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ORIGINAL ARTICLE

Comparison of antibacterial effectiveness between Sealapex and AH-plus sealer against *Enterococcus faecalis:* a systematic review of *in vitro* studies

ABSTRACT

Aim: *To summarize the outcome of in vitro studies comparing the antibacterial effectiveness of Sealapex and AH-plus sealer against E. faecalis.*
Methodology: *The research question was developed using the PICO methodology and studies were identified from three electronic databases in Medline, Scopus, and EBSCOhost (Dentistry; Oral Sciences Source) since inception up to November 2019. The title and abstract of the selected articles were independently reviewed by two reviewers based on the specified inclusion and exclusion criteria and extracted the data using the data extraction form. The quality of selected in vitro studies was appraised using revised Cochrane Risk of Bias tool.*
Results: *Sixteen studies satisfied the inclusion criteria and were included in this systematic review. Due to the lack of homogeneity in the data, meta-analysis could not be conducted. The quality of the evidence was "low", since every study had at least three questions related to high risk of bias. Different laboratory tests and protocols were used, their results were contradicting even for studies using the same laboratory tests and quality of evidence was found to be low. No study provided strong evidence, twelve studies provided moderate evidence, three studies provided limited evidence and one study provided conflicting evidence. The research question could not be meaningfully addressed.*
Conclusions: *No difference was observed in the antimicrobial efficacy of Sealapex and AH-plus root canal sealers against* Enterococcus faecalis. *There was an identification of poor quality relevant studies with contradicting results that indicates the need for development of standardized protocols for future in* vitro *studies.*

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Introduction

The main objective of the root canal treatment is to eliminate bacteria from the root canal system and prevent reinfection. However, due to complex root canal anatomy, bacteria can be found left in the root canal system even after thorough chemomechanical debridement. A number of microorganisms have been isolated from infected root canal system based on the type of infection and clinical manifestation of the disease (1). Endodontic infection can either be primary in nature due to the invasion of microorganisms or microbial by-products into the pulp tissue or secondary due to reinfection after root canal therapy or remnant or persistent infection. Although the occurrence of root canal failure is multifactorial, the persistence of microorganisms within the root canal system after treatment has found to be the most significant reason for endodontic failure (2). The microflora of primary infected canals with untreated apical periodontitis and secondary infected canals (failed endodontic treatment) differs in number and in phenotypes. In canals with secondary infection, facultative anaerobic and gram-positive bacteria predominate and comprised of one or two species per canal including *Enterococcus, Streptococcus, Peptostreptococcus*, *Actinomyces* species, *Fusobacterium nucleatum* and *Propionibacterium* (3).

Among these microorganisms, *Enterococcus faecalis (E. faecalis)* has been one of the most prevalent microorganisms isolated from failed root canal treated teeth (4, 5). Furthermore, *E. faecalis* has been more often associated with primary endodontic infections with asymptomatic chronic periradicular lesions than with acute periradicular periodontitis. Rôças et al. observed presence of *E. faecalis* nine times higher in endodontic cases with secondary infection than primary endodontic infection (6).

E. faecalis can survive harsh conditions as it can create biofilms and has the ability to penetrate into dentinal tubules (7). Bacteria in intracanal biofilms develop mechanisms to protect themselves against antibiotic medicaments, becoming 1,000 to 1,500 times more resistant compared to bacteria in planktonic form (8). In fact, calcium hydroxide, which is a very popular intracanal medicament used for elimination of the remaining bacteria after instrumentation and irrigation, can neither prevent *E. faecalis* from being organized in biofilms, nor eliminate these biofilms due to its adhering ability to the dentine that increases biofilms' resistance (9). The shaping and cleaning of the root canal system is followed by obturation using a core material and sealer necessary to establish a fluid tight seal of the root canal system (10). According to the Glossary of Endodontic terms which was developed by the American Association of Endodontists, a sealer is a radiopaque dental cement usually used in combination with a solid or semi-solid core material, to fill voids and seal root canals during obturation (11). The ideal properties of endodontic sealers were described by Grossman et al. (1988) (12). At this moment, there is no sealer that can fulfil all the above criteria and can be considered as the gold-standard although manufacturers may emphasize on various benefits of the sealer.

An informal market survey was conducted by compiling information from the sales teams from United Kingdom, European Union and Malaysian based dental distributors in order to distinguish the most popular sealers in the market. Dental suppliers in Scotland pointed to Sealapex as the most popular root canal sealer. AH-plus has also been a very well-studied sealer in the literature and found to be almost in every *in vitro* study investigating the antibacterial efficacy of sealers (13). Sealapex, a calcium hydroxide-based sealer produced by SybronEndo has shown to have antibacterial property that may facilitate quick periapical healing and hard tissue formation (14, 15). It is a catalyst/base system, introduced in two tubes or in a double barrel syringe (Sealapex Xpress). AH-plus, an epoxy-resin based sealer produced by

DENTSPLY DeTrey is a paste/paste system, introduced in two tubes or in a double barrel syringe (AH-plus Jet). According to manufacturers it has several advantages such as high radiopacity, high dimensional stability, good dentinal adherence and good sealing ability. Moreover, it does not release formaldehyde or cause tooth discoloration unlike its predecessor AH26 (16). Many *in vitro* studies have been done focusing on the antibacterial efficacy of sealers however, assessing the antimicrobial efficacy using *in vivo* studies could be difficult due to many confounding factors affecting the endodontic treatment outcome. As a result, the antimicrobial efficacy of the sealer type cannot be distinguished or separated on treatment outcome.

There has been only one unpublished systematic review registered in PROSPERO available comparing the antibacterial efficacy of bioceramic sealers with other root canal sealers against different types of bacteria including both *in vivo* and *in vitro* studies. However, bioceramic sealers are newly introduced and not widely used at the moment. Another published systematic review was found in the literature focussing on the antibacterial efficacy of various sealers against *E. faecalis* (17). However, this systematic review included studies which strictly used the direct contact test as a laboratory model and excluded studies that adopted other laboratory tests to assess antimicrobial efficacy. This could lead to loss of useful information which could ultimately lead to misleading conclusions concerning the antibacterial activities of sealers and potentially wrong clinical decisions. Besides, the authors carried out the initial search in 2015 and repeated the search in March 2016.

Therefore, the aim of this systematic review was to compare the antibacterial efficacy of Sealapex and AH-plus against *E. faecalis*. A scoping search was carried out by the authors of the current review which revealed additional relevant laboratory studies published in 2017, 2018 and 2019 were included in this review.

Materials and Methods

Review question

The research question was developed by using the Population, Intervention, Comparison, Outcome and study design (PICOS) framework. In the extracted permanent human teeth with *Enterococcus faecalis* (P), does Sealapex sealer (I) show better antibacterial property (O) compared to the AH-plus sealer (C) from *in vitro* studies (S). This systematic review was carried out in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (18). This study was carried out at the University of Central Lancashire, United Kingdom in collaboration with researchers at the International Medical University, Malaysia.

Search strategy

In order to identify the existing relevant papers needed to answer the research question, a specific process was undertaken as described below. Literature search was performed comprehensively using three electronic databases: Medline, Scopus and EBSCOhost (Dentistry; Oral Sciences Source) from inception to November 2019. The search terms are summarized in Table 1. Additional literature search was performed from the reference list of the eligible studies. Based on the journals publishing the content relevant to the topic, *Journal of Endodontics, International Endodontic Journal, Journal of Dentistry, Australian Endodontic Journal and Journal of Conservative Dentistry* were hand searched to identify any relevant studies.

Inclusion criteria

Inclusion criteria for this review were as follows. i) Time period: no time restriction was applied. ii) Population: papers that used *E. faecalis* among the test microorganisms were included. iii) Intervention and comparator: studies that used both AH-plus and Sealapex. iv) Type of studies: only *in vitro* studies. v) Types of outcome: the antibacterial efficacy of the sealers was assessed by determining the remain-

Table 1
Search strategy terms logic grid

Electronic databases search strategies			
	Pubmed	**EBSCO Dentistry & Oral Sciences Source**	**Scopus**
	(((((((((enterococcus faecalis) OR Enterococcus faecalis) OR e. faecalis) OR E faecalis) OR Biofilms) OR biofilm)) **AND** (((Sealapex) OR calcium hydroxide-based sealer) OR calcium hydroxide-based sealer cement)) **AND** (((((AH-plus) OR resin-based sealer) OR resin-based sealer cement) OR epoxy resin-based sealer) OR epoxy resin-based sealer cement)) **AND** ((antimicrobial) OR antibacterial)	((enterococcus faecalis) OR (Enterococcus faecalis) OR (e. faecalis) OR (E faecalis) OR (Biofilms) OR (biofilm)) **AND** ((Sealapex) OR (calcium hydroxide-based sealer) OR (calcium hydroxide-based sealer cement)) **AND** ((AH-plus) OR (resin-based sealer) OR (resin-based sealer cement) OR (epoxy resin-based sealer) OR (epoxy resin-based sealer cement)) **AND** ((antimicrobial) OR (antibacterial))	(enterococcus AND faecalis OR enterococcus AND faecalis OR e. AND faecalis OR e AND faecalis OR biofilms OR biofilm) **AND** (sealapex OR calcium AND hydroxide-based AND sealer OR calcium AND hydroxide-based AND sealer AND cement) **AND** (ah-plus OR resin-based AND sealer OR resin-based AND sealer AND cement OR epoxy AND resin-based AND sealer OR epoxy AND resin-based AND sealer AND cement) **AND** (antimicrobial OR antibacterial)
Total records	**19**	**87**	**53**

ing viable bacteria after the action/application of sealers, and always in relation to the laboratory method used. vi) Language: no language restrictions were applied. Applicable articles were included regardless of the language used, since support from translators was available.

Exclusion criteria
Exclusion criteria included, *in vitro* studies that did not include both AH plus and Sealapex assessing the antibacterial efficacy against *E. faecalis*, studies which assessed antibacterial efficacy against other species of bacteria, unpublished articles, review articles, *ex vivo* articles and systematic reviews.

Study selection and data extraction process
The title and abstract of the selected articles were independently reviewed by two reviewers (DN and SK) based on the specified inclusion and exclusion criteria.
The two reviewers had an almost perfect agreement with a Cohen's Kappa score of 0.9 (19). The reviewers independently read the selected articles for the review and extracted the data using the data extraction form exclusively developed for this study. This form consisted of following details: author, year, preparation of AH-plus and Sealapex, *E. faecalis* strain, control group, laboratory test, antibacterial evaluation, evaluation timing, statistical tests, findings and outcome. Any disagreement between the two reviewers was resolved by discussion with a third reviewer (AP).

Quality assessment of the included studies
The quality of each article was appraised using Cochrane Risk of Bias tool (RoB 2.0) (20). This tool was modified to include the contents based on the methodology employed in the included *in vitro* studies. The quality of included studies was assessed based on following domains: manufacture of materials, instructions of manufacturers followed, existence of control group, repetition of experiment, consistent measurements for repeated experiments, establishment of test methods, objective outcome measurement, direct outcome measurement, who undertook key parts of the experiment, blinding of assessors, use of appropriate statistical tests, report of variability. Two authors (DP and SK) independently evaluated and scored the articles based on the above domains. In case of disagreement, consensus was arrived in discussion with another reviewer (AP).
Haase (21) pointed the importance of quality assessment of the selected studies. Unfortunately, there was no validated tool

for bias assessment for *in vitro* studies. A decision was made to develop one by synthesizing the existing literature and applying necessary modifications to serve the individual purposes of this systematic review. More specific, a template questionnaire was developed by Neil Cook (2018), (Research Associate, School of Dentistry, University of Central Lancashire) based on six studies (22-27).
After individual bias assessment for each included study was completed, a traffic light system similar to the Cochrane Risk of Bias (RoB) tool was developed.

Results

Study Selection process
After completing the search of the three electronic databases, 19 articles were identified in Medline database, 87 articles in EBSCOHost (Dentistry, Oral Sciences Source), and 53 in Scopus database. In total 159 articles were identified after the electronic literature search.
After removing duplicates and abstract screening, 17 studies were found eligible for full text screening.
It was found that all of them met the inclusion criteria, however, two studies had three authors in common and used exactly the same numerical results for AH-plus and Sealapex (28, 29).
If both were included in the analysis the data would be double counted so it was decided that the most recent of the two studies should be excluded from analysis (28). In total 16 studies were included in this systematic review (29-44).
The process followed for study selection was presented via PRISMA Flowchart in Figure 1.
Due to the lack of homogeneity in the data, since studies used different bacteri-

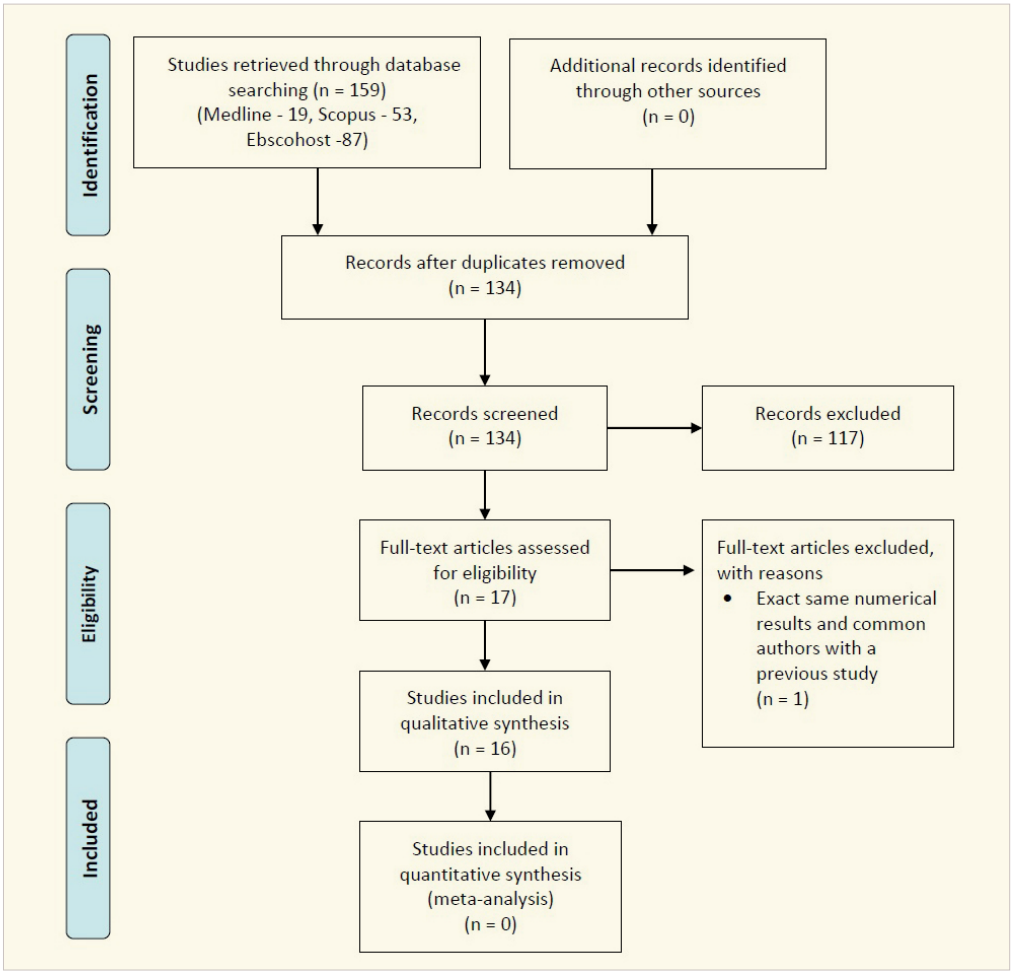

Figure 1.

al strains, different laboratory models to assess antibacterial efficacy and different evaluation times, a meta-analysis could not be conducted. Instead a narrative analysis was performed.

Characteristics of included studies

The studies included in this systematic review were published between 2000 and 2018. Six studies used the agar diffusion test as a laboratory method to assess the antibacterial efficacy of sealers against *E. faecalis* (31, 35-37, 42, 43), five studies used the direct contact test (32, 34, 38, 41, 44), three studies used both methods (29, 30, 33) and three studies used other methods such as membrane restricted contact test (34), time kill essay (39) and broth method (40). Following this, agar diffusion test and direct contact test can be considered as the most widely used laboratory method to assess the antibacterial efficacy of sealers.

Three studies have been found to use optical density and record results using spectrophotometer as antibacterial evaluation method (30, 33, 41). Whereas, six studies employed the method of determination of colony-forming units (CFU) (29, 32, 34, 38, 39, 44). Six studies evaluated the antibacterial activity thorough measurement of inhibition zones (31, 35-37, 42, 43) and one study measured *E. faecalis* growth as a function of sealers' concentration by broth method (40). Two studies employed both spectrophotometer and measurement of inhibition zones (30, 33) and one study determined by using CFU and measurement of inhibition zones (29).

The most commonly used strain of *E. faecalis* is the "ATCC 29212", which is used in nine studies (29, 30, 32, 35, 37, 39-42). Among the different evaluation times each study used, the most common evaluation time is 24 hours after sealers had been mixed and is included in ten studies (31, 33-37, 39, 42-44). Among the studies that used agar diffusion test as a laboratory method to assess the antibacterial effect of AH-plus and Sealapex against *E. faecalis*, three studies concluded that Sealapex has stronger antibacterial efficacy compared to AH-plus (31, 35, 36), and more specific two of them showed that the antibacterial efficacy of Sealapex is significantly higher (31, 36). On the other hand, three studies found that neither Sealapex nor AH-plus had any antibacterial effect against *E. faecalis* (33, 37, 42). Moreover, two studies found that AH-plus has stronger antimicrobial efficacy than Sealapex (29, 42). It must be pointed out though that one study showed that the difference found between the two sealers was not statistically significant (42), while on the contrary another study found a statistically significant difference between them (29). Finally, only one study found that there was no statistical difference between the antibacterial efficacy of the two sealers (30). Hence, contradictory results were shown from these three studies (29, 30, 32).

Among the studies that used the direct contact test as a laboratory method to assess the antibacterial efficacy of AH-plus and Sealapex, four studies showed that AH-plus had higher antibacterial efficacy compared to Sealapex (30, 33, 34, 41), and more specific, three of them showed that the antibacterial efficacy of AH-plus was significantly higher (33, 34, 41). It should be mentioned that one study showed that the difference was statistically significant for freshly mixed sealers only (41). On the other hand, three studies concluded that Sealapex had a statistically significant increased antibacterial efficacy compared to AH-plus (29, 32, 38).

Finally, only one study showed opposing results for different evaluation times (43). More specific, for freshly mixed sealers AH-plus showed statistically significant higher antimicrobial efficacy compared to Sealapex, but after 1, 3 and 7 days, Sealapex was more efficient. Total there were six studies measured the fresh mixed samples (33, 29, 39-41, 44), ten studies measured after 24 hours (31, 33-37, 39, 42-44), nine studies measured after 48 hours (29, 31-33, 36, 38, 41, 42, 44) and eight studies measured after 7 days (29, 30, 32, 38, 39, 41, 42, 44). The characteristics of the included *in vitro* studies were shown in Table 2.

Table 2

Characteristics of included studies in the systematic review

No.	Study	E. Faecalis strain	Control group	Laboratory test	Antibacterial evaluation	Evaluation timing	Statistical tests	Outcome
1	Poggio et al. [29]/2017/ Italy	ATCC 29212	ADT: Two plates without bacterial suspension, one without sealer DCT: Sealer-free saline suspension	ADT and DCT	ADT: Measurement of inhibition zones (mm) DCT: Determination of colony-forming units (CFU/ml)	ADT: After 48h DCT: After 6', 15' and 60' (set sealers-7 days)	Student's t- test	ADT: AH-plus showed significantly higher antimicrobial efficacy than Sealapex DCT: Sealapex showed significantly higher antimicrobial efficacy than AH-plus.
2	Cobankara et al. [30] /2004/ Turkey	ATCC 29212	NA	ADT, DCT	ADT: Measurement of inhibition zones (mm) DCT: Turbidometric measurement of bacterial outgrowth (optic density at 620nm)	ADT: After 7 days DCT: 12 measurements every 30mins for the first 6h and 12 measurements in the last 6h	ADT: Kolmogorov-Smirnov DCT: -	ADT: No statistical difference found between AH-plus and Sealapex DCT: AH-plus showed higher antibacterial efficacy than Sealapex.
3	Dalmia et al. [31]/2018/ India	MTCC 2093	NA	ADT	Measurement of inhibition zones (mm)	After 24h, 48h and 72h	ANOVA, Unpaired t-test	Sealapex showed statistically significant higher antimicrobial efficacy compared to AH-plus
4	Faria-Junior et al. [32]/2013/ Brazil	ATCC 29212 organised in biofilm	Biofilm not exposed to sealers	Modified DCT	Determination of colony-forming units (CFU/ml) in biofilm	After 5h, 10h and 15h (2 and 7 days after sealers had set)	Kruskal-Wallis and Dunn tests	Sealers set for 2 days, Sealapex showed statistically significant higher antimicrobial efficacy compared to AH-plus. Sealers set for 7 days, no statistical significance was detected between 0-5h, but Sealapex again showed statistically significant higher antimicrobial efficacy compared to AH-plus between 5-15h.
5	Heyder et al. [33]/2013/ Germany	DSMZ 20376	ADT: Chlorhexidine as positive control and distilled water as negative control DCT: culture medium as negative control and 100µl of bacterial suspension with baseline cell concentration mixed with 400µl Schaedler liquid medium as positive control	ADT and DCT only for sealers that showed good antibacterial effect in ADT	ADT: Measurement of inhibition zones (mm) DCT: Turbidometric measurement of bacterial outgrowth (optic density at 560nm)	ADT: After 48h (both for freshly mixed and set sealers) DCT: After 0, 2, 4, 6, 8, 12 and 24h	ADT: Mann-Whitney U-test DCT: two-tailed t-test	ADT: Neither AH-plus nor Sealapex showed antibacterial activity against E. faecalis (for fleshly mixed and set sealers) DCT: AH-plus significantly reduced E. faecalis, whereas Sealapex showed no antibacterial activity against E. faecalis.
6	Kayaoglou et al. [34] /2005/ Turkey	A197A	Teflon disc	DCT and membrane-restricted test	Determination of colony-forming units (CFU/ml)	After 24h	Student's t-test	AH-plus showed statistically significant higher antimicrobial efficacy compared to Sealapex
7	Leonardo et al. [35] /2000/ Brazil	ATCC 10541	NA	ADT	Measurement of inhibition zones (mm)	After 24h	NA	Sealapex showed stronger antibacterial efficacy than AH-plus

8	Mickel et al. [36]/2003/ USA	NA	amoxicillin disc (positive control)	ADT	Measurement of inhibition zones (mm)	After 24h and 48h	ANOVA, Tukey's test	Sealapex showed antibacterial effect against E. faecalis, while AH-plus did not show any antimicrobial effect. Difference is statistically significant.
9	Miyagak et al. [37]/2006/ Brazil	ATCC 29212	NA	ADT	Measurement of inhibition zones (mm)	After 24h	Non-parametrical test (without determining which)	Neither AH-plus nor Sealapex showed antibacterial effect towards E. faecalis
10	Rezende et al. [38]/2016/ Brazil	ATCC 51299	Dentine blocks with formed biofilm not exposed to sealers	DCT	Determination of colony-forming units (CFU/ml)	After 2, 7 and 14 days	Single-factor ANOVA model, Sapiro-Will, Kruskal-Wallis one-way test	Sealapex showed statistically significant higher antimicrobial activity than AH-plus in all the time periods
11	Sagsen et al. [39]/2009/ Turkey	ATCC 29212	A tube containing brain-heart infusion broth without bacteria, a tube containing brain-heart infusion broth with bacteria	Time kill essay	Determination of colony-forming units.	After 20', 24h, 7d and 9d	NA	AH plus is bactericidal at 20' and 24h and less bactericidal on 7th and 9th days. Sealapex is found bacteriostatic on the 7th and 9th days but without effect at 20' and 24h.
12	Shin et al. [40]/2018/ Republic of Korea	ATCC 29212	Cultures not treated with the spent culture medium	Broth method	Measurement of E. faecalis growth as a function of sealers' concentration	Before and after setting	Kruskal-Wallis and Mann-Whitney tests	Sealapex showed higher antibacterial activity than AH-plus.
13	Smadi et al. [41]/2008/ Jordan	ATCC 29212	Uncoated wells containing identical size inoculation, wells containing test materials without bacterial inoculation	DCT	Turbidometric measurement of bacterial outgrowth (optic density at 620nm)	After 20', 48h and 7d	Multiple t-tests	AH-plus showed statistically significant higher antibacterial efficacy than Sealapex when sealers were freshly mixed. No significant difference is found between them at 48h and one week tests.
14	Smadi et al. [42]/2008/ Jordan	ATCC 29212	Sterile saline	ADT	Measurement of inhibition zones (mm)	After 24h, 48h and 7 days	ANOVA, Tukey's test	Neither AH-plus nor Sealapex showed antibacterial effect against E. faecalis
15	Yasuda et al. [43]/2008/ Japan	ATCC 10541	Plate without sealers	ADT	Measurement of inhibition zones (mm)	After 24h	ANOVA, Tukey's test	AH-plus showed higher antibacterial efficacy than Sealapex, but difference is not statistically significant
16	Zhang et al. [44]/2009/ Canada	VP3-181	Bacterial suspensions on the wall of uncoated wells	Modified DCT	Determination of colony-forming units (CFU/ml)	Fresh sealers, set for 1, 2, 3 and 7 days	ANOVA, Tukey test	Fresh AH-plus significantly reduced E. faecalis numbers at 2' and eradicated them within 5'-20', whereas Sealapex started reducing E. faecalis significantly after 20'. Similar results were found after 1 day and 3 days of setting: Sealapex eradicated E. faecalis in 60' whereas AH-plus failed to kill E. faecalis in the same time. Seven days after mixing: Sealapex shows higher antibacterial efficacy eradicating E. faecalis at 20' and 60' whereas AH-plus shows slight antibacterial efficacy in 2', 5' and 20' and none in 60'.

Table 3

Risk of bias assessment of included studies

Author/Year/Country	Manufacture of materials	Instructions of manufacturers followed	Existence of control group	Repetition of experiments	Consistent measurements for repeated experiments	Establishment of test methods	Objective outcome measurement	Direct outcome measurement	Who undertook key parts of the experiment	Blinding of assessors	Use of appropriate statistical tests	Report of variability
Poggio et al. [29]/2017/Italy	+	+	+	+	–	+	–	+	–	–	+	+
Cobankara et al. [30]/2004/Turkey	+	+	–	+	?	+	–	+	–	–	+	–
Dalmia et al. [31]/2018/India	+	+	–	+	+	+	–	+	–	–	+	+
Faria-Junior et al. [32]/2013/Brazil	+	+	+	+	+	+	–	+	–	–	+	+
Heyder et al. [33]/2013/Germany	+	+	+	+	?	+	–	+	–	–	+	–
Kayaoglou et al. [34]/2005/Turkey	+	+	+	+	+	+	–	+	–	–	+	+
Leonardo et al. [35]/2000/Brazil	+	+	–	+	–	+	–	+	–	–	–	–
Mickel et al. [36]/2003/USA	+	+	+	+	?	+	–	+	–	–	+	–
Miyagak et al. [37]/2006/Brazil	+	+	–	+	?	+	–	+	–	–	+	–
Rezende et al. [38]/2016/Brazil	+	+	+	+	–	+	–	+	–	–	+	+
Sagsen et al. [39]/2009/Turkey	+	+	+	+	?	+	–	+	–	–	–	–
Shin et al. [40]/2018/Republic of Korea	+	+	+	+	+	+	–	+	–	–	+	+
Smadi et al. [41]/ 2008/Jordan	+	+	+	+	?	+	–	+	–	–	+	–
Smadi et al. [42]/2008/Jordan	+	+	+	+	?	+	–	+	–	–	+	+
Yasuda et al. [43]/2008/Japan	+	+	+	+	+	+	–	+	–	–	+	+
Zhang et al. [44]/2009/Canada	+	+	+	+	?	+	–	+	–	–	+	–

Quality of included studies

The studies were analysed using the modified Risk of Bias tool. A table with the traffic-light system was created to assess the overall quality of the included studies (Table 3).

After a thorough look of the traffic-light system it can be stated that the quality of the evidence was "low", since every study had at least three questions related to high risk of bias.

More specific, concerning how the sealers were manufactured, all studies reported that in detail, presenting tables with the ingredients of the sealers and preparing them according to the manufacturers' instructions. As a result, there was very low risk of bias in sealers' manufacturing and preparation.

This was expected as described in the background, sealers exist in the market in tubes that contain specific and standardized substances and were mixed in specific ratios which can be easily achieved.

As far as the existence of control group was concerned, only four studies did not

use a control group (30, 31, 35, 37), while twelve studies used a control group resulting in a relatively low risk of bias (29, 32-34, 36, 38-44). However, it should be mentioned that not all of them used both a positive and negative control group. The existence of control groups ensures the reliability of the results in experiments and the purpose of including them in the study design was to enhance the statistical validity of the dataset. The existence of both a positive and a negative control provides a reassurance that the experiment is designed and conducted properly. In addition, a very low risk of bias arises from repetition of experiments, since all studies repeated their experiments. Repetition of the experiments reduces the possibility that findings occurred by chance and as a result maximises their validity.

There is a higher risk of bias arising from consistency of measurements, since eight studies did not report the standard deviation when reporting their measurements and as a result it is unclear if their measurements were consistent (30, 33, 36, 37, 39, 41, 42, 44), three studies did not have consistent measurements (29, 35, 38), while only four studies had consistent measurements (31, 32, 34, 40, 42). Lack of consistency could mean that it is unlikely that results were significant. In other words, it minimises the validity of the findings.

There was a low risk of bias arising from the test methods used, since all studies used well established laboratory antimicrobial tests to assess the antimicrobial efficacy of the sealers. Each study described in detail the settings of the test and the methodology that is followed.

A low risk of bias was arising from direct outcome measurement, since all studies measured either absence of bacteria (inhibition zones) or presence of bacteria (colony forming units, optic density). Measurement of outcome in a direct way means that there were no cofounding factors that could possibly interfere and produce false results.

High risk of bias arises from objectivity in outcome measurement, blinding of assessors and the person or persons who undertook the key parts of the experiment.

None of the studies reported who conducted the experiments, how many people took part in the experiment, or if the assessors were blinded to the sealers. The importance of blinding lies in the concept of minimizing bias and consequently maximises the validity of the findings. However, it has to be mentioned that Sealapex has a slight grey appearance when mixed and AH-plus has a yellowish appearance when mixed, which arises the necessity of non-dentists or non-clinicians as assessors. Otherwise if assessors were dentists or clinicians, blinding is not possible since they could differentiate between the two sealers due to the different appearance in their shade.

There was a lower risk of bias arising from the use of appropriate statistical tests since only two studies did not perform any statistical analysis (35, 39), while all the rest did use statistical tests to identify any existing significant difference in their results (29-34, 36-38, 40-44). If a study identified a difference in the antimicrobial activity between the two sealers and did not perform statistical analysis, no conclusions can be drawn concerning whether this difference is real or occurred by chance. Therefore, the validity of the findings of such a study is minimised.

Finally, eight studies reported on variability of their measurements (29, 31, 32, 34, 38, 40, 42, 43), while the rest eight studies (30, 33, 35-37, 39, 41, 44) did not give any relevant information, resulting in a high risk of bias concerning this factor.

Without reporting variability, no conclusions can be drawn concerning how the data were spread. Hence, there was no evidence that measurements were repeatable and following this, results cannot be considered reliable. No study provided strong evidence, twelve studies provided moderate evidence (29, 31-34, 36, 38, 40-44), three studies provided limited evidence (30, 37, 39) and one study provided conflicting evidence (35).

Based on the assessment, none of the studies was of a high quality. Hence, it was decided not to exclude any study at this stage; instead an analysis including all studies was approached and the limitations arising from bias and low quality in relation to the interpretation of the results

and their implications for the clinical practice was discussed in this systematic review.

Discussion

This systematic review intended to give an answer to the question whether Sealapex had higher antibacterial efficacy against *E. faecalis* compared to AH-plus. Unfortunately, this question cannot be answered for the following reasons. Firstly, different laboratory tests gave contradicting results. Secondly, results were contradicting even for studies that used the same laboratory tests. Finally, the existing literature is constituted from studies of low quality, biased, that consequently produce untrustworthy findings. AH-plus and Sealapex are very well-studied sealers with respective advantages. AH-plus is slightly thixotropic, non-mutagenic, non-genotoxic, a weak sensitizer, easily removed if needed, and unlike its predecessor AH26, does not release formaldehyde or cause tooth discoloration (16, 45).

On the other hand, Sealapex also has its advantages by being biocompatible, ability to exhibit antibacterial effect immediately following manipulation and several days later and maintaining high pH in the medium (44, 46). The antimicrobial effect of epoxy resin-based sealers is due to the presence of bisphenol A diglycidyl ether or the release of formaldehyde during polymerisation. However, AH-plus does not release formaldehyde unlike AH-26 sealer (45, 47). In Sealapex, its antimicrobial property is attributed to the release of hydroxide ion which creates an alkaline environment (38). A pH level of more than 9 may reversibly or irreversibly inactivate cellular membrane enzymes of the microorganism resulting in loss of biological activity of the cytoplasmic membrane (48).

Agar diffusion test is a very commonly used laboratory method to assess sensitivity of bacterial species against antibiotic substances (49). During the test, cells of the bacterial species of interest were inoculated on nutrient agar petri dishes. Wafers containing the sample materials were placed in the centre of the petri dishes and were incubated at 37 °C for 18-24 hours. Evaluation is based on measurement of the inhibition zones, which were the areas around the wafers that bacteria have not grown enough to be visually detected. The larger the inhibition zones were, the more susceptible the bacterial species is to the test material.

This method has the advantages of being simple and easily conducted, without necessitating special equipment. However, several limitations have been attributed to this test method, which need to be taken into consideration. First of all, this test method follows a demanding procedure, which necessitates well controlled inoculum density, medium content, agar viscosity, agar plates' storage conditions, specimens' size and number (per plate), specimens' location and arrangement (on plate), incubation time and temperature, and adequate specimens' and agar contact. Secondly, it is insensitive and semiquantitative test method, which does not distinguish between bacteriostatic and bactericidal properties of the dental materials (9). Furthermore, the results produced by this method depend on the molecular size and diffusion constant of the antimicrobial component, the toxicity of the material against the bacterial species tested, the inoculum size, the incubation time and the degree of contact between the material and agar. Moreover, agar diffusion test can be applied only on water-soluble materials, because the antibacterial agent has to diffuse through agar which is in an aqueous form (50, 51). Solubility comes in contrast to the physical properties an endodontic sealer ideally should have. As a result, if a sealer contains an antimicrobial agent that it is insoluble it will not show inhibition zones and its antibacterial activity, although existing, will falsely be undetected.

Direct contact test is a laboratory method to assess antimicrobial efficacy of sealers and root canal filling materials, given the existing limitations of agar diffusion test and as an attempt to overcome some of them (9). According to Weiss et al. (1996), direct contact test is based on the turbido-

metric determination of bacterial growth in 96-well microtiter plates (9). The kinetics of the outgrowth in each well is monitored at 600 nm at 37 °C and recorded every 30 min using a temperature-controlled microplate spectrophotometer (9). This method is based on direct contact of the bacterial specie and the endodontic sealer and it has the advantage of being reproducible, quantitative method and uninfluenced by the size of the inoculum and the diffusion properties of the materials tested and the media used.

Other than agar diffusion and direct contact tests which were the most widely used and discussed methods, time kill assay, broth method and membrane restricted test were used as well. Time kill assay is a laboratory method used to assess the antibacterial efficacy of a material against a bacterial strain by determining the bactericidal or bacteriostatic activity of the test material in relation to time. It should be pointed out though, that the bacteriostatic effect may risk late failure as there is a risk of continued growth of surviving bacteria and potential loss of the antibacterial activity of endodontic sealers.

The broth method using the elute, is another method for determining susceptibility of bacterial species to antibiotic substances (17). However, the time kill assay and the broth method although validated as laboratory methods for assessment of antimicrobial activity, they have not been widely used for sealers. Their strengths and limitations associated to endodontic sealers have not been examined or discussed and there is no evidence to support their clinical relevance. Membrane restricted test is a non-contact test to assess antibacterial efficacy. It was developed and used for the first time for assessment of antibacterial efficacy of endodontic sealers in one of the studies (34). Further research is needed to validate this as a reliable method to assess the antibacterial activity of sealers and support its clinical relevance.

Having discussed the weaknesses of agar diffusion test in relation to endodontic sealers, even if the direct contact test can be considered a more reliable method to assess their antimicrobial efficacy, still results between studies were contradicting. Three studies showed that AH-plus had a significantly higher antibacterial activity compared to Sealapex (33, 34, 41) and out of these three, one study showed the result when freshly mixed (41) and two studies portrayed the results after 24 hours (33, 34). Moreover, in particular, this study showed no significant difference when tested after 48 hours and 7 days (41). The antimicrobial effect of AH-plus is due to the presence of bisphenol A diglycidyl ether (47). In contrary, three studies showed the exact opposite, namely that Sealapex had a significantly increased antimicrobial activity compared to AH-plus when tested after freshly mixed (29), from fifth to fifteenth hour (32) and during 2, 7 and 14 days (38). The possible reason to the contradiction is Sealapex has longer setting time and releases hydroxyl ions that is antimicrobial even up to 30 days as showed in a study comparing to Apexit plus (46, 52). Considering the above, there is no evidence to support the use of Sealapex against AH-plus, or vice versa in terms of their antibacterial efficacy against *E. faecalis*. Consequently, one cannot be considered superior to the other.

Concerning the different evaluation times most studies used, these times can be connected to the setting times of the sealers used. All studies in this review investigated multiple sealers, including the two sealers of interest. Sealapex takes 24 hours to set completely, which is longer time then the 8 hours AH-plus needs to set, so the evaluation after 24 hours which is the most popular evaluation time, can be considered important in terms of clinical practice. It is doubtful whether it is clinically relevant to evaluate the long-term antibacterial efficacy of the endodontic sealers. The reason why is connected to the physical properties an endodontic sealer should have. If a substance slowly releases antimicrobial agents, it will ultimately lose part of its initial mass, resulting in compromise of

its dimensional stability, strength, porosity and resistance to wear (9). This comes in contrast to the requirements an endodontic sealer should meet in order to fulfil its purpose during the process of obturation of the root canal system, which is to provide a tight-fluid seal, always in combination with the core filling material.

The standard strains that most studies used were usually preferred so that results can be reproducible. Nevertheless, bacterial susceptibility to antibacterial agents may differ between strains and clinical isolates (50). Moreover, assessing susceptibility of *E. faecalis* organised in biofilm can be considered more clinically relevant, since this bacterial species is usually found in biofilms in the root canal system (53). It is possible, that organisation of *E. faecalis* in a biofilm could further differentiate its antimicrobial susceptibility. This scenario is assessed only in one study though (32).

Hand searching did not reveal any other relevant studies in addition to the *in vitro* articles which were identified from the electronic searches. The quality assessment tool that was used has not been validated, but as previously mentioned there is no specific tool for bias assessment for *in vitro* studies. The fact that such a tool has not been developed yet, could be due to the different settings, methodologies and special characteristics of different types of *in vitro* studies, which makes it difficult to develop a single tool applicable for all types of *in vitro* studies. Nevertheless, an attempt was made to form a questionnaire including many questions and assessing as many aspects of the study design as possible so that any existing bias could be identified. The authors suggest that this tool will be adopted for future use when assessing the quality of *in vitro* antibacterial studies.

Generally, the most important disadvantage of the *in vitro* studies lies in the difficulty to extrapolate their results to the clinical situation or a randomised controlled trial. However, they were the first step in research for testing and assessing the properties of dental materials. In order to produce results that can be more easily related to clinical practice, laboratory methods need to simulate as much as practically possible the environment of the root canal system (54, 55). The reason why the antibacterial activity of root canal sealers against *E. faecalis* or any other bacterial species cannot be studied with clinical studies is the existence of confounding factors affecting treatment outcome. As confounding factors can be regarded for example the existence of the filling material, other surviving bacterial species in the root canal system that possibly interact with each other, the protocols used for disinfection of the root canal system and the complex anatomy of the root canal system. All these make it impossible to draw conclusions specifically about sealers' activity only. Nevertheless, high quality *in vitro* studies can provide the clinician with important information that can be taken into account when choosing between several available commercial sealers.

Microorganism in the root canal system are highly organized entities known as biofilms which is a form of protection for the planktonic bacteria towards antimicrobial agents (56, 57). Hence, studies using biofilm comprising of multiple microorganisms gives a higher impact factor in clinical relevance. When making a decision about the use of a specific sealer clinically, there are many aspects that need to be taken into consideration. All physical properties of the material need to be assessed to be as similar as possible to the ideal properties of an endodontic sealer as they have been described by Grossman et al. (12). Moreover, the antibacterial efficacy of the sealer against other bacterial species that can survive initial root canal treatment or were highly pathogenic should be taken into consideration, provided though that there were *in vitro* studies of high methodological quality and low risk of bias that can be used as evidence during clinical decision making. Furthermore, practical aspects of the daily clinical

practice such as easy and predictable placement of the sealer into the root canal system and cost can be considered essential when deciding which material is best to opt for.
All above aspects are important, and a balance needs to be found between properties and cost, and always in accordance to the individual challenges an endodontic case shows.

Conclusions

Due to the identification of poor quality relevant studies which also provided contradicting results, the above answer could not be addressed, and the two sealers of interest performed similarly against each other in terms of their antimicrobial efficacy against *E. faecalis*.

Clinical Relevance

Antibacterial effect of root canal sealers plays a crucial role in endodontic treatment. This systematic review provides an evidence of the antibacterial efficacy of Sealapex and AH-plus against *E. faecalis.* Based on the findings of this systematic review, freshly mixed AH-plus sealer showed higher antimicrobial efficacy compared to Sealapex but after 1, 3 and 7 days, Sealapex was found to be more efficient.
It also provides recommendation for future approach to carry out studies evaluating antibacterial efficacy of sealers.

Conflict of Interest

All authors declare that they have no conflicts of interest. In addition, all authors have read and approved the manuscript as submitted, are qualified for authorship, believe the submission represents honest work and take full responsibility for the reported findings. The study was self-funded.

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