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ORIGINAL ARTICLE

Relationship of MMP-9 with the clinical course of apical periodontitis and the main bacterial species in the oral microbiota

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Abstract

Bacterial products, host immune cells and cytokines have been reported to play an important role in the pathogenesis of apical periodontitis (AP). This study aimed to determine the main bacterial species in the microbiota as gram positive and negative and to compare the relationship between matrix metalloproteinase (MMP)-9 and tumor necrosis factor (TNF)-α with controlled patient groups. 60 patients with AP and extraction indication were included in the study. 30 systemically healthy volunteers without AP were selected as the control group. After access cavity preparation, an initial microbiologic sample (S1) was taken from the root canal. After atraumatic extraction of the tooth, a second microbial sample (S2) was taken from the extraradicular region. After bacterial DNA extraction, 16S rRNA gene primer was designed for sequence analysis. Bacterial community profiling was made by Sanger sequencing of the PCR products. In addition, serum MMP-9 and TNF-α levels were measured from all patients. TNF-α levels of the AP group were higher than the control group, while MMP-9 levels were found to be lower ($p=0.0264$ and $p=0.0146$, respectively). There was no difference in the main bacterial species isolated from the samples taken from the intracanal and extraradicular region of the tooth with AP ($p=0.714$). The main bacterial species in the intracanal region of the tooth with AP are similar to the main bacterial species in the extraradicular region. The pathophysiology of the tooth with AP is associated with low MMP-9 and high TNF-α, independent of the bacterial species in the intracanal and extraradicular regions.

Keywords Apical periodontitis · Matrix metalloproteinase · Tumor necrosis factor · Root canal · Bacteria species

Introduction

Apical periodontitis (AP) is a local inflammatory disorder of the periradicular tissue caused by bacterial infection of endodontic origin, which is characterized by periapical bone resorption. It is one of the most common diseases of the oral cavity and a common cause of tooth pain and loss, causing great discomfort in patients. The worldwide prevalence of people with at least one tooth with AP has been reported to range from 50 to 86% [1, 2]. Teeth with AP presents a microbiota characterized by a wide variety of combinations of bacteria, averaging 4–7 species per canal, predominantly anaerobic, with approximately equal proportions of Gram-positive (Gr(+)) and Gram-negative (Gr(−)) bacteria [3]. *Streptococcus, Enterococcus, Lactobacillus, Propionibacterium, Treponema, Bacteroides* and *Fusobacterium* are the most often encountered phyla in infected root canals [4–6]. There is clear evidence that microbial interaction plays an important role for the pathogenesis of AP [7]. However, there is insufficient evidence to determine which type of bacteria is more decisive in this microbial interaction. Microorganisms may cause direct tissue damage and modulate the immunological response by secretion of products, including enzymes, exotoxins and metabolic end-products [8, 9]. In addition to microbiological damage, host defense including immune response, tooth structure and collagen status are also critical in the development of AP pathogenesis [9].

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This collagen status is demonstrated by the measurement of various collagenolytic enzymes such as matrix metalloproteinases (MMP). MMP has a role in the degeneration of extracellular matrix, tissue repair, and remodeling by modulating cytokines/chemokines and it also has antiinflammatory properties [10, 11].

Inflammation due to infections in teeth as well as in other tissues of our body can be evaluated by measuring various cytokines. Interleukin (IL)-6 and tumour necrosis factor alpha (TNF- α) are the most frequently used proinflammatory cytokines [12]. Of these cytokines, TNF- α is a potent stimulator of bone resorption [12]. Current research is unsufficient in demonstrating whether gram-negative or -positive bacteria lead to a stronger inflammatory reaction.

In the light of the above information, the aim of the study was to determine the main bacterial species involved in the pathogenesis of AP as Gr(+) and Gr(−), and to compare their relationships with MMP-9 and TNF-α with controlled patient groups. In addition, the Gr(+) and Gr(−) bacteria species causing AP were also tested over MMP-9 and TNF-α levels to see which caused greater inflammation.

Materials and methods

Ethics committee approval

The study was approved by the Institutional Ethical Committee (#E-10840098-772.02-6757), and written consent was obtained from all participants.

Patient selection

Approximately 450 patients who applied to Istanbul Medipol University, Faculty of Dentistry, Endodontics Department for routine examination between March 2022 and February 2023 due to apical periodontitis complaints were examined and their demographic characteristics were recorded. The diagnosis was established according to the patient's history, electrical pulp test, clinical inspection including palpation and percussion tests and radiographic examination. The selection criteria for patients to be included in the study were as follows: having chronic apical periodontitis lesions and had an indication for extraction of the relevant tooth, having periapical radiolucencies' more than 2 mm, having intact coronal restorations without pulp exposure to the oral cavity; additionally selected teeth had adequate crown structures for isolation with a rubber dam, presented no periodontal pockets and attachment levels did not exceed 4 mm. The tooth to be sampled should not have been previous root canal treated. Patients who had any acute or chronic inflammatory disease, known infection status, history of trauma or surgical operation within 6 months that may affect MMP-9 levels, pregnant and/or lactating, malignity, morbid obesity, arthritis, immunologic diseases, cardiovascular diseases or use of vitamins with high biotin were not included in the study. Additionally, patients who used antibiotics or anti-inflammatory drugs within the last 6 months and patients with periodontal disease were excluded from the study. Of these patients, 60 patients with chronic apical periodontitis and extraction indication were included in the study. Extraction indication was due to prosthetic (insufficient crown-to-root ratio and teeth with uncertain prognosis due to lesion size) and restorative reasons (marginal staining, and/or leakage of the coronal restoration, teeth with cavity borders below the gingival line). A total of 30 systemically healthy volunteers who did not have AP were included in the study as the control group.

Demographic characteristics and clinical and radiographic evaluation

After the demographic characteristics (age, gender, smoking status) and the height and weight of the patients were measured. Body mass indexes [BMI = body weight (kg)/height squared (m^2)] were calculated with the Quetelet index. After clinical and radiographic examinations were performed, intraoral examination findings (number of teeth with root canal treatment, number of teeth with crown and/or composite restorations, number of missing teeth, clinical attachment loss and probing depth) of the patients were recorded. Although all teeth were diagnosed as chronic AP, it was noted that they were symptomatic and asymptomatic according to the percussion and palpation sensitivity responses in the intraoral examination. Clinical parameters such as calculus index, plaque index, probing depth, and clinical attachment level were measured for all teeth.

The oral hygiene status of participants was evaluated by the Simplified Oral Hygiene Index (OHI-S). The six surfaces examined for the OHI-S are selected from four posterior and two anterior teeth. This index has two components, the Debris Index simplified (DI-S) and the Calculus Index simplified (CI-S). CI-S and DI-S values ranged from 0 to 3. The DI-S scoring was as follows: score 0- no debris or stain present; score 1- soft debris covering not more than one-third of the tooth surface; score 2- soft debris covering not more than two-thirds of the exposed tooth surface; and score 3-soft debris covering more than two-thirds of the exposed tooth surface. The CI-S scoring was as follows: score 1- calculus covering not more than one-third of the tooth surface; score 2- calculus covering not more than two-thirds of the exposed tooth surface; and score 3-calculus covering more than two-thirds of the exposed tooth surface, and the OHI-S value was the sum of the CI-S and DI-S, thus the score ranged from 0 to 6. An oral hygiene index score of 0 to 1.2 was categorized as good oral hygiene; 1.3 to

2.9 was categorized as fair oral hygiene; and 3.0 to 6.0 was categorized as poor oral hygiene [13].

Panoramic dental radiographs were obtained from the patients whose intraoral examination was completed, and the number of teeth with AP was determined (VistaPano S, Durr Dental AG, Germany, operating at 73kVp and 10 mA with 13,500 ms exposure time in standard mode). For periapical radiographs Carestream RVG 5200 (RVG; Carestream Health Inc, Atlanta, CA, USA) system was used with an x-ray unit, setting of 70 kV, 8 mA. The bisecting angle technique was applied in obtaining the periapical images. Then, the radiographs were analyzed with Kodak Dental Imaging Software. From a radiographic evaluation of the teeth, the presence and severity of AP were assessed with the Periapical Index (PAI) scoring system, which was published by Ørstavik et al. [14]. This scoring system numbers the tooth for AP from 1 to 5 according to the radiolucency of the periapex on radiographs. Teeth scored as PAI 1 and 2 were not included in the study because of the lack of apparent periapical pathology and the AP group was composed of teeth scored as PAI 3, 4 and 5. When in doubt the higher score was given and for multi-rooted teeth, the highest score of each root was assigned as the PAI score of the tooth. The PAI scoring system was found insufficient due to grouping the lesions solely on size without taking the number of teeth affected into account. Therefore, we used the Apical Periodontitis Grading Scale (APGS) and Abscess Score (AS) system [15, 16]. APGS system categorizes patients into 3 grades, as those having 1 tooth with AP and severity of 3–4 were classified as grade 1 (mild); those having > 1 tooth and severity of 3–4 as grade 2 (moderate) and those with at least one tooth with a severity of 5 as grade 3 (severe). AS system categorizes patients into 3 grades. Patients who had at least 1 tooth with AP and severity of 3–4 according to PAI classification were categorized as AS 1 (mild); those having only 1 tooth with a severity of PAI 5 as AS 2 (moderate) and those with more than 2 teeth with a severity of PAI 5 as AS 3 (severe). Thus, a total of 60 AP patients without any periodontal involvement, were allocated to one of 3 classes (APGS 1, 2 and 3; AS 1, 2 and 3). The 30 systemically healthy individuals without periodontal disease (probing depth of < 3 mm and no radiographic bone loss) and without AP were included as the control group. Before microbial sampling, blood samples were collected from the patients for MMP-9 and TNF-α analysis. Blood samples were taken from the antecubital vein in a sitting position in a gel separator tube in the morning (between 08:00 and 09:00) after at least 8–10 h of fasting.

Bacteriological sampling from the root canal system and outer root surface

After plaque removal and rubber dam isolation, the operative field was cleaned with 3% hydrogen peroxide and disinfected with 2.5% NaOCl solution. Then, old coronal restorations and carious defects were removed, and an access preparation was completed when the root canal space was properly exposed. Afterwards, the tooth (including the pulp chamber), clamp and adjacent rubber dam were once again disinfected with 2.5% NaOCl, followed by inactivation with 10% sodium thiosulfate in order to avoid interference with bacteriological sampling. The working length (WL) was established 1-mm short of the apical foramen with an apex locator (Raypex6; VDW GmbH). Irrigation with sterile saline solution was performed in order to moisten the canal prior to sample collection. Next, the canal was left filled with saline, and a small hand instrument was placed at the WL and used to gently file the canal walls. An initial microbiologic sample (S1) was taken from the root canal with sterile paper points consecutively placed at the WL. Three sterile paper points were inserted into the root canal for sampling. Each paper point was left in the canal for about 1 min. Both the paper points and the endodontic hand instrument, without the handle, were transferred to cryotubes containing 300µL of PBS solution stored at − 20 °C. After the atraumatic extraction of the teeth, the external root surface was rinsed with sterile saline solution, and all attached soft tissue, including the apical periodontitis lesion from the root was curetted. Then, a second microbial sample (S2) was taken. Curetted dental tissue containing extraradicular biofilm was placed in cryotubes as described for S1. The samples were transferred to a genetic analysis laboratory for further analysis in the cold chain. In each case, a single root canal was sampled in order to confine the microbial evaluation to a single ecological environment. In multirooted teeth, the root with the periapical lesion was selected. If there were periapical lesions in all roots, the wider canal was selected. All examinations (including intraoral and radiographic) and microbial sampling procedures were performed by the principal investigator (B.O.).

Genomic DNA isolation and measurement of DNA concentration

DNA isolation from samples was performed using the QIAamp DNA Mini Kit (Qiagen, Germany). Before starting the mass isolation, 1 ml of sterile dH2O was added into the samples taken from the patients in 1.5 mL microcentrifuge tubes. Then, these samples were vortexed every ten minutes at 50–60 °C in the block heater and kept for 1 h. The liquids in the tubes were transferred to new tubes and centrifuged at maximum speed for 5 min. The upper supernatant was taken and transferred to tubes with paperpoints so that approximately 150–200 µl of liquid remained in the tube. The isolation was continued according to the protocol in the kit used. DNA concentrations of isolated DNA samples were determined after fluorometric measurements at 260 nm using Promega QuantiFluor device.

PCR (16S Universal primer)

In the study, a universal primer was designed for 16S rRNA genes. While a long 16S rRNA primer was designed for sequence analysis of all samples, one primer pair was designed to amplify a short region for use in ddPCR. This primer was selected from the conserved regions of the 16S rRNA gene and was designed to be used for all bacteria. After DNA extraction of samples with QIAamp DNA Mini Kit, approximately 1100 bp of 16S rRNA sequences was amplified by using universal E8F forward primer (5′-AGA GTTTGATCCTGGCTCAG-3′) and universal E1115R reverse primer (5′-AGGGTTGCGCTCGTTG-3′). After DNA was isolated from the samples taken from each patient at 2 different times (S1 and S2), 16S rRNA sequence analysis was performed to determine the bacterial diversity in the samples. Polymerase Chain Reaction (PCR) using 16S Universal long primer pair was performed as described below. Mytaq HS Taq polymerase (Bioline) was used as the enzyme. The final volume of PCR reactions for each isolated bacterial strain was adjusted to 25µL. The amplification reactions of 16S rRNA genes were performed with the following conditions. One cycle of predenaturation at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s which continue with a final extension step at 72 °C for 10 min. The PCR products were analysed by electrophoresis using 2% agarose gel (containing ethidium bromide) in Tris/BoratE/EDTA (TBE) buffer, with gels being analysed under ultraviolet light (at 140 V for 20 min). Their images were visualised under ultraviolet illumination.

Purification and sequencing of the 16S rRNA gene

After the PCR reactions, the purification of PCR products is done by hydrolyzing the excess primers and nucleotides with ExoSap-IT (Thermo, PN: 78,201.1.ML) containing Exonuclease I and Alkaline Phosphatase enzymes. 2µL of ExoSap-IT was mixed with 5µL of PCR product for each sample. The ExoSap reaction is performed at 37 °C for 15 min (enzyme activation) followed by 15 min (inactivation) at 80 °C. Sequencing reactions were performed using Bigdye Terminator v3.1 cycle sequencing kit (Thermo). The reactions were performed according to the manual kit for all isolated strains.

After purification of the products with Exosap, the sequence reaction was performed with BigDye Terminator v3.1Cycle Sequencing Kit (Thermo) under the following conditions. After the sequence PCR, BigDye products were purified by the colon method. Zymo ZR DNA Sequencing Clean-up Kit (Zymo Research, USA) was used for this process. All samples were purified in accordance with the protocol given in the kit and executed on the 3130XL genetic analyser.

Laboratory analysis

Blood samples were collected from the patients for MMP-9 and TNF-α analysis. The blood samples taken from the patients in a tube with a gel separator were kept at room temperature for 20–30 min to coagulate, and then centrifuged at 1800 g for 10 min. The sera obtained after centrifugation were aliquoted into eppendorfs and stored at − 80 °C until the time of analysis. The sera were then gradually warmed up by keeping them in the refrigerator at + 4 °C for 12 h. All samples were kept at room temperature for 30 min before the study.

ELISA measurement procedure of MMP-9 and TNF-α levels

BT lab branded Human Matrix Metalloproteinase 9 and Human Tumor Necrosis Factor-α ELISA Kits (Shangai, China) were used to measure MMP-9 and TNF-α levels. The sensitivity of the kits is 15.12 ng/L and 1.520 pg/mL, respectively, and the measuring range of the kit is 30–9000 ng/L and 3–900 pg/mL, respectively. The within-run coefficient of variation is < 10%. In the study, measurements were made using BIO-TEK EL × 500 ELISA Reader and BIO-TEK EL × 50 (USA) washing devices.

Power analysis and sample size calculations

The sample size for this study was determined by performing a priory power analysis (PS Power and Sample Size Calculations, Version 3.0) with the data of a study conducted by Lotfi A et al. [17] in which serum MMP-9 levels were compared in patients with oral squamous cell carcinoma. For the study consisting of two groups (α: 0.05, power: 0.80), it was determined that 9 experimental and 9 control subjects should be studied. In this study, the experimental group was determined as 60 and the control group as 30 in order to access parametric data and make comparisons for the group.

Statistical analysis

IBM SPSS statistics (version 25, USA) and MedCalc® statistical software (version 15.8, Belgium) were used for statistical analysis of the data. Chi-square Test was used to compare the categorical data of two independent groups in the study. Unpaired t test and Mann–Whitney U test were used to compare the parametric and nonparametric data of two independent groups, respectively. Receiver Operating Characteristic (ROC) analysis was performed to evaluate the diagnostic power of the serum MMP-9 test for the diagnosis of AP. After performing Correlation Matrix analysis to investigate the relationship between serum MMP-9, PAI score, OHI-S, TNF (total number of fillings), RCF, missing

teeth and number of crowns, Pearson correlation analysis was used in parametric data and Spearman correlation analysis was used in nonparametric data. Bar plot graphs showing the 1st quartiles, 3rd quartiles and median were used to display the nonparametric data. Box plot graphics were used to represent the mean and standard deviation of parametric data.

Results

AP and control groups were not statistically different in terms of age (34 ± 12 and 34 ± 10 years, respectively) and gender (M/F: 28/32 and 12/18, respectively) ($p > 0.05$). There was no statistical difference between the mean body mass index (BMI) values of the patients with AP and the control group (25.7 ± 4.2 vs. 25.4 ± 4.3 kg/m^2, respectively, $p = 0.7776$). Only 27% of patients with AP were symptomatic. The difference between the smoking status of patients with AP and control group was statistically insignificant (22 (45%) vs. 10 (33%), respectively, $p = 0.7550$). It was determined that the most common tooth with AP (MCT-AP: Most common tooth with AP) was tooth number 36 (mode) and its frequency was 7 in the group of 60 people (Table 1). Again, the frequency of AP was found to be 6 in teeth 34, 46 and 47. There was a statistically significant difference between the PAI scores of the AP and control groups [5(3–5) vs 1(1–2), respectively, $p < 0.0001$]. The APGS and AS-PAI scores of the AP group were found to be 3.0 (2.0–3.0) and 2.0 (1.0–3.0), respectively. The OHI-S value, which is a measure of oral and dental health, was found to be significantly worse in the AP group compared to the control group [4 (2–6) versus 1 (0–3), $p < 0.0001$] (Fig. 1). There was no statistical difference between the total number of fillings (NF) (Fig. 1), the number of root canal fillings (RCF), the number of missing teeth and the number of crown restorations (Fig. 2) of both groups ($p > 0.05$) (Table 2).

TNF-α levels of the patient group with AP were found to be statistically significantly higher than the control group [22.4 (8.4–47.8) pg/mL and 15.23 (8.20–53.42) pg/mL, respectively, $p = 0.0264$] (Fig. 3). MMP-9 levels of the patient group with AP were found to be statistically significantly lower than the control group [1434 (376–5600) pg/mL and 2158 (941–5475) pg/mL, respectively, $p = 0.0146$] (Fig. 3).

The number of smokers in the AP group was found to be 22 (40%). Although MMP-9 levels were found to be higher in smokers with AP compared to non-smokers, the difference was statistically insignificant [1333 (376–5036) vs. 1467 (830–5600) ng/L, p = 0.1270, Mann–Whitney *U* test]. Similarly, there was no statistical difference between the TNF-α levels of smokers and nonsmokers with AP [21.2 (8.4–47.8) vs. 24.8 (8.5–47.8) pg/mL, $p = 0.3263$, Mann–Whitney *U* test]. This finding should be re-examined with sufficient sample size.

The number of symptomatic patients with AP was found to be 16 (27%). There was no statistical difference between symptomatic and asymptomatic patients with AP in terms of MMP-9 and TNF-α levels [1513(1060–5036) and 1431(376–5600) ng/L for MMP-9, $p = 0.2010$;

Table 1 Clinical examination and laboratory findings of all patients with AP and control group

	AP Group	Control Group	*p* value
N	60	30	–
MCT-AP, mode (f)	36 (7)	–	–
PAI, score	5 ± 0.5 5(3–5)	1 ± 0.0 1(1–2)	[c] < 0.0001
APGS, score	2.9 ± 0.3 3.0(2.0–3.0)	0 ± 0	[ø] –
AS-PAI, score	2.2 ± 0.6 2.0(1.0–3.0)	0 ± 0	[ø] –
OHI-S,	4 ± 1 4(2–6)	1 ± 1 1(0–3)	[c] < 0.0001
Total number of fillings, n	3 ± 3 3(0–10)	4 ± 3 4(0–13)	[c] 0.1102
RCF, n	1 ± 2 1(0–8)	2 ± 2 1(0–9)	[c] 0.3975
Missing teeth, n	6 ± 5 6(0–23)	3 ± 3 3(0–8)	[c] 0.0024
Number of crowns, n	3 ± 5 0(0–18)	2 ± 5 0(0–19)	[c] 0.6733
MMP-9, ng/L	1925 ± 1326 1434(376–5600)	2781 ± 1652 2158(941–5475)	[c] 0.0146
TNF-α, pg/mL	23.7 ± 10.3 22.4(8.4–47.8)	19.19 ± 10.87 15.23(8.20–53.42)	[c] 0.0264

[c] Mann–Whitney Test. ø It is not possible to analyze the data because one column's SD is zero

In statistical comparisons, parametric data were shown as mean ± standard deviation and non-parametric data as mean ± standard deviation and median (min–max). *AP* apical periodontitis, *MCT-AP* most common tooth with AP, *f* frequency, *PAI* periapical index, *APGS* apical periodontitis grading scale, *AS-PAI* abscess score according to PAI, *BMI* body mass index, *OHI-S* simplified oral hygiene index, *RCF* number of root canal fillings, *MMP-9* matrix metalloproteinase-9, *TNF-α* tumor necrosis factor alpha, *S1* ıntracanal bacterial population, *S2* extraradicular bacterial population, *MBS-1st* the most common main bacterial species in the first place, *MBS-2nd* the most common main bacterial species in the second place,

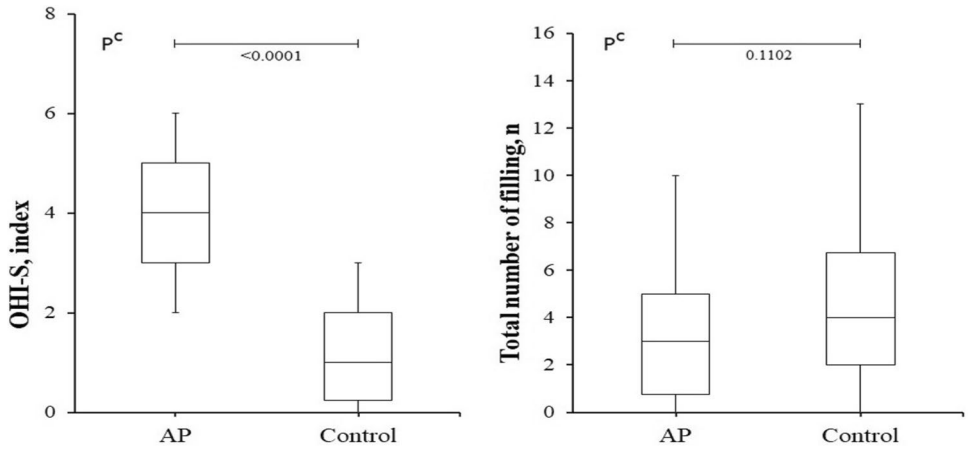


Fig. 1 Nonparametric bar plot graph of OHI-S, nonparametric and total number of filling values of AP and control groups. While a significant statistical difference was observed between the AP and control groups in terms of PAI score, no statistical difference was observed in terms of OHI-S and total number of filling values. [c]Mann–Whitney Test, *OHI-S* Simplified oral hygiene index

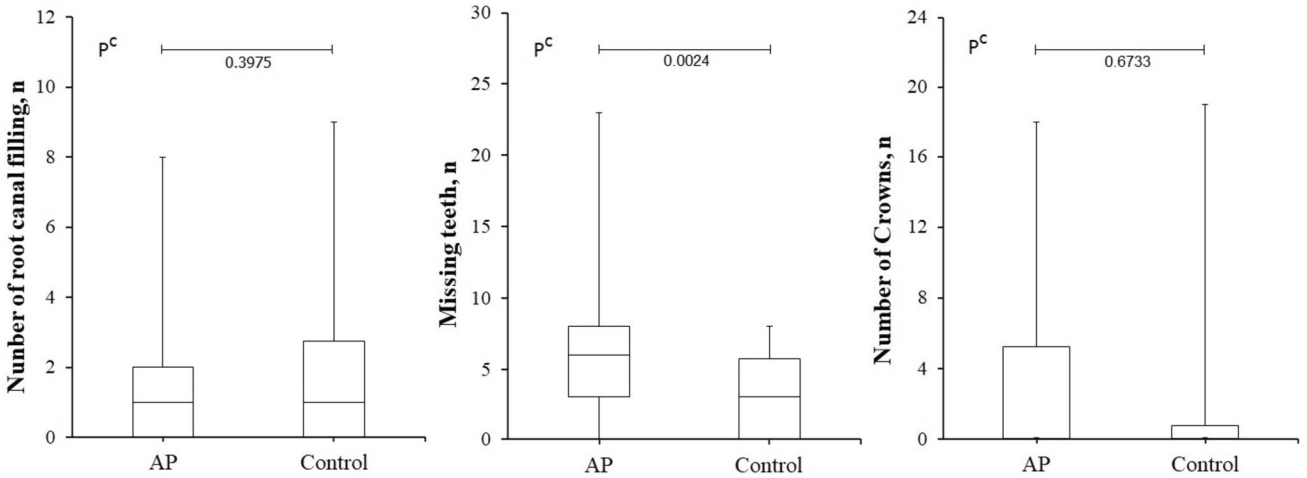


Fig. 2 Nonparametric bar plot graph of root canal filling, missing teeth and crown numbers of AP and control groups. While a significant statistical difference is observed between the AP and control groups in terms of the number of missing teeth, there is no statistical difference in terms of number of root canal filling and number of crowns. [c]Mann–Whitney Test, *RCF* Root canal filling

Table 2 Gram-negative and gram-positive bacteria species detected in the first and second most common order in samples taken from the intracanal and extraradicular regions of the tooth with AP

n: 60	S1			S2		
	MBS-1st	MBS-2nd	*p* value	MBS-1st	MBS-2nd	*p* value
Gram (−), *n*	32(53%)	23(38%)	[a]0.099	34(57%)	30(50%)	[a]0.464
Gram (+), *n*	28(47%)	37(62%)		26(43%)	30(50%)	
	S1-MBS-1st vs S2-MBS-1st			S1-MBS-2nd vs S2-MBS-2nd		
Comparison p	[a]0.714			[a]0.198		

[a] Chi-square test

S1 ıntracanal, *S2* extraradicular, *MBS-1st* the most common main bacterial species in the first place, *MBS-2nd* the most common main bacterial species in the second place, *AP* apical periodontitis

19.3(8.5–47.8) and 23.2(8.4–47.7) for TNF-α, $p = 0.5755$, respectively; Mann–Whitney *U* test].

In the patient group with AP, the most common first-order main bacterial species (S1-MBS-1st) detected from the samples taken from the intracanal region (S1) of the tooth with AP was found to be Gr(−) in 32 patients and Gr(+) in 28 patients (Table 2). The second most common major bacterial species (S1-MBS-2nd) was found to be Gr(−) in 23 patients and Gr(+) in 37 patients. There was no statistical difference between S1-MBS-1st and S1-MBS-2nd in terms of main bacterial species ($p = 0.099$).

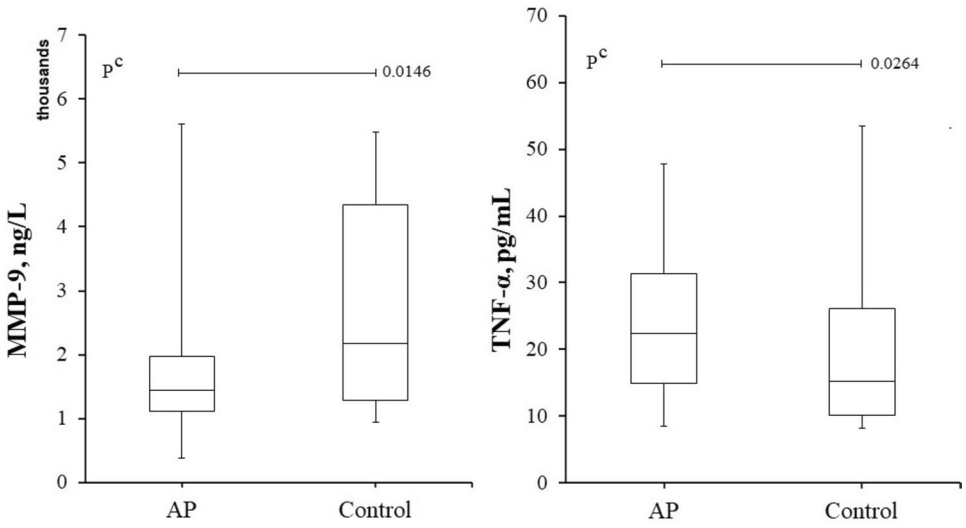


Fig. 3 Nonparametric bar plot graph of MMP-9 and TNF-α levels of AP and control groups. Significant statistical difference was observed between the AP and control groups for both biomarkers. [c]Mann–Whitney Test

There was no statistical difference between S2-MBS-1st and S2-MBS-2nd samples taken from the outer root surface (S2) of the tooth with AP in terms of main bacterial species ($p = 0.464$) (Table 2). Again, there was no statistical difference between S1-MBS-1st and S2-MBS-1st, and between S1-MBS-2nd and S2-MBS-2nd ($p = 0.714$ and $p = 0.198$, respectively).

According to the frequency of detection of S1-MBS-1st, the bacterial species were listed as follows: *Sphingomonas* species (n:14), *Lactobacillus* species (n: 12), *Enterobacter* species (n:12), *Streptococcus* species (n:11), *Fusobacterium* species (n:6) and *Enterococcus* species (n:5). According to the frequency of detection of S1-MBS-2nd, they were listed as: *Lactobacillus* species (n:19), *Streptococcus* species (n:12), *Enterobacter* species (n:11), *Sphingomonas* species (n:9), *Enterococcus* species (n:6), *Fusobacterium* species (n:3).

According to the frequency of detection of S2-MBS-1st, the bacterial species were listed as follows: *Fusobacterium* species (n:16), *Lactobacillus* species (n:11), *Sphingomonas* species (n:9), *Streptococcus* species (n:9), *Enterococcus* species (n:6), *Treponema* species (n:5) and *Enterobacter* species (n:4). According to the frequency of detection of S2-MBS-2nd, they were listed as: *Fusobacterium* species (n:13), *Lactobacillus* species (n:16), *Streptococcus* species (n:10), *Treponema* species (n:7), *Sphingomonas* species (n:6), *Enterococcus* species (n:4) and *Enterobacter* species (n:4). It was considered as an interesting finding that *Treponema* species could be detected only in the sample taken from the extraradicular region of the tooth with AP.

Detected *Sphingomonas* species are as follows: *S. mesophila, S. edaphi, S. jaspsi, S. parapaucimobilis, S. phyllosphaerae, S. olei, S. aracearum, S. aerolata, S. glacialis, S. melonis, S. echinoides, S. sanguinis, S. downei, S. sobrinus.* Detected *Fusobacterium* species are as follows: *F. nucleatum, F. periodonticum, F. necrophorum, F. hwasookii, F. watanabei, F. canifelinum, F. varium, F. gastrosuis, F. pseudoperiodonticum.* Bacteria belonging to *Lactobacillus* species are as follows: *L. paracasei, L. rhamnosus, L. casei, L. brevis, L. gasseri, L. salivarius, L. johnsonii, L. algidus, L. agilis, L. acidophilus, L. lactis, L. helveticus, L. gallinarum, L. delbrueckii, L. crispatus.* Bacteria belonging to *Enterococcus* species are as follows: *E. faecium, E. faecalis, E. italicus, E. durans, E. mundtii, E. devriesei, E. viikkiensis, E. gallinarum, E. casseliflavus, E. hermanniensis, E. lactis, E. ludwigii, E. hirae, E. cecorum.* Detected *Streptococcus* species are as follows: *S. mutans, S. equi, S. orisuis, S. devriesei, S. ratti, S. notomytis, S. milleri, S. sanguinis, S. pyogenes, S. gordonii, S. anginosus, S. intermedius, S. uberis, S. lutetiensis, S. equinus, S. thermophilus, S. parasanguinis, S. oralis, S. pneumoniae, S. mitis, S. australis, S. gallinaceus, S. constellatus, S. saliviloxodontae, S. agalactiae, S. tigurinus.* Detected *Enterobacter* species are as follows: *E. aerogenes, E. hormaechei, E. ludwigii, E. cloacae, E. asburiae, E. cancerogenus.* Bacteria belonging to *Treponema* species are as follows: *T. denticola, T. phagedenis, T. pallidum, T. vincentii, T. phagedenis, T. putidum.*

When the effect of the most common first-order main bacterial species (S1-MBS-1st) detected from the samples taken from the root canal in AP group patients on MMP-9 and TNF-α levels according to whether they are Gr(+) or Gr(−), no significant difference was found between Gr(-) S1-MBS-1st and Gr(+) S1-MBS-1st levels ($p > 0.05$) (Table 3). Likewise, for S2-MBS-1st determined from the samples taken from the outer root surface, the MMP-9 and TNF-α levels of Gr(−) S21-MBS-1st were not statistically different from Gr(+) S2-MBS-1st levels ($p > 0.05$) (Table 3). In other words, MMP-9 and TNF-α levels have no effect on the bacterial species in the intracanal and extraradicular region of the tooth with AP.

According to the correlation matrix results, a positive correlation was observed between PAI score and OHI-S and RCF and NF values ($r=0.789$ and $r=0.495$, respectively), while a negative correlation was found between MMP-9 and TNF-α levels. ($r=-0.517$). When these results are reanalyzed with appropriate statistical methods; statistically significant correlation values were obtained between PAI score and OHI-S values and RCF and NF values similar to the correlation matrix results (Spearman $r=0.760$, 95% CI 0.652 to 0.837, $p<0.0001$ and Spearman $r=0.5716$, 95% CI 0.408 to 0.699, $p<0.0001$, respectively). Similarly, a statistically significant moderately negative correlation was found between MMP-9 and TNF-α levels (Spearman $r=-0.534$, 95% CI -0.670 to -0.362, $p<0.0001$). The negative correlation between MMP-9 and TNF-α levels shows that MMP-9 levels are not independent of TNF-α levels and 39.5% of the subjects were affected by this relationship.

In the ROC analysis performed to determine the diagnostic power of the serum MMP-9 test for the determination of AP, the optimal cutoff was ≤ 2092 ng/L, the sensitivity was 81.7%, the specificity was 60.0%, and the area under the curve (AUC) was 0.659 ($p=0.0135$). (Fig. 4). The serum TNF-α test, on the other hand, had an optimal cutoff > 13 pg/mL, a sensitivity of 80.0%, a specificity of 50.0%, and an area under the curve (AUC) of 0.644 ($p=0.0268$) (Fig. 4). When the diagnostic performances of serum MMP-9 and TNF-α were compared, the difference between them was found to be statistically insignificant (AUC difference: 0.015, $p=0.8186$) (Fig. 5).

Discussion

AP, defined as the infection of the periapical alveolar bone of the tooth, is originated from necrotic infected dental pulp. The deterioration of the cortical bone as a result of the presence of infection and inflammation is seen as radiolucency in the periapical region and enlargement of the periodontal membrane around the apex in the radiograph used as a diagnostic criterion. The physiopathological process of AP is determined by the encounter between microbial organisms and the host defense system [18–20]. The host response includes a variety of cells (polymorphonuclear leukocytes, lymphocytes, monocytes/macrophages, and plasma cells), mediators, effector molecules, and antibodies. However, since polymorphonuclear leukocytes also secrete cytoplastic granules (lytic enzymes), they can cause structural damage to tissue cells and extracellular matrix [21]. Within the defense mechanism, the nature of the tissue forming the structure (collagen, fibroblasts, odontoblasts and mesenchymal cells) as well as localization and environmental factors are determinative in the formation of the lesion. The fact that the most common tooth with AP observed in this study was tooth number 36 followed by teeth numbered 34, 46 and 47 had a high incidence of AP, thus supporting this claim. Additionally, higher PAI score and OHI-S value of the AP group compared to the control group is also a finding in the same direction.

Behavioral aspects and hygienic measures play a big role in oral and dental health [22]. In this context, a comparison was made in this study to examine the effects of smoking in terms of dental health, and no difference was found between MMP-9 and TNF-α levels of smokers with AP compared to non-smokers. This finding needs confirmation with sufficient sample size. However, in the study of Shah and ElHaddad [22], it was found that frequent smokers perceive oral health problems more than other students, which explains our finding.

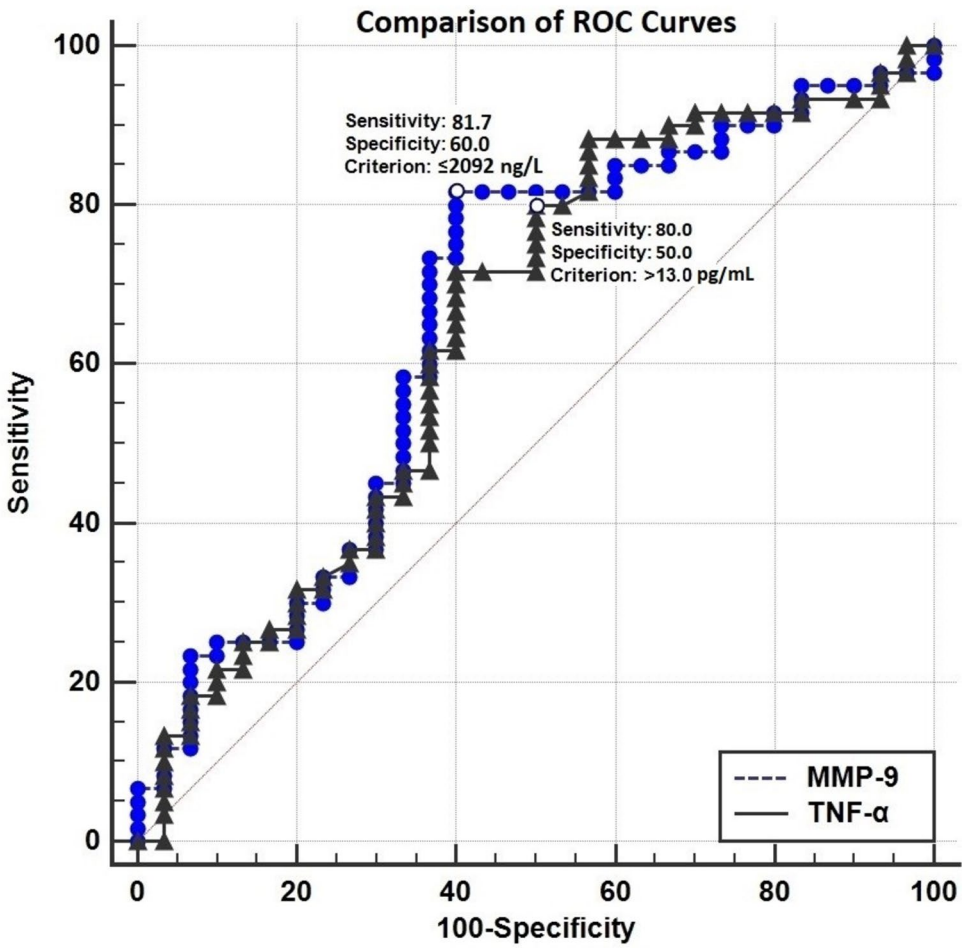
Evaluation of a painful tooth and whether the patient is symptomatic will ensure the appropriate diagnosis match of treatment. Apical periodontitis is classified as symptomatic apical periodontitis and asymptomatic apical periodontitis. Symptomatic apical periodontitis may result from infectious or aseptic inflammation [18]. Symptoms of this period include pain and tenderness. However, since the integrity of the bone, cementum and dentin has not been broken yet and

Table 3 The effect of the most common first-order main bacterial species (S1-MBS-1st) detected from the samples taken from the root canal in AP group patients on MMP-9 and TNF-α levels according to whether they are Gr(+) or Gr(−),

	S1-MBS-1st		*p* value	S2-MBS-1st		*p* value
	Gram (−)	Gram (+)		Gram (−)	Gram (+)	
N	32	28	–	34	26	–
MMP-9, ng/L	1686 ± 1068 1442(376–5036)	2197 ± 1546 1434(830–5600)	[c]0.5097	1987 ± 1302 1464(884–5520)	1843 ± 1379 1371(376–5600)	[c]0.2999
TNF-α, pg/mL	25.7 ± 10.7 25.1(8.5–47.8)	23.7 ± 10.6 23.2(8.4–47.8)	[c]0.1326	21.7 ± 9.8 19.9(8.5–47.8)	26.3 ± 10.5 24.9(8.4–47.8)	[c]0.0836

[c] Mann–Whitney test

S1-MBS-1st ın intracanal region, the most common main bacterial species in the first place, *S2-MBS-1st* ın extraradicular region, the most common main bacterial species in the first place



Classification variable: AP	Positive group (1): 60, Negative group (0): 30		
Variable	AUC	SE	95% CI
MMP-9	0.659	0.0643	0.551 to 0.756
TNF-α	0.644	0.0652	0.537 to 0.743
Difference between areas	0.015		
SE, (95% CI)	0.0630 (-0.109 to 0.138)		
z statistic	0.229		
Significance P	0.8186		

Fig. 4 Comparative ROC curve analysis graph comparing the performance of MMP-9 and TNF-α tests for the diagnosis of AP. Although the diagnostic performance of the MMP-9 test is statistically significant, it seems to have a weak performance for use alone. Although the diagnostic performance of the TNF-α test is statistically significant, it seems to have a weak performance for use alone. When the diagnostic performances of MMP-9 and TNF-α tests for the detection of AP are compared, it is seen that there is no statistical difference between them

the inflammation is limited to the periodontal ligament, the radiographic changes are not fully settled. This stage is an inflammatory process in which neutrophils and macrophages play an important role, intensifying cytokine release, vascular response, and osteoclastic bone resorption, and the lesion in the apical tissues may return or worsen, causing chronic apical periodontitis, which is usually asymptomatic [18, 23, 24]. These lesions can then become abscessed by spreading through the bone to other tissues. In symptomatic apical periodontitis, the tooth is sensitive and painful on percussion. However, because the pulp is necrotic in chronic apical periodontitis, there will be no response to sensibility tests. The presence of a radiolucent periapical lesion on radiographs makes the diagnosis of chronic apical periodontitis. In this study conducted in the light of the above information, the number of symptomatic patients with chronic AP was determined as (27%). This finding corresponds to the number of patients with symptomatic chronic apical periodontitis (in other words, secondary acute apical periodontitis). When bacterial types were examined according to the presence of symptoms, there was no relationship between specific microorganisms and symptom development. Similarly, no significant changes in the microbial composition according to the signs have been previously reported by certain authors [25, 26]. However, others demonstrated shifts in the microbial composition according to the signs [27]. We also found that there was no change in the biomarkers according to the symptoms. The fact that there was no difference between symptomatic and asymptomatic apical periodontitis patient groups in terms of MMP-9 and TNF-α levels showed that

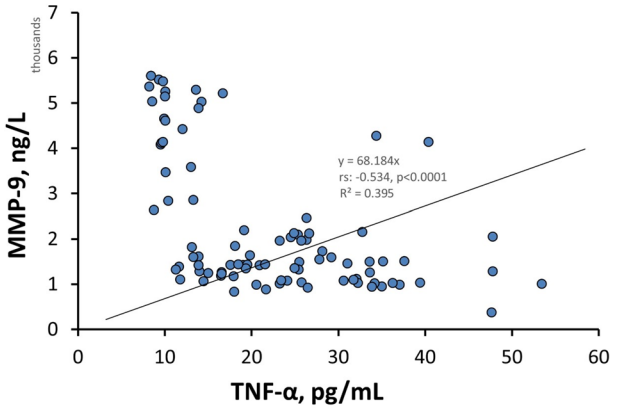


Fig. 5 Scatter plot showing the relationship between MMP-9 and TNF-α levels. It is seen that there is a moderate negative correlation between the two variables. rs: Spearman r

these two parameters are not alternative markers to radiological images for the differentiation of acute or chronic AP.

The pulp contains a connective tissue with a unique organization and location, a significant part of which is composed of collagen. Cells that make up the dental pulp (fibroblasts, odontoblasts and undifferentiated mesenchymal cells) are interconnected by a complex contact system and are responsible for the synthesis of the extracellular matrix (ECM). Collagen, the most abundant ECM protein (more so type III than type I collagen), forms a complex network to provide tensile strength and play an important role in the three-dimensional structure of the tooth [28, 29]. It is known that collagenases play an important role in the destruction and construction of this structure in pathological and physiological conditions. Matrix metalloproteinase (MMP)-9, one of these and a member of zinc-dependent endopeptidases, is known to be involved in remodeling processes in many pathological conditions including inflammation and fibrosis. MMP-9 activates cytokines and chemokines to regulate tissue remodeling while directly affecting the extracellular matrix proteins and all their components in the interstitial spaces, forcing them to be destroyed [30, 31]. After it was understood that MMPs play an important role in inflammatory conditions of periodontal, pulpal and periapical tissues and it was determined that MMP inhibition in periapical inflammation increases the lesion size [32, 33], measurements of MMPs expression or activity have been the target point in research. MMP-9 is one of the most popular of these. The fact that MMP-9 levels were found to be lower in patients with AP compared to the control group in this study shows that MMP-9 is important in understanding the pathophysiology of AP.

In a study conducted by Tjäderhane et al. [32], it was suggested that MMPs may have anti-infective and/or anti-inflammatory effects in periapical inflammation. In the same study, it was reported that an increase in lesion size was due to MMP's anti-inflammatory effect [32]. In our study, the MMP-9 levels were found to be lower in patients with AP compared to the control group, which is in line with above mentioned study. In addition, the detection of an inverse relationship between MMP-9 and TNF-α levels, which is a proinflammatory marker, is also evidence of the anti-inflammatory role of MMP-9.

When the effect of the main bacterial species (S1-MBS-1st) determined from the samples taken from the intracanal and extraradicular regions of the tooth with AP on MMP-9 and TNF-α levels according to whether they are Gr (+) or Gr (-), MMP-9 and TNF-α levels of Gr (-) ones were not found to be different compared to the levels of the Gr (+) bacteria. This finding showed that the two biochemical markers were not predictive of the main bacterial species causing AP. The possible reason for this was attributed to the fact that MMP-9 and TNF-α are associated with inflammation, necrosis and regeneration in tissue, independent of the bacterial species causing the infection. One of the limitations of this study is that intracanal measurement of MMP-9 and TNF-α cannot be performed due to insufficient sample volume.

More than 700 bacterial species with different virulence reside in the oral cavity, the composition of which may vary depending on harmful habits, diet (sucrose consumption) and environmental conditions (such as pH). Only some of this large microbial community (such as Streptococcus, Fusobacterium, Lactobacillus and Treponema) have the potential to cause pathogenicity in periapical tissues [34, 35]. It should also be noted that different species in the same genus may have different virulence. For example, *Streptococus mutans*, *Fusobacterium nucleatum*, *Treponema denticola*, *Lactobacillus casei* and *Enterococcus faecalis* species have been reported to have higher pathogenicity in terms of causing infection in teeth and dental tissues compared to other species [35–37]. Furthermore, one of the main factors associated with apical periodontitis is the presence of microbial biofilms. These biofilms can be observed inside the apical portion of the root canal system, or attached to the extraradicular region [38, 39]. When the microbiota inside the root canal (S1) and extraradicular region (S2) was examined in this study, no significant difference was found between them in terms of the bacteria being Gr (+) or Gr (−). As a result, it was evaluated as evidence that Gr (+) and Gr (−) bacteria cause disease at similar rates. Common bacterial phylotypes were found to be similar in both groups, which is in line with previous studies [39, 40]. Because extraradicular microorganisms derive from inside the root canals, it is reasonable to surmise that intraradicular and extraradicular infections share a similar composition [40].

Lactobacillus species, *Streptococcus* species, *Enterobacter* species and *Sphingomonas* species, which cause AP and

are most isolated from the canal, are the types of bacteria. These results suggest a high bacterial diversity in endodontic infections. One of the interesting findings of our study was the presence of *Treponema* species only on the extraradicular region, suggesting that these bacteria could not settle in the pulp of the tooth. Some previous studies similarly showed presence of *Treponema* on extraradicular regions [41, 42]. The invasion capacity of *Treponema* [41], helps the microorganisms evade the extracellular clearance debilitating the innate immune system [43]. This mechanism is characteristic of periodontal disease and could justify the presence of these bacteria in the periapical tissues. It has been demonstrated that *Treponema* spp. presents an aggressive behavior and virulence besides being capable of inducing the proinflammatory response seen as connective and bone tissue destruction and pain through the production of H2S by glutathione metabolism and acute cytokine release [44].

As a result of the ROC analyzes performed to determine the diagnostic performance of MMP-9 and TNF-α tests, it was determined that both tests had an important diagnostic value, especially in MMP. However, since the sum of the sensitivity and specificity values of both MMP-9 and TNF-α biomarkers is less than 170, they are not sufficient alone in the diagnosis of AP [45]. However, using such parameters together with other data in clinical approaches will increase the effectiveness of diagnosis and treatment based on diagnosis. Therefore, we think that although the MMP-9 parameter does not have a high diagnostic power, it may be more useful together with other supportive clinical indicators.

Conclusions

The main bacterial species in the intracanal region of the tooth with AP are similar to the main bacterial species [Gr(−) and Gr(+) bacteria] in the extraradicular region. The pathophysiology of the tooth with AP is associated with low MMP-9 and high TNF-α, independent of the bacterial species in the intracanal and extraradicular regions.

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Data availability The datasets used and/or analyzed during the current investigation are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Ethical approval This research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008). All interventions were undertaken with the understanding and written consent of each participant and according to the above-mentioned principles. The study has been independently reviewed and approved by an ethical board.

Informed consent Informed consent was obtained from all individual participants included in the study.

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