

## ORIGINAL ARTICLE

# *In vitro* efficacy of a non-instrumentation technique to remove intracanal multispecies biofilm

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## Abstract

**Aim:** The aim of this study was to assess the efficacy of a non-instrumentation technique to disinfect root canals infected by a human dental plaque-derived multispecies biofilm.

**Methodology:** Twenty-two mandibular incisors were accessed, autoclaved and inoculated with dental plaque. The Center for Disease Control biofilm reactor was used to promote contamination of the root canal space. In the conventional technique (control), the specimens were instrumented until size 35/04 and irrigated with 6% NaOCl. In the non-instrumentation technique, a glide path was established using K-files size 10–20 and specimens were immediately cleaned with the GentleWave System. Samples were obtained for culture and 16S rRNA gene sequencing. Differences in abundances of genera were evaluated using Kruskal–Wallis test, and differences in alpha diversity were compared using ANOVA. Alpha and beta diversity indices were calculated using mothur. The Shannon and Chao1 indices were used to measure alpha diversity. The Bray–Curtis dissimilarity was used to measure beta diversity. Differences in community composition were evaluated using analysis of similarity with Bonferroni correction for multiple comparisons.

**Results:** The total numbers of reads in biological samples ranged from 126 to 45 286. Significantly fewer reads were obtained from samples following cleaning by either method ( $p < .0001$ ), and significantly fewer reads were obtained in post-cleaning samples following conventional versus non-instrumentation cleaning regimen ( $p = .002$ ). Communities in pre-treatment samples were similar in both groups; however, significantly greater relative abundances of *Streptococcus*, *Veillonella* and *Campylobacter* were observed following cleaning using non-instrumentation technique (Kruskal–Wallis  $p = .009$ ,  $.033$ , and  $.001$ , respectively). Whilst no significant differences were observed in Shannon alpha diversity, the Chao1 index was significantly lower in post-cleaning samples.

**Conclusions:** Significant shifts in composition were observed following cleaning by using both regimens, but the impact of this change was greater following a conventional cleaning technique.

## KEYWORDS

biofilms, disinfection, next generation sequencing

## INTRODUCTION

Removal of bacteria and their by-products is essential for healing of apical periodontitis. This clinical step has been conventionally achieved through mechanical debridement and the use of proteolytic solutions such as sodium hypochlorite (NaOCl) (Schilder, 1974; Zehnder, 2006). Previous, *in vitro* studies have revealed that under certain conditions, chemical debridement can be achieved in the absence of instrumentation (Baumgartner & Mader, 1987; Lussi et al., 1993). In a highly cited study, Baumgartner and Mader (1987) observed that pulp tissue on non-instrumented canal walls can be completely dissolved by using 2.5% NaOCl. Biofilm-based studies have also observed that under direct contact test conditions, oral biofilms can be significantly removed using the same proteolytic solution (Ordinola-Zapata et al., 2012). Recently, a histological study (Lee et al., 2019) revealed that cleaning of the root canals was not dependent on the mechanical debridement. Specific clinical scenarios have revealed that neutralization of the root canal content can be performed using only irrigants and medicaments. Such cases include the disinfection of teeth with open apices and teeth candidates for guided endodontic repair procedures. These observations suggest that mechanical instrumentation could be avoided in teeth with reduced anatomical challenge, if an antibacterial and proteolytic irrigant can be delivered, activated and evacuated in an efficient way through the root canal system.

The Lussi's non-instrumentation technique (Attin et al., 2002; Lussi et al., 1993) represented one of the first attempts for an actual minimally invasive cleaning of the root canals system. More recently, the GentleWave system (Sonendo) has been proposed as a novel irrigation alternative to clean the root canal system after a minimal instrumentation (size 20 with a 0.06 taper) (Chan et al., 2019; Charara et al., 2016; Haapasalo et al., 2014; Molina et al., 2015). With the increased popularity of minimally invasive endodontics and machine-assisted irrigation, the question of whether the disinfection of the root canal procedure could be achieved without creating a taper preparation arises. Despite the initial evidence, the potential disinfection efficacy of a non-instrumentation technique is yet to be determined. Thus, the aim of this study was to assess the efficacy of a non-instrumentation technique to disinfect root canals infected by a human dental plaque-derived multispecies biofilm. We hypothesized that alpha and beta diversity is affected by the

endodontic treatment without differences between the experimental groups.

## MATERIAL AND METHODS

Twenty-two mandibular incisors extracted for causes not related to this study were collected (IRB protocol 00010445) and stored in 0.1% Thymol. Teeth were evaluated by micro-computed tomography for suitability for the study and discarded if resorptions, root cracks, open apices, previous root canal therapy, presence of multiple canals, long oval canals (aspect ratio >4) or existing indirect restorations were present. Conventional endodontic accesses cavities were prepared and #10 FlexoFiles (Dentsply) were introduced to confirm patency and to record specimen's canal lengths. Then, specimens were autoclaved and stored in distilled water. Two additional samples were used for electron scanning microscope analysis.

### Root canal contamination

The core component of the oral microcosm model used in this study is the Center for Disease Control (CDC) reactor (BioSurface Technologies, Bozeman, MT, USA). It incorporated a lidded vessel through which growth media (350 ml Columbia medium; Difco) could flow at a defined rate, and a stir bar was used to generate shear forces (Rudney et al., 2012). Prepared root canal specimens were coated with 0.1 ml of sheep blood to create an organic layer, followed by inoculation of subgingival human-derived dental plaque (100 µl), provided by one donor previously diluted in anaerobic transport medium (Anaerobe System, Morgan Hill, CA, USA). Samples were mounted onto a custom-built stand that could hold sixteen samples and could be removed aseptically. The internal temperature of the reactor vessel was maintained at 37°C and the stirring rate was set at 90 rpm. The reactor was first incubated under conditions of shear, without media flow, for 24 h. Columbia medium was then pumped through the reactor at a rate of 2.5 L/24 h flow rate for 14 days. Then, the teeth were removed for treatment and analysis.

### Root canal preparation and microbiological sampling

Before treatment, crowns were decontaminated with 6.0% sodium hypochlorite (Clorox) for 3 min and inactivated

with 10% sodium thiosulfate. The efficacy of the decontamination protocol was previously verified in a pilot study, confirming removal of external bacterial DNA (data not shown). Microbiological preoperative samples were taken right after decontamination procedure. Sterile Hedstrom files (Dentsply Maillefer) size 15–20 were used to create dentine shavings before instrumentation, and sterile paper points were used to collect preoperative intracanal microbiological samples. Before instrumentation, the foramens were blocked with sterile red wax to create a closed system. The teeth had previously been assigned randomly into two treatment groups, conventional (control) or non-instrumentation technique. In the conventional technique group, the specimens had their root canals prepared with Vortex Blue instruments (Dentsply), using a crown-down technique, within 0.5 mm of the apex. The final preparation size of all canals was 35/04. In the conventional technique group, irrigation was first performed delivering 10 ml of 6% NaOCl using a 30G side vented needle placed at 2–3 mm from working length. Passive ultrasonic irrigation (PUI) with additional 6 ml of NaOCl was also used to irrigate the canal for 60 s (EndoUltra; Vista Dental). Finally, the canals were irrigated with a final rinse of 1 ml of 17% EDTA. In the non-instrumentation technique, a glide path was established using K-files size 10–20 to the specimen's working length, and no rotary instruments were used in this group. After glide path, the GentleWave System was used using the extended cycle. This includes sealing the access (Soundseal; Sonendo), and an initial cycle with distilled water for 1 min, a 4-min cycle of 3% sodium hypochlorite and a final irrigation with 8% EDTA. In total, 400 ml of irrigants are used per cycle. After disinfection procedures, NaOCl was inactivated using 2 ml of 10% sodium thiosulfate for 3 min.

After root canal preparation, irrigation and inactivation, post-cleaning microbiological specimens were collected. Sterile Hedstrom files size 20 were used to generate dentine shavings, and the hand instrument was used in a filling motion for 30 s. Three sterile paper points were used to collect the post-cleaning samples. All microbiological samples (pre- and post-treatment) were immediately placed in liquid dental anaerobic transport medium (Anaerobe System) and vortexed for 10 s. Samples for DNA extraction were stored at  $-80^{\circ}\text{C}$  until analysis.

## Scanning electron microscopy

Two additional samples were processed to observe intracanal biofilms. Teeth were fractured in two parts, dehydrated and processed as described previously (Ordinola-Zapata

et al., 2014). The samples were viewed with a scanning electron microscope (6500; JEOL).

## Culture analysis

Ten-fold serial dilutions of the microbiological samples were prepared in sterile phosphate-buffered saline (PBS) and plated on Columbia agar plates. The plates were incubated at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  chamber for 72 h. Colony-forming units (CFU) were quantified and results were expressed using the logarithmic scale.

## DNA extraction and sequencing analysis

Bacterial DNA recovered from the microbiological samples was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen). PCR amplification and sequencing were done by the University of Minnesota Genomic Center. The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced using the 505F/806R primer set (Caporaso et al., 2012). DNA extracted from the elution buffer and a sterile water sample were also sequenced, as controls. Paired-end sequencing was done at a read length of 301 nucleotides (nt) on the Illumina MiSeq platform using their previously described dual-index method (Gohl et al., 2016). Raw data were returned as fastq files and deposited 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 et al., 2009). Sequences were first trimmed to the first 170 nt and paired-end joined using fastq-join software (Aronesty, 2013). 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 the SILVA database ver. 138 for downstream processing; chimeras were identified and removed using UCHIME ver. 4.2.40 (Edgar et al., 2011); and sequencing errors were further removed using a 2% pre-clustering step (Huse et al., 2010). 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).

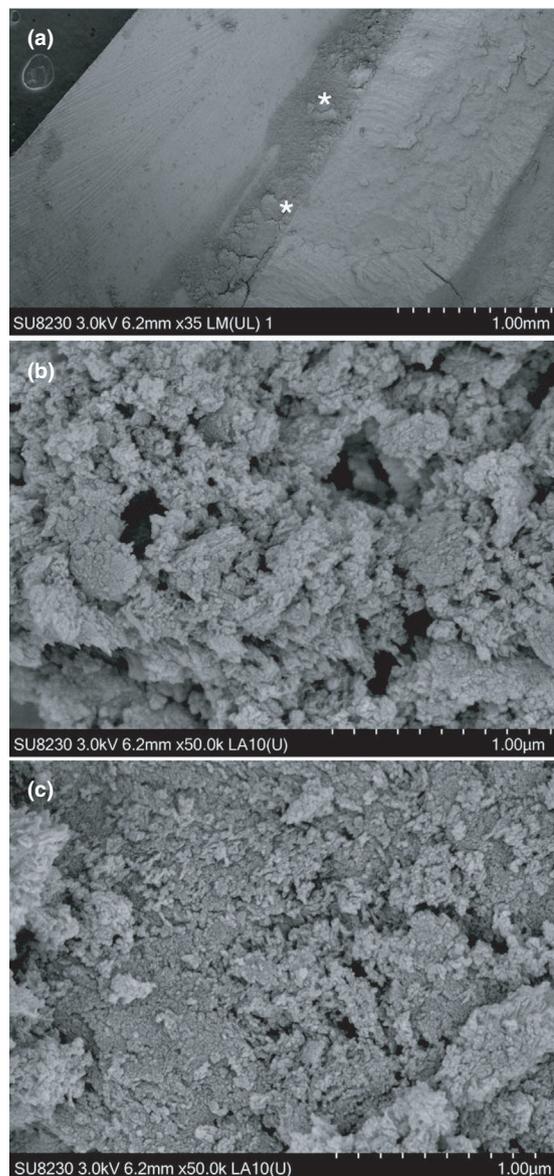
## Statistical analysis

Differences in abundances of genera were evaluated using Kruskal–Wallis test and differences in alpha diversity were compared using ANOVA with Tukey's *post hoc* test using XLSTAT ver. 2020.2.3 (Addinsoft). Alpha and beta diversity indices were calculated using mothur. The Shannon (Shannon & Weaver, 1949) (parametric) and Chao1 (Chao, 1984) (non-parametric) indices were used to measure alpha (within-sample) diversity. The Bray–Curtis dissimilarity (Bray & Curtis, 1957) was used to measure beta (between-sample) diversity and was visualized by ordination using principal coordinate analysis (Anderson & Willis, 2003). Differences in community composition were evaluated using analysis of similarity (ANOSIM) (Clarke, 1993), with Bonferroni correction for multiple comparisons. A covariance matrix correction to meet the assumptions of heteroscedasticity was used (White, 1980). Paired analyses were used when indicated. A power calculation was done to determine the sample size necessary to distinguish differences between pre- and post-treatment communities for future studies (taken as observed abundance of genera) using the HMP package in R software (la Rosa et al., 2012).

## RESULTS

The samples observed by scanning electron microscopy were characterized by the existence of a thick an amorphous biofilm layering covering the root canal wall surface. The presence of different morphotypes as cocci and rods could be identified (see Figure 1). The culture analysis showed that preoperative samples had  $1.91 \times 10^6$  and  $2.25 \times 10^6$  CFU in the non-instrumentation group and conventional group, respectively. After cleaning, only 2 out of 11 samples in the non-instrumentation group presented a positive but negligible culture (1 CFU each). In the conventional instrumentation group, all postoperative samples were negative for CFU formation.

Sequencing analysis showed that DNA extracted from the elution buffer and from sterile water control did not return quantifiable amplicon concentrations, confirming there was no contamination during sample processing. The total numbers of reads in biological samples ranged from 126 to 45 286, with negligible numbers of reads occurring in the negative controls (Table 1). Significantly fewer reads were obtained from samples following cleaning by either method (Tukey's *post hoc*  $p < .0001$ ), and significantly fewer reads were obtained in post-cleaning samples following conventional versus non-instrumentation cleaning regiment ( $p = .002$ ). The numbers of OTUs observed followed a similar pattern (Table 1). Whilst no significant differences were observed in Shannon alpha diversity (Figure 2a–b), the Chao1 index was



**FIGURE 1** Representative images of biofilm infected root canal walls. (a) The presence of organic structures on canal walls is discernible at relative low magnification (\*). (b–c) High magnification photomicrographs show colonization of the canal wall by diverse bacterial morphotypes

significantly lower in post-cleaning samples relative to pre-cleaning using the conventional or the non-instrumentation technique ( $p = .001$  and  $.002$ , respectively). The experimental flowchart can be found in Figure 3.

Pre-treatment biofilm communities were similar in both groups, being predominantly comprised of the bacterial genera *Streptococcus* and *Veillonella* (Figure 2c). Following both cleaning regimens, the relative abundance of *Acinetobacter* increased. Communities in pre-treatment samples were similar in both groups; however, significantly greater relative abundances of *Streptococcus*, *Veillonella* and *Campylobacter* were also observed following cleaning using non-instrumentation technique

**TABLE 1** Summary (mean  $\pm$  standard deviation) of sequencing data obtained amongst all samples

Time	Treatment	<i>n</i>	No. reads	Coverage (%)	OTUs
Pre	Conventional	11	38 090 $\pm$ 4844	98.4 $\pm$ 1.5	750 $\pm$ 634
	Non-instrument	11	36 833 $\pm$ 7340	99.0 $\pm$ 0.7	527 $\pm$ 330
Post	Conventional	11	2813 $\pm$ 4410 <sup>A</sup>	98.4 $\pm$ 1.3 <sup>A</sup>	42 $\pm$ 13 <sup>A</sup>
	Non-instrument	11	14 920 $\pm$ 10 873 <sup>B</sup>	99.6 $\pm$ 0.5 <sup>B</sup>	135 $\pm$ 143 <sup>B</sup>
Blank (water)		1	29	89.7	8

Note: Samples sharing the same letter did not differ significantly by ANOVA ( $p > .05$ ). Samples pre- and post-cleaning were evaluated separately.

(Kruskal–Wallis  $p = .009$ ,  $.033$ , and  $.001$ , respectively). Similarly, analysis of beta diversity showed no significant difference in pre-treatment communities (ANOSIM  $R = -.045$ ,  $p < .001$ ; Figure 2d). Significant shifts in composition were observed following cleaning by using both regimens, but the shift was greater using conventional cleaning technique ( $R = .913$  and  $.297$ ,  $p < .001$ , respectively). Significant differences in community composition, as noted above, were also observed in post-cleaning samples between both treatments ( $R = .354$ ,  $p < .001$ ). Communities characterized from negative control samples did not differ significantly from post-cleaning samples ( $p \geq .012$  at Bonferroni-corrected  $\alpha = .005$ ).

A power analysis to determine differences in pre- and post-cleaning based on the abundances of genera revealed that a sample size of 11 provided a power ( $1-\beta$ ) of 0.95 at  $\alpha = .05$ . Fewer samples ( $n = 10$ ) were slightly underpowered ( $1 - \beta = 0.72$ ).

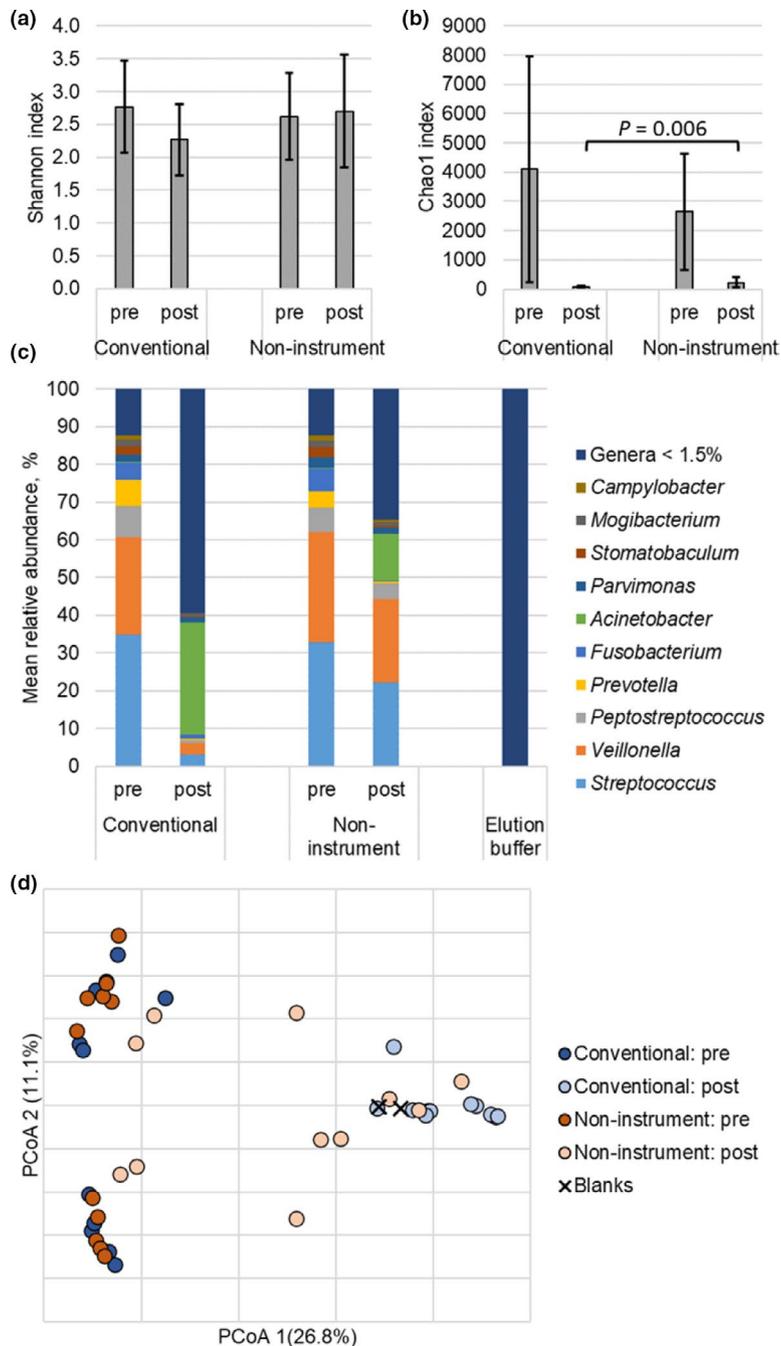
## DISCUSSION

The main challenge for the minimally invasive endodontic procedures is to improve the cleaning of the root canal space whilst decreasing the mechanical removal of tooth structure, which has driven the clinical practice of the root canal treatment for almost a century. In the present study, we investigated the efficacy of a non-instrumentation and the conventional technique in reducing microbial burden in an experimental root canal infection model. Efficacy was quantified using both traditional culture methods and next-generation sequencing (NGS). It is well-known that traditional culture methods enable identification of viable bacterial species, but it requires the incubation of the sample in specific selective conditions (Gupta et al., 2019). Although this allows the isolation of certain culturable bacterial species, it bias the characterization of the biofilm community composition towards species with less intricate culture requirements which readily proliferate under laboratory conditions (Dowd et al., 2008; Gupta et al., 2019; Wong et al.,

2021). Similarly, pre-selection of primers for PCR test skews microbial identification towards pre-targeted species and excludes the detection of unpredicted taxa (Sibley et al., 2012; Wong et al., 2021). Therefore, next-generation sequencing was used in this study to determine the order of nucleotides in targeted regions providing a more precise characterization of the whole community composition. Despite inability to distinguish between viable and non-viable cells, the relative abundance and diversity of the bacteria in a sample can be determined (Poretsky et al., 2014).

In our *in vitro* experimental model, root canals were contaminated with a human dental plaque-derived inoculum and incubated in a biofilm reactor for 2 weeks. NGS analysis revealed that the root canal biofilm was composed of more than 10 distinct bacterial genera. When compared to monocultures, multispecies microbial communities have been suggested to be more resistant against antimicrobial measures (Cavalcanti et al., 2017; Schwering et al., 2013), more virulent (Croxall et al., 2011; Korgaonkar et al., 2013) and more ready to form biofilms (Burmølle et al., 2006; Lories et al., 2020; Neelakantan et al., 2017), all of which can compromise treatment outcome. One concern with *in vitro* experimental root canal biofilm models is the lack of community characterization. Our characterization and analysis of pre-treatment microbiological samples, using NGS data, showed that a homogeneous and reproducible microbial community was obtained between the experimental groups (alpha diversity). Although our experimental biofilm model may not completely reproduce the flora of the root canal system—which can vary substantially between individuals, infection stages and types (primary and secondary/persistent) (Manoharan et al., 2020; Sassone et al., 2008; Siqueira & Rôças, 2009)—the model showed to be reproducible and composed of bacterial genera implicated in endodontic infections.

The biofilm reactor we used could not be operated under vacuum, limiting the incubation of our specimens to anaerobic environment. Nonetheless, as anticipated from previous studies (Bradshaw et al., 1996; Rudney et al., 2012), anaerobic species were identified upon community composition

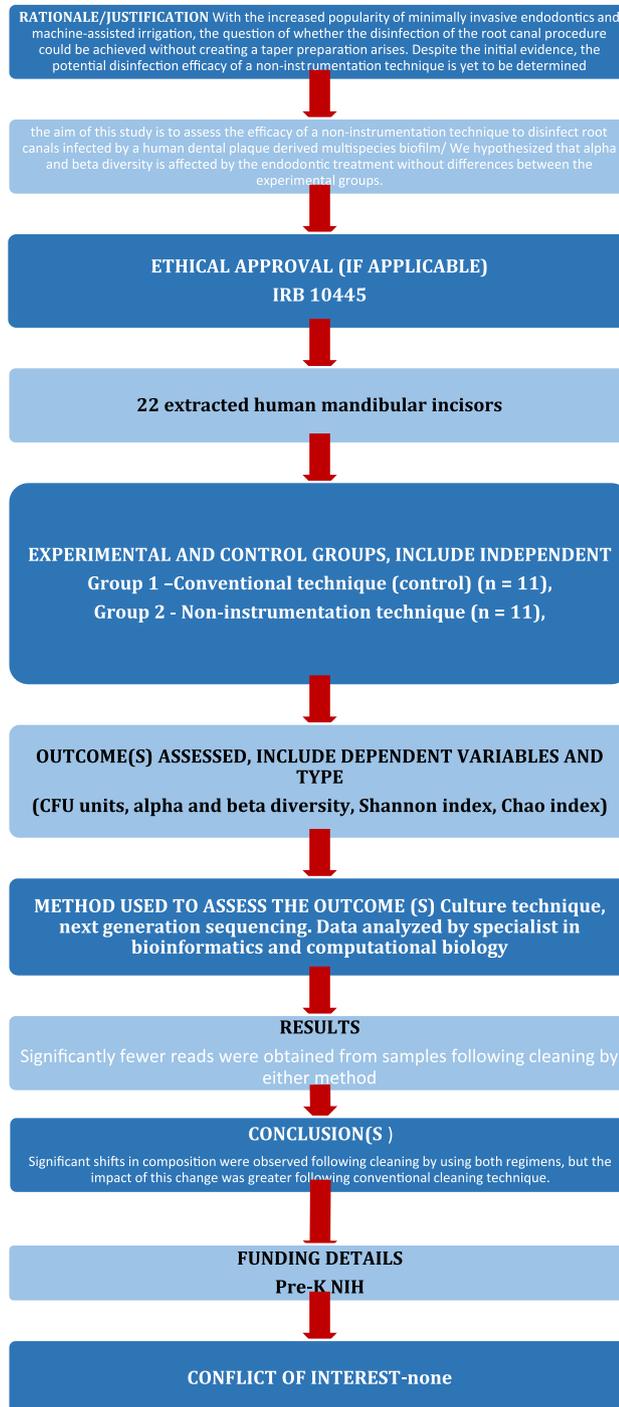


**FIGURE 2** Diversity and composition of microbial communities associated with root canal disinfection. (a) Shannon and (b) Chao1 alpha diversity indices. Error bars reflect standard deviation. Horizontal bar represents statistical differences. (c) Distribution of abundant genera amongst samples. Genera with a mean abundance <1.5% amongst all samples were consolidated. (d) Principal coordinate analysis of Bray-Curtis dissimilarity matrices

analysis of NGS data of pre-treatment samples. Previous studies have observed that obligate anaerobes can survive in aerobic environments when facultative species are also present (Bradshaw et al., 1996; Rudney et al., 2012). Rudney et al. (2012) hypothesized that anaerobic bacteria in a plaque-derived biofilm model can initially be protected by coexisting facultative bacteria, increasing their numbers once biofilm depth increased, or being favoured by the continued consumption of oxygen by facultative species. Importantly, the results cannot rule out the possibility that the incubation conditions may have contributed to modification in the proportion of fastidious anaerobes in the samples. Overall, this observation needs to be weighed against the fact that the

species with the highest relative abundance commonly found in endodontic infections such as *Streptococcus* spp., *Veillonella* spp., *Prevotella* spp., *Peptostreptococcus* and *Fusobacterium* spp. were retained in the microcosms of our pre-treatment samples (See Figure 2). In addition, previous studies have shown that upon challenge by sodium hypochlorite, multispecies biofilms grown aerobically or anaerobically did not differ significantly from each other, (Swimberghe et al., 2021), corroborating the use of the biofilm reactor and dental plaque-derived inoculum to generate homogeneous *in vitro* experimental multispecies biofilm communities.

Six per cent sodium hypochlorite is currently the preferred and most commonly used root canal irrigant by



\*From: Nagendrababu V, Murray PE, Ordinola-Zapata R, Peters OA, Rôças IN, Siqueira JF Jr, Priya E, Jayaraman J, Pulikkotil SJ, Camilleri J, Boutsoukis C, Rossi-Fedele G, Dummer PMH (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontology: a consensus-based development. *International Endodontic Journal* May 3. doi: 10.1111/iej.13542. <https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13542>.

For further details visit: <http://pride-endodonticguidelines.org/prile>

FIGURE 3 PRILE 2021 flowchart

endodontic practitioners (Dutner et al., 2012). Despite controversy on the benefits of using higher NaOCl concentrations (Dumitriu & Dobre, 2015; Retamozo et al., 2010), some *in vitro* and clinical studies report no significant

impact of the concentration on antimicrobial, tissue dissolution effectivity or on treatment outcome (Baumgartner & Cuenin, 1992; Siqueira et al., 2000; Verma et al., 2019). In the present study, NaOCl at 3% and 6% were used in the

non-instrumentation and in the conventional technique respectively. Full-strength NaOCl concentration and a large apical size (0.35 mm) were selected in the conventional group because it represents a common antimicrobial protocol to decontaminate root canals *in vivo*.

The microbial culture analysis suggested that both concentrations appear to induce comparable antimicrobial effectivity. One advantage of NGS analysis is that it improves the ability to quantify the effects of the chemical treatment on individual bacteria taxa. Qualitative and quantitative data can be statistically examined, increasing the robustness of the evaluation, to compare the diversity of species in a sample (alpha diversity), as well as differences in community composition between samples or environments (beta diversity). NGS analysis showed significant reduction in the numbers of reads between pre-treatment and post-cleaning, for both experimental groups. These data agree with our culture analysis results and suggest that both cleaning regimens were able to substantially reduce intracanal microbial burden, *in vitro*. Importantly, post-cleaning efficacy of the two experimental groups was not comparable. When compared to the conventional technique, the non-instrumentation regimen was less efficient, having significant greater numbers of sequence reads (Table 1;  $p = .002$ ). Even though the non-instrumentation protocol allows constant multisonic activation of large amounts of irrigants (400 ml), the lack of a tapered preparation possibly limited NaOCl bioactivity and reduced shear forces that may dislocate biofilms and are enabled by contact between irrigant and dentinal walls. Thus, as recommended by the manufacturer, it is still prudent to use the GentleWave system with a 20.07 preparation. Additionally, sequencing analysis also showed that the amount of *Streptococcus* spp., *Veillonella* spp. and *Campylobacter* spp. was significantly greater in the non-instrumentation technique, post-treatment samples (Figure 2c). It is possible that the reduced concentration of NaOCl available in the multisonic system or the lack of debridement of canal walls favoured the persistence of microorganisms that were able to invade dentinal tubules (Kwang & Abbott, 2014; Love & Jenkinson, 2002). In oral biofilms, the pioneer colonizers—such as members of the *Streptococcus* and *Veillonella* genera—participate in consortia that can attach to surfaces, establishing the initial biofilm community (Diaz et al., 2006; Jakubovics, 2015; Mark Welch et al., 2016) and, thus, may persist if their substrate is not mechanically dislocated and removed.

The determination of residual bacterial localization is a limitation of this study and is a drawback of all studies that use paper points to collect root canal microbiological samples. Even though paper points are the primary approach for collecting *in vivo* intracanal microbiological samples, the use of cryogrinding of *ex vivo* specimens may

be an alternative that allows for identification by microbiological analysis with distinction of root canal length localization (Siqueira et al., 2011). The use of mandibular incisors, which imposes a low anatomical challenge to both cleaning regimens, may also be considered a limitation of the present study. Mandibular incisors with only one straight root canal allowed for direct access of the ultrasonic tip to most of the root canal extent, including the apical third—which cannot be achieved in curved canals. These limitations may overestimate the antimicrobial effectivity of both cleaning regimens. Most importantly, it is hard to expand and draw parallels between the results of most *in vitro* root canal disinfection studies and clinical efficacy. Currently, there is still uncertainty about what are the subcritical bacterial load thresholds that are compatible with periapical healing (Siqueira & Rôças, 2008). Thus, whether the significant differences in the microbiome composition between the groups have a clinical meaning needs to be validated in future clinical research.

## CONCLUSION

Significant shifts in composition were observed following cleaning by using both regimens, but the impact of this change was greater following conventional cleaning technique.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors deny any conflicts of interest related to this study.

## AUTHOR CONTRIBUTIONS

Dina Mansour: data collection and writing; Ruoqiong Chen: laboratory work, DNA extraction, review, writing; Flavia Saavedra: writing, review and editing; Christopher Staley: writing, software, formal analysis; Alex S. Fok: funding acquisition, writing-review and editing; Ronald Ordinola-Zapata: conceptualization, methodology, funding acquisition, and writing.

## ETHIC STATEMENT

The use of extracted teeth was approved by the local ethics committee (IRB#00010445).

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available. Raw data were returned as fastq files and deposited in the Sequence Read Archive under BioProject accession number SRP3286793.

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