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Microbiological Analysis of Primary Infected Root Canals with Symptomatic and Asymptomatic Apical Periodontitis of Young Permanent Teeth

SUMMARY

Background/Aim*: Understanding the composition of bacteria in infected root canals is important for ameliorating the treatment strategies that lead to the elimination of pathogens and infection control, but also prevent reinfection. Aim of this study was to investigate microbial composition of primary infected root canals with apical periodontitis of young permanent teeth, originating form school children in Serbia, and its association with clinical symptoms.* ***Material and Methods****: To determine the bacterial composition of infected root canals in children, 35 endodontic samples were obtained. The identification of cultured bacteria was performed by MALDI-TOF MS analysis. The presence or absence of clinical symtoms were recorded.* ***Results****: Facultative anaerobes were 2,2 times more frequent than obligate anaerobes. The most common facultative anaerobes belonged to following genera, Streptococcus (58 isolates), Actinomyces (10) and Enterococcus (8), while predominant obligate anaerobes, belonged to genera Veillonella (15), Prevotella (9) and Fusobacterium (8). The most common clinical isolates recovered from infected root canals with symptomatic apical periodontitis were Veillonella parvula (10) and Fusobacterium nucleatum (7), while from the asymptomatic ones, they were Streptococcus mitis/Streptococcus oralis (5). Prevalence of Parvimonas micra, Prevotella buccae and Streptococcus constellatus within the root canals might be associated to clinical symptoms.* ***Conclusions:*** *Species of genera Streptococcus and Veillonella were the most common isolates from primary infected root canals with apical periodontitis in Serbian school children. Facultative anaerobes were predominant over obligate anaerobes. The prevalence of obligate anaerobes was much higher in symptomatic compared to asymptomatic root canal infections. No specific bacterial strain might be associated to a single examined clinical symptom (pain, tenderness to percussion or swelling), but majority of the strains are associated to all of the examined three symptoms.*

Key words: Oral, Microbiome, Infected Root Canal, Primary Infection, Pain, Symptom

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Introduction

Primary infected root canals with apical periodontitis are untreated root canals, colonized for the first time by microorganisms[1], which exacerbate the functions of the root canal system and surrounding tissues. Microbiota within such canals was investigated in many studies and diversity in its constituents was confirmed[1-4]. In recent years, such investigations were in focus[5-8], probably as they represent a good starting point in development of novel material or approach in infected root canal treatment[9-11]. Knowing microbiota constituents provides an opportunity to distinguish sensitivities among the pathogens, which is crucial for development of potent antibacterial agents, efficient towards all pathogens, and therefore capable to prevent reinfection.

Some studies examining microbiota within root canals suggested different factors influencing its composition, including eating and oral hygiene habits but also geographic location and socioeconomic status of the patients[12-14]. So far, microbial composition of the infected root canals was reported for adult patients from China, Brazil, Netherlands, Sweden, Italy, UK, USA and Japan[12-15]. There was only one study on the microbial composition of permanent teeth in children in Turkey[16]. Up to our knowledge, there is no information in literature about microbiota in young permanent teeth. Based on difference in bacteria recovered from asymptomatic and symptomatic cases, some of the mentioned studies claimed composition of microbial community responsible for clinical symptomatology[9,16]. Analyzing correlation between clinical symptoms such as pain, swelling and tenderness and microbiota, Cogulu et al. (2008)[16] revealed that the symptoms could be associated with the incidence of following bacteria within the infected root canals: *Enterococcus faecalis, Fusobacterium nucleatum, Streptococcus* spp., *Prevotella intermedia* and *Parvimonas micra*.

Apart to suggestion that geographic location may influence microbiota composition, it could be also suggested that geographic location influences the therapeutic approach. For example, some cultural or historical patterns, in particular the society, can contribute to the acceptance of antimicrobial agents of natural origin similarly as they do to the use of antibiotics [17-19]. Consequently, in discovery of any new antibacterial, detailed investigation on microbiota and its relationship to clinical symptoms has to proceed, and the society members must be opened to accept and support a novel therapeutic approach.

For the onset of such a complex investigation, the present study focused on the biodiversity of the microbiota in the infected root canal of pediatric patients. Therefore, the aim of this study was to investigate the microbial composition of infected root canals with apical periodontitis of young permanent teeth in Serbian school children and to associate clinical symptoms with particular bacterial species.

Material and Methods

Patient selection and clinical features

A total of 35 children, with an indication for treatment of infected single root canal of the young permanent teeth with apical periodontitis, were included in this study.

The excluding criteria were:

- Patients who had deciduous teeth root canal infection.
- Patients who received antibiotic therapy in the preceding 3 months.
- Patients who had a systemic disease.
- Patients with a tooth that could not be fully isolated with a rubber dam.

Anamnestic information and medical history not related to endodontic treatment were not included in this study. Only the presence of clinical symptoms (pain, tenderness to percussion and swelling), or lack of the symptoms were monitored.

For all patients participating in this study, informed consent was obtained, which allowed microbial sampling. The study was approved by the Ethical Committee No 36/7.

Specimen sampling

A total of 35 endodontic samples were obtained from the young permanent teeth with apical periodontitis during the first root canal therapy visit. The samples were collected from clinically and radiographically proven infected root canals. They were obtained from single root canals only. Prior sampling, tooth were disinfected first with 3% hydrogen peroxide, then 2.5% sodium hypochlorite solution, and finally 2% chlorhexidine, each of them being used for 30 seconds. After disinfection, the tooth was isolated with a rubber dam. Coronal restorations and the carious lesions were completely removed. The access cavity was formed and root canal accessibility and diameters were checked with a pathfinder (Pathfinder stainless steel File, Kerr Endodontics, Kerr Dental, Orange, California, USA) and 20 K-file (Kerr Dental, Orange, California, USA), respectively, without using any irrigant. Samples were collected by placing sterile paper points in the full length of the canal cavity for 60s after wise paper point was placed in a tube containing 1 ml of thioglycollate broth (Sigma-Aldrich, USA) transport medium[16,20]. The samples were immediately sent to a microbiology laboratory where they were seeded on Brucella Agar (bioMerieux, France) supplemented with 5% defibrinated sheep blood, 5 mg/L hemin, and 1 mg/L menadione (both purchased from Sigma-Aldrich, USA), and cultivated in anaerobic conditions at 37ºC for up to 7 days. After incubation all morphologically different bacterial colonies were subcultured to obtain pure culture for Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS, VITEK® MS bioMerieux, France).

MALDI-TOF mass spectrometry analysis

Pure colony of fresh bacterial isolate (after 18h to 72h incubation) was smeared on VITEK® MS-DS target slide spot. 1µl of matrix solution (CHCA matrix, bioMerieux, France) was added on dried smear. Prepared samples were exposed to laser shots, evaporated and ionized, and travel through electric field in vacuum tube to linear detector which generates mass spectrum. This spectrum was compared with spectrum in knowledge base (VITEK® MS V3.0 knowledge base) and bacterial species of analyzed sample was identified if good confidence value was obtained (60 to 99,9% probability). For isolates with low discrimination or no identification

results, the procedure was repeated, but with an additional step: 70% formic acid was added before matrix solution, for more successful protein extraction. For isolates with second unsuccessful identification result no further testing was performed.

Results

Out of all 35 examined infected root canals with apical periodontitis, 21 was with symptomatic and 14 with asymptomatic apical periodontitis. Number of recovered isolates from symptomatic infected root canals was 86, and from asymptomatic 62, together counting 148 isolates. Among them, 45 bacterial species belonging to 22 genera were identified (Table 1). Out of presented bacteria, 102 were facultative and 46 obligate anaerobes. Within the facultative anaerobes, the most commonly isolated belonged to genera *Streptococcus* (58 isolates), *Actinomyces* (10) and *Enterococcus* (8), while within the obligate anaerobes, the most common isolates belonged to genera *Veillonella* (15), *Prevotella* (9) and *Fusobacterium* (8).

Table 1. Isolates from infected root canals

Facultative anaerobes			***Obligate anerobes***		
Genus	***Species***	**No**	***Genus***	***Species***	**No**
	*S. mitis/ S. oralis**	12	***Veillonella***	*V. parvula*	13
	S. salivarius	9		*V. dispar*	2
	S. sanguinis	8		*P. buccae*	3
	S. gordonii	7	***Prevotella***	*P. denticola*	4
	S. parasanguinis	6		*P. oralis*	2
Streptococcus	*S. anginosus*	4	***Fusobacterium***	*F. nucleatum*	8
	S. intermedius	3	***Parvomonas***	*P. micra*	4
	S. constellatus	3	***Bifidobacterium***	**	5
	S. cristatus	2	***Propionibacterium***	*P. avidum*	1
	S. mutans	2	***Cutibacterium***	*C. acnes*	1
	S. pneumonia	2	***Atopobium***	*A. parvulum*	1
Actinomyces	*A. odontolyticus*	7	***Leptotrihia***	*L. buccalis*	1
	*A. oris/ A. viscosus**	3	***Porphyromonas***	*P. gingivalis*	1
	E. faecalis	5			
Enterococcus	*E. hirae*	1			
	E. faecium	1			
	E. durans	1			
	G. haemolysans	2			
Gemella	*G. sanguinis*	2			
	G. morbillorum	1			
Lactobacillus	*L. rhamnosus/ L. casei / L. acidophilus**	4			
	L. delbruecki	1			
Eikenella	*E. corrodens*	3			
Capnocytophaga	*C. ochracea*	1			
	C. gingivalis	2			
Staphylococcus	*S. hominis*	1			
	S. epidermidis	2			
Lactococcus	*L. garvieae*	1			
	L. lactis	1			
Rothia	*R. mucilaginosa*	1			
	R. dentocariosa	1			
Klebsiella	*K. oxytoca*	2			
Escherichia	*E. coli*	1			

* these species colud not be distinguish with MALDI-TOF MS method.
** *Bifidobacterium* spp could not be identified to the species level

Bacterial composition within infected root canals with symptomatic and asymptomatic apical periodontitis

Out of 148 strains in this study, 32 (71%) different bacterial species and 86 (58%) clinical isolates were originally obtained from the infected root canals with symptomatic and 35 (77%) species and 62 (42%) clinical isolates from the infected root canals with the asymptomatic apical periodontitis. Number of isolated obligate anaerobes in the symptomatic canal infections was 30, while in the asymptomatic ones it was 16. Prevalence of facultative anaerobes in the infected root canals with symptomatic apical periodontitis was 56 and in the root canals with asymptomatic apical periodontitis it was 46 (Figure 1).

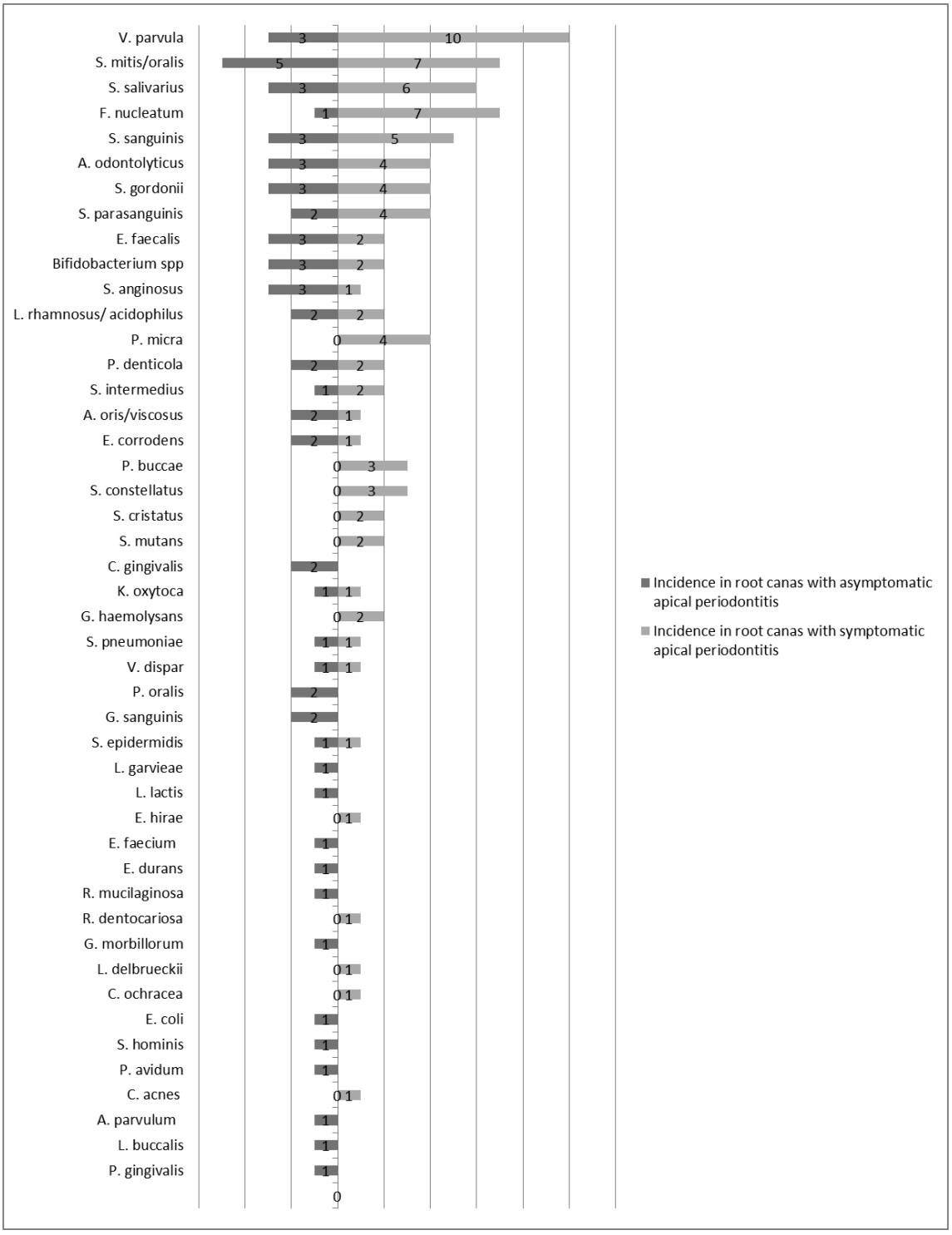

Figure 1: Incidence of recovered species in symptomatic and asymptomatic infected root canals

The most commonly isolated bacteria in the root canals with symptomatic apical periodontitis were *V. parvula* (10), *F. nucleatum* (7), *S. mitis/S. oralis* (7), *S. salivarius* (6) and *S. sanguinis* (5), while in the canals with asymptomatic apical periodontitis it was *S. mitis/ S. oralis* (5).

Among the twelve different species recovered only from the symptomatic root canal infections, the highest incidence showed *P. micra* (4), *P. buccae* (3) and *S. constellatus* (3). On the other hand, 15 bacterial strains were recovered only from the canals with symptomatic apical periodontitis but all of them were in low prevalence (Figure 1).

Clinical findings and bacterial distribution within the infected root canals

Out of 30 different bacterial species isolated from the infected root canals with symptomatic apical periodontitis 19 species were associated with all clinical symptoms monitored in this study (Figure 2). Isolates recognized in infected root canals in early stage of the development of symptomatic apical periodontitis, which characterizes pain and tenderness to percussion, were *S. cristatus, C. acnes, L. delbrueckii* and *S. epidermidis*. Isolates recognized in root canals with progression of infection (swelling) were *S. pneumonia, E. hirae, A. oris/A. visocosus, C. ochracea, R. dentocariosa, E. corrodens* and *V. dispar.*

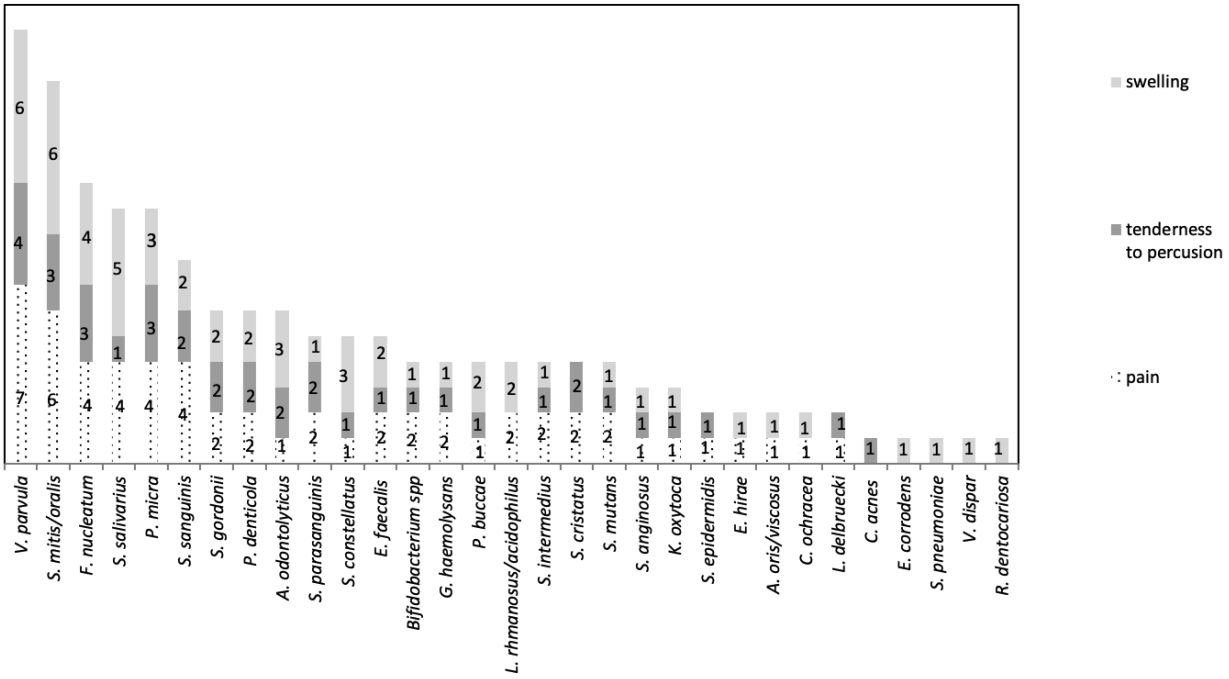

Figure 2: Incidence of isolated species per examined clinical symptom

Discussion

The biodiversity of the bacterial composition in primary infected root canals of young permanent teeth with apical periodontitis and its association to clinical symptoms of pediatric patients from Serbia was investigated and for the first time presented in this study. Biodiversity in infected root canals was presented in several studies[15-16,20-23]. For determining the root canal biofilm constituents, some studies used cultivable[15,20-21], while others used molecular methods[16,22-23]. The cultivable methods detect live bacterial cells within the biofilm, while the molecular determine total biofilm composition, whether the cells are dead or live. In this study, for identification of live constituents of biofilm formed in the infected root canals a MALDI-TOF analysis (cultivable method) was applied, as the identified isolates could be preserved and their sensitivity towards antibacterial agents could undergo profound examinations.

In general, the bacterial community within infected root canals is represented by facultative and obligate anaerobic species [8, 16, 25]. In our study, the facultative anaerobes were 2,2 times more frequently isolated than the obligate anaerobes, which was in line with the dominance of facultative anaerobes observed by Ercan et al. (2006)[1]. The most commonly isolated facultative anaerobic species in our study belonged to genera *Streptococcus* and was namely *F. nucleatum* which is in accordance with the results presented by Siqueira et al (2002)[22] and Guimarães et al (2012)[23]. Certain isolates from the primary infected root canals of Serbian children (Table 1), including *A. viscosus, Bifidobacterium* spp., *E. faecalis, F. nucleatum, Lactobacillus* spp., *P. micra,*

P. gingivalis, S. mutans and *S. mitis*, were identical to isolates recovered from the canals of adults originating from China, USA, Brazil, Netherlands, Sweden, Italy, UK, and Japan[12]. However, several other species that have never been reported before were recovered in our study, and they belonged to following genera: *Gemella, Lactococcus, Rothia, Leptotrichia, Cutibacterium, Capnocytophaga, Klebsiella* and *Escherichia* (Table 1). Observed differences in microbial community within the infected root canals between the patients originating from different countries contribute to previous statements [13-14] that geographic location plays a significant role in it, in addition to the socio-economic status. It should be noticed that literature suggests information about root canal microbiota in adult patients. Little is known about the microbial composition of the infected root canals in children. So far, only one study reported bacterial community composition in the infected root canals of primary school children[16], but not stating permanent teeth from which isolates were recovered as young permanent teeth. Our results were in line with their, regarding the common isolate *E. faecalis* but not regarding *P. gingivalis*, which we isolated only once.

Overall, in this study, more isolates were identified from the root canas with symptomatic in comparison to the root canals with asymptomatic apical periodontitis. Opposite to findings of Jacinto et al. (2003)[15] that symptomatic infected canals correlate with bigger species diversity, the species isolated from symptomatic patients in our study were less variable (Figure 1). The prevalence of the obligate anaerobes in this study was almost two times higher in symptomatic in comparison to asymptomatic infected canal (Figure 2), which is in line with finding of Jacinto et al. (2003)[15] that higher portion of the obligate anaerobes correlate with intensive clinical symptoms.

In this study, the most commonly isolated species in the symptomatic root canal infections were following obligate anaerobes: *V. parvula* and *F. nucleatum*, followed by three *Streptococcus* spp., *S. mitis/S. oralis*, *S. salivarius* and *S. sanguinis* (Figure 1). Our results are in line with those of Jacinito et al. (2003)[15] and Sassone et al. (2008)[26], who also isolated the obligate anaerobes *F. nucleatum* and *Veillonella* spp., the most frequently from the symptomatic root canal infections. Further, in our study *S. mitis/ S. oralis* had the highest prevalence in the asymptomatic infected canal infections while in the study of Jacinito et al. (2003)[15] it was *S. sanguinis*. Such findings implicate once again that the anaerobes are associated to symptomatic infection.

Comparison of bacteria recovered from the infected root canals with symptomatic and asymptomatic apical periodontits was conducted also in an attempt to reveal if any bacterial species could serve as a "marker" for either of the two kinds of infections. The obligate anaerobes *P. micra*, *P. buccae*, and the facultative *S. constellatus*, were all absent in the asymptomatic and frequent in symptomatic root canal infections (Figure 1). However, the mentioned bacteria might not be sufficient to cause symptomatic infection alone, but their presence in specific consortiums might be a crucial in the development of symptoms; similar was suggested by Skučaitė et al (2009)[27].

Pain, swelling and tenderness to percussion are the basic three clinical symptoms of an apical periodontitis. Although severity of the symptoms also depends on general patient's health as well as on its tooth intracanal microbiota, several studies tried to attribute them to specific bacteria, recovered from the canals[9, 14-16, 28]. Similar to other findings [9, 15-16, 29], the majority of our isolates that were attributed to all symptoms, included bacteria from *Prevotella* spp. and *Actinomyces* spp. On the other hand, although *E. corrodens, V. dispar, S. pneumonae, R. dentocariosa* and *C. acnes* were recovered from the infected canals with apical periodontitis followed by a single symptom, this specific symptom could not be attributed to either of them as their incidence was low (Figure 2).

Conclusions

To the best of our knowledge, this study is a first one in which the effort was made to identify microbiota constituents within the primary infected root canals with apical periodontitis of pediatric patients in Serbia, with a particular focus to associate them with basic clinical symptoms (pain, swelling and tenderness to percussion). The most commonly isolated bacterial species from the canals belonged to genera *Streptococcus* and *Veillonella.* The facultative anaerobes predominated over the obligate anaerobes in both, the symptomatic and asymptomatic root canal infections. The obligate anaerobes prevailed in the symptomatic infections. Prevalence of *P. micra, P. buccae* and *S. constellatus* within the canal was attributed to clinical symptoms. On the other hand, no specific bacterial isolates could be attributed to particular clinical symptom, although the majority of them were attributed to all three symptoms.

In a further study of the infected root canal microbiota, a larger number of young patients should be engaged, including children from various geographic localities, as it would provide a more profound understanding of the possible relationship between the microbiota constituents and clinical symptoms they cause, and enable prompter application of the appropriate therapeutic approach.

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