppendorf tubes.

Next, two physical techniques for exposing the microfossils were tested: the first was the disaggregation by ultrasound, done in distilled water, at room temperature, in Cristófoli Ultrasonic Tub (USC12038L188018), for progressively longer times at 42Khz, which was not very effective. Alternatively, the calculi were crushed, and the fragments were fixed to the stubs with carbon tape (Ted Pella Inc.) for microscopic examination.

The assemblies were then taken to the SEM at the Herta Mayer Cellular Ultrastructure Laboratory, Carlos Chagas Institute of Biophysics, of the Federal University of Rio de Janeiro, where they were coated with gold for better visualization of microfossils. Scanning electron microscopy was performed with the technical support of the SEM laboratory. Due to problems with one of the microscopes, part of the material had to be examined in a Zeiss EVO MA10, and part in a Quanta 250 (FEI Co.), obtaining images with different magnitudes according to the case (Figure 4).

The best image of each specimen was analyzed. To this end, each was divided into quadrants, for microfossil quantification. Only those whose outlines could be fully visualized were considered. Each individualized microfossil was measured as follows: the rod-shaped ones at their two maximum orthogonal diameters, and the cocci at their maximum diameter. The measurements were compared with the morphology of the current bacteria known and described according to Bergey's current manual of systematics of archaea and bacteria [58] and the descriptions in Zhou and Li (2020) [59]. The results allowed them to be grouped into morphotypes.

An average of three counts in each specimen was considered. The density of microfossils per area was calculated by dividing the total number of microfossils (cavities + intact microfossils) by the planimetric value of the photographed area, to control the bias introduced by the different magnifications. The results were compared and discussed.

3. results and discussion

The analysis of dental calculi from Cabeçuda shell mound from a microbiological point of view, considered only morphological and morphometric aspects of the microfossils visible on the fractured surfaces. This condition brought great limitations to the study. In addition, even the good SEM images limited the three-dimensional view of the microfossils, a consequence was that only simple morphotypes were applicable. Remaining attached to the matrix of the calculi, the microfossils were poorly described, unless when free from the calcified layers.

Even so, the good external preservation of the microfossils confirmed the promising scenario in this line of research. It is remarkable how their outer morphology was preserved in the ancient material, allowing the contours and volumes of the microorganisms to be maintained, as described in previous works by Charlier et al. (2010) [42] and Power et al. (2014) [43]. The images of the scanning electron microscopy show that the microfossils presented themselves in two ways: intact corpuscles formed by closed curved surfaces, corresponding to the external contours, or mineralized membranes of bacterial cells; and cavities delimited by the same contours, i.e., mineralized membranes. The fragmentation of the samples for assembly under the microscope also removed parts of the microfossils, sometimes releasing intact microfossils, sometimes fracturing them, exposing the inside. The observation of open and fractured microfossils confirmed that the process of calcium phosphate deposition mineralized only the envelopes of microorganisms, leaving their interior empty, as described by Hilson (1990) [15].

Uma imagem contendo no interior, foto, coberto, colorido

O conteúdo gerado por IA pode estar incorreto.Uma imagem contendo no interior, segurando, grande, mão

O conteúdo gerado por IA pode estar incorreto.

Figure 4 - Scanning electron microscopy of human dental calculi from the Cabeçuda shell mound. Note the intact and fragmented microfossils, still embedded in the phosphocalcium matrix of the calculus. Above, MN1673 specimen photographed under a QUANTA 8,000x microscope. Below, MN1743 photographed under a ZEISS microscope, 9.430x (Photos: Hertha Mayer Laboratory/UFRJ)

The possibility of confirming the results by biomolecular techniques [60], although considered, has been limited by the costs of applying this methodology. On the other hand, the microfossils preservation, losing cellular contents and nucleic accids, brings limitation to paleogenetic studies. In the absence of intracellular structures, the results could represent only the contamination by old and recent microorganisms from the environment, as reported in previous studies [61]. In recent years, however, new technologies seem to be offering a more favorable scenario, and this path can be resumed [12].

On the other hand, microfossils preservation drew attention to their fragility under the apparent resistance of calculus. Their delicate structures are a challenge to those studies that need them intact for proper morphological study. Since the mineralization processes in the calculus, in addition to the calcification in vivo, continues in the soil [15], the formation of calcite in the bony, dental, and calculus matrix interstices are frequent. It is important to consider in future investigations that an increase in the hardness of the matrix may occur, increasing the difficulty for sample disaggregation.

The possibility of different calcium phosphate recrystallization of minerals in the samples accounts for the differences in the hardness of the calculi, and therefore in their resistance to ultrasonics. The minerals most frequently deposited in life, in the formation of the calculus, would be bruxite (hardness 2.5 on the Möhs scale), whose basic formula is Ca8HPO4.2H2O, and whitlockite (hardness 5 on the Möhs scale), whose basic formula is Ca8(HPO4)2. In the shell mound substrate, rich in skeletal remains of fauna, more than in other sites, the percolation of fluvial, rainwater and tidal water favor the migration of ions. It may contribute to the gradual replacement of original minerals by apatite (also hardness 5.0 on the Möhs scale), whose generic formula is Ca5(PO4)3(OH, F, Cl). This substitution certainly gives greater hardness to the matrix and, consequently, to the calculus, which now offers greater resistance to disaggregation. The Cabeçuda, a shell mound whose substrate is formed by a predominance of mollusk shells, with a reduced proportion of soil, a taphonomy process of this type would find even more conditions to occur. The observation of great variation in the resistance of stones to dissolution processes by physical and chemical methods has been observed in other studies, and even for their dissolution, different times of exposure to acid solution have been employed for various materials [30, 62].

Confirming a large number of bacterial microfossils preserved in most of the 20 samples, except two of them, MN 1674 and MN 1942, was an important result. It draws attention to the potential of this material. Cases in which microfossils were rare or absent may represent parts of the calculus where the matrix predominated, such as in areas of rapid precipitation of calcium salts, or of specimens where, due to specific conditions, the preservation of microfossils has been impaired.

The number of intact microfossils in each image ranged from 48 to 300, and the number of broken microfossils in each image ranged from 32 to 190. Adding the amounts of both, the proportion of bacteria in each stone surface examined ranged from 0.056 bacteria per μm² to 0.83 bacteria per μm² (Figure 5). These results confirm that there may be different proportions of microfossils in specimens from the same site. Differences in diet, such as in periods of higher protein intake, form ammonia radicals that base the mouth environment, favoring and accelerating the precipitation of calcium salts and faster calculus formation. Another possible cause for variation in diet may be the existence of different diets, according to cultural patterns related to sex or age, for instance. Isotopic studies, such as by Colonese et al. (2014) [63], seeking to reconstruct paleo nutrition in shell mounds from Brazil, suggest that even in a single site there would have been variability in the diet between different individuals, which would certainly impact the formation of dental plaque and the speed of calculus formation [4], including for factors such as gender or age. In the case of Cabeçuda, in only eight specimens, it was possible to estimate the sex. The small number of observations avoids establishing a correlation, however, the results concentrated lower values of microfossil density in skeletons estimated to be female.

When analyzing samples from cemeteries, we must consider that the specimens studied are not always synchronous, their lives and deaths may represent different periods of the group's life. Thus, variations in the individual diet may represent different strategies for obtaining food, especially in a fishing-gatherer group. In the same way, there may be differences related to the entry of individuals from other areas or groups buried in the same site. It is also possible to find differences related to specific personal events since differences determined by cultural patterns and divisions of subsistence activities are not uncommon. Isotopic studies for coastal populations in Brazil suggest such differences [64], and further bioarchaeological studies in larger series could detail interpretations of the formation of dental calculi in shell mound builder groups.

The detail of the location, the size of the calculus, the more precise record of the pictured area of the calculus surface, the plane corresponding to the broken face examined, and its proximity to the enamel surface, among other variables, can also explain the differences. The succession of microbial communities that form dental plaque conforms to an architecture that may explain quantitative variations and morphotypes. In special cases, worn-out calculi, or calculi that were partially lost during their lifetime, may present peculiar variations in their composition, and even abrupt transition zones. In future studies, it would be desirable to be able to define in advance the portion of the calculus to be removed, as well as to standardize the fracture plane for microscopic analysis, ensuring greater comparability of the results.

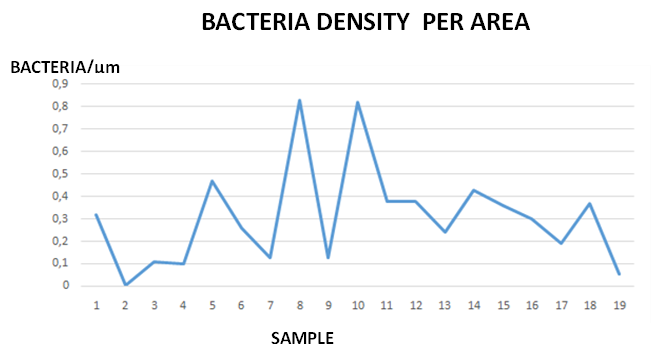


Figure 5 – Variation in the density of microfossils by area in the human adult dental calculi from people of the shell mound of Cabeçuda, Laguna, Santa Catarina, Brazil.

About the general aspects of the results, the pictures showed no inclusions that could be mineral debris, pollens, phytoliths, fibers, starch, or other elements introduced along with food or other. However, considering the results of Jorge (2018) [62] for Cabeçuda, the almost total absence of starch was confirmed to characterize this material. Although pictures of intrusive materials of this type have been recorded in matrices of other shell mound dental calculus photographed similarly [65], the preparation of the samples in the present study, without dissolution and decalcification of the samples, would make the presence of evidence of this type.

The results obtained with the measurement of the microfossils were grouped into five morphotypes. The comparison of these morphotypes with the described metric characteristics [58, 59], allowed us to propose taxa represented in the microbial biofilm of these calculi corresponding to the following possible taxa: Type "A" - consistent with rod-shaped ones - 0.5μm to 0.8μ m wide/1.0μm to 3.5μm long, corresponding to straight bacterial corpses, similar to rod-shaped, either isolated or in chains: *Lactobacillus* spp., *Porphyromonas* spp., *Actinomyces* spp.. Type "B" – consistent with rod-shaped ones - 0.5μm to 0.8μm/1.3μm to 3.8μm long, corresponding to curved rod-shaped ones, either isolated or in chains: *Lactobacillus* spp., *Leptotrichia* spp. Type "C" – probably cocci – diameter varying from 0.5μm to 1.3μm, isolated: *Rothia* spp., *Neisseria* spp., *Staphylococcus* spp. Type "D" – probably cocci – diameter varying from 0.5μm to 0.8μm, spherical bacterial corpus in chains: *Rothia* spp., *Neisseria* spp., *Vellonella* spp., *Peptrostreptococcus* spp., *Streptococcus* spp. Type "E" - probably cocci – diameter varying from 0.5μm to 0.8μm, doubled or tetrads groups of spherical bacterial corpus: *Rothia* spp.; *Neisseria* spp., *Staphylococcus* spp.

Since the way of preparing the calculations for microscopy did not allow full three-dimensional visualization of the microfossils, it was admitted that some of the proposed gender, *Lactobacillus* among the rod-shaped, and *Rothia*, *Neisseria*, and *Staphylococcus* among the cocci, could be classified in more than one morphotype, depending on the perspective in which they were visualized and their three-dimensional arrangements.

Although they are described by different authors [6, 19, 66, 67], their possible presence in the human dental calculus of the Cabeçuda shell mound was proposed based only on general morphological aspects. Limitations resulting from the image perspective should be considered.

Since the measurements could be made only in part of the microfossils, because part of them were either broken or embedded in the phosphocalcium matrix, the amount of microfossils available for analysis was reduced, which also hindered the recording of variability. The complete disaggregation of the calculus, initially attempted by ultrasound, would certainly favor the morphotype count to be increased, making its measurement and identification more precise. Following laboratory tests will help with technique adjustments and possible successful results in the future. Although taphonomy aspects should probably be considered, the complete exposure of the microfossils, after release of the matrix, would certainly allow the observation of additional morphological details and variability, in addition to allowing the more precise estimation of their proportion in each sample.

The possible presence of *Streptococcus*, with known cariogenic potential, deserves consideration. It should be remembered that the mere presence of the taxon would be insufficient to determine the occurrence of caries, which would make it important to investigate the proportion in which microfossils morphologically compatible with this genus occur, and why they may, or may not, exercise their cariogenic potential. Knowing the number of possible morphotypes and the way they are distributed in the samples studied would help to interpret the findings [66].

Despite the care with which the microfossils were examined, the morphotypes identified did not coincide with shapes that could suggest *Vibrio* sp. These Vibrios have been described for decades in the biofilms that form dental plaques [19, 34, 67, 68, 69], and their presence and caries-modulating role have been reiterated by Santos (1978) [39] in Brazil. Other problems caused by saline water *Vibrio* have been reported in public health [56]. However, despite the hypothesis that motivated the study in this coastal sample, no image analyzed suggested morphology that resembled *Vibrio*. Previously, a microfossil with a compatible aspect was observed in a SEM image of a specimen of the shell mound from Forte Marechal Luz, also from Santa Catarina, Brazil [65].

The calculi used in the present study represents only supragingival areas of the teeth, which may explain the absence of the Vibrio morphology. Biofilm is variable in different areas of the teeth and mouth, the anatomic location of this genus is the subgingival area [70]. New experiments should be carried out to improve techniques for preparation and analysis of the calculus.

It is important to continue looking for ways to confirm the presence of Vibrio in the samples from this archaeological site. That possible paleoepidemiologic evidence of these additional mechanisms would justify the low frequency/absence of dental caries in the shell mounds of Brazil. The new and promising results obtained with omics techniques and the possibilities of decontamination, excluding the errors resulting from the presence of microorganisms of recent origin, for example, point to other possible investigations as suggested by some authors [12, 13, 71]. Despite the promising scenario, the scarcity of samples and the restrictions on destructive analyses, have still hindered the continuation of research,

Taphonomy processes modify dental calculus, making them sometimes more resistant, thus experiments to improve calculi disaggregation techniques should consider differentiated solubilization techniques. Protocols with temperature variations, or using calcium chelators, ultrasound, and other combined resources should allow the adaptation of techniques applicable to different materials. Studies on the composition and hardness of calculi, physicochemical processes in soil, and recrystallizations of minerals present in cavities of bones, teeth, and others from archaeological sites, will certainly bring knowledge to this area. Finally, identification of the minerals present in the calculi may be used as a basis for protocols aimed at differential solubility [72], which allow the separation of matrix and microfossils, favoring their quantification and morphological analysis. Studies in this line should be programmed to continue in this line of research.

4. Conclusion

The study allowed new questions for this line of investigation. Although it was not possible to confirm the presence of the Vibrio morphology, its presence cannot be refuted either. Therefore, this work opens doors for the continuity of investigations.

The morphological preservation of microfossils in millennial dental calculi draws attention to the potential of this type of material for the study of the microbial biofilm of teeth, and its interest in expanding knowledge related to oral health in ancient human groups. This is a promising line to be explored. The complexity and role of these communities are great, and it is necessary to expand the knowledge of their relationship with our pathophysiological and physiological processes, a conception that is also applicable to the oral microbiome and its relationship with human health and diseases of the present, as well as of the past.

Some directions can be proposed based on our results, aiming at continuing the studies and improving the protocols for the analysis of dental calculus. To proceed in this line, the experiments must be oriented towards the release of the microfossils embedded in the matrix without damaging them, to allow their qualitative, quantitative, and other morphological analysis. If a good technique for this is achieved, it will be possible to know the composition of dental plaques in different individuals and groups and seek to better understand their relationship with caries and other events. The taphonomy processes that affect the preservation of dental calculi should be better documented, and their differences, on a case-by-case basis, would help the adequacy of protocols and customization of laboratory procedures, according to the archaeological sites studied. Finally, considering the challenges for the disaggregation of dental calculus, future studies based on imaging techniques such as microtomography should be tested in this line of investigation.

Likewise, the contribution of other approaches and methodologies, mainly genomics and proteomics, should be sought, in order to explore the integration of results, reducing biases and contaminations, and reinforcing the interpretation of results.

Finally, it is worth remembering that the dramatic loss of the collection of human skeletons excavated in the 1950s in the shell mound of Cabeçuda, on the occasion of the accident that destroyed a large part of the archaeological collections of the National Museum, is a permanent motivation for the continuity of investigations and improvement of the questions and methodologies that will allow us to continue to tell the history of health in the past.

Ethical approval

It is not applicable.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declares that no generative AI technologies such as large language models (ChatGPT, copilot, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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