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PALEOMICROBIOLOGICAL STUDY IN DENTAL CALCULUS: *Streptococcus mutans*

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Abstract

Morphological types of bacterial remains preserved in ancient tartar of teeth from extinct human groups, which included some communities of coastal gatherers, fishermen, hunters, and farmers, and those practicing a mixed economy, were analyzed. Previous studies have shown the presence of bacteria in ancient tartar. The aim of this work was to determine whether *Streptococcus mutans* was present in ancient populations (500-12,000 years old). Teeth samples were from ancient skulls obtained from different anthropological collections: the north and south of Chile (before the Spanish conquest), Palencia, Spain, and an eastern Mediterranean region (Levant). Optical microscopy showed Gram positive and Gram negative bacteria. Scanning electron microscopy identified morphological types of bacteria. Transmission electron microscopy enabled categorization of bacterial structures. Fluorescence microscopy helped label and identify *S. mutans*, using polyclonal antibodies.

Bacterial morphotypes were related to different subsistence patterns. Hunters, fishermen, and gatherers had a less diverse flora with bacillary and coccal morphotypes. Agricultural groups showed greater diversity with additional filamentous and spiral morphotypes. The best preserved ultrastructural feature was the cell wall. The existence and colonization capacity of the mutans-like streptococci preserved in tartar was established for the ancient populations studied, with the exception of Cerro Sotta (south of Chile). Hence, their occurrence could not be related to diet or subsistence pattern.

Key Words: Ancient tartar, *Streptococcus mutans*, paleomicrobiology.

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Introduction

The oral microflora, which constitutes the bacterial dental plaque, remains relatively stable for a long time in healthy sites of the oral cavity. This stability, termed eubiosis, is the result of a dynamic balance of interaction among microbial species, including both synergism and antagonism between the species and the host (Marsh, 1989). It is widely known that disturbance of this balance may result in the initiation and later progression of oral pathologies, such as caries and periodontal disease (Loesche, 1986a; Wolff *et al.*, 1994).

Infectious pathologies affecting the host tissues can be due to an imbalance in the host-parasite relationship, thus altering the resident microflora, for example, the pathogenic ability of *Streptococcus mutans* may be increased by sucrose consumption (Van Houte, 1980; Loesche, 1986b).

From a historical perspective, the host susceptibility or resistance to a particular infectious agent is the result of a process of adjustment and co-evolution between them (Fitzgerald, 1985; Mitchell, 1991). This investigation is not a direct, step-by-step study, since the microorganisms and the damaged host tissues after death are rarely well-preserved.

However, the mineralized nature of the tartar (dental calculus) in ancient teeth of ancestral origin preserves the included microorganisms, and thus offers possibilities for investigation.

The purpose of this study was to determine the types of bacterial microorganisms present in the calcified matrix of ancient dental tartar, as well as to gain insight into the origin, ecology, and changes in the oral microflora. In addition, these parameters are analyzed in relation to the subsistence patterns of the particular extinct human populations (Dobney and Brothwell, 1986; Linossier *et al.*, 1988).

Materials and Methods

Tartar samples

Tartar samples from living human beings, used as

control (n = 15) were taken from first and second upper molars and lower incisors of patients, ages 21 to 71 years old.

Ancient tartar samples of Chonos skulls, 490 BP (Before Present), (n = 20) were taken from upper molars (first and second), lower molars (first and second), and lower incisors. The skulls were collected by O. Ocampo and E. Aspillaga and kept in the Department of Anthropology, University of Chile. They belonged to a Chilean indigenous population inhabiting Chiloé, in the south of Chile, before the Spanish conquest. Chonos aborigines were hunter-fishermen and gatherers.

Tartar samples of Atacameños skulls, 900-300 BP (n = 10) also were taken from upper and lower molars and lower incisors. These skulls belonged to Chilean indigenous population located in San Pedro de Atacama north of Chile, before the Spanish conquest. They are kept in the museum, Gustavo Le Paige, and were kindly lent by Mrs. M. A. Costa. Atacameños people were agriculturalists.

Tartar from one skull, 3400 BP, was obtained from one upper molar. This skull was found by J. Bird in Cerro Sotta, Chile and now kept in the American Museum of Natural History (AMNH), New York. Cerro Sotta was a Patagonian region in the south of Chile, before the Spanish Conquest. The aborigines in this region were hunter-gatherers and fishermen.

Another sample from one upper molar was obtained from a skull, called "Man of Acha," 9,000 BP. That skull is kept in the Museo San Miguel of Azapa, Arica, north Chile. It was lent to us by V. Standen and I. Muñoz. Indigenous in this area were gatherers and hunters.

The bacterial morphotypes present in the tartar samples obtained from the skulls of Chilean aborigines were compared with those of other extinct populations from different geographic regions.

Tartar samples of four skulls, 1,300-600 BP from La Olmeda were taken from one upper canine and one upper molar and from lower incisors and molars. La Olmeda is a medieval burial site in Palencia, Spain. The aborigines of this region were principally agriculturalists.

A sample from a first upper molar was obtained from one skull, 6,000-5,500 BP of Sant Pau del Camp, a Neolithic burial site located in Barcelona, Spain. These people had a mixed economy: agriculturalist, hunter and gatherer.

Five skulls from the Eastern Mediterranean, 12,000 BP, that belonged to a Mesolithic culture (Natufian) were also studied. Tartar samples were taken from upper molars and lower incisors. Part of this human group possibly migrated to the Iberian peninsula about 40,000 years ago (Howells, 1992). They were agriculturalists.

Sample treatment

Dental tartar samples were obtained with a steel curette and collected in sterile plastic bags, or Eppendorf tubes. Individual fragments of each sample were treated as follows:

Gram and cetylpyridinium staining: Smears of tartar samples, grounded and resuspended in 10 mM phosphate buffer (pH 7.4), were prepared on microscopy slides and Gram stained to detect the presence of structures and morphotypes, comparable to those of contemporary bacteria. Cetylpyridinium stain, which is specific for glycosaminoglycans (Chardin *et al.*, 1990), was used in order to detect peptidoglycan of bacterial cellular walls in the samples.

Scanning electron microscopy (SEM): Samples of dental calculus were adhered with neo-Lube resin (Hulow Industries, Huron, Michigan) to a metallic support, and shadowed with approximately 50 nm of gold in a vacuum evaporator (Polaron Equipment, Ltd., Doylestown, PA). Observations were made using a Siemens Autoscan SEM, with a 20 kV accelerating voltage. The samples were observed at the most appropriate angle, which was usually 45°. Micrographs were taken at magnifications of 3,000x, 6,000x and 7,200x.

Transmission electron microscopy (TEM): Tartar samples from Atacameños, medieval and Natufian specimens were prepared for TEM. These samples were fixed with 4.0% glutaraldehyde, buffered in 0.1 M sodium cacodylate, pH 7.4, for 3 hours. After washing, the specimens were dehydrated in serially graded ethanol solutions and then embedded in EMBED 812 (Electron Microscopy Sciences, Ft. Warrington, PA). The procedures were as follows: Acetone: EMBED 812, 1:1, 12 hours, Acetone: EMBED 812, 1:2, 24 hours, EMBED 812, 100%, 24 hours. 60 nm sections were cut with the Porter-Blum Ultramicrotome equipped with a diamond knife. Some sections were decalcified, stained with 1% tannic acid for 30 minutes, and 4% uranyl acetate for 5 minutes, and later analyzed by TEM using a Zeiss A-109 electron microscope (Zeiss, Oberkochen, Germany). Before TEM, 1-μm sections, stained with Toluidine blue, were examined by optical microscopy.

Indirect immunofluorescence (IIF) technique: To investigate if the bacterial specimens observed in the ancient tartar by TEM were antigenically related to modern plaque bacteria, all the samples were submitted to the conventional indirect immunofluorescence (IIF) technique. Smears of ancient tartar were prepared by grinding the samples between two slides. The resulting tartar powder was suspended in sterile saline solution and placed on the IIF slide. The smears were incubated using rabbit hyperimmune antibacterial antibodies, kindly

donated by Dr. D. Lopatin (University of Michigan, Ann Arbor, MI). Among these, highly specific antibodies to *Streptococcus mutans* (rabbit anti-*S. mutans* absorbed with *S. sanguis*), *Streptococcus sanguis*, *Porphyromonas gingivalis*, *Actinomyces viscous*, *Treponema dentícola*, *Treponema vincentii* and *Prevotella intermedius* were used. The second antibody was a fluorescein isothiocyanate (FITC), coupled with an affinity purified, anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO).

A mounting medium, containing 9 parts glycerol and 1 part phosphate buffered saline (PBS, pH 8.6) was used. Coverslides were sealed with nail varnish. Observations were made using a Nikon Microphot-FXA UV (ultraviolet) microscope (Nikon Inc. Instrument Group, Melville, NY).

Results

Optical microscopy studies

Bacterial forms in the ancient tartar samples were first observed by optical microscopy using Gram and Cetylpyridinium stains. The observations of the Natufian tartar sample (12,000 BP), showed coccal (a) and bacillary forms of bacteria (b), which were Gram positive (1,000x); some Gram negative specimens also appeared in the samples. Cetylpyridinium staining revealed the presence of cellular walls quite similar to those of contemporary bacteria (Fig. 1).

Scanning electron microscopy (SEM) studies

SEM examination of contemporary tartar from a subject older than 30 years, showed many coccal (a), bacillary (b) and spiral (c) forms of bacteria (Fig. 2), revealing the number and diversity of bacterial morphotypes present in the tartar of living individuals.

The Chonos tartar sample (490 BP), showed some bacterial forms inside "printed" cavities in the calcified tartar matrices. The most predominant forms were bacillary (a) and coccal (b) (Fig. 3).

The Atacameños tartar sample (900-300 BP), revealed some filamentous (a) and coccal (d) forms as well as some short bacilli (b), and some thinner bacilli (c) (Fig. 4).

In the tartar sample of a Cerro La Sotta skull (3,400 BP), the existence of a fractured microbe emerging from a cavity in the tartar (a) is easily detectable. Also observable, is a small fractured specimen (b) beside some bacillary (c) and some coccal forms (d) (Fig. 5).

The tartar sample from the skull of the "Man of Acha" (9,000 BP), exhibited many bacillary forms (a), and some scattered coccal forms (b) (Fig. 6).

The Medieval tartar found in Spain from La Olmeda skulls (1,300-600 BP), revealed some spiral (a), filamentous (b), coccal (c) and short bacillary (d) forms (Fig. 7).

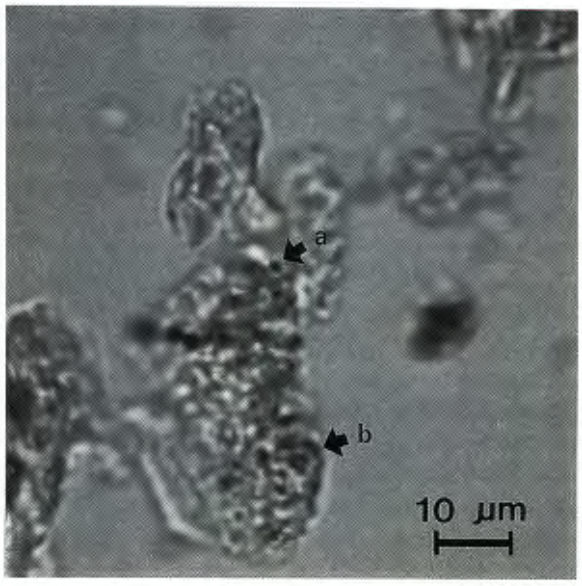


Figure 1. Gram stain of a 12000 BP tartar sample with coccal (a) and bacillary (b) forms.

The tartar sample from skulls of the Spanish neolithic period (6,000-5,500 BP) revealed coccal (a, b), filamentous (b), and bacillary (c) forms (Fig. 8). Figure 9 corresponds to a Mesolithic culture (Natufian, 12,000 BP), the tartar sample examined by SEM contained both spiral (a, b) coccal forms (c, d), and bacillary form (e).

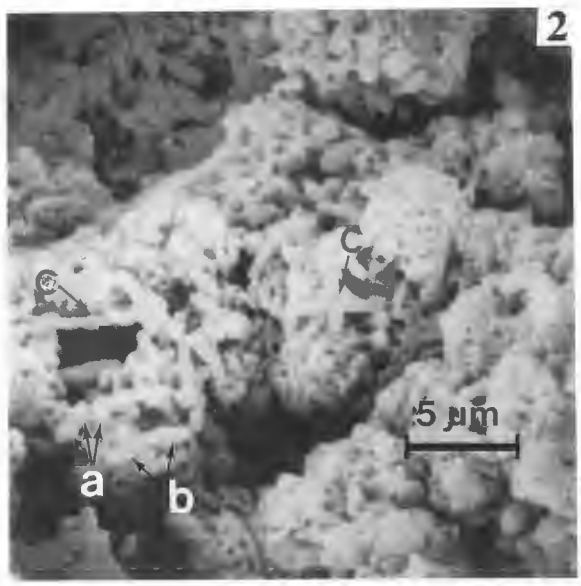
In summary, the results of the SEM study of ancestral tartar samples showed different morphological types of bacteria, particularly in the Spanish and Natufian groups). Spiral forms were observed only in samples from La Olmeda (Spain) and the eastern mediterranean (Natufian) (Table 1).

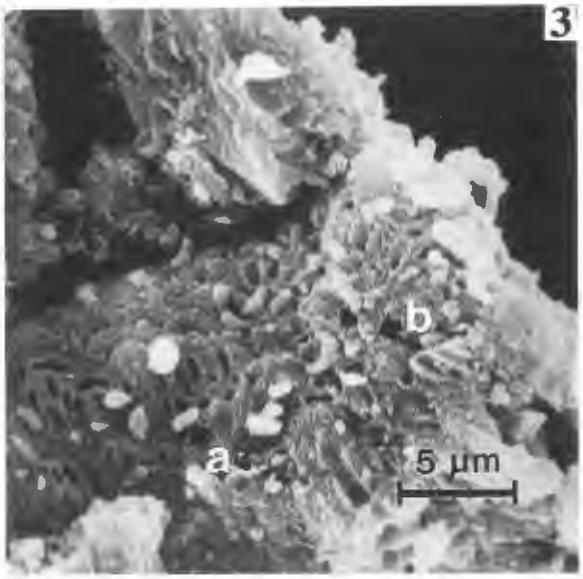
TEM studies

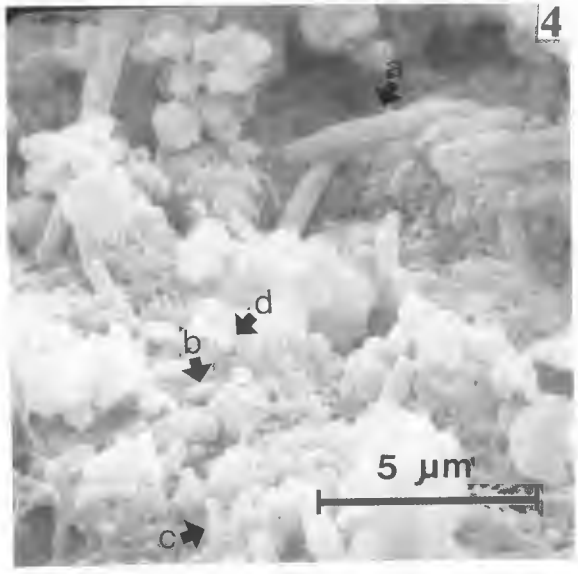
TEM examination (100,000x) of the decalcified Atacameños tartar sample (900-300 BP) revealed bacterial walls (a, b) and cellular membranes (c) similar to those described in contemporary bacteria (Fig. 10A). A decalcified tartar sample from the medieval period (1,300-600 BP), showed well-defined cell walls (a) of bacteria and a cell division (c) (Fig. 10B).

The Natufian decalcified tartar sample (12,000 BP), showed the presence of a fairly well-defined cellular wall (Fig. 11); TEM (380,000x) of modern *S. mutans*, used as control, showed the typical aspect of the cell wall (a) and cell membrane (b) of bacteria (Fig. 12).

In Table 2, we summarize the observations obtained by TEM in relationship with some structures preserved in ancestral tartar. This provides information on the characteristics of the flora shown in the samples.







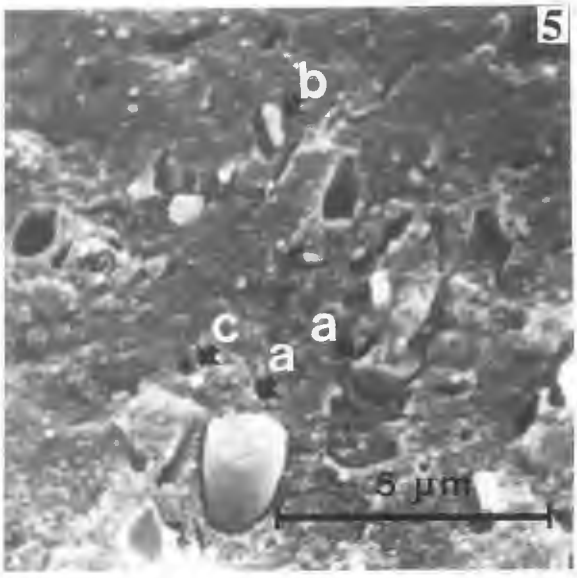


Figure 2. Scanning electron micrograph of a modern tartar sample. Coccal (a), bacillary (b) and spiral (c) forms.

Figure 3. Scanning electron micrograph of a 490 BP Chonos tartar sample with bacillary (a) and coccal (b) forms.

Figure 4. Scanning electron micrograph of a 900-300 BP Atacameños tartar with filamentous (a), short bacillary (b), thin bacillary (b) and coccal (d) forms.

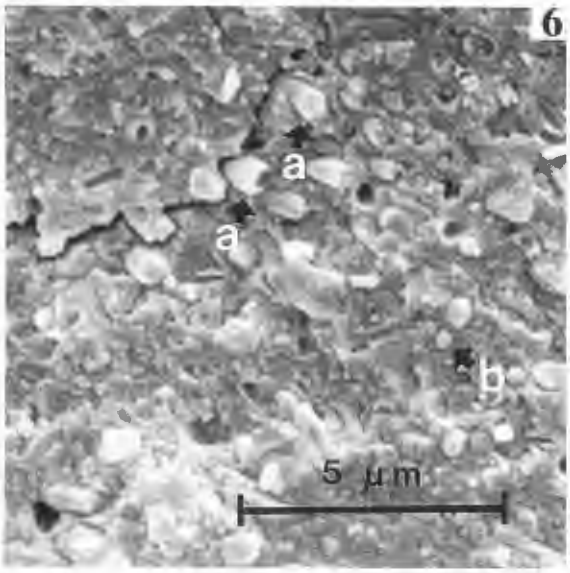
Figure 5. Scanning electron micrograph of a 3400 BP Cerro Sotta tartar sample. Fractured specimens (a) emerging from cavities in the tartar. Bacillary (b) and coccal (c) forms.

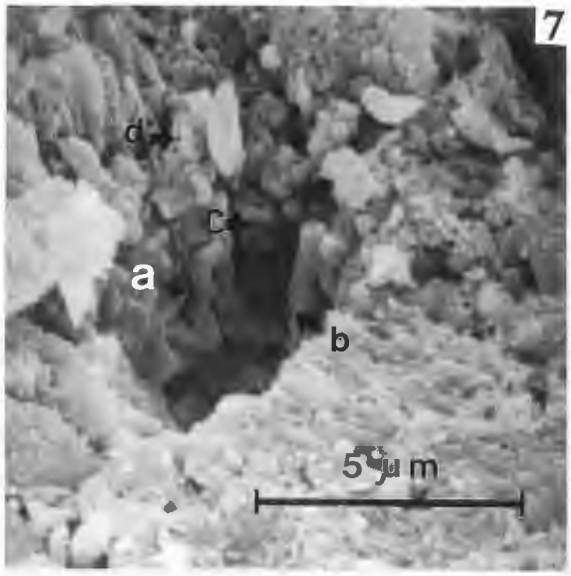
Indirect Immunofluorescence Studies

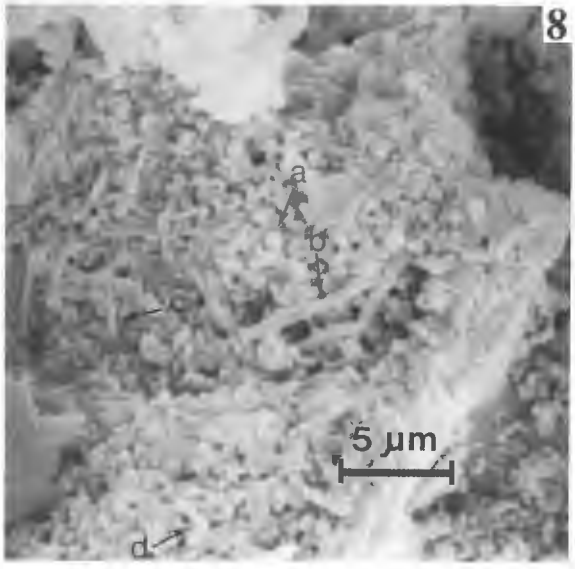
Figure 13 shows the Natufian tartar (12,000 BP), observed, using IIF. The specimen exhibited a positive reaction to the monospecific polyclonal antibody for *S. mutans*, similar to that observed for modern salivary *S. mutans*, used as control (Fig. 14).

Discussion

SEM and TEM figures clearly reveal preserved bacterial fossils in ancient tartar of human remains. Gram and Cetylpyridinium stains results further support the presence of preserved bacteria.







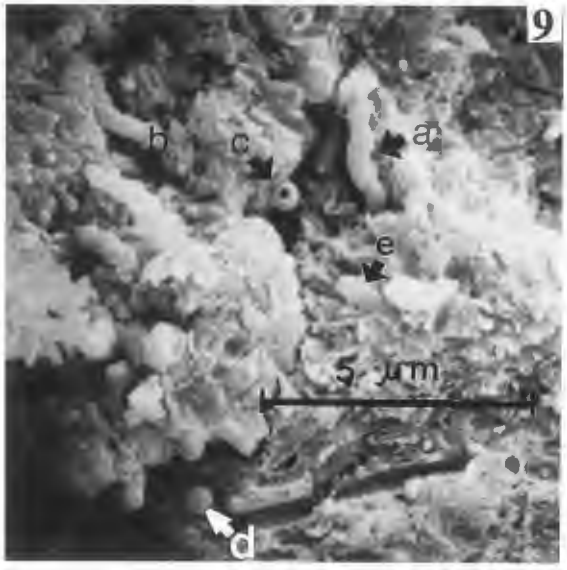


Figure 6. Scanning electron micrograph of a 9000 BP "Man of Acha" tartar sample with bacillary (a) and coccal (b) forms.

Figure 7. Scanning electron micrograph of a 1600-1300 BP tartar sample from the medieval period (Spain). Spiral (a), filamentous (b), coccal (c) and short bacillary (d) forms are present.

Figure 8. Scanning electron micrograph of a 6000-5500 BP tartar sample from the Neolithic period (Spain). Coccal (a,d), filamentous (b) and bacillary (c) forms are present.

Figure 9. Scanning electron micrograph of a 12000 BP Natufian tartar sample with spiral (a,b), coccal (c,d) and bacillary (e) forms.

In this study, the fragments of archeological calculi scanned by SEM at magnifications higher than 2,000, show large numbers and diversity of microorganisms present in the samples. These microorganisms represent constituents of the original dental plaque, which became mineralized during its calcification process, as shown by previous studies (Dobney and Brothwell, 1986; Linossier *et al.*, 1988, 1994). Our results seem to demonstrate

Table 1. Presence of bacterial forms in different ancient tartar samples.

Sample	Bacillary	Coccal	Filamentous	Spiral
Control*	+	+		+
490 BP Incisors	+	+		
900-300 BP Atacameños	+	+	+	
3,400 BP Cerro Sotta	+	+		
9,000 BP Man of Acha	+	+		
1,600-1,300 BP Medieval Period Spain	+	+	+	+
6,000-5,500 BP Neolithic Period Spain	+	+	+	
12,000 BP Natufian	+	+		+

+: Presence of bacterial forms BP: Before Present *: Actual tartar

Table 2. Presence of some bacterial structures revealed by TEM studies in human groups whose subsistence patterns were agriculture.

Age and Origin	Cell wall	Cell membrane
900-300 BP Atacameños	+	+
1,300-600 BP The Medieval Spanish Period	+	
12,000 BP Natufian Culture	+	
Actual Tartar	+	+

+: Presence of bacterial structure; BP: Before Present

that the microbes in the ancient tartar were preserved in a similar way to those found in the modern tartar (Lustmann *et al.*, 1976; Friskopp and Hammarström, 1980).

A wide variation in the composition of the microflora present in samples of individuals from different extinct human groups was observed, facts that could be related with different subsistence patterns, according to anthropological studies by Harris (1992) of these human

Figures 10-14 on the facing page 1011

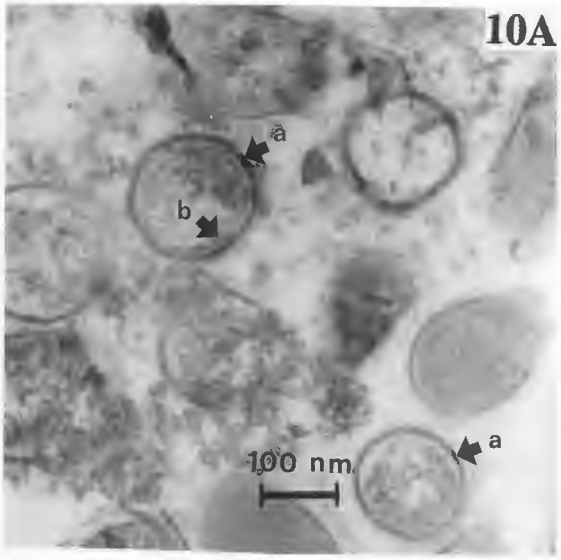
Figure 10. Transmission electron micrograph of decalcified tartar samples. (A) Atacameños culture; cell walls (a) and cell membrane (b) are visible; (B) Medieval Spanish period; cell wall (a) and a cell division (c) can be seen. Both figures are at the same magnification.

Figure 11. Transmission electron micrograph of a decalcified tartar sample (Natufian culture) showing a cell wall (a).

Figure 12. Transmission electron micrograph of an isolated *S. mutans* sample used as control. Cell wall (a) and cell membrane (b).

Figure 13. Indirect immunofluorescence micrograph (original in color) of a tartar sample (12000 BP). The positive reaction was obtained with anti-*S. mutans* antiserum.

Figure 14. Indirect immunofluorescence micrograph (original in color) of a *S. mutans* sample isolated from saliva showing a positive reaction with anti-*S. mutans* antiserum.


10A
a
b
a
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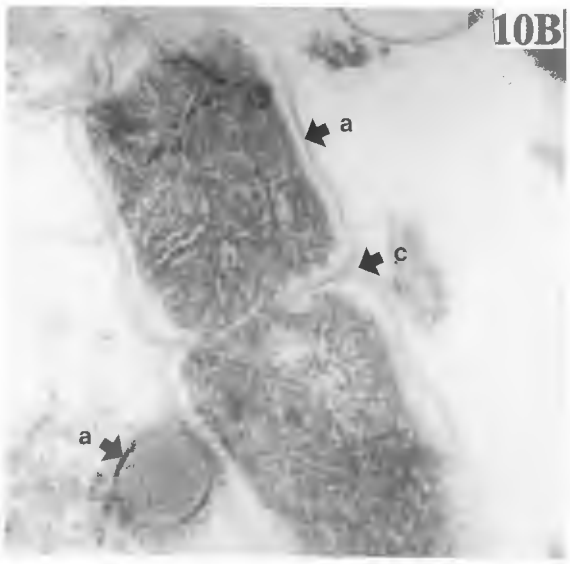
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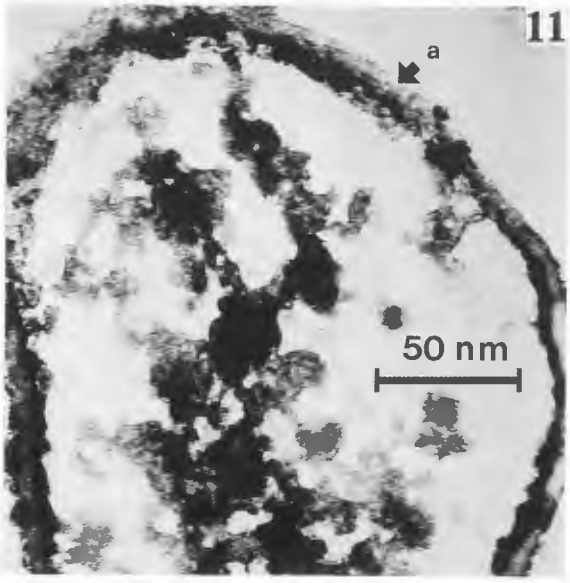
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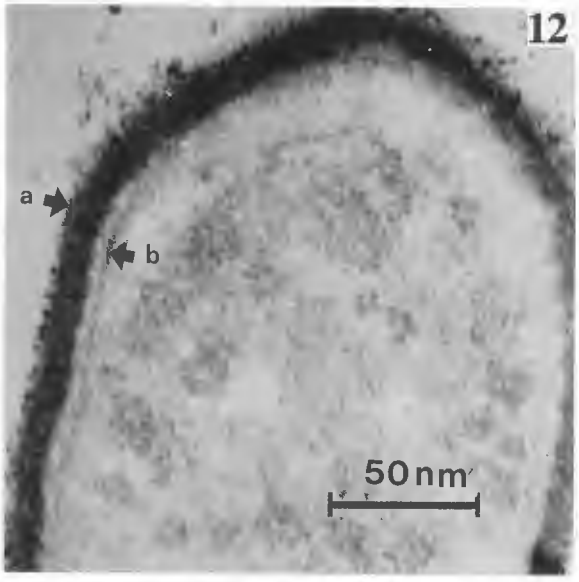
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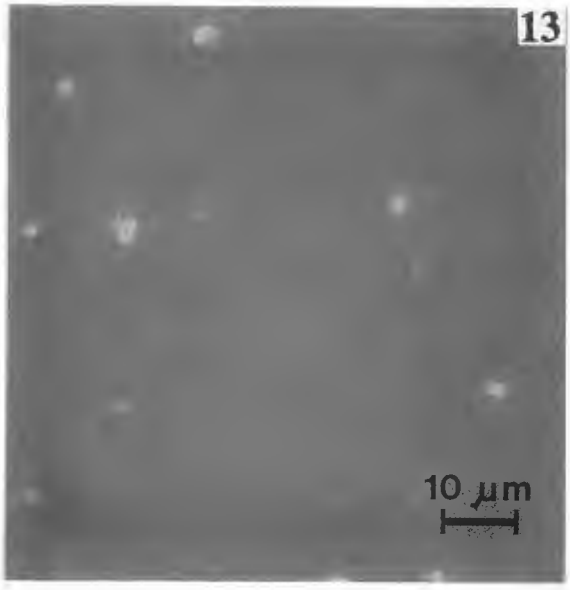
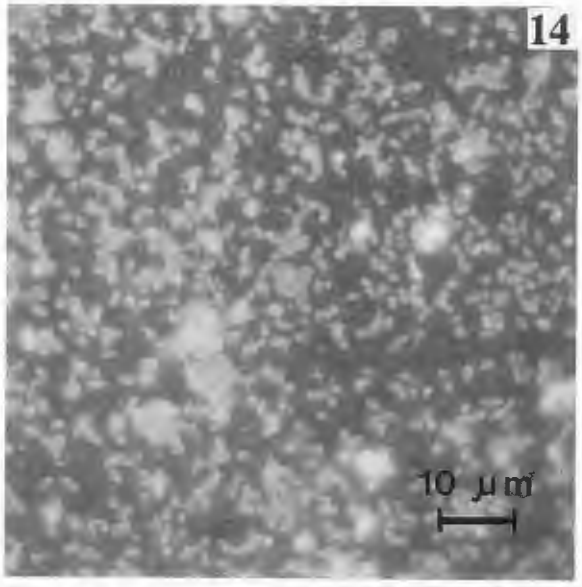

13
10 µm


14
10 µm

groups. The tartar samples from Chonos, Acha and Cerro La Sotta belonged to hunters, fishermen and gatherers, located in restricted geographical zones. Apparently, they had less diversity of microbial morphotypes than the groups of Sant Pau del Camp, a Spanish burial site of the Neolithic period which had a mixed economy: agriculturalist, hunter-gatherer or fisherman (Lalueza and Pérez, 1993; Lalueza *et al.*, 1993). These samples exhibited a diversity of densely arranged microbial morphotypes, which can be explained in part by the inclusion of wild cereals to their subsistence pattern. Tartar samples from the agricultural human groups namely, Atacameños, La Olmeda and Natufian, from different and distant geographic locations, showed a remarkable diversity of microbial flora, expressed as a wide variety of morphotypes (Hole, 1992). The spiral morphotypes, in particular those found in the La Olmeda and Natufian groups, are in agreement with the morphological description by Dobney and Brothwell (1986). The composition of the microflora in the tartar from farmers can also be attributed to their dietary habits, which also can account for the most prevalent occlusal dental caries observed in these individuals in relation to hunters and gatherers (Moore and Corbett, 1971). It is well known that some cereals contain significant amounts of sucrose (Coykendall, 1976). The diet of the hunters-gatherers and fishermen was abrasive, a situation that was aggravated by the habit of using the mouth as a leather tanning tool. Both of these factors are involved in the attrition of the occlusal surfaces of the teeth, and the loss of these surfaces as food retentive zones, this could explain the absence of carious lesions in those teeth (Linossier *et al.*, 1994). If the unbalance of the oral microflora is determined by diet, the low level of mutans streptococci suggested by this work would be consistent with the find that ancient populations did not consume refined sucrose. Moreover, that was probably the reason that the type of dental caries occurring in those days was mainly found at the occlusal and the root surfaces (Coykendall, 1976). When sucrose became an important component of the diet, the oral microflora changed in quantity and quality, so at present, with a population that consumes large amounts of sucrose, a substantial increase of smooth dental decay is seen (Mouton and Robert, 1995).

TEM "*in vitro*" (Sidaway, 1980) and "*in vivo*" (Ruzicka, 1984) studies of dental calculi revealed Gram positive bacterial structures such as the bacterial wall, cytoplasm and cell membrane. This latter structure could not be observed in dental tartar bacteria from the Spanish middle age and Natufiant culture. These observations indicate that the bacterial wall is the most permanent structure through time (Table 2). Our results in control samples were in agreement with the work of Moro *et al.* (1986), on TEM observations of *S. mutans* isolated from the mouth, in fresh cultures.

The IIF technique allowed us to detect ancient bacteria that reacted with anti *S. mutans* polyclonal antibodies. Quite probably, the same antigens involved in adherence could be preserved. At present, some of these antigens, probably proteins, can be shared with other species such as protein I//II, which is an important factor in the adherence of *S. mutans* to the salivary glycoproteins surrounding the teeth (Bleisweis and Oyston, 1993). If some of the cell antigens of *S. mutans* can be resistant to environmental changes, this is probably due to the structure of the cell wall of the Gram positive bacteria.

Our results clearly indicate that species antigenically similar to *S. mutans* was present in almost all types of tartar samples, except Cerro Sotta, as it was assessed by rabbit anti-*S. mutans* antisera, absorbed with *S. sanguis*. However, it was not possible for us to relate its presence with the dietary habits of the ancient population studied here, nor with their pattern of subsistence.

The polyclonal antibodies to *S. mutans*, *S. sanguis*, *P. gingivalis*, *A. viscous*, *T. denticola*, *T. vincentii* and *P. intermedius* did not give positive results, facts that require further investigation. The serological diagnosis of ancient bacteria is a very useful tool for determining the genus of bacteria. Although the results of this study only represent a muestral group of extinct communities, and the available information on the ancient dental plaque is yet incomplete, the results of this study help complete and support previous observations in the field of oral paleomicrobiological and paleopathological research.

Finally, there are many questions to be answered: Is it possible to isolate and to analyze bacterial antigens in ancient samples? Could it be possible to relate the presence of bacterial morphotypes or bacterial antigens, in dental plaque or calculus, with diet or cultural changes? Are the antigens found in the tartar a biological marker to trace human migration patterns of early man? New technological advances in, for example, SEM, TEM, epi-immunofluorescence microscopy, or DNA fingerprinting, could help answer these questions in the coming years. This would not only benefit science but the health of human beings.

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Discussion with Reviewers

Reviewer V: The authors offer the inclusion of increased sucrose content as a potential explanation for the clearly observed differences in bacterial morphotypes with different subsistence patterns. Are there alternative explanations or contributory factors?

Authors: We do not yet have any satisfactory explanation. Nevertheless, we propose, in the near future, to study two groups of individuals with different subsistence patterns: for example, one with an agricultural based nourishment and the other based on fishing and hunting (Eskimos).

T. Fassel and Reviewer VI: Would the authors comment on the potential problem of present time contamination of ancient samples?

Authors: We only observed fungus, *Bacillis substilis*, Sarcinas sp., which are typical environmental contaminants. On the other hand, we always took care of taking the samples for study from the center of the dental tartar under rigorous aseptic conditions.

T. Fassel: There is a much lower level of labeling in Figure 13, the ancient sample, than in Figure 14, the present day sample. While the ultrastructural preservation of ancient samples are remarkable, could the authors comment further on processes that would lead to loss of ultrastructural as well as antigenic preservation of ancient samples? Also, do any future research plans include any attempt to label electron microscopy samples?

Authors: The level of labeling in Figure 13 was much lower than in Figure 14 because the determination was made directly in tartar samples, while in Figure 14, the samples were fresh bacteria obtained from saliva. With regard to the processes that would lead to the loss of ultrastructure as well as antigenic preservation of ancient samples, we think that the environmental conditions of the samples are very important. Samples from the desert zones are much better preserved (for example, those obtained from Atacama Desert); also, the salinity ensures a good preservation. We plan in the near future to assess the bacterial specificity through immuno SEM and immunocytochemistry by TEM. This would allow us to evaluate antigenic structure and stability with age.