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Microbial analysis of endodontic infections in teeth with post-treatment apical periodontitis before and after medication

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Abstract

This study aimed to determine the intraradicular microbiota of previously root canal-treated teeth with apical periodontitis and to investigate the antibacterial effectiveness of different intracanal medicaments. Sixteen patients with post-treatment apical periodontitis were allocated into two groups according to the intracanal medicament used: calcium hydroxide (CH) and 2% chlorhexidine gluconate gel (CHX) group. Total bacterial loads, as well as the amount of *Enterococcus faecalis* (*E. faecalis*) were determined before (S1) and after (S2) chemomechanical preparation and finally, after intracanal medication (S3) by means of ddPCR. The unpaired *t* test was used to compare parametric. S3-total bacteria copy number of the CH group was lower than the CHX group ($p < 0.05$). There was no statistical difference between the CHX- and the CH groups in terms of *E.faecalis* copy number ($p > 0.05$). But in terms of total bacteria, CH is better than CHX. Consequently, CH can be used to optimise the antibacterial efficiency of chemomechanical preparation in previously root canal-treated teeth with apical periodontitis.

KEYWORDS

chemomechanical preparation, droplet digital, *Enterococcus faecalis*, intracanal medicaments, microbiology, post-treatment apical periodontitis, total bacterial load

INTRODUCTION

Post-treatment apical periodontitis is an inflammatory process of the periradicular region caused by endodontic infection and is usually seen when treatment procedures have not reached a satisfactory standard for the control and elimination of infection. [1] It is primarily caused by bacteria persisting in or reinfecting the root canal system after initial endodontic therapy. [1] Therefore, the main microbiological goal of the endodontic treatment and re-treatment of teeth with apical periodontitis is to eradicate bacterial infection. [2] The main steps of the endodontic treatment responsible for antimicrobial control are the chemomechanical preparation and intracanal medication. Numerous studies have demonstrated that both steps are highly effective in controlling the root canal infection. [2,3] Intracanal medicaments have long been used to augment the chemomechanical procedure. Use of intracanal medicaments during the inter-appointment period has been widely used to reduce the remaining bacteria in the root canal system and to retard the growth of new bacteria. [2,3] But there are still cases in which bacteria survive the effects of these procedures and put the treatment outcome at risk. [4] Therefore, it is of utmost importance to disclose the main bacterial taxa that can endure the antibacterial steps to understand their role in treatment outcome and to set the grounds for establishment of more effective and predictable measures to deal with them.

Strategies to combat infection must be based on a thorough knowledge of the microflora. However, when the microbiota of the necrotic dental pulp was meticulously investigated [4], data on the intracanal microbiology of

failing root canal treatments are scarce. Furthermore, the great majority of these studies evaluating the antibacterial effects of endodontic retreatment procedures have been conducted by using culturing techniques, which are unable to detect many difficult-to-grow or uncultivable bacteria and have low diagnostic sensitivity. [5] Alternative methods for identification of intracanal bacteria, such as polymerase chain reaction (PCR) have been proposed which can overcome the aforementioned shortcomings. Few studies have used culture-independent molecular methods to overcome these shortcomings of conventional culture-dependent techniques in endodontic retreatment cases. [6] To date, culture methods have aided the disclosure of bacterial colonisation in 44 – 85% of the samples collected solely from reinfected root canals, [7] whereas molecular analysis has verified the presence of bacteria in 65–100% of root canal samples. [8] Recently a novel method of culture-independent molecular microbiology technique has become available. This is droplet digital PCR (ddPCR). ddPCR is a method for performing digital PCR that is based on water–oil emulsion droplet technology. A sample is fractionated into 20,000 droplets and PCR amplification of the template molecules occurs in each individual droplet. [9] ddPCR may facilitate noncoding RNA measurement, especially in liquid biopsy, since it has proved to be more sensitive, to offer highly reproducible results and to be less susceptible to inhibitors than conventional quantitative PCR (qPCR). [9] In comparison with qPCR, the ddPCR technique has some favourable features: (i) it performs absolute quantification based on the principles of sample partitioning in uniform droplets and Poisson statistics correction for multiple target molecules per droplet, thus overcoming the normalisation and calibrator issues; (ii) It has shown increased precision and robustness and sensitivity in detecting low target copies; (iii) it is relatively insensitive to potential PCR inhibitors; (iv) it measures the absolute number of microRNA copies per microliter of reaction, with confidence intervals, large dynamic range and a high throughput; (v) it is easy to use such as a conventional RT-qPCR; and (vi) it can show superior diagnostic performance than conventional RT-qPCR. [9,10]

So far, no study has used this technology to evaluate the bacteriologic conditions in root canal-treated teeth. Hence, the aim of this clinical study was to determine the intraradicular microbiota of previously root canal-treated teeth with apical periodontitis using ddPCR analysis and to investigate the antibacterial effectiveness of different intracanal medicaments [calcium hydroxide (CH) and 2% chlorhexidine gluconate gel (CHX)] in increasing the effectiveness of the chemomechanical preparation.

METHODS

Study design, settings and sampling

The study was designed as a parallel, randomised clinical trial. It was approved by the institutional review board/ethical committee in the Faculty of Dentistry –Istanbul Medipol University (#2018/265). The protocol was registered at www.clinicaltrials.gov (#NCT04978545). Recruitment and completion of the operative procedures for the study participants were done by the co-investigator (Y.E.H) from January 2019 to October 2019.

Eligibility criteria

The study population consisted of 16 patients (6 women and 10 men, aged 19–63 years, mean age 34.43) presenting to the endodontic clinic at Istanbul Medipol University Dental School, for nonsurgical endodontic retreatment of teeth with apical periodontitis lesions. Sixteen previously root canal-treated teeth exhibiting clinical and radiographic evidence of chronic apical periodontitis lesions were included in this study. Radiographically, the diameter of the periapical radiolucency ranged from 2 to 7 mm. Teeth with post-treatment apical periodontitis had endodontic therapy completed more than 2 years earlier and required retreatment. Termini of the root canal fillings ranged from 0 to 4 mm short of the radiographic apex, with no overfilling. [11] The teeth had intact coronal restorations, with no obvious exposure of the root filling material to the oral cavity. Selected teeth had enough crown structure for adequate isolation with a rubber dam and showed an absence of periodontal pockets or attachment level deeper than 4 mm. Exclusion criteria were also applied, as follows: teeth from patients who had received antibiotics within the previous 3 months or who had any general disease, teeth that could not be properly isolated with rubber dam, teeth with absence of coronary sealing, teeth with periodontal pocket depth >4 mm and teeth with crown/root fracture. Only one tooth was included from each patient.

Randomisation and blinding

A random sequence of numbers [1–16] was generated by the principal investigator (S.E.) using computer software (http://www.random.org/) and arranged in two equal columns according to the intracanal medicament (A and B). The numbered papers were packed in opaque envelopes. After completion of chemomechanical preparation, each

patient selected an envelope and was allocated either to the CH group (A) or the CHX group (B). The patients and outcome assessors were blinded to the assigned treatment group throughout the study. The procedures and the purpose of the study were explained to all patients. Informed consent was obtained from all patients before entering the study.

Root canal treatment procedures and sampling

Rubber dam and an aseptic technique were used throughout the endodontic retreatment. After plaque removal and rubber dam isolation, the operative field was cleaned with 3% hydrogen peroxide and disinfected with 2.5% NaOCl solution. Then, all coronal restorations, posts and carious defects were removed, and an access preparation was completed when the root canal filling 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. Sterility control samples (SR1) were taken from the tooth surface with a sterile Omni Swab (Whatman FTA, Sigma-Aldrich) with an ejectable head. Paper points were transferred to cryotubes containing phosphate-buffered saline (PBS) solution stored at −20°C. 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. Two of the canals included in this study were from single-rooted teeth, 3 were buccal canals in maxillary premolars, 1 palatal canal in maxillary molar, and 10 distal canals in mandibular molars.

Old root fillings were removed using Gates-Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland) and endodontic files without the use of chemical solvents. The working length (WL) was established 1-mm short of the apical foramen with an apex locator (Raypex6; VDW GmbH, Munich, Germany), and then periapical radiographs were taken to ensure that all filling material was removed. Irrigation with sterile saline solution was performed in order to remove any remaining materials and 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 minute. Both the paper points and the endodontic hand instrument, without the handle, were transferred to cryotubes containing 300 µl of PBS solution stored at −20°C. The samples were transferred to genetic analysis laboratory for further analysis in cold chain.

Root canals were prepared by using the Revo S files and irrigated with 2.5% NaOCl. The canals were apically enlarged to size 35 (AS35) at the working length. Between each instrument change, the root canal was irrigated with 5 ml of 2.5% NaOCl solution. Hence, a total of 30 ml of the irrigating solution was used. After instrumentation was completed, the smear layer was removed with 1 ml 17% EDTA, which was left in the canal for 3 min, followed by 2.5% NaOCl. The root canal was dried with sterile paper points and flushed with 2 ml of 10% sodium thiosulfate for 1 min to inactivate the NaOCl solution. Next, a sample (S2) was taken from the canals as described for S1. Before placing the intracanal medicament, the root canals were rinsed for the final time by using 2 mL of sterile saline for both groups (CH and CHX) to avoid the formation of a precipitate. Then, the canal was dried and medicated with either the $Ca(OH)_2$ or 2% chlorhexidine gel based on the study group. In the CH group, calcium hydroxide powder (Calxyl; OCO Products, Dirnstein, Germany) was mixed with saline in a ratio 1:1, and the paste was inserted into the canal by using lentulo spirals (Malleifer-Dentsply). In the CHX group, chlorhexidine gluconate gel 2% (Gluco-Chex 2% gel, Cerkamed, Stalowa Wola, Polland) was placed into the root canals of this group with an Ultradent Capillary Tip (Ultradent products). All root canals of the 2 above groups were sealed with a 1-mm cotton pellet and at least 3 mm layer of temporary filling material (Cavit G; 3 M ESPE AG, Seefeld, Germany).

After 7 days, the tooth was isolated, the temporary restoration was removed, and disinfection procedures of the operative field were performed following the same protocol used in the first visit. A new control sample of the dental crown and dentin surrounding the pulp chamber was obtained (SR2). The medication was removed with 5 ml of saline solution and by carefully filing the canal with a master apical file. For the groups using $Ca(OH)_2$, the calcium hydroxide's antimicrobial activity was neutralised with 0.5% citric acid for a period of 1 min, which was then removed with 5 ml of saline solution. Thus, for the groups with CHX gel, the medicament was neutralised with a mixture of 0.3% L-α-lecithin and 3% Tween 80. Next, the root canals were irrigated with 2 ml of saline solution. The post medicament sample (S3) was obtained in the same way as the pre-medicament sample was collected and sent for the PCR analysis.

After the final sampling procedure, smear layer was removed by irrigation with 5 ml 17% EDTA and then, a

final rinse with 10 ml distilled water. Completion of the root canal treatment proceeded with root filling using lateral condensation of gutta-percha. Access cavities were restored with composite resin (Z250, 3 M Corporation, Saint Paul, MN, USA), and a final radiograph was taken.

Total bacterial loads, as well as the amount of *Enterococcus faecalis* were determined before instrumentation, after instrumentation and use of the intracanal medicaments, by means of ddPCR.

Genomic DNA isolation and measurement of DNA concentration

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) following the protocol recommended by the manufacturer. [12] Before DNA isolation, samples (the tubes with paper points) were digested at 50–60°C BY vortexING for 30s every 10 min in order to ensure disaggregation of all bacteria into the PBS solution. Afterwards, the paper points were aseptically removed from the suspension and the bacterial suspension was pelleted by centrifugation for 10 min at 5000 g. The pellet was then resuspended in 180 μl buffer ATL supplied by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) and 20 μl proteinase K (20 mg/ml) was added. Samples were incubated for 3 h at 56°C. Subsequently, total bacterial genomic DNA was isolated according to the protocol of the QIAamp DNA Mini Kit. The final volume of DNA solution of each sample was 150 μl and was taken into account during calculation. DNA concentration (absorbance at 260 nm) was determined with a spectrophotometer (Promega Quantifluor).

Amplification of 16S rRNA genes

Primers for Universal and Enterococcus 16S rRNA genes were designed in this study. After DNA extraction of samples with QIAamp DNA Mini Kit, 700–800 bp of 16S rRNA sequences were amplified by using universal E8F forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal E1115R reverse primer (5′-AGGGTTGCGCTCGTTG-3′) 59. The final volume of PCR reactions for each isolated bacterial strain was adjusted to 25 μl. The amplification reactions of 16S rRNA genes were performed with the following conditions. 1 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 140V for 20 minutes). Their images were visualised under ultraviolet illumination. In addition, the control and optimisation of primers to be used for ddPCR was also done in conventional PCR.

Purification and sequencing of the 16S rRNA gene

After the PCR reactions, the purification of PCR products is done by hydrolysing the excess primers and nucleotides with ExoSap-IT (Thermo, PN: 78201.1.ML) containing Exonuclease I and Alkaline Phosphatase enzymes. 2 μl of ExoSap-IT was mixed with 5 μl 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 by using Bigdye™ Terminator v3.1 cycle sequencing kit (Thermo). The reactions were performed according to the kit manual for all isolated strains.

After purification of the products with Exosap, the sequence reaction was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo) under the following conditions. After the sequence PCR, BigDye products were purified by 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 analyzer.

Droplet digital PCR (ddPCR)

Droplet Digital PCR (ddPCR) was performed using primers designed according to the 16S rRNA region specific to the total bacteria and *Enterococcus faecalis* species, after sequencing, absolute quantitation from the bacterial species found in the paper-point sample. Primer pairs were 16S-F-5′-AGGGAATCTTCSGCAATGGG-3′ and 16S-R-5′-ACGCCCAATAAATCCGGACA-3′ for total bacteria and ENT-F-5′-CGCTTCTTTCCTCCCGAGT-3′ and ENT-R-5′-GCCATGCGGCATAAACTG-3′ primer pairs for *E. faecalis*. In the PCR reaction, amplicons amplified with unmarked primer pairs were analysed by labelling with Eva-Green dye. For absolute quantitation of *Enterococcus* and total 16S rRNA, PCR was performed with two primer pairs from the same sample. 20 μl of PCR mix containing 10 μl of 2X ddPCR EvaGreen Supermix (Bio-Rad, cat. no. 1864034), 9 μl of nuclease-free water, 0.25 μl of both forward and reverse primer and 2 ng of DNA from each sample Thermal cycling conditions were: 95°C for 5 min, then 40 cycles of 95°C for 30 s and 60°C for 1 min and two final steps at 4°C for 5 min and 90°C for 5 min with a 4°C

infinite hold. After PCR was completed, the sealed plate was transferred into the plate holder of the QX200 Droplet Reader (Bio-Rad, cat. no. 1864003).

Statistical analysis

Data analysis was performed with IBM SPSS Statistics 25 IBM (Corp, Chicago, USA), InStat3 GraphPad Statistics and MedCalc version 15.8 Software. The unpaired *t* test was used to compare parametric data of independent groups with two groups in the study and the Mann–Whitney *U* test was used to compare nonparametric data. In order to examine whether there is a relationship between parameters, Pearson correlation analysis was used for data showing parametric distribution in statistical analysis and Spearman correlation analysis for nonparametric data.

RESULTS

All 16 patients were included in the statistical analysis; the process of patient enrolment and progress through each phase of the trial is shown in Figure 1. Two teeth were single-rooted and 14 were multirooted. Six patients presented for treatment with acute pain. The remainder had no spontaneous pain, except for 2 patients who gave a history of previous pain. Tenderness to percussion was present in 8 teeth and a sinus tract was detected in one patient. Sixteen teeth were restored with a permanent coronal restoration. Previous gutta-percha fillings were present in all teeth. Root canal fillings were short from the radiographic apex in all teeth. Termini of the previous root canal fillings ranged from being 2 to 4 mm short of radiographic apex in most of the included cases (3 mm short in 4 cases and 4 mm short in 6 cases). The remaining 6 cases had root canal fillings 2 mm short of the apex. Upon radiographic examination, 6 teeth had well-condensed root fillings, whilst 10 had poorly obturated canals.

Sanger sequencing was performed for the determination of microbiota in samples taken from the patients. The PCR reaction was set up with primer pairs specific for the 16S rRNA gene. Agarose gel electrophoresis was performed, and the bands were checked in order to see whether the target regions of the reaction were performed under appropriate conditions and to see if they have successfully grown. The 16S rRNA gene sequences were obtained as a result of sequencing and were carried out by BLAST analysis to determine the most similar type of bacteria and also 4 different species with less similarity. The most similar bacterial species are shown in Table 1. In S1 samples, the most abundant genera were *Enterococcus* (37.5%) occurring in 6 of 16 samples, *Streptococcus* (25%) in 4 of 16, *Enterobacter* (18.7%) in 3 of 16, *Eubacterium* (6.2%) in 1 of 16, *Lactobacillus* (6.2%) in 1 of 16 and *Bacillus* (6.2%) in 1 of 16 (Table 1).

Absolute quantitation of total bacteria and 16S rRNA gene primer pairs specific to *E. faecalis* were performed in S1, S2 and S3 samples of the patients using the ddPCR method. The ddPCR reaction was compared in terms of the copy number of both populations in the samples by placing equal nanograms of Genomic material. Thanks to the sensitivity of the ddPCR method, clearer and more consistent results were obtained. The quantitation results are shown in the table below (Table 2).

When the groups were examined in terms of gender, age, number of root canals (NRC), number of patients with symptoms (NPS), SR-1 sterility control total bacteria (SR1-SCTB), SR-2 sterility control total bacteria (SR2-SCTB), S1-droplet (S1-total bacteria copy number) and S2-droplet (S2-total bacteria copy number), it was found that there was no statistical difference between the CHX group and the CH group ($p > 0.05$) (Table 2). The Periapical Index (PAI) score of the CHX group was found to be lower compared with the CH group ($p < 0.05$) (Figure 2). On the contrary, the S3-droplet (S3-total bacteria copy number) of the CHX group was higher than the CH group ($p < 0.05$) (Figure 3). These findings show us that although the PAI score is worse, good results can still be obtained even in patients in the CH group (Figure 4).

There was no statistically significant correlation between the number of root canals, the PAI score, and the S3-droplet count (Spearman r = 0.1467, 95% CI = −0.3902 to 0.6091; $p = 0.5878$ and Spearman r = −0.2326, 95% CI = −0.6622 to, respectively. 0.3121; $p = 0.3861$). Similarly, there was no significant correlation between the PAI score and the S3-droplet copy number (Spearman r = −0.2326, 95% CI = −0.6622 to 0.3121; $p = 0.3861$).

When the groups were examined in terms of S1-ECN (S1- *E. faecalis* copy number), S2-ECN (S2- *E. faecalis* copy number) and S3-ECN (S3- *E. faecalis* copy number), it was found that there was no statistical difference between the CHX group and the CH group ($p > 0.05$) (Table 3).

DISCUSSION

Since it has been reported in previous studies that classical chemomechanical preparation alone is not sufficient, the present study aimed to investigate the antibacterial effects of different intracanal medicaments that will improve the efficiency of classical chemomechanical preparation. The effect of the intracanal medicaments on total bacteria and *E. faecalis* in the microbiota was determined and quantitated by the ddPCR method. Whether or not medicaments

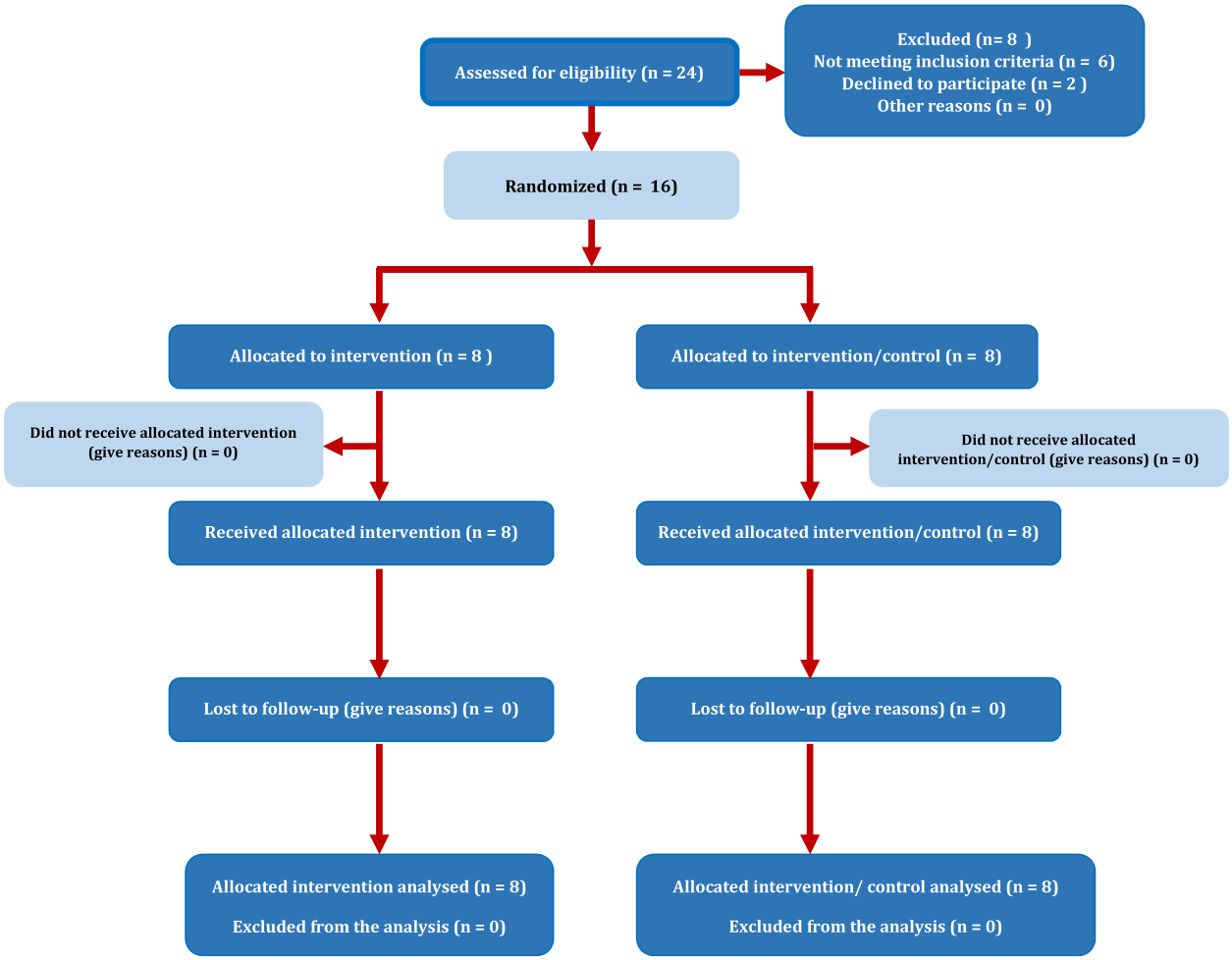

FIGURE 1 Consolidated Standards of Reporting Trials (CONSORT) flow chart

with different properties showed a different efficacy in their use in S3 was compared. This study also aimed to investigate the intraradicular microbiota of previously root canal-treated teeth with apical periodontitis.

First, the sequence analysis of 16S rRNA genes was performed by the Sanger sequencing method for the determination of microbiota in S1, S2 and S3 samples taken from patients, and the most common bacterial species in microbiota were determined by the BLAST analysis. According to the results of the BLAST analysis, the most common bacterial species found were *Enterococcus* and *Streptococcus*. This finding shows that these two species are the most frequently isolated species from the failed root canals, which is in agreement with earlier studies. [13,14] In the present study, the effect of the intracanal medicaments on total bacteria and *E. faecalis* in the microbiota was determined. *E. faecalis* was selected as the test microorganism because it shows resistance to elimination from the root canal and is also associated with the etiopathogenesis of persistent apical periodontitis. [9,13,14] In this study, we calculated the amount of *E. faecalis* in S1, S2 and S3 samples and it seems that this pathogenic organism decreased in two groups as expected.

Culture-based methods have been useful in identifying the bacteria or bacterial combinations that are important in various types of root canal infection. [15] However, these methods have obvious limitations such as low sensitivity, and they are time-consuming and laborious. [6] Furthermore, extensive expertise is needed to identify certain bacteria. [6] Therefore, it appears that culture-based methods can underestimate the diversity of endodontic microbiota.

In this study, the quantitation of total bacteria and *E. faecalis* in 5 different samples (SR1, S1, S2, SR2 and S3) taken from teeth were measured using the ddPCR absolute quantitation method, which is a new and highly sensitive method to accurately quantify copy number alterations in genomic DNA recovered from tissue samples. [9] In order to make a meaningful comparison, the amount of samples put into the PCR must be equal. Therefore, the DNA concentration of each sample was measured and all of them were added to the reaction at 1 ng/μl. Patients with a high rate of total bacteria and *E. faecalis* in S1 samples caused

TABLE 1 Prevalence of bacteria from the root canals evaluated. Prevalence of main bacterial species in S1, S2 and S3 samples in all 16 patients

	CHX Group		
Patient no	**S1**	**S2**	**S3**
1	Enterococcus Faecalis, 99.39%	Enterococcus Faecalis, 98.15%	Enterococcus Faecalis, 99.37%
2	Enterobacter Species, 97.66%	Uncultured Organism, 95.12%	Enterobacteriaceae Bacterium, 99.09%
3	Enterobacteriaceae Species, 96.41%	Lactobacillus Rhamnosus, 94.98%	Streptococcus Mitis, 93.78%
4	Enterococcus Species, 95.94%	Enterococcus Italicus, 94.93%	Enterococcus Casseliflavus, 93.24%
5	Uncultured Enterococcus Sp., 92.87%	Enterococcus Species, 93.17%	Enterococcus Faecali, 92.13%
6	Streptococcus Oralis, 90.74%	Uncultured Enterococcus Species, 92.82%	Enterococcus Gallinarum, 91.44%
7	Uncultured Eubacterium Species, 85.60%	Uncultured Streptococcus Species, 81.63%	Streptococcus Australis, 90.74%
8	Streptococcus Oralis, 81.21%	Neisseria Flavescens, 75.16%	Streptococcus Species, 78.52%
Patient no		**CH Group**	
	S1	**S2**	**S3**
1	Enterococcus Faecium, 97.82%	Streptococcus Species, 94.40%	Enterococcus Faecium, 99.84%
2	Bacillus Species, 95.45%	Enterococcus Italicus, 94.00%	Fusobacterium Nucleatum, 97.20%
3	Enterococcus Faecalis, 94.70%	Streptococcus Australis, 93.71%	Streptococcus Oralis, 95.85%
4	Streptococcus Gallinaceus, 92.59%	Enterococcus Hermanniensis, 92.05%	Enterococcus Italicus, 95.48%
5	Lactobacillus Acidophilus, 92.38%	Enterococcus Species, 91.93%	Streptococcus Species, 92.96%
6	Uncultured Streptococcus, 87.99%	Lactobacillus Ultunensis, 91.60%	Enterococcus Italicus, 91.71%
7	Enterobacter Species, 84.97%	Streptococcus Pneumoniae, 83.08%	Streptococcus Oralis, 78.94%
8	Enterococcus Italicus, 76.16%	Streptococcus Marmotae, 71.81%	Streptococcus Mitis, 69.69%

a complete or very high reduction in total bacteria and *E. faecalis* in S2 and S3 samples after irrigation and medication procedures. With ddPCR, which is a much more sensitive method compared with cell count and qPCR methods, clearer and more consistent results were obtained with a copy number. However, concern has been raised over the inability of the molecular methods to differentiate between viable and nonviable organisms. Particularly with the use of PCR-based methods, the amplified copies of DNA from dead bacteria may lead to exaggerated conclusions. [16] Although the detection of dead cells can be an advantage in certain studies, it poses a major problem when one is investigating the immediate effectiveness of antimicrobial treatment [6]. Another limitation of the present study was its small sample size. Only a limited number of tests could be carried out due to the high expenses of the molecular analysis. In addition, patients who allowed CH and CHX applications were included in the study. Further research will be conducted with larger samples in the light of this first study.

All S2 samples taken after chemomechanical preparation with NaOCl showed a significant reduction in the levels of total bacteria and *E. faecalis* during retreatment. This is in agreement with several previous studies that have shown the effectiveness of preparation procedures in reducing bacterial populations from root canal-treated teeth. [17] The choice of using 2.5% NaOCl in our study was based on the fact that no significant differences in the intracanal antibacterial effects have been observed when comparing it with the higher concentrations. [18] Copious irrigation with NaOCl may maintain a chlorine reserve that is sufficient to eliminate bacterial cells and compensate for the effect of concentration. [18]

The mean number of total bacteria copy number in canals after 1-week medication with CHX were higher than after instrumentation and irrigation with NaOCl, but lower than before treatment. It appears that 1-week medication with CHX in a canal after instrumentation and irrigation may maintain, at most, the endodontic environment in a relative 'stable' status during the interappointment period. CHX could not further disinfect canals. Therefore, the clinical procedure of 1-week medication with CHX may not be very successful, comparing with instrumentation and irrigation.

For the CH group, total bacteria were found to be less at S3 than that of the CHX group. Although the mean PAI

TABLE 2 Distribution of Patients' Characteristics and Comparison of data between groups

	All patients	Patients treated with Chlorhexidine (CHX Group)	Patients treated with Calcium hydroxide (CH Group)	*p* values
N	16	8	8	-
Gender, F (%)	3 (37.5%)	3 (37.5%)	3 (37.5%)	[a]0.9569
Age, year	35 ± 13 35 (19–75)	36 ± 18 30 (19–75)	35 ± 8 35 (27–50)	[a]0.4621
NRC, *n*	3 ± 1 3 (1–4)	2 ± 1.1 2.5 (1–4)	3 ± 0.7 3.5 (2–4)	[a]0.0716
NPS, *n* (%)	3 (37.5)	2 (25)	4 (50)	[a]0.4198
PAI Score, *n*	4 ± 0.9 4 (3–5)	4 ± 0.7 3.5 (3–5)	5 ± 0.7 5 (3–5)	[a]0.0342
SR1- SCTB, *n*	18 ± 5.2 18 (11–27)	17 ± 5.1 17.5 (11–27)	18 ± 5.7 17.5 (11–27)	[a]0.1724
SR2- SCTB, *n*	21 ± 11.8 20 (6–60)	20 ± 16.8 15.5 (6–60)	21.3 ± 3.9 21.5 (14–27)	[a]0.0657
S1-Droplet number/nl	41 510 ± 47 337 19 430 (30–146 200)	22216 ± 32 888 14200 (30–101 000)	60805 ± 53564 62 720 (2156–146 200)	[a]0.2345
S2-Droplet number/nl	8209 ± 9361 5010 (238–38 740)	6324 ± 4656 5010 (968–15580)	10 094 ± 12 569 7090 (238–38 740)	[a]0.8785
S3-Droplet number/nl	13333 ± 12 467 9060 (620–40 500)	19 330 ± 13 472 16 760 (4900–40 500)	7335 ± 8328 2927 (620–23 460)	[a]0.0499
S1-ECN/nl	7603 ± 14 354 311 (0–55 960)	7072 ± 19 755 2.9 (0–55 960)	8133 ± 7115 6660 (2.4–18 800)	[a]0.0650
S2-ECN/nl	75 ± 190 4 (0–756)	38 ± 70 4.8 (0–196)	113 ± 263 4.4 (0–756)	[a]0.9581
S3-ECN/nl	70 ± 218 4 (0–882)	125 ± 308 1.5 (0–882)	14 ± 16 8.1 (0–42)	[a]0.7129

Abbreviations: F, female; NRC, number of root canals, NPS, number of patients with symptoms; SR1-SCTB, SR1-sterility control total bacteria; SR2-SCTB, SR2-sterility control total bacteria; S1-droplet, S1-total bacteria copy number (in 20 nanolitre: nl); S1-ECN: *E.faecalis* copy number in S1; S2-droplet: S2-total bacteria copy number (in 20 nanolitre: nl); S2-ECN, *E.faecalis* copy number in S2; S3-droplet, S3-total bacteria copy number (in 20 nanolitre: nl); S3-ECN, *E.faecalis* copy number in S3.

[a]Mann–Whitney test. Nonparametric data are given as mean, standard deviation and median (min-max). If *p* value is less than 0.005, the difference is significant.

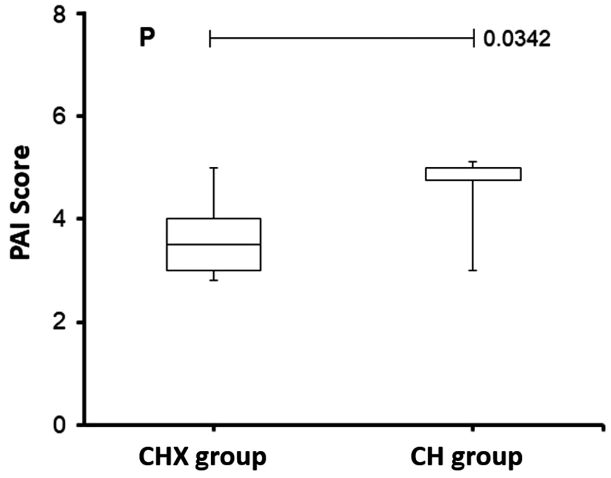

FIGURE 2 Evaluation of groups in terms of PAI score. CH group had a significantly higher PAI score. Mann–Whitney *U* test was used for statistical comparison

values of the patients in the CH group were higher than that of the CHX group, CH was found to be more effective against the total bacteria. Despite high PAI values, CH provided a more effective disinfection than CHX. Although mechanical instrumentation with NaOCl irrigation led to a considerable reduction in bacterial load, complementing conventional endodontic treatment with CH appears to be very effective in reducing the complete microbiota. Most studies showed a decrease in the number of positive cases from S2 to S3, [19,20] which is in agreement with the present study.

After the quantitation experiments, the results were compared with the sequence analysis results. For most of the samples, the quantitation and sequence results turned out to be perfectly consistent. *E. faecalis* was not found in the Sequence analysis results of S1, S2 and S3 samples of 3 patients (2in CHX and 1 in CH groups), and the ddPCR quantitation result was found to be zero or very close to

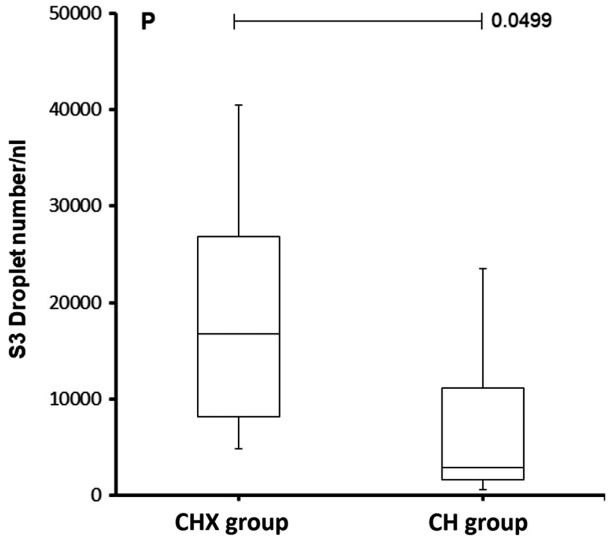

FIGURE 3 Evaluation of the groups in terms of the number of S3 droplets. It was seen that the number of S3 droplets of CHX is higher than that of group CH. Mann–Whitney *U* test was used for statistical comparison

zero as expected. On the other hand, ddPCR quantitation was made in S1 samples of 2 patients (in CH group) who did not show similarity to *E. faecalis* in the sequence analysis. In the samples of these 2 patients, the total bacterial count was very high, and the BLAST analysis showed that streptococcus species was the mostly seen bacteria type. The reason for not finding similarity with *E. faecalis* as a result of the sequence is because of the change in the sequence order, which increases due to the density and excess of bacteria. Furthermore, although there are different enterococcal species for some samples, the results of quantitation showed that *E. faecalis* was not found. The reason for this is that the microbiota, which varies from sample to sample and from patient to patient, increases the difference in sequence analysis. It can be also due to the primer usage, which is designed especially for *E.* faecalis. [13]

Although changes in the copy number of *E. faecalis* in the S2 and S3 samples were similar in most patients, these ddPCR results show that there were some differences. For example, whilst some patients had an increase in the *E. faecalis* copy number, a serious decrease in the *E. faecalis* copy number was also observed. By way of illustration, there was a 4-fold increase from S2 to S3 in one patient in the CHX group and a 10-fold increase from S2 to S3 in one patient in the CH group. On the other hand, from S2 to S3, one patient had an 18-fold decrease in the CH group and a 3-fold decrease in the other. There was also a 4-fold decrease in one patient in the CHX group. The reason for this may be that the methods used differ from patient to patient or because of the differences in microbiota. As a result, dividing the copy number of *E. faecalis* with the copy number of the total bacteria yielded a decrease in percentage in the majority of patients.

No difference was found between the CHX and CH groups in terms of the *E. faecalis* copy number in S1 and S2. This situation shows that the distribution of the groups is similar. Similarly, no significant difference was found between the two groups in terms of the *E. faecalis* copy number at S3. This result shows us that CHX and CH are similar in terms of efficacy against *E. faecalis*. When we interpret this result together with the results of the total bacterial activity, we conclude that the use of CH will be better.

Our findings are in accordance with several studies that also show that CH is effective in killing the majority of bacteria in the root canal system. [19,20] However, some controversies exist about its effectiveness against *E. faecalis*. [21] A few number of in vitro studies showed the effectiveness of CH paste against *E.* faecalis. [20,22] On the other hand, several previous studies have also shown that *E. faecalis* in the dentine was not affected by CH, [21,23] which is in line with the results of this study. Mechanisms involved in the resistance of *E. faecalis* to CH can be explained by several mechanisms, including the buffering capacity of dentin, neutralisation of the medication by bacterial by-products, localisation of bacteria in inaccessible areas of the canal, insufficient medication time, intrinsic bacterial resistance to the medications or alteration of bacterial gene expression that allows them to survive the environmental changes. [19,20] However, it is inappropriate to directly relate these *in vitro* findings to our *in vivo* data. Be that as it may, it could still be argued that the PCR findings of our study do not necessarily testify to the presence of viable bacteria in post-treatment canals, and they may reflect molecular traces of remnants of the nonviable organisms that colonised the canals.

CHX's gel formulation was preferred as an intracanal medicament in this study because of its low toxicity on periapical tissues, solubility in water, viscosity that keeps the active agent in contact with the root canal walls and prolonged antimicrobial activity. [24] Studies have shown that chlorhexidine alone, although it has substantivity, is not a good microbial barrier. [25] A clinical study evaluating the antimicrobial effectiveness of medication with 1% CHX gel for 2 weeks found 1% CHX gel ineffective in completely eliminating aerobic and facultative anaerobic microorganisms. [25] However, another study found that medication with 1% CHX gel for a week caused a considerable amount of microbial load reduction (82.17% reduction in colony-forming units). [26] Due to these conflicting results, the present study evaluated CHX's antibacterial effectiveness with a highly sensitive molecular method and it was found that the total bacteria copy number in the canals after 1 week of medication with 2% CHX gel

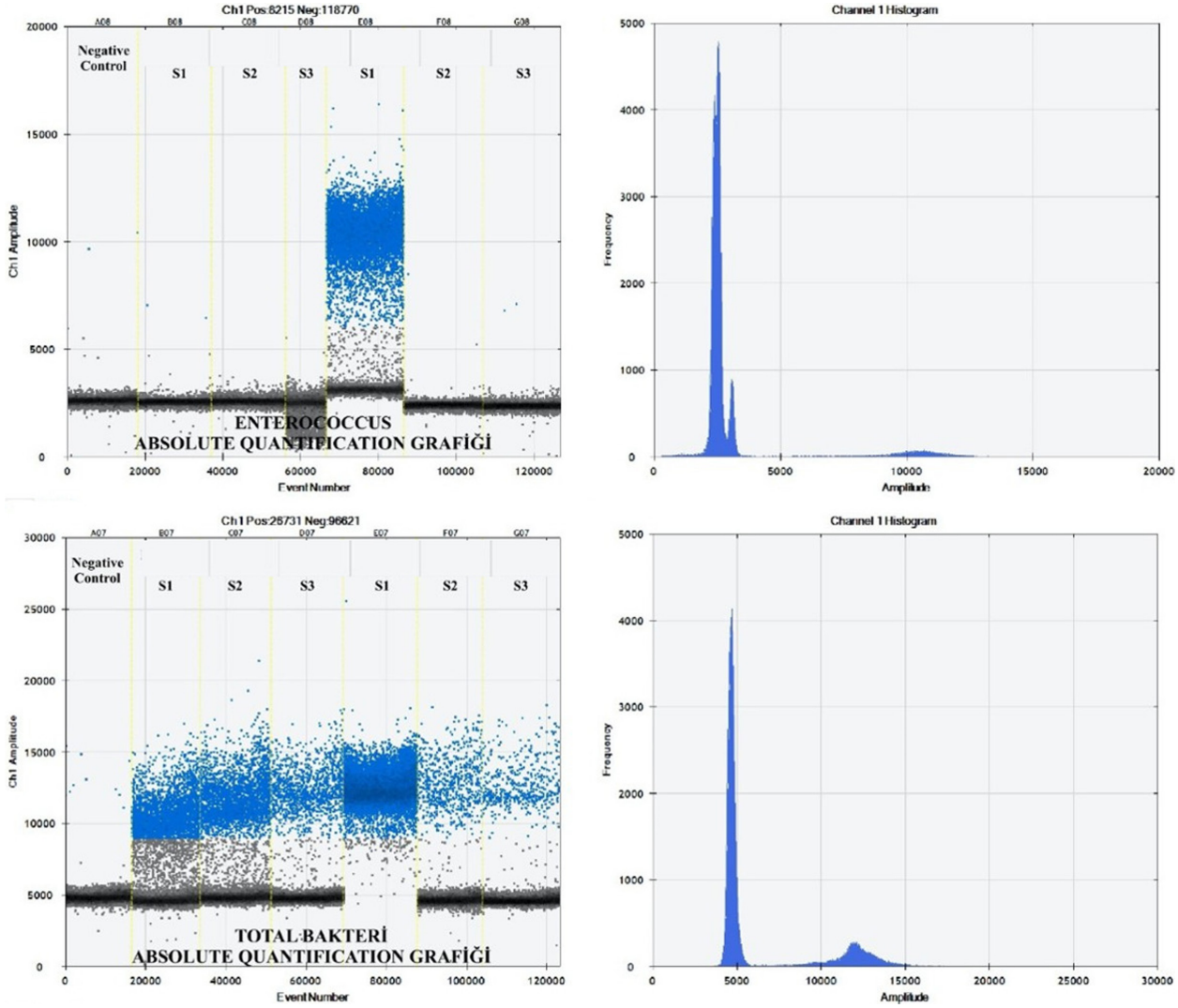

FIGURE 4 Absolute quantification graphics for both total bacteria and *E.faecalis*

(S3) was higher than after chemoinstrumentation at S2. Furthermore, CHX was also found ineffective against *E. faecalis*. Culture-based studies have shown CHX's high antimicrobial efficiency to eliminate *E. faecalis*. [23] However, when evaluating its presence by means of the SYTO 9/propidium iodide technique, CHX does not seem to be able to kill 100% of common oral bacteria including *E. faecalis*. [27] Moreover, a recent study [28] showed that the number of viable E. faecalis found after a 30-day exposure to CHX was similar to the number of viable bacteria in the saline solution group. Those observations are corroborated with our findings. Therefore, it can be hypothesised that the dead cells or the exopolymeric matrix of an aged biofilm might have neutralising substances. [29]

Analyses of reductions in total bacteria copy number levels demonstrated that chemomechanical preparation with NaOCl as the irrigant was highly effective. Total bacteria copy number in CH group further reduced after medication with CH. However, this number increased in CHX group when compared to chemomechanical procedures. There was a significant reduction in the copy number of *E. faecalis* from the pre instrumentation to the post-instrumentation samples in both the experimental groups thus demonstrating the antimicrobial efficacy of NaOCl 2.5% against *E. faecalis*. This was in accordance with the earlier studies that have proven NaOCl significantly effective against *E. faecalis*. [8,30] Moreover, the copy number of *E. faecalis* did not further reduce after both medications. No difference was observed when comparing CH and CHX either with regard to *E. faecalis* copy number in S3. However, all cases still yielded a small amount of *E. faecalis* droplets both after chemomechanical instrumentation and medication. This does not mean the persistence of *E. faecalis*. This can be explained by the limitation of the DNA-based methods inability to distinguish between live and dead cells, which may result in an overestimation of bacterial targets in root canals, especially in post-treatment samples.

The present study confirms the polymicrobial nature of endodontic infections. Early studies attempted to correlate some bacterial species with symptoms, [31] but it has been demonstrated that the same species can also be found in asymptomatic cases, even in higher prevalence. [6] Similarly, the same species were highly prevalent in nearly all clinical conditions evaluated in this study, and none of these most prevalent taxa were positively associated with symptoms. Furthermore, none of the 16 teeth where Enterococcus spp and Streptococcus spp were mainly present, had a flare-up at the second visit. Only one patient had tenderness to percussion and moderate pain at the 2nd visit, in which *F. nucleatum*, known for its pathogenic potential and isolation from symptomatic cases, was found in the canal. [31] Therefore, it seems that factors other than the presence of a given putative pathogenic species might influence the development of symptoms. [32,33] These factors include the presence of unequally virulent clonal types of the same species, microbial synergism or antagonism in the mixed root canal bacterial community (which can also influence virulence), number of microbial cells, host resistance to various infections, concomitant herpesvirus infection, etc. [32,33] Further studies are required to investigate whether these more virulent communities develop right from the beginning of the infection process or are a result of a shift in the community composition because of an environmental change which remains to be determined.

There is a need for clinical longitudinal molecular studies to investigate a possible causal relationship between any specific bacterial taxa persisting after endodontic treatment and treatment failures. So far, results from culture studies have shown that nonspecific bacterial presence at the time of root canal filling is considered a risk factor. [34] In the present study, the bacterial diversity was substantially reduced after irrigation except for few taxa such as *Streptococcus*, *Enterococcus* and *Fusobacterium* species. Although these taxa persisted after chemomechanical preparation and intracanal medication, we found that the microbial load decreased significantly, and no clinical symptoms were present on the 2nd visit. This situation can be explained as even the DNA from dead bacteria that cannot be detected by other serological methods (e.g. ELISA assay) was detected by a highly sensitive nucleic acid detection method ddPCR, used in this study. [35] Our findings corroborate previous data that showed that DNA may persist in infected teeth even after bacterial death. [35] Considering the results obtained for total bacteria copy number, the amount of dead bacteria that remained after chemomechanical preparation appeared to be very small. The possibility that these taxa may influence the outcome of retreatment cannot be discarded, and future studies should address this. Other less prevalent taxa enduring chemomechanical preparation were also identified in both CH-treated and CHX-treated teeth. This calls for a need to improve disinfection before placing root canal filling.

CONCLUSIONS

The present study showed that *Enterococcus* and *Streptococcus* species were the most frequently isolated species in teeth with post-treatment apical periodontitis. Mechanical instrumentation with NaOCl lead to a considerable reduction in bacterial load. For the CHX group, its placement in S3 did not provide any superiority in terms of total bacterial reduction during chemomechanical preparation. However, complementing conventional endodontic treatment with CH appears to be very effective in reducing the complete microbiota. On the other hand, intracanal medication with either CH or CHX did not efficiently eliminate *E. faecalis* from infected root canals. But in terms of total bacteria, CH shows superiority. The decrease in bacterial prevalence from S2 to S3 in the overall sample highlights the importance of using CH after chemomechanical procedures to predictably control root canal infection.

CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

The study was conceived and designed by YEH and SE. YEH clinically carried out the experiments. SE did the statistical analyses. SE and YEH prepared the manuscript and reviewed together.

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