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Original Article

Oral Microbial Flora in Bulgarian Adolescents with Moderate Plaque-induced Gingivitis

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Abstract

Introduction: In children and adolescents, the most common periodontal disease is the plaque-induced gingivitis.

Aim: The aim of this study was to reveal the bacterial species associated with supragingival plaque of Bulgarian adolescents diagnosed with plaque-induced gingivitis.

Materials and methods: Supragingival plaque samples from 70 healthy subjects with moderate plaque-induced gingivitis (37 females and 33 males), aged 12-18 years, were obtained and examined microbiologically.

Results: A total of 224 microorganisms were isolated. Gram-negative bacteria were predominant compared to Gram-positive [132 (59%) vs. 92 (41%), $p<0.001$]. Aerobic microorganisms were detected more often than anaerobic (151; 67.5% vs. 73; 32.5%, $p<0.001$). The *Streptococcus mutans* group and *Neisseria spp.* were isolated from all adolescents. The frequency of isolation of *C. albicans* was relatively lower – 11 (15.7%). The anaerobes showed much greater microbial diversity (12 pathogen groups were isolated). Gram-negative rods were isolated from 57 of the adolescents (isolation frequency 81.4%). *F. varium, P. melaninogenica*, *P. intermedia* and *P. assacharolyticus* were detected respectively in 12 (16%), 9 (12%), 8 (11%) and 7 (10%) samples. The less frequently isolated anaerobes were Gram-positive cocci, Gram-negative cocci, *Bacteroides uniformis* and *Bifidobacterium spp*. together.

Conclusion: The most frequently isolated microbiota in our study is part of the normal oral bacterial flora. The presence of anaerobes such as *Prevotella, Fusobacterium, Bacteroides* and *Porphyromonas* reflects the gradual change of the flora to more complex one. The results of quantitative and qualitative evaluation of the plaque of adolescents with moderate plaque-induced gingivitis may contribute to the selection of the prevention and treatment of this disease.

Keywords

adolescents, dental plaque, microbiology, plaque-induced gingivitis

INTRODUCTION

Microbial biofilms are communities of bacteria and are common in the human body and in the environment. The dental plaque has been identified as a biofilm. Plaque microflora produces extracellular matrix and the bacterial communication is presented by a complex interaction network.[1] These microorganisms also produce toxins and pro-

teolytic enzymes which evoke local immune reaction and symptoms of gingival inflammation (redness, edema, swelling and bleeding on provocation), as reviewed by Cobb.[2]

The microbial biofilm is complex and the prevalent bacterial populations vary according to its maturation. Gram-positive aerobic *Streptococcus spp*. are most common in the initial stage of plaque formation. The number of Gram-negative anaerobes is increasing in a matured plaque biofilm.[1-3] The presence of Gram-negative microbiota could be related to gingivitis.[4]

Gingivitis is a reversible inflammatory disease, which occurs as a response of the human organism to plaque bacteria and their products. Gingivitis has a high rate of occurrence (50-90% of adults are affected). The inflammation is limited to gingival soft tissues and does not result in clinical attachment loss.[2,5,6]

There is a substantial body of research focused on the prevalent microbiota in healthy adults[5], adults with periodontitis[7], adults with gingivitis[3], and to our knowledge only one study[8] focuses on adolescents. However, the study was performed on subjects with periodontitis, analyzing samples of subgingival plaque.

In children and adolescents, plaque-induced gingivitis is the most common form of periodontal disease. The age range gingivitis occurs most often is 11-13 years (80%).[9] The composition of the bacterial flora in adolescents with plaque-induced gingivitis is not studied enough.

AIM

The aim of this study was to reveal the bacterial species associated with supragingival plaque of Bulgarian adolescents diagnosed with moderate plaque-induced gingivitis.

MATERIALS AND METHODS

Ethical approval

All procedures in the study involving human participants were performed in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study design was approved by the Ethics Committee of the Medical University - Plovdiv, Bulgaria (No 3/12.10.2010). The protocol was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice guidelines, and national laws.

All procedures were conducted after a written informed consent was signed by the parents and verbal consent was obtained from the subjects.

Preliminary screening for plaque-induced gingivitis was conducted in 1391 students at the Sts Cyril and Methodius Humanitarian High School in Plovdiv, Bulgaria. During the initial examination, the oral health was evaluated in accordance with WHO instructions as described by Petersen et al.[10] Gingival index (GI) and plaque index (PlI) were evaluated as described by Löe.[11] The score criteria are summarized in **Table 1**.

Subjects

Criteria for inclusion in the study: physically and mentally healthy children aged between 12 and 18 years of both genders, with moderate plaque-induced gingivitis (GI=2). Adolescents, meeting the following criteria, were excluded: 1) treatment with an orthodontic appliance; 2) severe deformities of jaws and teeth; 3) severe plaque-induced gingivitis; 4) smokers. Carious lesions were treated, poor dental restorations were corrected and calculus was removed, if present.

Sample preparation

Dental plaque samples were obtained by scraping the dried tooth surface near the gingival margin of mandibular central incisors using sterile curettes. They were allocated in transport medium (tryptic soy broth, Lioflichem - Italy) and delivered immediately to the Department of Microbiology and Immunology, Faculty of Pharmacy at the Medical University in Plovdiv for microbiological examination.

Methods

Bacterial isolation was performed by specimen inoculation in 5% sheep blood agar and chromogenic Candida agar (Lioflichem) in aerobic conditions. Simultaneous anaerobic cultures in Schaedler Agar with 5% sheep were inoculated in special anaerobic pouches (bioMerieux-France). Isolated anaerobic bacteria were identified on the basis of API 20A identification system (bioMerieux-France).

Statistical analysis

Data were analyzed using GraphPad InStat software 3.10 version. Descriptive statistics were used, and the proportions were compared using the chi-square test. The level of significance was set at $p<0.05$.

RESULTS

Seventy healthy subjects (37 females and 33 males), aged 12-18 years, were randomly selected after being diagnosed clinically with moderate plaque-induced gingivitis (GI=2). The results of preliminary oral health examination (GI and PLI) of all adolescents are shown in **Table 2**.

The quantitative microbiologic analysis resulted in the isolation of 224 microorganisms from all groups. Aerobic microorganisms were detected more often than anaerobes

(151 total isolates vs. 73 isolates, $p<0.001$). No significant difference was found between the two genders regarding the distribution of aerobic and anaerobic microorganisms ($p>0.05$). However, all isolated aerobes belonged to 3 genus groups. *S. mutans group* and *Neisseria spp*. were isolated from all samples (isolation frequency 100%). As shown in **Table 3**, the frequency of isolation of *C. albicans* was relatively lower – 11 (15.7%).

The anaerobes showed much greater microbial diversity (**Fig. 1**). A total of 12 pathogen groups were isolated. *Fusobacterium varium, P. melaninogenica* and *P. intermedia* were the most frequently isolated species, present respectively in 12 (16%), 9 (12%) and 8 (11%) samples. *P. bivia, B. uniformis, Bifidobacterium spp*. and other anaerobic Gram-positive rods were with same relatively low isolation frequency 3 (4%). *P. assacharoliticus* and other anaerobic Gram-negative rods were present in 7 (10%) of the samples. *B. fragilis* and *S. intermedius* were detected in 5 (7%) of the adolescents. Anaerobic Gram-negative cocci showed the same isolation frequency.

Table 1. Score criteria for gingival index (GI) and plaque index (PLI), as described by Löe (10)

Score criteria for gingival index (GI)			
Appearance	**Bleeding**	**Inflammation**	**Points**
Normal	No bleeding	None	0
Slight change in color and mild edema with slight change in texture	No bleeding	Mild	1
Redness, hypertrophy, edema and glazing	Bleeding on probing/ pressure	Moderate	2
Marked redness, hypertrophy, edema, ulceration	Spontaneous bleeding	Severe	3
Score Criteria for PLI			
Appearance			**Points**
No plaque			0
A film of plaque adhering to the gingival margin and adjacent area of the tooth, which cannot be seen with the naked eye, but only by using disclosing solution or by using probe.			1
Moderate accumulation of deposits within the gingival pocket on the gingival margin and/or adjacent tooth surface, which can be seen with the naked eye.			2
Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.			3

Table 2. Gingival index (GI) and plaque index (PLI) in Bulgarian adolescents aged 12-18 years

GI	Number of adolescents	Percentage of all examined students
0.0-1.0	1190	85.5
1.1-2.0*	140	10.1
2.1-3.0	61	4.4
Total	1391	
PLI	**Number of adolescents**	**Percentage of all examined students**
0.0-1.0	456	32.8
1.1-2.0	681	48.9
2.1-3.0	254	18.3
Total	1391	

*The table presents the results from the initial examination of 1391 students. Some of them were excluded and only 70 students met the criteria for inclusion in the study. See section Material and methods for more details.

Table 3. Aerobic microorganisms isolated from 70 adolescents with plaque-induced gingivitis (GI=2). All samples were positive for more than one bacterial species

Gram	Microorganisms	Number of positive isolates	Percentage of total positive samples (%±SE)	Isolation frequency of all adolescents (%±SE)
Gram/+/	*Streptococcus mutans group*	70	31.3±3.09	100
	Candida albicans	11	4.9±1.44	15.7±4.35
Gram/-/	*Neisseria spp*	70	31.3±3.09	100

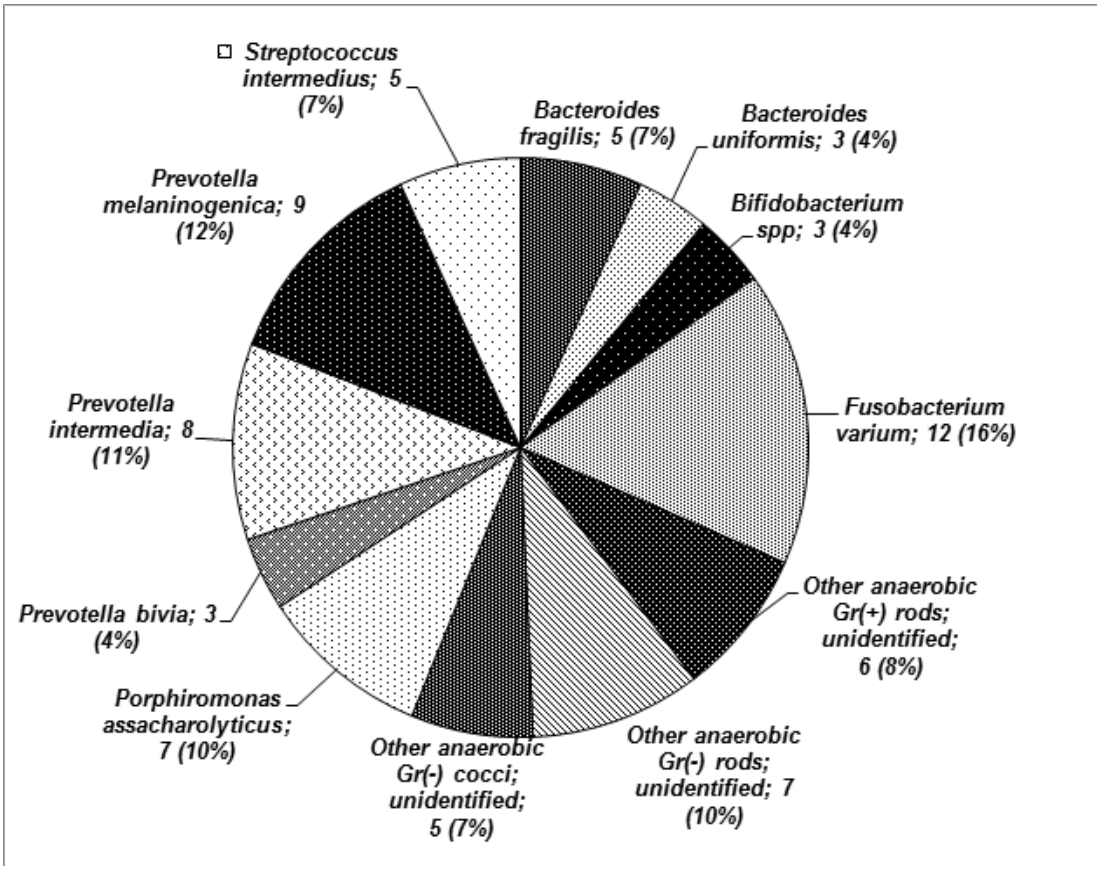

Figure 1. Isolation frequency of 73 anaerobic isolates (number; %) from 70 adolescents with plaque-induced gingivitis. 70 samples were analyzed, all of them positive for more than one bacterial species.

Analyzing the prevalence of the pathogens by genera, we can clearly indicate *Prevotella* as the most often isolated anaerobic bacteria (23, 27%). *Fusobacterium* and *Bacteroides* were also detected relatively often. *Bifidobacterium* (3, 4%) and other anaerobic Gram-positive rods (6, 8%) were rarely detected (**Fig. 1**).

Comparing the distribution of different gram-stain categories microorganisms, we found that Gram-negative bacteria were predominant compared to Gram-positive of the total isolates (132, 59% vs. 92, 41%; $p< 0.001$). No significant difference was found between the two groups regarding the distribution of Gram-positive and Gram-negative microorganisms ($p>0.05$).

DISCUSSION

The hormonal changes during puberty influence the host response to dental plaque microorganisms and the oral microbial flora, resulting in increased rate of periodontal diseases in adolescents and adults.[12] A possible explanation of the higher gingival diseases incidence in adolescents is the altered response of the individual to the oral microbial flora. In this study, a preliminary dental examination of 1391 high school students was performed and 531 of them were diagnosed with plaque-induced gingivitis (frequency of 38.17%).

It should also be taken into consideration that plaque-induced diseases occur at sites already colonized by microorganisms (normal bacterial flora). The bacterial homeostasis could be influenced by changes in diet, dentition or saliva production.[13] *Streptococcus spp., Neisseria spp*., anaerobic Gram-positive and anaerobic Gram-negative rods are often recovered from approximal surfaces and gingival crevices of teeth, as reviewed by Marsh.[13] *S. mutans* and other acidogenic bacteria are capable of producing acids using dietary sugars and play a major role in dental caries development.[14-17]

The high isolation frequency of *Streptococcus mutans* group bacteria, and *Neisseria spp*. in our study is not surprising. *Streptococcus spp*. were reported as one of the first microbiota isolated in the first stage of development of bacterial biofilm.[2]

S. viridans group consisted of *S. mutans* group, *S. salivarius* group, *S. anginosus* group, *S. mitis* group, *S. sanguinis* group, and *S. bovis*.[18] In our study these microorganisms were combined as *S. mutans* group. Aas et al.[5] reported *S. sanguinis* and *S. gordonii* (part of *S. sanguinis* group) as often detected in tooth surface samples of healthy subjects

and *S. mitis* as a predominant species in buccal epithelium samples.

Oxygen-consuming microbiota (e.g. *Neisseria subflava*) provide a suitable environment for the growth of anaerobic microorganisms[13] and explains the high isolation frequency of *Neisseria spp*. in our study.

Moore et al. reported *Actinomyces, Streptococcus, Fusobacterium, Veillonella*, and *Treponema* genera as main bacteria isolated from adults with early gingivitis.[3] Later trial of the authors compared the isolates in children and adults.[19] They found a significant difference in the bacterial isolates from children and adults. However, these authors also reported that regardless of the participants' age, the members of *Fusobacterium, Actynomyces*, and *Bacteroides* genera increased with increasing GI score.

According to Socransky and Haffajee, in subjects with gingivitis, *Prevotella intermedia, Fusobacterium nucleatum, Porphyromonas gingivalis,* and *Tannerella forsythia* were detected more often and in higher numbers.[4] Some of those bacteria (*Fusobacterium nucleatum, Porphyromonas gingivalis, and Prevotella intermedia*) are present also in healthy subjects.[1]

In our study, the most prevalent anaerobic genera isolated in supragingival samples were *Prevotella* (27%), followed by *Fusobacterium* (16%). Our findings are in accordance with those of Salako et al.[20] These authors analyzed supragingival plaque samples from healthy children, aged 3-12 years. Fifty percent of the anaerobic isolates were species of *Prevotella* genus and 18% were *Fusobacterium spp*. The lower isolation rate of *Prevotella spp*. in our study may be related to the increased variety of other anaerobes.

Porphyromonas gingivalis, a frequently isolated pathogen in subjects with severe and advanced periodontitis, is rarely detected in healthy subjects or individuals with gingivitis. Accordingly, we failed to recover *P. gingivalis* from adolescent subjects.

However, the progression of gingivitis to periodontitis depends not only on the presence of specific bacteria but also on the individual susceptibility of the host.[21]

Moore and Moore reported that the variety of oral flora could be influenced by environmental and genetic factors.[22]

Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, Fusobacterium nucleatum, *Prevotella intermedia/nigrescens, Porphyromonas gingivalis, Peptostreptococcus micros,* and *Streptococcus intermedius* are often isolated when the periodontal disease is accompanied by loss of connective tissue attachment and alveolar bone, as reviewed by Zambon.[23] Out of these species, only *Prevotella intermedia* and *Streptococcus intermedius* were recovered from the samples in our study.

CONCLUSION

The most frequently isolated microbiota in adolescents with moderate plaque-induced gingivitis, are *Streptococcus mutans group, Neisseria spp., Prevotella spp*., and *Fusobacterium varium*. All these are part of the normal bacterial flora. The presence of anaerobes such as *Prevotella, Fusobacterium, Porphyromonas* and *Bacteroides* outlines the gradual change of the flora to a more complex one. Nevertheless, the presence of these anaerobes does not necessarily mean progress of gingivitis to periodontitis. Additional factors – oral hygiene status, diet, hormonal changes and genetic endowment, could influence the host response to dental plaque.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Микробная флора полости рта у подростков из Болгарии с бляшковым гингивитом средней степени тяжести

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Абстракт

Введение: Наиболее частым заболеванием пародонта у детей и подростков является бляшковый гингивит.

Цель: Цель этого исследования состояла в том, чтобы идентифицировать виды бактерий, связанных с наддесневой бляшкой, у несовершеннолетних лиц из Болгарии, у которых диагностирован бляшковый гингивит.

Материалы и методы: Была взята и исследована микроскопическая наддесневая бляшка у 70 здоровых лиц с бляшковым гингивитом средней степени тяжести (37 женского пола и 33 мужского пола) в возрасте от 12 до 18 лет.

Результаты: Всего было изолировано 224 микроорганизма. Грамотрицательные бактерии были более распространены по сравнению с грамположительными [132 (59%) против 92 (41%), p <0,001]. Аэробные микроорганизмы присутствовали чаще, чем анаэробные (151; 67,5% против 73; 32,5%, p <0,001). Группы Streptococcus mutans и Neisseria sp. были изолированы у всех подростков. Частота изолирования C. albicans была относительно низкой - у 11 лиц (15,7%). Анаэробы показали гораздо большее микробное разнообразие (было изолировано 12 патогенных групп). Грамотрицательные палочки были изолированы

у 57 подростков (частота изоляции 81,4%). F. varium, P. melaninogenica, P. intermedia и P. Assacharolyticus были обнаружены в 12 (16%), 9 (12%), 8 (11%) и 7 (10%) образцах соответственно. Реже изолированными анаэробами были грамположительные кокки, грамотрицательные кокки Bacteroidesiformis и Bifidobacterium spp. вместе взятые.

Выводы: Наиболее часто изолированная микробиота в нашем исследовании является частью нормальной бактериальной флоры полости рта. Присутствие анаэробов, таких как Prevotella, Fusobacterium, Bacteroides и Porphyromonas, указывает на постепенное изменение флоры на более сложную. Результаты количественной и качественной оценки характеристик бляшек у подростков с бляшковым гингивитом средней степени тяжести могут способствовать выбору профилактики и лечения этого заболевания.

Ключевые слова

бляшковый гингивит, подростки, микробиология, зубной налёт