Types of microorganisms in proximal caries lesion and ozone treatment

Janet N. Kirilova, Snezhanka Z. Topalova-Pirinska, Dimitar N. Kirov, Elitsa G. Delivereska and Lilia B. Doichinova

Department of Conservative Dentistry Faculty of Dental Medicine, Medical University, Sofia, Bulgaria; Department of Prosthetic Dentistry Faculty of Dental Medicine, Medical University, Sofia, Bulgaria; Department of Oral and Maxillofacial Surgery Faculty of Dental Medicine, Medical University, Sofia, Bulgaria; Department of Pediatric Dentistry Faculty of Dental Medicine, Medical University, Sofia, Bulgaria

ABSTRACT
The purpose of this clinical study was to investigate the types of microorganisms in deep proximal caries lesions and the efficacy of elimination of microorganisms after a 24 s ozone application to proximal cavity lesions prepared for restoration. Sixteen caries-active volunteers (female: 8, male: 8; age range: 35–55), with proximally situated deep caries lesions on premolars or molars, were included in the study. Each patient underwent two microbiological investigations. First, the caries decay before treatment was assessed. Second, the dentine tissue was assessed after the removal of necrotic tooth decay and 24 s ozone application. The prevalence of oral streptococci was determined. In addition, we isolated Candida albicans, Enterococcus faecalis and Peptostreptococcus spp. from deep proximal caries lesion in caries-active patients. All microorganisms found in the deep proximal caries lesions were destroyed following 24 s ozone application. Our findings suggest that S. anginosus group, C. albicans and E. faecalis should be considered as an index for caries activity in caries-active patients, but additional studies are necessary to confirm this suggestion. This study demonstrated that 24 s of gaseous ozone application to the deep proximal caries lesions effectively eliminated microbial species.

INTRODUCTION
Caries management is a complex multifactorial process. Caries-active patients require a wide range of prophylactic treatment because they develop new initial and secondary caries lesions despite regular visits to dental offices. Maintaining strict oral hygiene and restoration with fluoride-containing products can elicit improvements; however, this does not prevent the development of new caries lesions. In the search for alternative antibacterial mechanisms, researchers have embraced ozone therapy as a hopeful prospect for the future [1]. Ozone therapy is an alternative method for caries treatment that has provided reliable results [2–4].

Ozone exhibits antibacterial properties and has been studied and applied in dental practice for treatment of dental caries, chronic apical periodontitis and chronic generalized periodontal disease [5, 6]. Ozone destroys microorganisms by rupturing their cell membrane within two seconds of contact. Lynch [3] reported that ozone application for 10 s can treat early carious lesions because it would potentially destroy up to 99% of microorganisms, such as bacteria, fungi and viruses, in the caries lesions and over the tooth surface. To date, no serious side effects have been reported during the use of ozone. Martinelli et al. [7] and Megahed et al. [8] report that gaseous and aqueous ozone significantly reduce the cultivated microbial flora, in particular Streptococcus mutans. Ozone destroys the integrity of bacterial cells via oxidation of phospholipids and lipoproteins and the inactivation of bacteria, viruses, fungi, yeast and protozoa [9].

The data regarding the duration of ozone administration on the dentine surface are inconsistent. Baysan and Lynch [2] report that ozone application for 10–20 s destroys many microorganisms that are found in the primary lesions of tooth roots. A further study argues that 60 s ozone application results in the removal of most cariogenic microorganisms [10]. While the antibacterial properties of ozone and its role in creating suitable conditions for restoration of dental
tissue mineral structures provide a strong argument for its use in the management of dental caries, some studies contradict these favourable results [11, 12].

The purpose of this clinical study was to investigate the types of microorganisms in deep proximal caries lesions and the efficacy of their elimination from cavities prepared for restoration after a 24-s ozone application.

**Subjects and methods**

**Study group**

This study included 16 patients and examined 432 teeth (female: 8, male: 8; age range: 35–55), 151 of which (34.95%) were intact, 111 (25.69%) were affected by deep proximal caries lesions, 134 (31.02%) with restorations and 36 (8.33%) with dental crowns. All the assessed deep proximal caries were acute. The mean number of teeth with primary or secondary caries was 6.9.

**Ethics statement**

The study protocol was approved by the Scientific Ethical Committee at the Medical University of Sofia, Bulgaria, prior to the initiation of the study (KENIMUS – No. BK-297/14.04.2015 and No. 13/23.4.2015). All subjects provided written informed consent for participation in this study, according to the Helsinki Declaration II.

**Study setup**

A routine clinical check-up was performed during the first patient visit. Data from this check-up were entered onto the individual patient card. For precise diagnostics of caries lesions, a fluorescent method was used with a blue-light emitting device FaceLight (W&H, Austria). We selected 22 teeth with deep caries lesions along the proximal surfaces of 7 premolars and 15 molars for microbiological tests.

During the second visit, material was obtained for two microbiological examinations. The chosen tooth (molar or premolar) was cleaned and soft and solid plaques were removed with a silicon brush and paste. The caries lesion was opened from the occlusal side using a sterile turbine bur. The first sample N1 was obtained with a sterile round steel bur #012 (Komet, Germany) to differentiate the microorganisms in the caries mass.

After removal of the damaged tooth structures, the cavity was rubbed three times with sterile cotton swabs soaked in 3% hydrogen peroxide. Gas ozone application was made for 24 s. An ozone generator Prozone (TIP TOP TIPS Sarl, Switzerland) was used with a coro-tip for direct cavity disinfection. The maximum ozone production per patient was 5 × 24 s. During this study, no patient was exposed to an ozone doze per day limit that has been established according to the manufacturer. After ozone application, the second sample N2 was obtained for microbiological testing. The material was obtained with a sterile round steel bur #012 (Komet, Germany) from the clinically healthy dentine and by rubbing with a sterile cotton swab soaked in distilled water. The samples were fixed using a Stuart transportation culture medium (Stuart Transport Medium, Oxoid) and transported to the microbiological laboratory.

**Laboratory procedures**

We cultured the aerobic microorganisms taken from caries samples using Columbia blood agar (Oxoid); MacKonkey agar (Oxoid); CHROM agar Candida (BBL) and Glucose broth (BulBio). The following cultures were used for anaerobic microorganisms: Schaedler Agar with 5% of sheep blood + Vancomycin, Kanamycin (BD); Schaedler Agar with Vitamin κ1 and 5% of blood (BD); fluid thioglycollate medium (BD); Komkova broth (BulBio) and supplemented murellena blood agar (BD). Anaerobic microorganism cultures were grown in JAR GASPAK 100 HOLDING (BD) with a gas mixture provision using the BD GasPak Anaerobe pouch system and subsequent incubation at 37°C for 5–7 days. During the anaerobic identification process, Disc Taxo ANAEROBE SET (BD) and CRYSTAL Anaerobic ID (BD) were applied. Biochemical identification and antimicrobial sensitivity testing of the aerobic isolates was performed using the Vitek 2 Compact (BioMerieux, France) automated system and conventional methods. Sheep blood agar (SBA) was utilized for the quantitative culture. One colony was the SBA equivalent to 1000 CFU’s per millilitre of suspended material.

**Statistical analyses**

Means and standard deviations (SD) were calculated. According to the data, the distribution was not normal so Kruskal–Wallis and Mann–Whitney nonparametric tests were used. Kruskal–Wallis tests were used to compare the means of seven different groups of microorganisms only from proximal carious lesion and Mann–Whitney U test was used to compare means between different groups. Bonferroni correction was employed to correct for multiple comparisons. The
results were analysed using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA).

**Results and discussion**

In deep proximal caries lesions, there were different microorganisms. They were divided as follows: Group 1 – Oral streptococci; Group 2 – Gram-positive cocci; Group 3 – Peptostreptococcus spp.; Group 4 – Candida; Group 5 – Staphylococcus spp.; Group 6 – Gram-negative rods; Group 7 – Leptotrichia. Table 1 summarizes the species differentiation and frequency of isolated microorganisms from 16 individuals and 22 caries lesions prior to caries mass removal and following caries dentine elimination (a FaceLight control) and ozone gas application for 24 s in the cavity. There was no concurrence in the microorganism strains when examining two different proximal caries in a single patient.

The microbiological test analyses, performed using Kruskal–Wallis and Mann–Whitney U tests are presented in Tables 2 and 3.

The Kruskal–Wallis test shows that there was a significant difference in the distribution of the number of isolated microbial species in the samples (p < 0.05). The Mann–Whitney test confirmed that there was a significant difference between the oral streptococci group (S. mutans, Streptococcus mitis, Streptococcus salivarius and Streptococcus anginosus) when compared with other species isolated from caries lesions (p < 0.001) and were the most common microorganism found (90.90%).

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**Table 1.** Frequency and species affiliation of the microorganisms isolated from deep proximal caries lesions.

| Isolated microorganisms | Samples* from teeth |
|-------------------------|---------------------|
|                         | Sample N1 | Sample N2 |
|                         | n = 22    | n = 22    |
| Group 1 - Oral streptococci - 20 (90.91%) |          |           |
| Mutans group - 4 (18.18%) |          |           |
| Streptococcus mutans    | 3 (++++)  | –         |
| Streptococcus cricetus  | 1 (++++)  | –         |
| Mitis group 5 (22.73%) |          |           |
| Streptococcus oralis    | 1 (++++)  | –         |
| Streptococcus parasanguinis | 1 (++++) | –         |
| Streptococcus sanguinis  | 2 (++++)  | –         |
| Streptococcus gordonii  | 1 (++++)  | –         |
| Salivarius group 2 (9.09%) |          |           |
| Streptococcus salivarius| 1 (++++)  | –         |
| Streptococcus vestibularis| 1 (++)   | –         |
| Anginosus group - 9 (40.90%) |          |           |
| Streptococcus constellatus| 3 (++++) | –         |
| Streptococcus intermedius | 4 (++++) | –         |
| Streptococcus anginosus  | 2 (++++)  | –         |
| Group 2 - Gram-positive cocci - 5 (22.72%) |          |           |
| Enterococcus faecalis   | 5 (++++)  | –         |
| Group 3 - Peptostreptococcus spp. - 8 (36.36%) |          |           |
| Peptostreptococcus prevotii | 4 (++)   | –         |
| Peptostreptococcus anaerobius | 2 (++)  | –         |
| Peptostreptococcus magnus | 1 (++)   | –         |
| Peptostreptococcus micros | 1 (++)   | –         |
| Group 4 Candida - 8 (36.36%) |          |           |
| Candida albicans        | 8 (++++)  | –         |
| Group 5 Staphylococcus spp. - 4 (18.18%) |          |           |
| Staphylococcus epidemicus | 3 (++)   | –         |
| Staphylococcusaemolyticus| 1 (++)   | –         |
| Group 6 Gram-negative rods - 3 (13.63%) |          |           |
| Prevotella intermedia   | 1 (++++)  | –         |
| Prevotellaoralis        | 1 (++++)  | –         |
| Prevotellamelaninogena   | 1 (++++)  | –         |
| Group 7 Leptotrichia - 2 (9.09%) |          |           |
| Leptotrichia buccalis   | 2 (++++)  | –         |
| Others                  |          |           |
| actinomycyes naeslundii - 1 (4.56%) | 1 (++++)  | –         |
| Acinetobacter Lwoffii - 1 (4.56%) | 1 (+)   | –         |
| Streptococcus bovis - 1 (4.56%) | 1 (+)   | –         |
| Corynebacterium aquaticum - 1 (4.56%) | 1 (+)   | –         |

*Note: Sample N1 (microorganisms from the caries lesions) and Sample N2 (microorganisms from the prepared cavity after ozone application). (-), <10⁵ CFU·mL⁻¹; (+), −10⁵ to 10⁵ CFU·mL⁻¹; (++), 10⁴ CFU·mL⁻¹; (+++), 10³–10⁴ CFU·mL⁻¹; (++++) and >10³ CFU·mL⁻¹.

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**Table 2.** Kruskal–Wallis analysis for mean of various groups.

| Bacterial species          | Group* | n   | Mean rank | SD    |
|----------------------------|--------|-----|-----------|-------|
|                            | 1 2 3 4 5 6 7 | 22  | 123.00    | 0.294 |
|                            | 22     |     | 70.50     | 0.428 |
|                            | 22     |     | 81.00     | 0.492 |
|                            | 22     |     | 77.50     | 0.477 |
|                            | 22     |     | 67.00     | 0.395 |
|                            | 22     |     | 63.50     | 0.351 |
|                            | 22     |     | 60.00     | 0.294 |

*See Table 1.*
Our data agree with Safwat et al. [11], who found that gaseous ozone had a strong antimicrobial effect on cariogenic bacteria both in vitro and ex vivo and can be used as an adjuvant in caries therapy, unlike other studies [13] that have shown that 20% of S. mutans, 6.7% of Fusobacterium nucleatum, 13.4% of Bifidobacterium spp. and 6.7% Eubacterium spp. remain alive after a 6-hour ozone treatment. This study showed that 24 s gaseous ozone application destroyed all microorganisms isolated from the caries dentine, including S. mutans, Candida albicans, Peptostreptococcus spp., Actinomyces naeslundi and Enterococcus faecalis. These results were more favourable than the respective results of Johansson et al. [14], who reported that application of gaseous ozone for 60 s is needed to destroy these microbial species. However, exposure to ozone for such an extended period of time disintegrates saliva proteins; therefore, we recommend 24 s application to significantly reduce A. naeslundi and S. mutans. Baysan et al. [15] report a reduction in S. mutans following 10 s ozone treatment. This agrees with our study, which found no living bacterial cells of S. mutans after 24 s ozone treatment. The difference in the duration between this study and Baysan et al. [15] could be explained by differences in specific features of the devices and methodologies used. For example, Baysan et al. [15] use ozone-generating device (HeolOzone, USA) in their study. This allows some hermetic encapsulation during the ozone delivery in the cavity, while the coro-tip of Ozotop that we used does not lie tightly to the tooth.

In another comparative in vivo study of the oral microbiota, Johansson et al. [14] determined that the role of oral streptococci as a primary caries pathogen appears less pronounced in populations with prevention programmes. In agreement with this, we found a larger percentage of S. anginosus when compared with S. mutans. While this difference was not statistically significant, it may be associated with overall dental hygiene. Furthermore, a significant correlation between caries lesion development and S. mutans has been found [16]. The present study found S. mutans in 18.18% of patients. This result is lower than that predicted based on previous studies. This may be because the samples were obtained from the superficial layers of deep caries lesions.

In this study, we showed that 24 s gaseous ozone application of prepared cavities before their restoration eliminated S. anginosus. The presence of these microorganisms and their larger availability in this study could be explained by the fact that the caries samples were extracted from superficial layers. However, we argue that this study describes species characteristic of caries lesions microbial flora that coincide with those described by Bjørndal and Larsen [17].

S. mitis includes the microorganisms S. oralis, S.parasanguinis, S. sanguinis and S. gordonii. In this clinical study, there were found in 22.73% of examined samples before ozone application. Bjørndal and Larsen [17] report that S. mitis is an etiological factor in the development of tooth caries. In addition, S. parasanguinis is one of the main species to show early colonization of tooth surfaces. This study showed that these microorganisms were destroyed following ozone treatment for 24 s.

Aas et al. [18] report various anaerobic microorganisms present in deep caries lesions. In addition to S. mutans, species such as Veillonella, Lactobacillus, Propionibacterium, Actinomyces spp., Bifidobacterium and Atopobium spp. may play an important part in caries lesion progression. In this study, we found abundant microflora. There was a statistically significant difference in the group of oral streptococci; however, we also found Enterococci and C. albicans. We hypothesise that it is the combination of multiple microorganisms that contribute to the development of caries lesions in caries-active patients.

E. faecalis is not part of the normal oral cavity microflora and may be related to pathology [19]. We found E. faecalis in 22.72% of proximal caries lesions. This result is similar to findings by the Konidhi et al. [19]. They report that 27.5% of examined caries-active children from Tunis are positive for E. faecalis in the mouth. The identified enterococci were in 55.8% of the caries lesions and only 2% in non-caries positive children. Radman et al. [20] determined this microorganism in 80% of deep caries lesions. Aas et al. [18]

### Table 3. Comparison between all tested groups of microorganisms using Mann–Whitney test.

| Group | p value | Group 2 | p value | Group 3 | p value | Group 4 | p value | Group 5 | p value | Group 6 | p value | Group 7 | p value |
|-------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Group 1 | – | 0.0001* | – | 0.0003* | – | 0.0001* | – | 0.0001* | – | 0.0001* | – | 0.0001* |
| Group 2 | – | – | 0.3415 | – | 0.7594 | – | 0.1899 | – | 0.7003 | – | 0.4166 | – | 0.6649 |
| Group 3 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Group 4 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Group 5 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Group 6 | – | – | – | – | – | – | – | – | – | – | – | – | – |

*See Table 1.
argue that the presence of *E. faecalis* is associated with failures in performing of endodontic treatment and secondary endodontic infections. Our results of *E. faecalis* in the deep proximal caries lesion from caries-active patients could be an indicator of caries activity in the patients. *E. faecalis* is resistant to many antibacterial treatments, such as calcium hydroxide and mineral trioxide aggregate [21]. This study showed that 24 s gaseous ozone application successfully eliminated these organisms from caries lesions prepared for restoration.

*C. albicans* is typically found in the gastrointestinal tract [22]. *C. albicans* can become pathogenic when there is an imbalance of oral microflora or immunosuppression, causing stomatitis and periodontitis. The results in this study showed presence of *S. mutans* and *C. albicans* in the caries lesions of 18.18% and 36.36%, respectively. These data are significantly different from Fragkou et al. [23]. They report the frequency of *S. mutans* and *C. albicans* as 66.11% and 18%, respectively. They argue that caries-active children have more frequent and significantly larger amounts of streptococci and candida when compared with caries-free children. Anther investigation reports candidal carriage among the early childhood caries group for 84% of cases, which is significantly higher than the non-early childhood caries group of 24% of cases [24]. Conversely, some studies do not find a difference in oral microbiota between caries-active and caries-free children [25]. It has been suggested that differentiation of the candida species is a determining factor in the identification of the aetiology of dental caries. This is because *Candida dubliniensis* was not found in caries-free children; however, it was isolated in one quarter of the examined carious active children. Pereira et al. [26] suggest that the available data tend to imply that Candida may play a pivotal role as a secondary agent perpetuating the carious process, especially in dentinal caries. The results of this study also confirm this trend.

*Peptostreptococcus* spp. are Gram-positive anaerobic cocci. When we examined the deep caries lesions, we found these microorganisms were present in 36.36% samples. Previous studies have shown that these micrococci are found in dental plaques, caries dentine, subgingival plaque, dento-alveolar abscesses and advanced periodontal diseases, usually in mixed cultures. Their role as pathogens is unclear [27]. Nevertheless, our study eliminated these microorganisms after 24 s ozone treatment.

*Staphylococcus* are not typically regarded as cariogenic microorganisms; however, their strains were present in 18.18% cases of caries in this study and were eliminated following 24 s ozone treatment. We hypothesise that this is a result of their presence in the patients’ saliva. In a previous *in vitro* study, a single ozone application was effective at reducing *Staphylococcus* spp. [27, 28]. *Actinomyces* spp. are related to the proximal tooth caries, in particular with radicular caries [27]. However, *Actinomyces naeslundii* was isolated in only one caries lesion (4.54%) in this study and it was eliminated by ozone treatment.

**Conclusions**

Our findings suggest that *S. anginosus* group, *C. albicans* and *E. faecalis* should be considered as an index for caries activity in caries-active patients, but additional studies are necessary. This study demonstrated that 24 s of gaseous ozone application to the deep proximal caries lesions effectively eliminated microbial species.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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