

Antibacterial and antibiofilm activity over time of GuttaFlow Bioseal and AH Plus

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The sealers' antibacterial and antibiofilm activities against *Enterococcus faecalis* were evaluated by direct contact test (DCT) and confocal laser scanning microscopy (CLSM), respectively, after 1 day, and 1 and 4 weeks of aging. Cell viability was determined by adenosin triphosphate (ATP) assay after DCT. The parameters evaluated for the antibiofilm property were total biovolume and percentage of green cells in *E. faecalis* biofilms. The data from the bioluminescence ATP assay as well as the total biovolume and green percentage were analyzed by non-parametric tests, Kruskal-Wallis for global comparison and Kolmogorov-Smirnov for each two variables. Results of the DCT and CLSM for all parameters evaluated show that the antimicrobial activity of AH Plus decreased over time, whereas GuttaFlow Bioseal had an opposite property, increasing its antibacterial activity as the material aged.

Keywords: AH Plus, Antimicrobial activity, Endodontic sealers, GuttaFlow Bioseal

INTRODUCTION

The purpose of endodontic therapy is to remove microorganisms from infected root canals and prevent reinfection¹. Although instrumentation and irrigation during biomechanical preparation significantly reduces the microbiota within the infected root canal to a level compatible with healing², complete elimination is impossible^{3,4}. Furthermore, the procedure does not necessarily impede a secondary infection. For this reason, the use of a biocompatible root canal sealer that hermetically seals the root canal and also possesses long-term antimicrobial and antibiofilm properties could help reduce residual infection or create an environment that hinders bacterial colonization⁵.

AH Plus (Dentsply DeTrey, Konstanz, Germany), an epoxy-based resin sealer, widely used in clinical practice and for comparison in investigation, is considered the gold standard because of its good physicochemical properties, biocompatibility and tissue tolerance⁶⁻⁸. However, it is not bioactive and lacks osteogenic potential⁹. While it has demonstrated some antimicrobial properties, the antiseptic capacity of AH Plus is limited after setting^{10,11}.

A recently marketed silicone-based sealer, GuttaFlow Bioseal (Coltène/Whaledent, Altstätten, Switzerland) is said to improve upon the biological properties of its predecessors, GuttaFlow and GuttaFlow 2. It is a mixture of gutta-percha powder, polydimethylsiloxane, platinum catalyzer and zirconium dioxide. It also incorporates calcium silicate particles in its composition, allowing it to be used in environments contaminated with fluids, and facilitating the release of calcium ions necessary for the *in situ* nucleation of apatite deposits^{12,13}. This can

be seen as an attractive strategy to obtain a bioactive gutta-percha sealer and may prove useful in endodontic and regenerative therapy¹³. The new product exhibits adequate physicochemical properties⁷ such as good dentin penetrability¹⁴ and a higher cytocompatibility than AH Plus¹⁵. The antimicrobial activity of GuttaFlow Bioseal is unknown to date.

The aim of this study was to investigate *in vitro* the antibacterial and antibiofilm activity of GuttaFlow Bioseal and AH Plus, after 1 day, and 1 and 4 weeks of aging.

MATERIALS AND METHODS

Table 1 shows the specifications (manufacturer, lot number and composition) of the tested materials. GuttaFlow Bioseal (Coltène/Whaledent) and AH PlusTM (Dentsply DeTrey) were assessed and prepared according to manufacturers' recommendations.

The bacterial strain used in this study was *E. faecalis* ATCC 29212. For the antimicrobial and antibiofilm tests, an initial bacterial suspension of approximately 1×10^7 colony-forming units (CFU) per milliliter in brain–heart infusion (BHI) broth (Scharlau Chemie, Barcelona, Spain) was adjusted using a turbidimeter.

In this investigation the antibacterial and antibiofilm activity was determined by the direct contact test (DCT), and the total biovolume and percentage of green cells by confocal laser scanning microscopy (CLSM), respectively. The results in terms of antibacterial activity reflect the ability of the material to kill bacteria that come in contact with it, while the antibiofilm activity denotes the antimicrobial capacity of the material over time, preventing the formation of biofilm.

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Table 1 Endodontic sealers and their manufacturers

Endodontic sealer	Lot number and expiration date	Manufacturer	Composition
GuttaFlow Bioseal	H51755 2018-09-30	Coltene/Whaledent, Altstätten, Switzerland	Gutta-percha powder, polydimethylsiloxane, platinum catalyst, zirconium dioxide, silver (preservative), coloring, bioactive glass ceramic
AH Plus	1610000531 2018-08-31	Dentsply DeTrey, Konstanz, Germany	Bisphenol A/F epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments dibenzylidiamine, aminoadamantane, silicone oil

Antibacterial activity test

To test the antimicrobial activity of materials, a modified DCT¹⁶⁾ was used. A 96-well microtiter plate (Nunclon Delta Surface, Nunc, Roskilde, Denmark) was held vertically and an area of established dimensions on one side of the wells was delimited by measuring to points at the edge of the wells separated by 4 mm. The area was coated with approximately 30 μ L of each sealer using a sterile syringe needle system (BD Plastipak, Becton Dickinson, Madrid, Spain) and a cavity liner applicator. Once the sealers were set, they were subjected to an aging process through the addition of 250 μ L of phosphate-buffered saline (PBS) in each well, then kept at 37°C for 1 day, 1 and 4 weeks¹⁷⁾. Three microtiter plates were similarly prepared and tested for each experimental period ($n=12$ /group).

After each time period, the plates were disinfected by ultraviolet irradiation for 2 h. The plate was positioned vertically, and a 10- μ L aliquot of the initial bacterial suspension was placed on the surface of each sealer. Bacterial suspensions placed on the wall of uncoated wells served as the positive control. After incubation for 1 h at 37°C, with 95% relative humidity to ensure direct contact between the bacteria and tested materials, 220 μ L of sterile BHI was added to each well.

Cell viability was determined by means of the adenosine triphosphate (ATP) assay (BacTiter-Glo™, Promega, Madison, WI, USA)¹⁸⁾. For the ATP assay, 100 μ L of bacterial suspension was added to 100 μ L BacTiter-Glow reagent in a 96-well white plate (Greiner, Monroe, NC, USA) followed by incubation at room temperature for 5 min. The luminescence produced was measured with a luminometer (GloMax™, Promega) and expressed as an absolute value of relative light units (RLUs) in each group.

Antibiofilm activity test

Disks of each sealer were prepared under aseptic conditions in sterile silicone molds, 5 mm in diameter and 1.5 mm high, and stored in an incubator at 37°C for 48 h to achieve complete setting. After aging for 1 day, 1 and 4 weeks, the samples were placed in 24-well plates containing 2.7 mL of BHI and 0.3 mL of the initial bacterial suspension per well, and incubated at 37° for 3 weeks. The BHI was refreshed every two days. Five samples of each sealer were tested, with each sealer

group placed in a different plate.

Once the biofilms had formed, the samples were rinsed with 0.9% saline solution, stained with Syto-9/Propidium iodide (PI) (Live/Dead, BacLight, Invitrogen, Eugene, OR, USA)¹⁹⁾ for 15 min and were observed under a confocal laser scanning microscope (CLSM, Leica TCS-SP5 II, Mannheim, Germany). Syto-9 is a green-fluorescent stain, labeling both live and dead microorganisms. PI is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes (dead microbes). Four microscopic confocal volumes from random areas were obtained from each sample using a 40 oil lens, 1 μ m step-size and a resolution of 512×512 pixels. Each picture represented an area of 387×387 μ m. The scanning was performed from the top of the biofilm to the dentin surface. For quantification purposes bioImage_L software was used²⁰⁾. The parameters evaluated in each group were the total biovolume expressed in μ m³ and the percentage (%) of green population (live cells).

Statistical analysis

Results of the ATP assay, total biovolume and green cells percentage were analyzed by non-parametric tests, Kruskal-Wallis for global comparison and Kolmogorov-Smirnov for each two variables. The level of significance was 0.05.

RESULTS

The results of the antibacterial and antibiofilm tests with AH Plus and GuttaFlow Bioseal are given in Tables 2 and 3. The DCT showed that GuttaFlow Bioseal exerted antimicrobial activity with respect to the control, increasing this efficacy according to the aging time of the material. The antimicrobial activity of AH Plus decreased over time, although no significant differences were seen between 1 and 4 weeks.

A total of 120 CLSM operative fields (3D stacks) were evaluated in the antibiofilm test (20 stack/group/period). Total biovolume increased over time in AH Plus, and decreased for GuttaFlow Bioseal. No significant differences were shown in % green cells for AH Plus while there were statistical differences at 4 weeks for GuttaFlow Bioseal with respect to 1 day and 1 week. Representative images of the biofilms grown on the surface of the sealers were found in Fig. 1.

Table 2 Aging effect on antibacterial activity using DCT of GuttaFlow Bioseal and AH Plus against *E.faecalis* in terms of bioluminescence determined with ATP assay (RLUs)

		Antibacterial activity		
		AH-Plus	GuttaFlow Bioseal	Comparisons** <i>p</i> value
ATP assay Relative Light Units (RLUs)	1 day	5,915.75 (954.88) ^a	22,855.33 (5295.49) ^a	<0.001
	1 week	21,329.41 (8780.10) ^{b,c}	13,797.50 (5294.23) ^b	0.01
	4 week	19,979.41 (2831.91) ^b	11,416.50 (3030.99) ^b	<0.001
	Control	27,805.00 (4697.37) ^c	27,805.00 (4697.37) ^c	—
	Comparisons* <i>p</i> value	<0.001	<0.001	—

*Global comparison determined by Kruskal Wallis test. Read vertically, the same superscript letters do not show statistically significant differences compared by Kolmogorov-Smirnov test. **Comparison with the Kolmogorov-Smirnov test. Mean (standard deviation); *n*=12/group.

Table 3 Aging effect on antibiofilm activity of GuttaFlow Bioseal and AH Plus

		Antibiofilm activity		
		AH-Plus	GuttaFlow Bioseal	Comparisons** <i>p</i> value
Total biovolume (μm^3)	1 day	27,029.85 (20,288.38) ^a	136,760.9 (109,071.66) ^a	<0.001
	1 week	50,737.9 (23,206.05) ^b	92,232.50 (66,073.52) ^a	0.005
	4 weeks	79,548 (37,483.89) ^c	25,246.45 (16,898.57) ^b	<0.001
	Comparisons* <i>p</i> value	<0.001	<0.001	—
Green cells (%)	1 day	52.38 (18.29) ^a	80.83 (8.99) ^a	<0.001
	1 week	56.13 (18.11) ^a	71.56 (26.96) ^a	<0.001
	4 weeks	60.68 (19.61) ^a	41.81 (21.33) ^b	0.082
	Comparisons* <i>p</i> value	0.342	<0.001	—

Global comparison determined by Kruskal Wallis* test. For each of the two variables, read vertically, the same superscript letters show differences not statistically significant as compared by Kolmogorov-Smirnov test. **Comparison between AH Plus and GuttaFlow Bioseal at each time by Kolmogorov-Smirnov test.

Mean (standard deviation) of total biovolume (μm^3) and percentage of green cells of *E.faecalis* 3 week- biofilms observed under confocal laser scanning microscopy (CLSM); (*n*=20 stack/group/period).

DISCUSSION

The use of an endodontic sealer possessing long-term antimicrobial capacity could be determinant for the success of an endodontic treatment, since it would help diminish the residual microbial load after chemico-mechanical preparation and impede the formation of new biofilms^{5,10}. The aim of this study was to evaluate the antimicrobial efficacy of a new bioactive endodontic sealer, GuttaFlow Bioseal, after aging. AH Plus was selected as a control because it is a well-known sealer used in most studies of this nature^{11,13}. Furthermore,

silicone-based sealers showed no antimicrobial activity against *E. faecalis*¹¹.

Given that *E. faecalis* is one of the most frequently detected bacterial species in persistent periapical lesions, it is most commonly used in *in vitro* studies to evaluate the antimicrobial efficacy of root canal sealers^{11,21}. Its ability to penetrate the dentinal tubules and form biofilms—even in unfavorable conditions—and its resistance to antimicrobials allows it to remain in root canals after endodontic treatment²². Therefore, evaluating the antibacterial property of endodontic sealers against this bacterium is relevant from a clinical

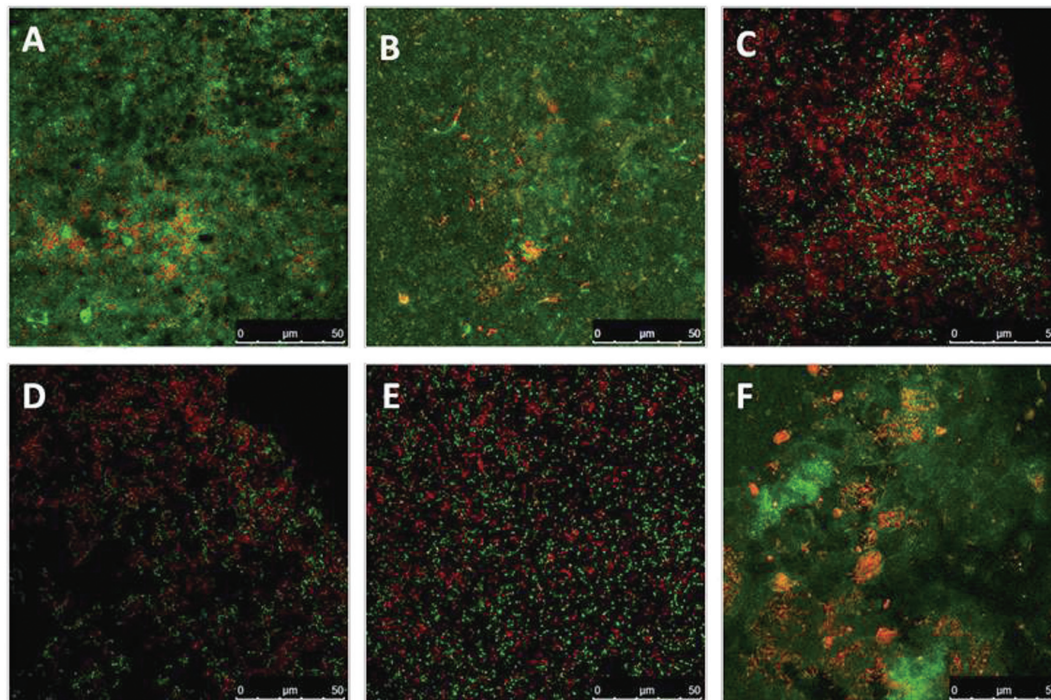


Fig. 1 Representative images of bacterial growth with CSLM. GuttaFlow Bioseal at 1 day (A), 1 week (B) and 4 weeks (C). AH Plus at 1 day (D), 1 week (E) and 4 weeks (F).

standpoint¹¹.

In this study, the antibacterial activity of the sealers was assessed by means of DCT. It is a reproducible method that can be used in standardized aging studies, to quantify the bactericidal efficacy of insoluble materials, simulating contact with microorganisms^{11,16,17}. Cellular viability after the DCT was measured by the determination of ATP—an easy and rapid method of quantifying bacteria that is viable, but non-culturable (VBNC) in traditional culture media²³. The bioluminescence ATP assay has sufficient sensitivity for bacteria detection (between 10 and 100 cells) in root canal infection and can discriminate between positive and negative cultures²⁴.

To determinate the antibiofilm property of sealers, an approach based on CLSM was used, since it is a simple, reproducible and highly sensitive method for quantifying the approximate amount of cells adhered to a surface. The CLSM offers information about the cell viability at the same time. For both assays, the materials were tested after fully setting and over time, after 1 day, and 1 and 4 weeks. It is known that freshly mixed materials have a greater antibacterial effect than those fully set and aged; therefore, aging time is a parameter that must be taken into account when evaluating antimicrobial activity¹⁷.

Globally, AH Plus showed a reduction in its antimicrobial activity overtime, which is compatible with the results of previous studies^{11,25,26}. DCT data indicated that at 24 h after setting, AH Plus achieved a reduction

percentage of RLUs close to 80% compared to the control. This value dropped to 23.28 and 28.14% at 1 and 4 weeks, without significant differences between these two periods. The bactericidal activity of AH Plus against *E. faecalis* by DCT was recently reviewed¹¹. Although somewhat diverse conclusions so far have been drawn, most results reflected a positive antimicrobial effect before setting, which declines or even disappears after 2 and 7 days of setting and aging. The study by Sagsen *et al.*²⁷ alone evaluated the long-term (30 days) antibacterial capacity of AH Plus, concluding that the bactericidal activity was only effective up to 24 h after setting. Once the material was set, it lost its antimicrobial activity. This short-term antimicrobial capacity of AH Plus may have to do with the bactericidal effect of formaldehyde released in small amounts during the setting process²⁸; or with the toxicity of non-polymerized components, such as amines or epoxy resins²⁹. This fact also has been attributed for explaining the reduced antibiofilm activity of AH Plus in previous studies¹⁰, and is compatible with the results obtained in the present investigation respect to the antibiofilm activity test. *E. faecalis* was able to grow on the surface of AH Plus, with the lowest values of total biovolume at 1 day; the values increased over time with significant differences among the three time periods recorded. However, the percentage of viable cells for AH Plus did not vary over time.

To date, although it has been shown to have adequate physicochemical properties¹³⁻¹⁵, no information on antimicrobial activity of GuttaFlow Bioseal is

available in current scientific literature. According to DCT results in this study, the ATP assay indicated an increased antimicrobial activity over time, from a 17.81% reduction in RLUs at one day, to a 58.94% reduction at 4 weeks. This property could be considered opposite of the effect of AH Plus. Similarly, regarding its antibiofilm capacity, after 1 day of aging, GuttaFlow Bioseal showed the highest biovolume values and percentage of green cells (80.83%). However, after 4 weeks, both variables decreased; the total biovolume to one-fifth, the percentage of viable cells to one-half (41.8%), which confirmed that the antimicrobial capacity increased up to 4 weeks. GuttaFlow Bioseal contains calcium silicate particles, which provide alkalizing activity through the continuous release of calcium ions after setting¹³. The alkaline environment has an antimicrobial effect within the root canal⁵ and can prove beneficial for the healing process, since the pH of the periapical region would be increased, contributing to the formation of hard tissue through the activation of alkaline phosphatase¹³.

Given the ability of GuttaFlow Bioseal to kill *E. faecalis* and inhibit the formation of biofilms determined in this study, as well as its adequate physico-chemical and biological properties^{7,13-15}, this silicone-based sealer appears to be a promising material in root canal treatment. A recent study demonstrated that GuttaFlow Bioseal provided better apical sealing than Roeko Seal Automix and GuttaFlow 2 used in teeth with wide (apical diameter 40) and wet apices³⁰, pointing to an added advantage for its clinical use. However, further research need to be carried out to investigate how long the sealer's antibiofilm capacity can last after 30 days, at what point it totally disappears, and how its properties change over time depending on the oral cavity environment of each patient.

CONCLUSION

GuttaFlow Bioseal showed increased antibacterial and antibiofilm activity at 1 and 4 weeks as determined by DCT and CLSM, while AH Plus indicated an opposite property in which its antimicrobial activity decreased over time.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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