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Comparative analysis of Streptococcus mutans adhesion on direct 3D-printed versus thermoformed orthodontic aligners: an in- vitro study using tetrazolium reduction assay

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Abstract

Background

The increasing adoption of clear aligner therapy, particularly with the advent of direct 3D printing technologies, necessitates a comprehensive understanding of how fabrication techniques influence microbial adhesion and biofilm development.

Objective

To quantitatively compare Streptococcus mutans biofilm formation on direct 3D- printed aligners and thermoformed aligners using a tetrazolium chloride (TTC)-based metabolic assay.

Materials and Methods

Forty aligner specimens were allocated into two groups (n = 20each): direct 3D-printed aligners and thermoformed aligners. A standardized S. mutans suspension (10^6 CFU/mL) was used to induce biofilm formation over a 14-day incubation period under controlled conditions with periodic media replenishment. Adherent biofilms were detached via sonication and quantified using TTC reduction assay, with absorbance measured at 500 nm. calibration curve correlating absorbance to log CFU was constructed. Statistical analysis included Shapiro–Wilk normality testing and independent samples t-test ($\alpha = 0.05$).

Results

Mean log CFU/sample values were 5.41 ± 0.22 for the 3D-printed group and 5.34 ± 0.19 for the thermoformed group. Statistical analysis revealed no significant difference between groups ($t = 1.056$, $p = 0.297$).

Conclusion

The manufacturing technique of direct 3D printing versus thermo forming did not significantly influence the adhesion of S. mutans to aligners under the conditions tested These findings suggest that factors beyond fabrication method, such as surface finishing and intraoral conditions, may play a more decisive role in microbial colonization.

Trial registration

Not applicable. This study is an in vitro laboratory study and does not involve human participants.

BACKGROUND

Clear orthodontic aligners are becoming a popular choice compared to fixed orthodontic appliances. They offer better aesthetics, comfort, and the ability to remove them, which helps patients stick to their treatment plans. This is especially true for adults and teenagers[1]. The thermoformed design allows the aligners to fit snugly against the teeth and gums. This means they can move teeth effectively without irritating soft tissues, improving the overall experience for patients[2] [3]. However, wearing aligners for long periods, usually 20 to 22 hours a day, creates a setting that favors microbial growth, biofilm formation, and changes in the oral microbiome[4].

Building up oral biofilm on orthodontic devices is a well-known risk factor for problems like enamel erosion, cavities, gum disease, and inflammation, especially when oral hygiene is not adequate during treatment[5]. Although aligners are removable, they can create sheltered areas that shield bacteria from being cleaned away or washed out by saliva. This can lead to bacteria sticking around on their surfaces[6]. Recent studies have shown that important oral bacteria, like *S. mutans* and Candida albicans, can attach to clear aligner materials and form biofilms, which suggests they could harbor harmful microorganisms[7]. The surface traits of orthodontic materials, like roughness, porosity, and chemical makeup, greatly affect biofilm formation as rougher surfaces tend to trap and hold more bacterial burden[8].

The bacterial model chosen for this study is Streptococcus mutans, which is highly specific due to its ability to form biofilms and adhere to surfaces. GtfB and GtfC, two glucosyltransferase enzymes, are essential for the production of extracellularly enriched proteins that facilitate bacterial attachment, cell-to-cell adhesion, and the formation of a stable biofilm matrix[9]. Due to these characteristics, S. mutans is an ideal candidate for study of how well the bacteria adhere to dental materials and on polymeric surfaces. Bacterial adhesion to different dental restorative materials has been extensively studied using S. mutans in past studies, which suggests its usefulness as an organism standard for studying material-related bacterial colonization[10, 11].

Traditional thermoformed aligners are made by using pressure or vacuum to shape thermoplastic sheets over dental models. This process can introduce irregularities that help bacteria adhere [12]. In contrast, recent advances in 3D printing allow for the direct creation of orthodontic aligners, which can lead to better precision, customized mechanical properties, and possibly different surface features that may affect how bacteria attach[13].

An important methodological aspect of the present study is the use of a tetrazolium-based assay to quantify the viability of *S. mutans* associated with clear aligner surfaces. Conventional methods used to evaluate bacterial colonization of orthodontic materials, particularly colony-forming unit (CFU) counting, depend on the recovery and growth of viable bacteria after removal from the tested surface. Although CFU provides valuable information about cultivable microorganisms, it may underestimate the bacterial population when cells are strongly attached to the material or are present in different physiological states. Tetrazolium-based assays provide a complementary approach by measuring the metabolic

activity of bacteria through the reduction of tetrazolium salts into measurable formazan products, thereby providing a quantitative indication of metabolically active cells within the biofilm[14]. This approach is particularly relevant to clear aligners, where bacteria can adhere directly to the polymer surface and develop a structured biofilm that may be difficult to completely recover for subsequent culture-based enumeration. Previous dental-material studies have successfully used tetrazolium assays to quantify the metabolic activity of *S. mutans* biofilms associated with resin-based materials[10].

However, the direct measurement of the viability of S. mutans using quantitative metabolic assays is relatively underdeveloped, especially when utilized as a measure of comparison between commercially available materials used in fabrication of clear aligners. Hence, the current work explores the use of the tetrazolium-based methodology for evaluation of bacteria metabolic activity on clear aligners, which could be considered a quick and quantitative method for assessing the adhesion of bacteria on different aligner materials and could be useful to detect differences in the behavior of bacteria on different materials even if differences in bacterial numbers are slight.

Colorimetric tests with tetrazolium salts, such as 2,3,5-triphenyl-tetrazolium chloride (TTC), offer a dependable and sensitive way to spot active bacteria by turning them into red formazan compounds. This method allows for a quantitative assessment of bacterial viability and metabolic activity

The TTC assay is widely used in microbiology to measure bacterial growth, viability, and biofilm activity due to its simplicity and sensitivity, which allow for indirect measurements of bacterial metabolic function[15]. This color change reaction provides a way to visually and spectrophotometrically detect bacterial colonization. It has been successfully used to measure microbial activity on various materials in lab settings, as active bacteria can reduce TTC to a colored formazan that can then be measured[16]. Adding TTC-based indicators to orthodontic aligners could introduce a new way to detect bacterial buildup and indirectly track oral hygiene during treatment. Even with the increasing use of both traditional thermoformed and 3D-printed aligners, there is a little gap in literature directly comparing microbial adhesion and biofilm growth between these two types There has been limited research on the comparison of microbial adherence and biofilm formation in the two different aligners. Additionally, there is a shortage of simple, objective, and reproducible methods for assessing bacterial activity on aligner materials, especially with the rise of new manufacturing techniques. The aim of this in-vitro study is to investigate if orthodontic aligners can change color in response to bacterial adhesion using a TTC-based test as an indirect measure of oral hygiene. The study will also quantitatively compare bacterial adhesion and activity levels between conventional thermoformed and directly fabricated 3D-printed aligners.

From these objectives, the null hypothesis of the current research will be that there would be no significant visible color changes in biofilm formed on orthodontic aligners using TTC-based assay. This indicates that this method may not be useful for measuring bacterial adhesion or assessing oral hygiene in a lab setting. Moreover, there would be no significant difference in bacterial adhesion between traditional thermoformed aligners and direct 3D-printed aligners using TTC-based assay.

MATERIALS AND METHODS

1. **Study Design**: this research is a comparative controlled laboratory based *in-vitro* study. The study utilized a two-arm comparative experimental model focused on the technique used to make the aligners. All experiments were conducted under standardized lab conditions to ensure consistency and minimize bias.
2. **Study Setting**: This *in-vitro* study was conducted at the Department of Orthodontics, Pharos University in Alexandria. The study was approved from the research ethics committee of pharos university (approval number PUA 02202606023519). All procedures followed controlled microbiological and biomaterial lab conditions according to standard protocols.
3. **Sample size calculation**: The sample size was calculated using a previous study [7] by MedCalc statistical software. Assuming an alpha of 0.05 and an 80.0% power, a beta error of 0.1 was determined. The typical guideline is to reject the null hypothesis H_0 if the *p*-value is below 0.05. The minimum sample size was set at 40 samples, with 20 samples in each group.
4. **Aligner Specimen Fabrication**: Two groups of orthodontic aligner specimens were created: Group A: conventional thermoformed aligners, these aligners were made from standardized medical-grade thermoplastic sheets using pressure-forming techniques according to the manufacturer's guidelines. Group B: direct 3D-printed aligners, these aligners were created using dental-grade biocompatible photopolymer resin through additive manufacturing with a standardized 3D printing protocol. All aligner specimens were standardized in terms of thickness, surface area, and geometrical shape. This standardization aimed to reduce variability in bacterial adhesion and activity measurements.

Location of Fig. 1

1. **Bacterial Strain and Culture Conditions**: A mono-species bacterial model was used in this study, focusing on *Streptococcus mutans* (ATCC 700610) due to its known role in early dental biofilm formation and relevance to oral hygiene.
2. **Preparation of Standardized Bacterial Suspension**: A standardized suspension of *S. mutans* was prepared using serial ten-fold dilutions. First, an aliquot of 1 mL of the primary bacterial suspension was added to a sterile tube with 9 mL containing sterile saline. This was repeated to create a dilution series from 10^{-1} to 10^{-6}. To find the baseline bacterial concentration, the pour-plate method was used. Aliquots of 0.1 mL from the chosen dilutions were transferred into sterile plates. Then, 15 to 20 mL of molten Tryptic Soy Agar (TSA) with bacitracin was cooled to 45 to 50°C to keep the bacteria alive and poured into each plate. The plates were gently swirled to distribute the inoculum evenly and left to solidify at room temperature. They were then incubated at 37 ± 2°C for 24 to 48 hours. After incubation, plates with count between 30 and 300 colonies were selected to calculate the baseline colony-forming units (CFU).
3. **TTC Assay Calibration**: A 1% stock solution of 2,3,5-triphenyltetrazolium chloride (TTC) was prepared by dissolving 1 g of TTC in 100 mL of distilled water, the solution was sterilized using membrane filtration with a 0.22 µm pore size. It was then stored at 4°C and kept away from light. A

standard calibration curve was prepared by adding an aliquot of 1 mL of the stock TTC solution to 10 mL of standardized bacterial dilutions recovered from biofilm previously formed on aligners, resulting in a final TTC concentration of 0.01%. These mixtures were incubated at 37°C for 2 to 6 hours. After incubation, the absorbance was measured at 500 nm using a spectrophotometer.

4. **Biofilm Formation on Aligners**: Each aligner specimen was transferred into a sterile tube containing 10 mL of Tryptic Soy Broth (TSB) with bacitracin. To start biofilm formation, an aliquot of 1 mL of the standardized *S. mutans* inoculum, adjusted to 10^6 CFU/mL, was added to each tube. The samples were incubated at 37°C for two weeks. To support continuous bacterial growth and strong biofilm formation, the culture media was replaced every 48 hours.
5. **Harvesting and Quantification of Biofilms**: After the incubation period, the aligners were carefully washed to remove any non-adherent planktonic cells. Each aligner was then transferred into a new tube that contained 9 mL of sterile saline solution. To detach the adherent biofilm cells from the aligner surfaces, the tubes underwent sonication. Then the aligners were removed from the solution and an aliquot of 1 mL of TTC stock solution was added to each tube. This mixture was then kept at 37°C for 2 to 6 hours and the cells' metabolic activity was measured by reading the absorbance at 500 nm against the standard calibration curve using a spectrophotometer.
6. **Statistical Analysis**: Normality of data was checked using Shapiro Wilk test, the bacterial log counts were normally distributed. Comparison between the two groups, thermoformed aligners and direct 3D-printed aligners, was done using *t-test*: two sample assuming equal variances. The significance level was set at *p*-value ≤ 0.05. Data was analyzed using IBM SPSS (Statistical Package for Social Science), version 23 for Windows, Armonk, NY, USA.

RESULTS

The TTC assay produced measurable absorbance at 500 nm following incubation with S. mutans suspensions of standardized concentration. The calibration experiment indicated an association between bacterial concentration (X) and absorbance (Y); the calibration curve followed the following equation Y = 0.186X - 0.5848, coefficient of determination (R^2) = 0.9925, number of calibration points was 3, which demonstrate a good analytical performance of the assay. Absorbance readings from the 10^{-4} dilution and higher dilutions were below the detection limit of this method, indicating that estimates outside the validated range should not be interpreted quantitatively. The calibration curve is presented in Figure 2.

Location of Figure 2

Following 14 days of incubation, TTC reduction was measured for all 40 specimens and the absorbance values were converted to estimated $\log_{10}$ CFU-equivalents per specimen using the calibration curve. The mean estimated bacterial burden was 5.41 ± 0.22 $\log_{10}$ CFU-equivalents/specimen for directly 3D-printed aligners and 5.34 ± 0.19 $\log_{10}$ CFU-equivalents/specimen for thermoformed aligners. The between-group difference was 0.07 $\log_{10}$ units, with higher values in the 3D-printed group. An independent-samples t-test did not detect a statistically significant difference (t = 1.056, p = 0.297; n = 20 per group). Thus, the

data do not provide evidence of a difference in TTC-detectable metabolic activity between the two aligner groups under the experimental conditions used. The group summaries and statistical comparison are provided in Table 1 and Figures 3–4. Because TTC measures metabolic reduction rather than colony formation directly, the values were described as CFU-equivalents estimated from the calibration curve for determination of CFU of *S. mutans* recovered from aligner specimens.

Location of Table 1

Location of Figure 3

Location of Figure 4

DISCUSSION

This in-vitro study compared S. mutans-associated metabolic activity on directly 3D-printed and thermoformed orthodontic aligner specimens after 14 days of mono-species incubation. The mean estimated burden was numerically higher on 3D-printed specimens than on thermoformed specimens (5.41 ± 0.22 versus 5.34 ± 0.19 $\log_{10}$ CFU-equivalents/specimen), but the difference was not statistically significant ($t = 1.056$, $p = 0.297$). Accordingly, the present findings do not demonstrate that either fabrication approach supports greater S. mutans colonization under the conditions tested.

The calibration experiment supports the feasibility of detecting TTC reduction associated with S. mutans suspensions by spectrophotometry at 500 nm. Tetrazolium salts are reduced by metabolically active cells to colored formazan products, providing an indirect measure of cellular metabolic activity. This principle is consistent with prior work optimizing tetrazolium-based assays for biofilm assessment, including the study by Sabaeifard et al. [16], and with reviews describing the strengths and limitations of metabolic viability assays [12, 14]. Nevertheless, assay signal is influenced not only by cell number but also by metabolic state, incubation conditions, reagent concentration, and potential interactions with the tested material. Therefore, TTC absorbance should not be treated as a direct viable-cell count without reporting calibration performance and validating the conversion against an independent reference method, such as viable plate counts. The coefficient of determination (R^2) of the calibration curve was 0.9925

The absence of a detected difference between groups is biologically plausible because microbial attachment is governed by several interacting variables, including surface topography, surface free energy, polymer chemistry, wettability, and post-processing. Additive manufacturing may produce layer-related features, whereas thermoforming can alter the surface of the original sheet during forming; however, the direction and magnitude of these effects cannot be inferred from fabrication method alone. Previous literature has linked material characteristics and surface roughness with biofilm development [8][17], but the present study did not directly measure roughness, wettability, or surface chemistry. Consequently, it would be speculative to conclude that print lines and thermoforming irregularities provide equivalent bacterial attachment sites. The results instead indicate that, for the specific materials

and preparation protocols examined, any difference in TTC-detectable S. mutans activity did not reach statistical significance.

LIMITATIONS AND FUTURE DIRECTIONS

Several limitations should be considered when interpreting these findings. First, the experiment used a single bacterial species, whereas oral biofilms are polymicrobial and are influenced by saliva, acquired pellicle, dietary substrates, oral hygiene, and changing environmental conditions. Second, the static 14-day incubation model does not reproduce the dynamic conditions of aligner use, including intermittent removal, cleaning, salivary flow, and mechanical wear. Third, the study compared fabrication categories, but the manuscript does not provide surface finishing, surface roughness, wettability, or surface chemistry; these factors may confound any apparent effect of manufacturing method. Future studies should include multiple clinical isolates or a defined multispecies biofilm, artificial saliva or salivary pellicle, clinically relevant cleaning and exposure cycles, and physicochemical characterization of the tested surfaces. Any proposed color-changing clinical application of TTC would require separate evaluation of material safety, optical stability, esthetics, and whether the indicator meaningfully improves patient hygiene behavior.

Declarations

Ethics approval:

This *in-vitro* study was conducted at the Department of Orthodontics, Pharos University in Alexandria. The study was approved from the research ethics committee of pharos university (approval number PUA 02202606023519).

Consent to participate:

not applicable

Consent for publication:

not applicable

Competing interests:

there are no Competing Interests

Funding:

This research received no external funding.

Author Contribution

YR, SM, RA and SW conducted the experiments. KA conceived the idea of comparing the aligners types. WL conceived the idea of using tetrazolium chloride for the determination of bacterial load. MA and NA supervised the experimental work. NA and YR drafted the manuscript. All authors revised and approved the final manuscript.

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Availability of data and materials:

All data generated or analyzed during this study are included in this published article

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Tables

Table 1 is available in the Supplementary Files section.

Figures

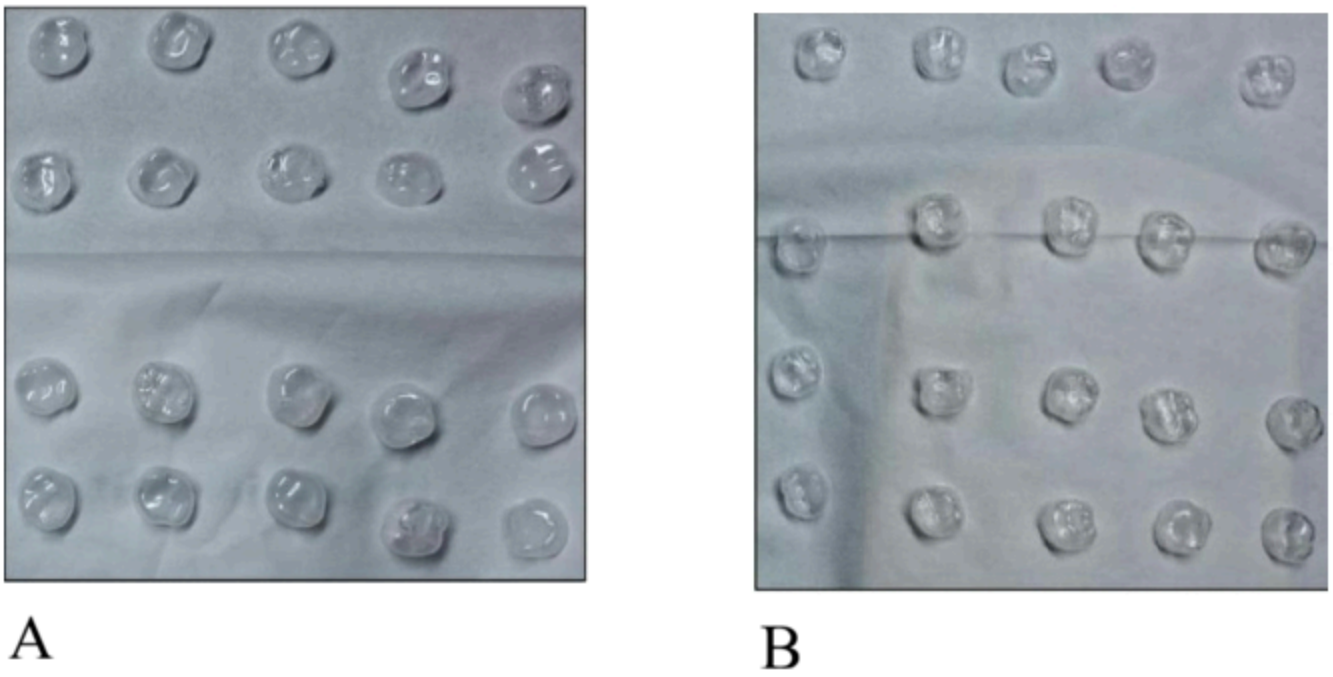


Figure 1

Conventional thermoformed aligner (A) and Directed 3D-printed aligner (B)

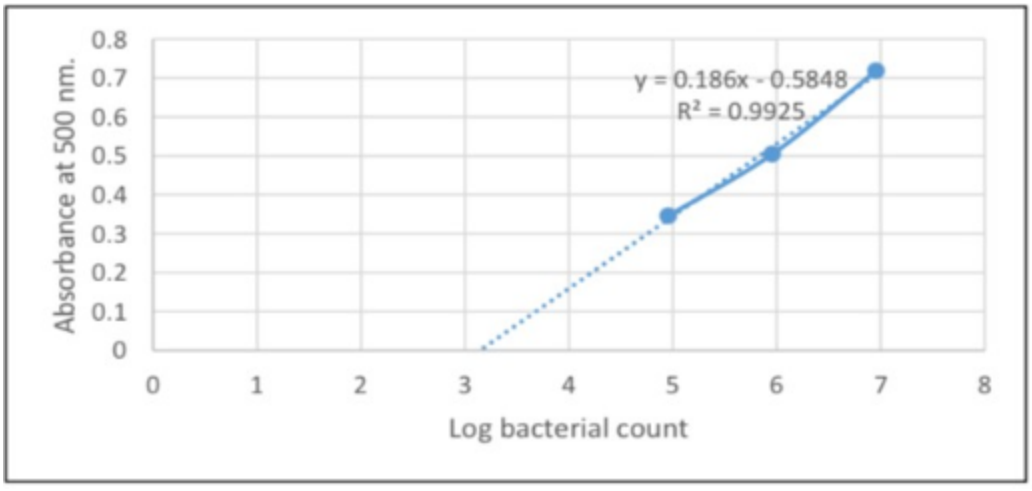


Figure 2

Standard curve of log bacterial count versus absorbance

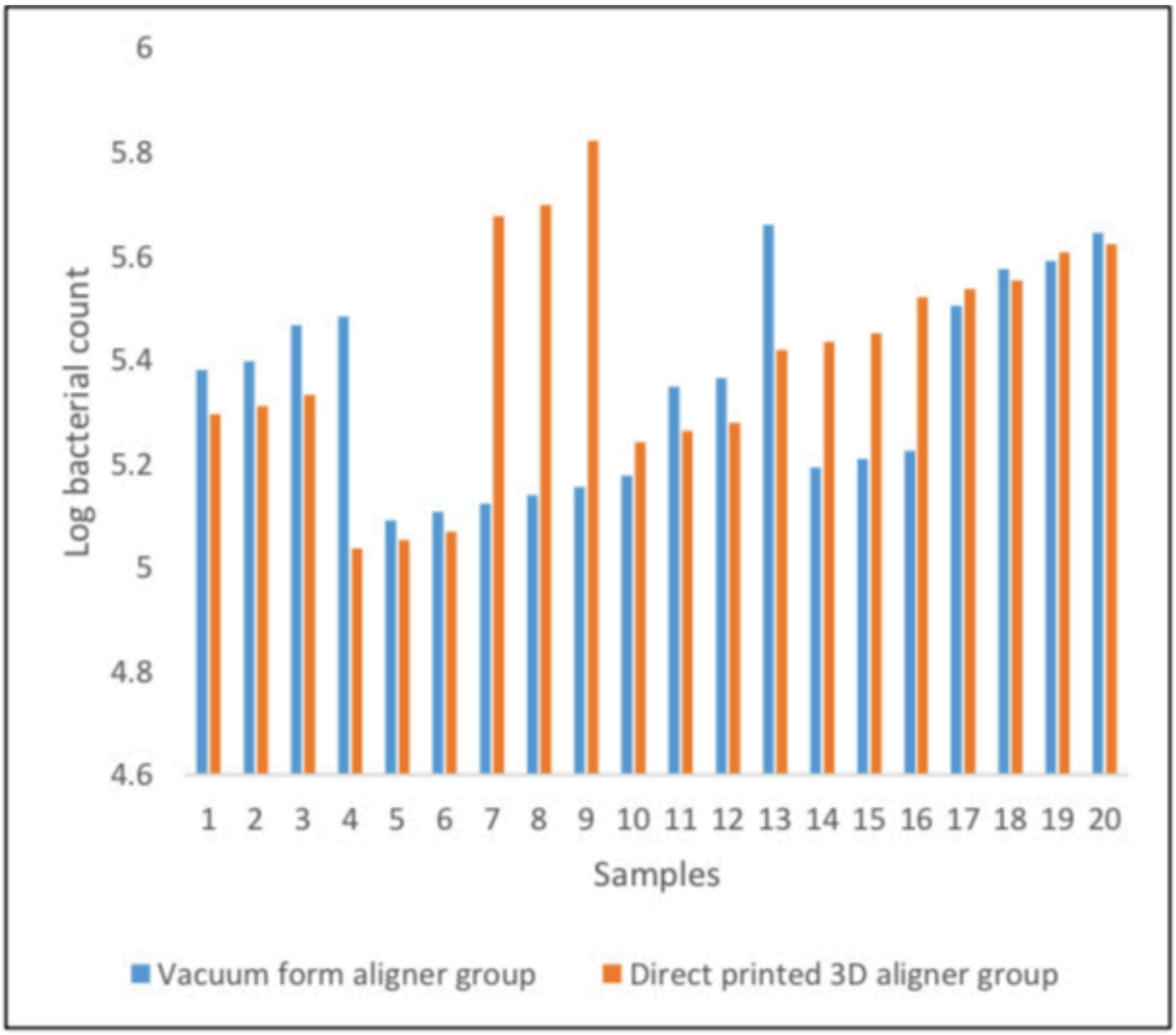


Figure 3

Log bacterial count of vacuum form and direct printed 3D aligners

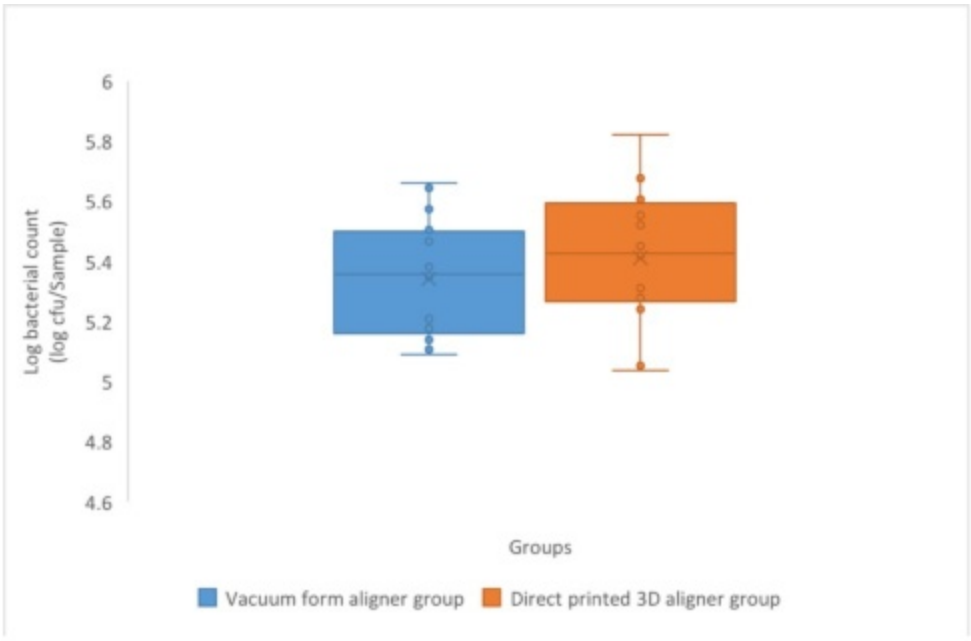


Figure 4

Mean log bacterial count of vacuum form and direct printed 3D aligners

Supplementary Files

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- Table.docx