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Multispecies biofilm removal by a multisonic irrigation system in mandibular molars

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Abstract

Aim: The aim of the study was to assess biofilm removal efficacy of GentleWave System and passive ultrasonic irrigation (PUI).

Methodology: Twenty-two human mandibular molars with Vertucci's type II configuration in the mesial root were selected. Teeth were autoclaved, inoculated with dental plaque and incubated in a CDC biofilm reactor for two weeks. The mesial roots were instrumented up to 20.06 file (V-Taper) for the GentleWave group and up to 35.04 file (Vortex Blue) for PUI group. Irrigation was performed using GentleWave and PUI irrigation protocols ($n = 11$). Dentine debris on paper points samples were obtained for quantitative real-time polymerase chain reaction (qPCR) and 16S ribosomal RNA gene sequencing (next-generation aequencing—NGS). For qPCR, a non-parametric test ($\alpha = 0.05$) was used. Next-generation sequencing data were analysed using mothur, with alpha diversity calculated as the Shannon and Chao1 indices and Bray–Curtis dissimilarities were used for beta diversity. Differences in alpha diversity and abundances of genera were evaluated using Kruskal–Wallis test. Differences in community composition were evaluated using analysis of similarity with Bonferroni correction for multiple comparisons.

Results: Quantitative real-time polymerase chain reaction results showed that the reduction estimated in percentages for both groups was equivalent ($p > .05$). NGS analysis showed that both techniques promoted a significant reduction in reads and OTUs number ($p < .05$). Shannon alpha diversity and Chao1 index showed no differences between pre- or post-treatment samples for both groups ($p > .05$). Additionally, pre-treatment communities differed from post-treatment samples in both groups regarding bacterial taxa reduction (ANOSIM R = 0.50 and 0.55, $p < .001$).

Conclusions: Bacterial reduction in mesial roots of mandibular molars prepared to 35.04 with PUI was similar to those prepared to 20.06 with a multisonic irrigant activation system.

KEYWORDS
biofilms, GentleWave, microbiome, next-generation sequencing, ultrasonic irrigation

INTRODUCTION

The main goal of endodontic treatment is to promote the healing of periapical tissues affected by apical periodontitis. To accomplish this goal, the operator needs to eradicate or at least reduce the bacterial concentration in the root canal system (Siqueira & Rôças, 2022a, 2022b). However, disinfection may be challenging when bacteria are organized in multispecies matrix-enclosed communities called biofilms, especially in teeth with complex anatomies. These bacterial structures can colonize the canal walls, ramifications and isthmuses (Ricucci & Siqueira, 2010).

Traditionally, culture methods have been used to assess the bacterial composition and decontamination of the root canal system (Siqueira & Rôças, 2022a). This method allows a semi- or absolute quantification of culturable bacteria. However, a significant amount of microorganisms in the root canal space cannot be cultured under laboratory conditions. The development of The Human Genome Project (Venter et al., 2001) allowed the subsequent development of databases (i.e., SILVA) for use in conjunction with next-generation sequencing (NGS) technologies (van Dijk et al., 2018; Zhong et al., 2021). NGS is a fifth-generation laboratory tool of microbiological analysis for the study of endodontic infections. This method provides vast information about bacterial communities and their profiles (Manoil et al., 2020; Siqueira & Rôças, 2022a).

The introduction of NGS brought new knowledge related to the composition of secondary endodontic infections. During the last 2 decades culture and closed-end methods such as PCR showed that *Enterococcus faecalis* was the most common bacteria associated with secondary or persistent endodontic infections (Molander et al., 1998; Pinheiro et al., 2003; Rôças et al., 2004; Siqueira & Rôças, 2004; Sundqvist et al., 1998). However, this knowledge has been challenged by recent studies that revealed a complex bacterial composition present in failed cases (Anderson et al., 2013; Bouillaguet et al., 2018; Keskin et al., 2017; Sánchez-Sanhueza et al., 2018; Siqueira et al., 2016; Tzanetakis et al., 2015). Given the complex microbial composition of secondary root canal infections, it is necessary to develop novel tooth models inoculated with multispecies biofilm, in order to challenge endodontic disinfection procedures.

Recently, a new system that combines multisonic and negative apical pressure (GentleWave, Sonendo Inc.) was introduced for cleaning and disinfecting root canals (Haapasalo et al., 2014) using minimal preparation sizes, i.e., using small files with sizes #15 or #20 tip (Coaguila-Llerena et al., 2022). This system creates hydrodynamic cavitation in the root canal space, and the implosion of microbubbles creates an acoustic field of broadband frequencies that travels through the fluid to the entire root canal system (Sigurdsson et al., 2018). To date, the decontamination efficacy of infected root canals irrigated with this method in molars has not been proved using a relevant infection model. This study aimed to assess the root canal decontamination of two irrigation techniques (GentleWave and passive ultrasonic irrigation—PUI) in mandibular molars infected with a multispecies biofilm model. The null hypothesis was that there would be no differences between both decontamination protocols.

MATERIALS AND METHODS

This study is reported in accordance with the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al., 2021). The PRILE 2021 flowchart is presented in Figure 1.

Specimen selection

A power calculation was performed in a previous study to determine the sample size necessary ($n = 11$) to distinguish differences between pre- and post-treatment communities (taken as observed abundance of genera) using the HMP package in R software (la Rosa et al., 2012; Ordinola-Zapata et al., 2022). After Ethics Committee approval (IRB protocol 00010445), 22 permanent human mandibular molars, extracted for reasons not related to this study, were used. Teeth were selected following these inclusion criteria: intact apices, no extensive restorations, absence of calcifications, root canal curvature between 20 and 40°, and type II Vertucci's configuration in mesial roots confirmed by Micro-CT scanning (Skyscan 1176; Bruker-MicroCT). After the access cavity and patency, the working length (1 mm short of apical foramen) was confirmed by using an ISO size 10 K-file (Dentsply). Afterwards, teeth were autoclaved and stored in distilled water.

Root canal contamination

The multispecies biofilm was obtained by using a human subgingival dental plaque sample from a single healthy donor (BPE 0–1), which was obtained from interdental spaces of mandibular molars using sterile inoculating needles (Fisher Scientific), as previously reported (Ordinola-Zapata et al., 2022). The sample was placed and diluted in an anaerobic transport medium (Anaerobe System). The root canals were coated first with 0.1 ml of sheep blood (HemoStat Laboratories) to promote the formation of an organic layer, and then 100 μl of the diluted dental plaque

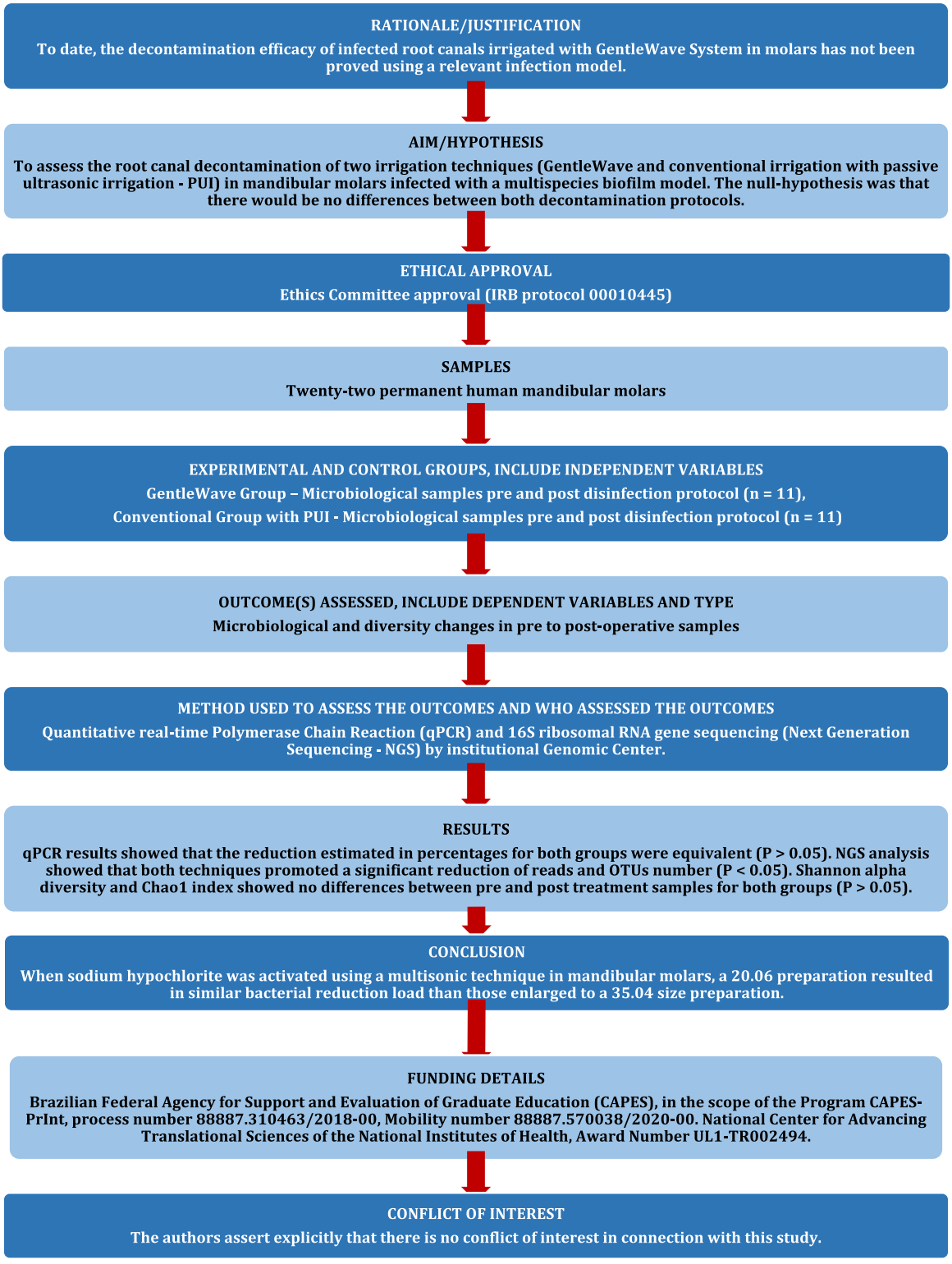

FIGURE 1 PRILE 2021 flowchart. *From*: Nagendrababu et al. (2021). For further details visit: http://pride-endodonticguidelines.org/prile/

was introduced. The teeth were mounted on a custom-built stand. This stand was placed in the Center for Disease Control (CDC) reactor (BioSurface Technologies), which produces conditions for an oral microcosm model (Figure 2). All the reactor components were autoclaved. The reactor has a lidded vessel that allows a defined flow of the 350 ml Columbia medium (Difco, BD) and has a stir bar that generates shear forces (Rudney et al., 2012). The internal temperature of the CDC vessel was set at 37°C and a 90 rpm stirring rate. Initially, the reactor was

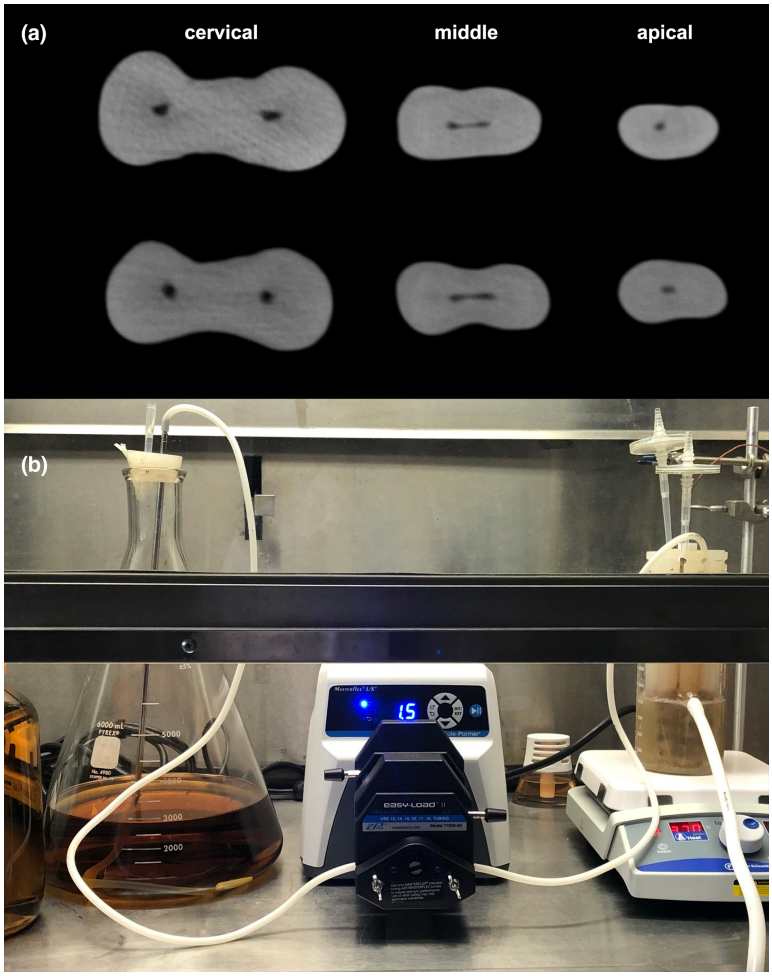

FIGURE 2 (a) Representative cross-sectional micro-CT images of two samples scanned at 35 μm voxel size, showing a type II Vertucci's configuration in the mesial roots. (b) Center for Disease Control (CDC) reactor (BioSurface Technologies) used in the present study.

incubated under shear conditions, without media flow, for 24 h; then, Columbia medium was pumped into the reactor at a 2.5 L/24 h flow rate for 2 weeks. The presence of an amorphous biofilm layer covering diverse substrates including the root canal wall surface has been previously validated (Li et al., 2014; Ordinola-Zapata et al., 2022; Rudney et al., 2012). The teeth were then removed for treatment.

Root canal preparation, final irrigation protocols and microbial sampling

The external surface of the crowns of accessed teeth was decontaminated with sodium hypochlorite (NaOCl) at 6% (Clorox) for 3 min and then with 10% sodium thiosulfate for 3 min. The decontamination protocol was previously verified in a pilot study confirming the removal of bacterial DNA (data not shown). In the sequence, the mesial canals were instrumented with ISO size 15 Hedstrom files (Dentsply Sirona) using a filling motion for 30 s to generate dentine shavings. Then, sterile paper points were placed to absorb the root canal content with shavings (preoperative sample). The teeth were randomly distributed into two groups: GentleWave ($n = 11$) and PUI ($n = 11$). For the GentleWave group, a minimal instrumentation was performed with a 20.06 variable taper file (V-Taper, SSWhite) following manufacturer recommendations. For the PUI group, conventional instrumentation up to 35.04 file (Vortex Blue, Dentsply Sirona) was performed. All canals were irrigated with 10 ml of 3% NaOCl (Clorox, diluted and titrated from a 6% NaOCl solution) during the instrumentation process using a 3-ml syringe and a 30G side-vented needle 2–3 mm short of working length. All apices were sealed with cyanoacrylate and sterile red wax to obtain a closed system for irrigation. The agitation protocols were:

PUI group: For final irrigation, 3% NaOCl was activated in each canal (MB and ML), 2 mm short of the working length, using a 20.02 ultrasonic tip coupled to a piezoelectric device (EndoUltra, Vista Dental). A total of 2 ml of the solution was activated for 20 s, the procedure was repeated three times for a total final irrigation time of 1 min. The total NaOCl volume of final irrigation per canal was 6 ml. The same procedure was repeated for 17% EDTA.

GentleWave group: The access of all teeth was sealed using a barrier (Soundseal, Sonendo). The pulp chamber floor was gauged to have the procedure instrument tip

1–2 mm above the pulp floor. The irrigation protocol consisted in 1-min cycle with distilled water, 4-min cycle with 3% NaOCl, 1-min cycle with 8.5% EDTA and finally 1-min cycle with distilled water.

After final irrigation, all canals were irrigated with 2 ml of 10% sodium thiosulfate for 3 min to inactive the NaOCl carry-over effect. Post-operative microbiological samples from both groups were obtained using ISO size 15 Hedstroem files in a filing motion for 30 s (Dentsply Sirona). Sterile paper points were used to obtain the samples (post-operative sample). The pre- and post-operative samples (paper points) were placed in an anaerobic transport medium (Anaerobe System) and vortexed for 10 s.

Quantitative real-time PCR analysis, DNA extraction and sequencing analysis

The DNeasy® PowerSoil® Pro Kit (Qiagen) was used to extract the DNA. The institution Genomic Center processed the samples for quantitative real-time PCR (qPCR) quantification and results were expressed in molecules/µl. For DNA sequencing, the V3-V4 hypervariable region of the 16S ribosomal RNA (rRNA) gene was amplified and sequenced using paired-end sequencing at 301 nucleotides (nt) read length on the Illumina MiSeq platform by the dual-index method. Raw data returned as ".fastq" files and uploaded in the Sequence Read Archive under BioProject accession number SRP328673.

Amplicon processing and analysis

Sequence data were processed and analysed using mothur ver. 1.41.1 (Schloss, 2020). Sequences were first trimmed to the first 250 nt and paired-end joined using fastq-join software. Quality trimming was performed at a threshold of 35 over a sliding window of 50 nt. In addition, sequences with homopolymers >8 nt, ambiguous bases, or >2 mismatches from primer sequences were removed. High-quality sequences were aligned against SILVA database ver. 138.1 for downstream processing. Chimeras were identified and removed using UCHIME ver. 4.2.40. Sequencing errors were further removed using a 2% preclustering step. Operational taxonomic units (OTUs) were binned at a similarity of 99% using the furthest-neighbour algorithm and were classified against the version 18 release from the Ribosomal Database Project (Cole et al., 2009). Different databases were used for alignment and classification due to processing considerations described previously (Schloss & Westcott, 2011).

Statistical analysis

For qPCR, data were transformed to Log values and the percentage of reduction between pre- and post-operative samples was analysed using Mann–Whitney *U* test (GraphPad Software). For microbiome analysis, Kruskal–Wallis test was used to evaluate differences in alpha diversity and abundances of genera, with Dunn's *post-hoc* test and Bonferroni correction for multiple comparisons using XLSTAT ver. 2020.2.3 (Addinsoft). The Shannon and Chao1 indices were used to measure alpha (within-sample) diversity. Paired analyses were used when indicated. The Bray–Curtis dissimilarity was used to measure beta (between-groups) diversity and was visualized by ordination using principal coordinate analysis. Differences in community composition were evaluated using analysis of similarity (ANOSIM), with Bonferroni correction for multiple comparisons (Clarke, 1993).

RESULTS

The analysis with qPCR showed that the PUI group promoted a reduction from 5.95 molecules/µl $\log_{10}$ (pre-operative) to 1.75 qPCR molecules/µl $\log_{10}$ (post-operative), whilst GentleWave had 5.6 molecules/µl $\log_{10}$ (pre-operative) and 0.44 molecules/µl $\log_{10}$ (post-operative). The reduction estimated in percentages indicates that both groups were equivalent ($p > .05$) **(**Table 1**)**. Multiple diversity assessments revealed that pre-operative samples in both groups had no significant difference in the bacterial composition. NGS analysis showed that fewer reads and OTUs were obtained from post-operative samples in both experimental groups ($p < .0001$). Shannon alpha diversity and Chao1 indexes showed no differences for both groups ($p > .05$) (Table 2). Both groups promoted significant reduction in relative abundances of *Parvimonas* and *Prevotella*. The PUI group promoted significant reduction in relative abundance of *Fusobacterium* (Figure 3). Beta diversity analysis showed no differences in pre- or post-treatment communities in both groups (ANOSIM R = 0.08 and 0.02; $p = .088$

TABLE 1 qPCR molecules/µl $\log_{10}$ of multispecies biofilm before and after treatment with PUI or GentleWave treatment

Groups	Pre	Post	Reduction (%)
PUI	5.95 (0.68)	1.75 (0.96)	78.5 (51.8–100)^{A}
GW	5.60 (0.83)	0.44 (0.70)	100 (66.9–100)^{A}

Note: Values are expressed as mean (SD). Percentage reduction is expressed in terms of median and range. Similar uppercase letter in the column represents no significant differences between groups.

Abbreviations: GW, GentleWave system; PUI, passive ultrasonic irrigation.

TABLE 2 Mean and standard deviations of sequencing data from all samples, including Shannon and Chao1 alpha diversity indices

Treatment	Time	Reads	Coverage (%)	OTUs	Shannon	Chao 1
PUI	Pre	85711 ± 23230^{A}	100 ± 0.00	631 ± 202^{A}	3.15 ± 0.13^{A}	1974 ± 743^{A}
	Post	19068 ± 33679^{BC}	99 ± 0.02	51 ± 37^{B}	2.37 ± 0.64^{BC}	63 ± 55^{B}
GW	Pre	58016 ± 27957^{AB}	99 ± 0.00	439 ± 191^{A}	2.98 ± 0.24^{AB}	1297 ± 630^{A}
	Post	4632 ± 13247^{C}	87 ± 0.30*	27 ± 27^{B}	2.20 ± 0.68^{C}	40 ± 60^{B}
	P value	<.0001	.344	<.0001	<.0001	<.0001

Note: Different uppercase letters in each column indicate significant differences by Dunn's *post-hoc* test ($p < .05$).

Abbreviations: GW, GentleWave system; OTUs, Operational taxonomic units; PUI, Passive ultrasonic irrigation.

*Two post-treatment samples had negligible amount of DNA and reads; coverage was 0 and 0.71.

and .025, respectively; Bonferroni-corrected $\alpha = 0.008$). Additionally, pre-treatment communities differed from post-treatment samples in both groups regarding bacterial taxa reduction (ANOSIM R = 0.50 and 0.55, $p < .001$) (Figure 3).

DISCUSSION

The present study assessed the multispecies biofilm removal ability of GentleWave and PUI in mesial roots of human mandibular molars. The null hypothesis was accepted because no differences were found between both treatment strategies. The 16S rRNA gene presents a combination of conserved, variable and hypervariable regions, the latter of which has made it the most sequenced taxonomic marker for the characterization of microbial community diversity (D'Amore et al., 2016). In the present study, the biofilm removal was assessed by 16S rRNA amplicon sequencing using NGS technology, which is an open-ended analysis. This allows the detection of the vast majority and most dominant bacteria in a root canal sample (Manoharan et al., 2020; Siqueira & Rôças, 2022b). Thus, it has become a standard method in basic biology (Siqueira & Rôças, 2022a; van Dijk et al., 2018).

The production of multispecies biofilm was performed using the CDC reactor. Although biofilm composition is different for each individual, the reactor generates reproducible microcosm biofilms closely representative of the oral microbiota (Li et al., 2014; Rudney et al., 2012). The microbial community retains more than 60% of the inoculated species under a controllable homogeneous environment (Rudney et al., 2012). Additionally, specimens can be incubated at predetermined times for biofilm assessment (Li et al., 2014). It is important to consider that an "old" biofilm (weeks) differs from a "young" one (days) in terms of biomass/thickness, cell count and antimicrobial resistance (Swimberghe et al., 2019). In the present study, the incubation time was 2 weeks because it has been shown that a polymicrobial biofilm reaches its maturation within this timeframe (Stojicic et al., 2013). In this study, the biofilm was dominated by 10 bacterial taxa including *Streptococcus, Parvimonas, Fusobacterium, Prevotella, Veillonella, Mogibacterium, Slackia, Selenomonas, Stomatobaculum* and *Lancefieldella* which represented a significant proportion of the microbial population. These species have been found consistently in cases of primary and secondary endodontic infections (Manoharan et al., 2020; Manoil et al., 2020). More specifically, a study proposed that the genera *Streptococcus, Prevotella, Parvimonas, Fusobacterium* and *Veillonella* are non-motile bacteria that play an important role in microbiome cargo-transport which shaped the spatial organization of a microbial community (Shrivastava et al., 2018). Similar to a previous study (Ordinola-Zapata et al., 2022), the *Streptococcus* and *Veillonella* taxa persisted in post-operative samples, regardless of the disinfection protocol. This can be explained by the high concentration of these bacteria before treatment, and the adhesion of these bacteria to the dentinal substrate (Do et al., 2015; Love & Jenkinson, 2002), which would allow deep colonization of the dentinal tubules, and therefore making their removal difficult.

Multispecies biofilm removal was assessed in human molars considering two different instrumentation approaches: large and minimal apical size instrumentation. For the PUI group, instrumentation was performed up to 35.04 instrument because it promotes a significant reduction in endotoxin levels (Marinho et al., 2012) and allows free placement of the irrigation tip in the canal (Van Der Sluis et al., 2007). For GentleWave, a minimal instrumentation was performed as recommended by the manufacturer (GentleWave Datasheet, 2022). In the present study, the 20.06 file was used for GentleWave as previously reported (Grigsby et al., 2020). Although the tip size is small, the taper is 0.06, which may favour the irrigant flow (Boutsioukis et al., 2010). It is important to note that the standardization of both groups in anatomic and microbiological terms did not remove the intrinsic differences in the irrigation protocols assessed. It was not possible to match the irrigation protocols for PUI and GentleWave groups in terms of canal size, taper

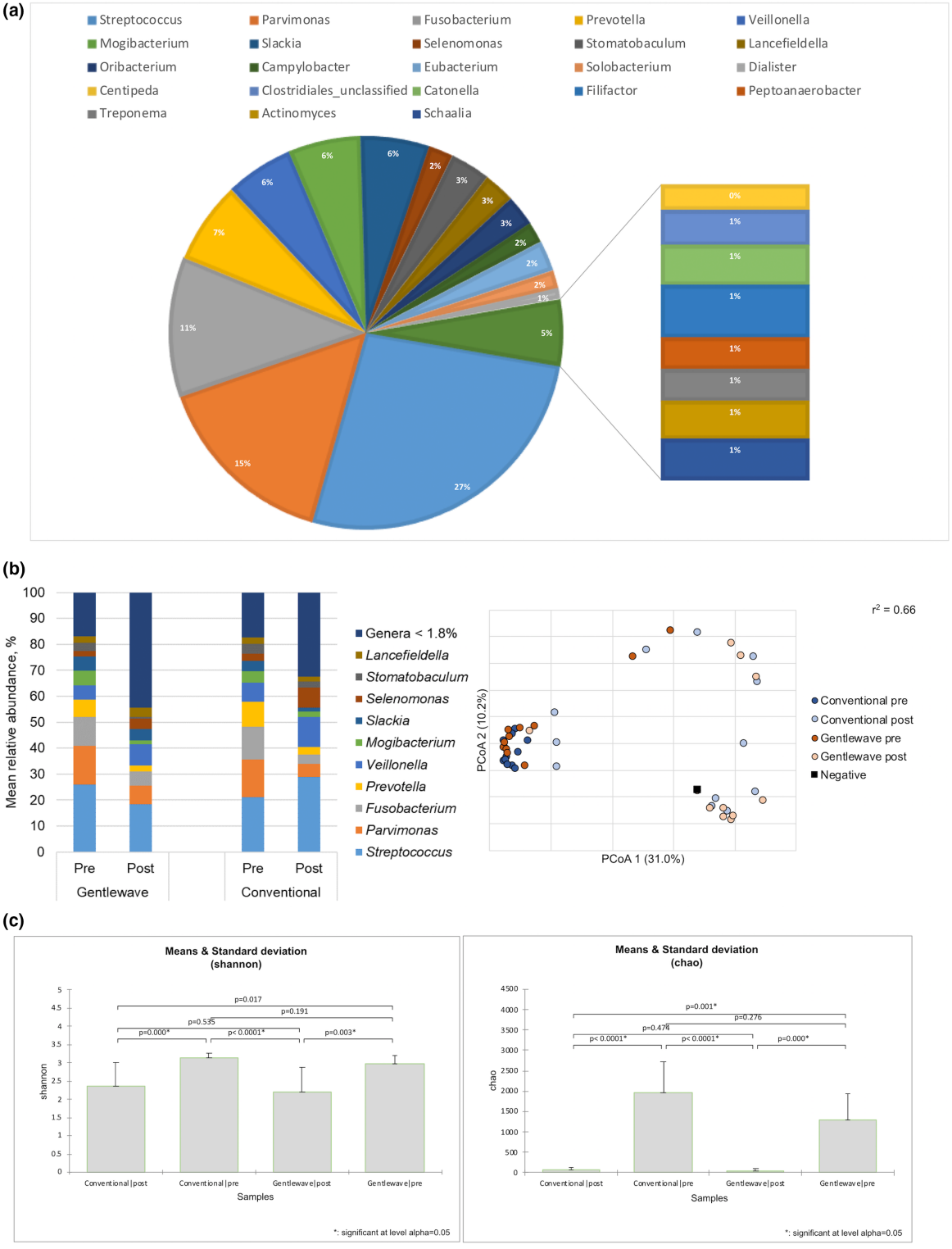

FIGURE 3 (a) Composition of microbial communities associated with the root canal disinfection model used in this study (preoperative samples). (b) Distribution of abundant genera amongst groups. Genera with a mean abundance <1.8% amongst all samples were consolidated. Principal coordinate analysis of Bray–Curtis dissimilarity matrices (beta diversity). (c) Shannon and Chao1 alpha diversity. Conventional = PUI.

and irrigant volume. Thus, results must be interpreted carefully. GentleWave uses approximately 45 ml/min flow rate (Haapasalo et al., 2014), being 180 ml of NaOCl, and approximately 270 ml total volume of irrigant solutions, whereas 10 ml of needle irrigation plus 6 ml of ultrasonic irrigation per canal were used

in the PUI group. Trying to match the irrigation protocols is impractical, because the PUI irrigation may need approximately more than 10–20× of continuous activation at a rate of 6 ml/min in each canal to match at least the NaOCl volume. This increase in activation time increases the chances of ultrasonic tip separation and other procedural errors (i.e., ledge and uncontrolled dentine removal) (Retsas et al., 2016). In addition, a 30-min ultrasonic activation per canal might be considered unrealistic and impractical for clinical use. The instrumentation of the canals was also not standardized, being 20.06V for the GentleWave group and 35.04 for the PUI group. In this regard, #15 (Zhang et al., 2019) or #20 (Sigurdsson et al., 2018) apical sizes have been used as minimal instrumentation sizes in previous studies. The gene sequencing analysis showed that there was no difference between GentleWave and PUI groups, regardless of the instrumentation size. Furthermore, a previous study showed that there was no difference between the use of GentleWave using minimal (15.04) and conventional (35.04) instrumentation in the reduction of *E. faecalis* lipoteichoic acid (Velardi et al., 2022). In this regard, a study revealed that the apical diameter is not a relevant factor when the irrigant is activated (Lee et al., 2019). Based on our results, the instrumentation up to 20.06V in mesial roots of mandibular molars in combination with a multisonic irrigation protocol could be advantageous considering the reduced amount of pericervical dentine removal and the decreased chances to create instrumentation error procedures.

Some limitations were observed in the biofilm model used in this research. The magnitude of reads and OTUs per sample is significantly higher than the amount of OTUs found in clinical cases by a magnitude of 20–100× (Manoil et al., 2020). This shows that the in-vitro model could be more challenging to disinfect than an infected tooth with a necrotic pulp. On the other hand, the model allowed us to obtain a standardized microcosm that is representative of the primary root canal infection. Another limitation is that the results should be considered as a surrogate, because the success of endodontic treatment not only relies on the antimicrobial effect of the treatment but also on pre-operative and post-treatment clinical factors. Thus, this model simulation cannot unambiguously establish causality between success and treatment failure.

CONCLUSION

Bacterial reduction in mesial roots of mandibular molars prepared to 35.04 with PUI was similar to those prepared to 20.06V with a multisonic irrigant activation system.

AUTHOR CONTRIBUTIONS

Hernán Coaguila-Llerena: data collection, methodology and writing; Ronald Ordinola-Zapata: conceptualization, methodology, funding acquisition and writing; Ruoqiong Chen: laboratory work, DNA extraction, review; Christopher Staley: writing, software, bioinformatic analysis; Matthew Dietz: bioinformatic analysis; Gisele Faria: review, editing, funding acquisition.

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CONFLICT OF INTEREST

The authors assert explicitly that there is no conflict of interest in connection with this study.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

ETHICS STATEMENT

The experiment was approved by the Ethics Committee approval (IRB protocol 10445, CAAE: 37090820.3.0000.5416).

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