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Prevalence and determinants of oral microflora among Portuguese adolescentes

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KEYWORDS
Aggregatibacter actinomycetemcomitans;
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Oral microflora

Abstract
Introduction: The aim of the present study was to determine the prevalence and determinants of salivary *Streptococcus mutans*, *Lactobacillus* and *Aggregatibacter actinomycetemcomitans* and the associated risk of development of dental pathologies on a sample of Portuguese adolescents.
Materials and methods: A cross-sectional study was designed including a final sample of 447 adolescents aged between 12 and 18 years old, attending a public school in Sátão, Portugal. A self-administered questionnaire was filled out by the adolescents. Clinical examination of oral health status was carried out and saliva collection was accomplished by the passive drool method. The identification of the different types of bacterial strains was accomplished using the Polymerase Chain Reaction technique.
Results: The prevalence of *S. mutans* in the studied sample was 99.5% *Lactobacillus*, 80.8% *S. mutans* and 15.2% *A. actinomycetemcomitans*. The presence of *S. mutans* was associated with gender (male = 76.1% vs female = 83.6%; *P* = .04) and dental pain in the presence of severe dental caries (77.3% vs 87.8%; *P* = .006). The infection with *A. actinomycetemcomitans* was associated with age (< 15 years = 12.3% vs ≥ 15 years = 20.3%; *P* = .03) and residence area (rural = 18.2% vs urban = 11.0%; *P* = .04), and may be related with a higher risk of periodontal disease development in adulthood.
Conclusions: A. actinomycetemcomitans infection was found to be associated with socio-demographic variables, suggesting that, if not clinically well identified and treated, may cause serious oral diseases during adulthood. It has been described that the oral microflora is one of the main etiological factors for dental caries and periodontal diseases development, but cannot be considered in an isolated manner.

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Introduction

The oral cavity is inhabited by different bacterial species that play vital roles in maintaining oral health or in shifting to a diseased state such as dental caries and periodontal disease.[1,2] Dental caries consists of a post-eruptive bacterial infectious disease characterized by a progressive demineralization process that affects the mineralized dental tissues. It is considered the most prevalent oral disease and the main responsible for tooth loss (teeth decay) among the population.[3,4] A carious lesion initiates with the production of organic acids by the microorganisms of the oral cavity that metabolize the extracellular carbohydrates of the individual's diet.[5,6] The presence of the produced organic acids will decrease the pH in the interface between the tooth surface and the bacterial plaque, which allows the development of the demineralization process on the tooth enamel.[4] When the oral cavity has a pH below 5,5 (considered the critical pH), the dental tissue begins to saturate, causing an initial lesion that will be the precursor of the dental carie.[7] Prevention methods have the main goal of decreasing the time of exposure of the tooth tissues to the low values of pH. Therefore, it is strictly necessary the frequent removal of bacterial plaque to avoid the increased contact with tooth surfaces.[8]

The researcher Paul Keyes developed a diagram that describes the multifactorial aetiology of dental caries. In this diagram, we can observe that there are three main etiological factors that are essential for the initiation and development of the disease: susceptible host; cariogenic oral microflora; *substratum* that depends on the host's diet, which is then metabolized by the microorganisms that constitute the bacterial plaque.[5]

Dental caries, as an infectious disease, correlates directly with bacterial strains that co-exist in the oral cavity, like *Streptococcus mutans* and *Lactobacillus*.[9] The cariogenic properties of *S. mutans* and *Lactobacillus* are widely recognised and, as significant odontopathogens, the former group is linked to enamel lesion formation while the latter is associated with cavity progression.[10] *Aggregatibacter actinomycetemcomitans* is one of the most well studied periodontal bacterial strains. It stays in the periodontal pocket of the oral cavity and damages tooth supporting tissues, being considered as the major cause of periodontitis.[11] Kaplan and colleagues found that all *A. actinomycetemcomitans* strains possess strong virulence potential.[12] A study developed by Hart et al, indicated the main bacterial species or groups that could be implicated in caries onset and progression, and both *S. mutans* and *Lactobacillus* were reported as been linked with both onset and progression.[13]

Aas et al, reported a distinctive predominant bacterial flora of the healthy oral cavity that is highly diverse and subject specific. It is crucial to fully identify/recognize the human microflora of the healthy oral cavity before we can understand the role of bacteria in oral disease.[14] Furthermore, it has concurrently been clearly established that social, economic, cultural, ethnic, and environmental factors also play an important role in the formation of dental caries and (also) influence the individual oral microflora highly correlated to oral health behaviours.[15]

The aim of the present study was, therefore, to investigate the prevalence of salivary *S. mutans*, *Lactobacillus* and *A. actinomycetemcomitans* and their influence on the risk of development of dental caries and the association with socio-demographic aspects among a sample of Portuguese adolescents.

Materials and methods

Material collection

A sample of 447 adolescents aged between 12 and 18 years old, attending a public school in Sátão, Portugal, was enrolled in this study. All samples were obtained from September to December of 2012. Questionnaires without information about gender and age were excluded of the study as well as the adolescents whose parents did not sign the informed consent before data collection.

All participants in this study filled out a self-administered questionnaire focusing socio-demographic variables, social and daily habits and oral health behaviours. Questions about socio-demographic variables such as gender (male/female), age, school grade at the moment of the study, residence area (urban/rural), parents' educational level (choosing the higher educational level between father and mother), parents' professional situation (employed/unemployed) and the number of rooms and people living in the house were used to determine the crowding index.

This research has been performed in accordance with the Declaration of Helsinki and was submitted and approved by the Ethics Committee of the Health School and Research Centre for Education, Technology and Health Studies of the Polytechnic Institute of Viseu, Portugal (CI&DETS). The information collected by the questionnaires was provided voluntarily and confidentially, and the anonymity of the information collected was guaranteed by telling the adolescents not to sign their names or write down any other form of identification in any part of the questionnaire. Data collection was made only for adolescents whose parents signed an informed consent that explained the objectives of the study. After collection, the questionnaires were numbered, stored and processed by computer. The results do not refer to nominal adolescents or contain any information that may identify any of the participants.

Clinical sample characterization

Clinical examination of oral health status was carried out according to the World Health Organization (WHO) criteria.[16] The teeth were clinically examined with dental instruments using visual-tactile method with the use of a dental mirror and a probe (approved by the WHO for caries diagnosis) and took place in the classroom under standardized conditions recommended by the WHO. Cotton rolls and gauze were available to remove moisture and plaque when necessary. There was only one observer that registered the results of each clinical observation during the study. The recorded variables of the clinical examination were caries experience, using the decayed, missing and filled permanent tooth index (DMFT) as oral health indicator, which consists of the sum of teeth decayed, teeth missing due to dental caries and teeth filled for each analysed adolescent. Each tooth would be classified with only one of the following codes: 0, sound

crown or root, showing no evidence of either treated or untreated caries; 1, indicates a tooth with caries; 2, filled teeth, with additional decay; 3, filled tooth with no decay; 4, tooth that is missing as a result of caries; 5, a permanent tooth missing for any other reason than decay; 6, teeth on which sealants have been placed; 7, indicate that the tooth is part of a fixed bridge; 8, this code is used for a space with a non-erupted permanent tooth where no primary tooth is present; 9, erupted teeth that cannot be examined; T, indicates trauma in the presence of a fractured crown.

Bacterial strain identification

Saliva collection was accomplished by the passive drool method and placed into a polypropylene tube until about 2 mL of saliva in each tube *per* adolescent. Next, DNA was extracted using the MagNA Pure LC DNA Isolation Kit (Bacteria, Fungi) (Roche), quantified with Nanodrop (Thermo Lifesciences), and bacterial DNA was amplified using the Multiplex Polymerase Chain Reaction (PCR) kit (Qiagen) with primers specific for the bacterial strains analysed. To validate primers specificity, DNA bands with the expected molecular from 3 positive-cases were excised from the agarose gel, purified with ULTRAPrep® Agarose Gel Extraction kits (AHN), and sequenced with BigDye Terminator Sequencing Kit (Applied Biosystems). The obtained DNA sequences were compared with a control and with NCBI database (http://www.ncbi.nlm.nih.gov/). Primers employed in this study are as described: *Lactobacillus* (GGAATCTTCCACAATGGACG and CGCTTTACGCCCAATAAATCCGG); *S. mutans* (TCGCGAAAAAGATAAACAAACA and GCCCCTTCACAGTTGGTTAG); *A. actinomycetemcomitans* (AAACCCATCTCTGAGTTCTTCTTC and ATGCCAACTTGACGTTAAAT).[17-19]

Statistical analysis

Data analysis was carried out using SPSS for Windows (version 18.0). Prevalence was expressed in proportions and was compared by the Chi-square test. The significance level established the inferential statistics was 5% ($P < .05$).

Results

The sample used in this research was composed by 447 adolescents (38.3% were males and 61.7% females), all between the ages of 12 and 18 years old, from a public school of Sátão, Portugal. When analysing the parents' educational level, we observed that 4.3% have parents that only frequented school to or less than 4th grade, 53.5% stayed in school from the 5th to the 12th grade and 15.0% went to a superior degree after finishing the 12th grade. Crowding index < 1.0 is presented among 71.4% of adolescents, while 14.1% are equal to 1 and only 4.5% > 1.0, which indicates possible overcrowding at home. The analysis of the distribution of the sample by residence area revealed that the majority of adolescents lives in rural areas (65.3% vs 34.7%) (Table 1).

The prevalence of *S. mutans* in the studied sample was 99.5%, *A. actinomycetemcomitans,* 80.8%, *Lactobacillus* 15.2%. When analysing the combinatorial presence of two or more bacterial strains, we observed the following: *S. mutans* and *Lactobacillus*: 80.5%; *Lactobacillus* and *A. actinomycetemcomitans*: 15.0%; *S. mutans* and *A. actinomycetemcomitans*: 12.1%; *S. mutans*, *Lactobacillus* and *A. actinomycetemcomitans*: 12.1%.

Looking for the association between the bacterial species and gender, we observed a significant difference among gender (higher in females) for *S. mutans* (male = 76.1% vs female = 83.6%; $P = .04$) and the co-infection with both *S. mutans* and *Lactobacillus*. Focusing on socio-demographic variables, we found that infection with *A. actinomycetemcomitans* was significantly associated with both age (< 15 years = 12.3% vs ≥ 15 years = 20.3; $P = .03$) and residence area (rural = 18.2% vs urban = 11.0%; $P = .04$). The same situation was observed in adolescents infected with *A. actinomycetemcomitans* and any of the other bacterial species mentioned above. Taken together, these results demonstrate that *A. actinomycetemcomitans* may be the main bacterial strain associated with socio-demographic variables independently of the presence of the other bacterial strains analysed in the present study. These results may also be associated with a higher prevalence of periodontal disease among older adolescents and those that present worse socioeconomic status that live in rural areas as verified in Table 2.

Our study revealed the lack of a significant correlation between worse oral health behaviours and the presence of the various bacterial species analysed. However, some bacterial strains were associated with dental pain, which occurs in the presence of severe dental caries. We observed that adolescents who suffered one or more episodes of dental pain due to dental caries had a higher rate of *S. mutans* infection (episode of dental pain = 87.8% vs no dental pain = 77.3%; $P = .006$) and both *S. mutans* and *Lactobacillus* as seen in Table 3. The results demonstrate that dental pain might be associated mainly with *S. mutans* infection rather than with *Lactobacillus* infection, considering that 99.5% of the sample used in this study showed infection with this bacterial strain.

Despite the observed association between dental pain and the presence of *S. mutans* alone or in combination with *Lactobacillus,* no significant association was found between the presence of the bacterial species studied and the risk of dental caries development. In fact, we detected the presence of the three studied species not only among adolescents that have active caries during the time of intra-oral observation but also in caries-free individuals.

Discussion

The results obtained in this research demonstrate that the salivary presence of *S. mutans*, *Lactobacillus*, and *A. actinomycetemcomitans* is not associated with the development of dental pathologies in our community sample of adolescents. Dividing the sample in caries-free adolescents and caries-active adolescents, we observed that both groups have similar levels of infection by *S. mutans* and *Lactobacillus*. Nevertheless, our findings go against some studies that correlate the presence of *S. mutans* and *Lactobacillus* with a higher risk of caries formation.[20,21] Also Fysal et al, observed that the number of *S. mutans* and *A. actinomycetemcomitans* determined by microscopic counts was twice as high in caries patients.[22] On the other hand, and strengthening our results, Reyes et al demonstrated that *Streptococcus*

Table 1 Socio-demographic characterization of the studied sample of Portuguese adolescents

	Male, n (%)	Female, n (%)	Total, n (%)
	171 (38.3)	276 (61.7)	447 (100.0)
Age			
12	31 (18.2)	27 (9.8)	58 (13.0)
13	29 (17.0)	38 (13.8)	67 (15.0)
14	18 (10.5)	34 (12.3)	52 (11.6)
15	28 (16.4)	27 (9.8)	55 (12.3)
16	16 (9.4)	34 (12.3)	50 (112)
17	16 (9.4)	29 (10.5)	45 (10.1)
18	33 (12.3)	87 (24.3)	120 (26.6)
Grade			
7	34 (19.9)	26 (9.4)	59 (13.4)
8	26 (15.2)	38 (13.8)	64 (14.3)
9	19 (11.1)	33 (12.0)	52 (11.6)
10	30 (17.5)	38 (13.8)	68 (15.2)
11	19 (11.1)	32 (11.6)	51 (11.4)
12	44 (25.2)	109 (39.4)	153 (34.1)
Parents' educational level			
≤ 4 grade	3 (2.0)	16 (6.9)	19 (4.3)
5-12 grade	105 (61.4)	134 (48.6)	239 (53.5)
> 12 grade	30 (17.5)	37 (13.4)	67 (15.0)
Without information	33 (19.1)	89 (31.1)	122 (27.2)
Crowding index			
< 1.0	110 (64.3)	209 (75.7)	319 (71.4)
1.0	25 (14.6)	38 (13.8)	63 (14.1)
> 1.0	8 (4.7)	12 (4.3)	20 (4.5)
Without information	28 (16.4)	17 (6.2)	45 (10.0)
Residential area			
Rural	100 (58.5)	192 (69.6)	292 (65.3)
Urban	71 (41.5)	84 (30.4)	155 (34.7)

Table 2 Association between bacterial strains *Streptococcus mutans* (SM), *Lactobacillus* (LA) and *Aggregatibacter actinomycetemcomitans* (AA) and socio-demographic variables

	Gender			Age (years)			Residential area		
Bacterial strain	Male, n (%)	Female, n (%)	*P*	< 15, n (%)	≥ 15, n (%)	*P*	Rural, n (%)	Urban, n (%)	*P*
SM	121 (76.1)	219 (83.6)	.04	161 (79.3)	107 (77.5)	.4	169 (79.0)	78 (99.0)	.5
LA	158 (99.4)	261 (99.6)	.6	203 (100.0)	136 (98.6)	.2	213 (99.5)	126 (99.2)	.6
AA	19 (11.9)	45 (17.2)	.09	25 (12.3)	28 (20.3)	.03	39 (18.2)	14 (11.0)	.04
SM + LA	121 (76.1)	218 (83.2)	.05	161 (79.3)	107 (77.5)	.4	169 (79.0)	99 (78.0)	.5
LA + AA	18 (11.3)	45 (17.2)	.07	25 (12.3)	27 (19.6)	.05	39 (18.2)	13 (10.2)	.03
SM + AA	14 (8.8)	37 (14.1)	.07	21 (10.3)	20 (14.5)	.2	32 (15.0)	9 (7.1)	.02
SM + LA + AA	14 (8.8)	37 (14.1)	.07	21 (10.3)	20 (14.5)	.2	32 (15.0)	9 (7.1)	.02

Table 3 Association between bacterial strains *Streptococcus mutans* (SM), *Lactobacillus* (LA) and *Aggregatibacter actinomycetemcomitans* (AA) and oral health behaviours and dental pain

	Oral hygiene			Dental appointment			Dental pain		
Bacterial strain	≤ 1/day, n (%)	≥ 2/day, n (%)	*P*	No, n (%)	Yes, n (%)	*P*	No, n (%)	Yes, n (%)	*P*
SM	56 (80.0)	205 (78.2)	.5	117 (81.8)	219 (79.9)	.4	218 (77.3)	122 (87.8)	.006
LA	70 (100.0)	260 (99.2)	.6	143 (100.0)	272 (99.3)	.4	282 (100.0)	137 (98.6)	.1
AA	9 (12.9)	44 (16.8)	.3	22 (15.4)	42 (15.3)	.5	46 (16.3)	18 (12.9)	.2
SM + LA	56 (80.0)	205 (78.2)	.5	117 (81.8)	218 (79.6)	.3	217 (77.0)	122 (87.8)	.005
LA + AA	9 (12.9)	43 (16.4)	.3	22 (15.4)	41 (15.0)	.5	46 (16.3)	17 (12.2)	.2
SM +AA	9 (12.9)	32 (12.2)	.5	18 (12.6)	33 (12.0)	.5	36 (12.8)	15 (10.8)	.3
SM + LA + AA	9 (12.9)	32 (12.2)	.5	18 (12.6)	33 (12.0)	.5	36 (12.8)	15 (10.8)	.3

species were present in caries-active and also in caries-free individuals. In this study, the authors also observed that dental caries can occur in the apparent absence of the *bacterium Streptococcus* and can even be associated with healthy states.[1] Parisotto et al explored the association between caries development, colonization with caries-associated microflora, and immunity as children begin the transition to mixed dentition. In baseline level there was no significant differences in *S. mutans* and *Lactobacillus* between caries-free and caries-active groups.[23] Beighton confirmed that dental caries may develop in the absence of these species and their presence does not necessarily indicate dental caries activity.[24] Additionally, Wolff et al demonstrated that there are no significant differences of oral bacterial strains between caries-free and caries subjects.[25]

A question may arise from the present study: can an association of other bacterial strains with *S. mutans*, *Lactobacillus* and *A. actinomycetemcomitans,* potentiate and increase the risk of oral disease development? Looking at the oral health behaviours, no differences were observed between oral hygiene habits and the presence or absence of the three bacterial strains in study. Our results are different from those obtained by Plonka et al that determined that the presence of *S. mutans* and *Lactobacillus* would increase with children's age and was also associated with worse oral health behaviours.[26] Levels of *S. mutans* and *Lactobacillus* were found to be strongly associated with socioeconomic status among Palestinian children in East Jerusalem. The relatively high prevalence of cariogenic bacteria suggests that oral care prevention and treatment demands special attention from the health care institutions and authorities.[15]

In the present study, *A. actinomycetemcomitans* was associated with age and residence area. Adolescents from rural areas showed a higher prevalence of this bacteria in comparison with the ones who live in urban areas. Paolantonio et al demonstrated that *A. actinomycetemcomitans* colonization in children and adolescents from central Italy is affected by socioeconomic and cultural factors, namely residence area (urban vs rural areas), and that these factors may also affect the periodontal condition of the subjects.[27] The same situation is verified in the presence of *Lactobacillus* and *A. actinomycetemcomitans*, *S. mutans* and *A. actinomycetemcomitans* and in the presence of all three bacterial strains. This fact can be considered important, because the adolescents that present *A. actinomycetemcomitans* can have, in the near future, a higher risk of periodontal disease development.[28,29] Therefore, special attention should be given to adolescents living in rural areas, which present worse oral health behaviours and more difficulties in attending frequent dental appointments biannually.

We verified that adolescents who suffered one or more episodes of dental pain due to dental caries had a higher incidence of *S. mutans* alone or in combination with *Lactobacillus*. This may be justified by the fact that adolescents that suffer dental pain have worse dental caries lesions and more retentive sites on the tooth surface that may increase the levels of anchored *S. mutans*, as previously suggested by Thaweboon et al.[30] However, it is important to understand that this association between the prevalence of certain bacterial strains and the occurrence of dental pain does not occur in an isolated manner. Even knowing that *S. mutans* is one of the main bacterial strains responsible for dental caries development, we must have into account other aetiological factors, such as the consumption of sweet foods and oral hygiene habits which have been clearly described as being significantly associated with the severity of dental caries development.[4,5]

Conclusions

The presence of oral microflora is clearly one of the main etiological factors for dental caries and periodontal diseases development, but cannot be considered in an isolated manner. For the development of oral disease, various other factors need to be present such as high and daily sugar intake, inadequate oral hygiene habits and even genetic susceptibility. *A. actinomycetemcomitans*, even among adolescents, can be considered associated with socio-demographic variables, and, if not clinically well identified and treated, may cause serious oral diseases during adulthood. Adolescents who suffered one or more episodes of dental pain due to dental caries had a higher incidence of *S. mutans*.

Probably the imbalances in the resident microflora may be the ultimate mechanism of oral disease development. Oral diseases can appear in the presence of changes of the oral bacterial communities' structure and that may be related with the shift from health to disease. The enormous diversity of oral microbiota allowed for a better understanding of oral micro ecosystem, and these pathogenic populations present in the oral cavity provide new insights into the aetiology of oral diseases and suggest new targets for interventions of the disease. The present study indicates that epidemiologi-

What we know about the theme

- The oral cavity is inhabited by different bacterial species that play vital roles in maintaining oral health or in shifting to a diseased state such as dental caries and periodontal disease.
- *Aggregatibacter actinomycetemcomitans* is one of the most well studied periodontal bacterial strains.
- Social, economic, cultural, ethnic, and environmental factors play an important role in the formation of dental caries and also influence the individual oral microflora.

What we get out the study

- *A. actinomycetemcomitans*, among adolescents, can be considered associated with socio-demographic variables, and, if not clinically well identified and treated, may cause serious oral diseases during adulthood.
- Adolescents who suffered one or more episodes of dental pain due to dental caries had a higher incidence of *Streptococcus mutans*.
- It has been described that the oral microflora is one of the main etiological factors for dental caries and periodontal diseases development, but cannot be considered in an isolated manner.

cal surveys with the assessment of etiologic risk factors are crucial tools for oral health planners and clinicians in order to implement a risk-based preventive strategy.

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Conflicts of interest

The authors declare that there are no conflicts interests.

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