IN VITRO ANTIMICROBIAL ACTIVITY OF VARIOUS ENDODONTIC IRRIGANTS IN THE ELIMINATION OF ENTEROCOCCUS FAECALIS

In vitro antimikrobiálna aktivita rôznych endodontických výplachových roztokov pri eliminácii *Enterococcus faecalis*

Ján KOVÁČ[1], Lívia SLOBODNÍKOVÁ[2], Silvia BITTNER FIALOVÁ[3]

[1]Department of Stomatology and Maxillofacial Surgery of the Faculty of Medicine, Comenius University, and the St. Elisabeth´s Hospital, Heydukova 10, 812 50 Bratislava, Slovak Republic, prednosta doc. MUDr. D. Hirjak, PhD.
[2]Institute of Microbiology of the Faculty of Medicine, Comenius University, and the University Hospital in Bratislava, Sasinkova 4, 811 08 Bratislava, Slovak Republic, prednosta doc. MUDr. A. Liptáková, PhD., MPH
[3]Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University Bratislava, Odbojárov 10, 832 32 Bratislava, Slovak Republic, prednosta prof. PharmDr. P. Mučaji, PhD.

Abstract
Irrigants play a crucial role in the eradication of microorganisms in the complex root canal system. Eliminating intracanal *Enterococcus faecalis* is challenging because of its ability to penetrate deep into dentinal tubules and its high resistance to many chemicals. The aim of this study was to evaluate the sensitivity of *E. faecalis* obtained from dental infections to antimicrobial irrigants used in endodontics to help prevent treatment failures. Four irrigants were tested against six clinical *E. faecalis* isolates and *E. faecalis* CCM 4224 in a disk diffusion test, and in three modifications of bactericidal assays. Inactivation of all tested enterococcal strains was detected by chlorhexidine digluconate at all tested concentrations (1 %, 2.5 %, and 5 %), by octenidine dihydrochloride, and by 10 % sodium hypochlorite. Na_2EDTA, and NaClO at 1 %, 2.5 %, and 5.25 % concentrations were effective only in some of the used assays, or were not effective against all the tested strains. Octenidine and chlorhexidine seems to be the most effective root canal irrigants for eradication and prevention of enterococcal infections in endodontics *(Tab. 6, Ref. 29). Text in PDF www.lekarsky.herba.sk.*
KEY WORDS: chlorhexidine digluconate, *Enterococcus faecalis*, sodium hypochlorite, Na_2EDTA, octenidine dihydrochloride.
Lek Obz 2025, 74 (6): 229-235

Abstrakt
Výplachové roztoky zohrávajú kľúčovú úlohu pri eradikácii mikroorganizmov v zložitom systéme koreňových kanálov. Odstránenie vnútrokanálového *Enterococcus faecalis* je náročné kvôli jeho schopnosti preniknúť hlboko do dentínových tubulov a jeho vysokej odolnosti voči mnohým chemikáliám. Cieľom tejto štúdie bolo vyhodnotiť citlivosť *E. faecalis* získaného z dentálnych infekcií na antimikrobiálne iriganciá používané v endodoncii, v snahe predchádzať zlyhaniam liečby. Testovali sa štyri iriganciá proti šiestim klinickým izolátom *E. faecalis* a *E. faecalis* CCM 4224 v diskovom difúznom teste a v troch modifikáciách baktericídnych testov. Inaktivácia všetkých testovaných enterokokových kmeňov sa zistila pri chlórhexidín diglukonáte vo všetkých testovaných koncentráciách (1 %, 2,5 % a 5 %), oktenidín dihydrochloride a 10 % hypochlorite sodnom. Na_2EDTA a NaClO v koncentráciách 1 %, 2,5 % a 5,25 % boli účinné len v niektorých z použitých testov, alebo neboli účinné proti všetkým testovaným kmeňom. Oktenidín a chlórhexidín sa ukázali byť najúčinnejšími výplachovými roztokmi koreňových kanálov na eradikáciu a prevenciu enterokokových infekcií v endodoncii *(Tab. 6, Ref. 29). Text v PDF www.lekarsky.herba.sk.*
KĽÚČOVÉ SLOVÁ: chlórhexidín diglukonát, *Enterococcus faecalis*, hypochlorit sodný, Na_2EDTA, oktenidín dihydrochloride.
Lek Obz 2025, 74 (6): 229-235

Introduction

Enterococci belong to natural inhabitants of the intestinal and genital mucosa of humans. On the other hand, they have been recognized as potentially pathogenic for humans since the beginning of the last century - they have ability to cause various extraintestinal infections in a predisposed host, and they are also implicated in infections of the dental root canal system (1, 2, 3). The source of enterococcal dental infection is probably exogenous, as they are only transient colonizers of the oral cavity, but with ability to adhere to- and survive in the dental plaque. From there they can invade damaged dental root spaces (4, 5, 6). Enterococci make up only a small proportion of the initial dental root canal flora, which is dominated by anaerobic Gram-negative bacteria. However, they are frequently isolated

from obturated root canals of teeth with chronic periapical pathological process (7, 8). The most frequent *Enterococcus* species, isolated from infected root canals and periradicular abscesses is *Enterococcus faecalis* (9).

Several E. faecalis virulence factors have already been recognized, which facilitate the colonisation of dentin and the necrotic, or improperly filled dental root canal. These factors stimulate the inflammatory response with potential damage to periapical tissue (10, 11). Enterococcal biocides with suppressing activity towards the other root canal colonizers, together with the ability of enterococci to survive long-lasting starvation, and the natural resistance of enterococci to several antimicrobial drugs, including lincosamides, may result in their selection in this specific environment, with a consequent infection (10, 12).

The primary goal of endodontic therapy is the reduction or elimination of microorganisms and their by-products from the root canal system. Proper canal cleaning, shaping, and irrigation significantly reduce and sometimes eliminate bacteria from dental root canals (13, 14). *E. faecalis* is considered to be one of the most resistant species in the oral cavity, capable to survive extreme conditions, and is one of the possible causes of root canal treatment failure (11). From this point of view, it is very important to have information about the susceptibility of this bacterium to antimicrobial irrigant solutions used in dental practice. Sodium hypochlorite, ethylene-diamine-tetraacetic acid disodium salt (Na_2EDTA), and chlorhexidine digluconate are classical endodontic irrigant solutions with various degree of antimicrobial activity (15, 16, 17). Octenidine hydrochloride, with a broad-spectrum antimicrobial effects covering both Gram-positive and Gram-negative bacteria, fungi and several viral species, is the main component of Octenisept®, an antiseptic used for skin burns, wound disinfection and as a mouth rinse (18).

The aim of the present study was to evaluate the antimicrobial potential of the above mentioned irrigant solutions against one of the most resilient microbes in the oral cavity – *Enterococcus faecalis*.

Material and methods

Biological samples

Samples for microbiological examination were collected from patients with deep dental caries, infected dental root canals, or periapical infections (Tab. 1). The affected hard dental tissue was collected by manual excavator, and the infected content of the root canals was sampled by a pulpextractor and root canal instrument Hedstroem Files (VDW GmbH, Germany). In cases where the clinical condition and X-ray findings necessitated the tooth extraction, the whole tooth was sent for examination. All samples were immediately transported in the nutrient broth (Imuna Pharm, Slovak Republic) to the microbiological laboratory.

Table 1. Aerobic culture results of patients with dental infections.
‡ Tooth extraction, when the clinical condition made the conservative preservation impossible

Patients	Age	Diagnosis	Aerobic culture results
1	38	Pulpitis	Viridans streptococci
2	7	Dentoalveolar abscess‡	Viridans streptococci
3	31	Periodontitis apicalis	Viridans streptococci
4	32	Dentoalveolar abscess‡	Viridans streptococci
5	40	Dentoalveolar abscess‡	Viridans streptococci
6	37	Periodontitis apicalis	Negative
7	9	Periodontitis apicalis	Viridans streptococci, *Candida albicans*
8	32	Caries dentis profunda	*Candida albicans*
9	43	Periodontitis apicalis	*E. faecalis*, viridans streptococci
10	16	Dentoalveolar abscess‡	Viridans streptococci
11	49	Periodontitis apicalis	*E. faecalis*, Gram-negative rods, *Neisseria* sp.
12	72	Periodontitis apicalis	*Staphylococcus* sp. cogulase-negative
13	72	Periodontitis apicalis	*Staphylococcus* sp. cogulase-negative
14	57	Dentoalveolar abscess‡	*Staphylococcus* sp. cogulase-negative
15	66	Dentoalveolar abscess‡	Viridans streptococci
16	57	Periodontitis apicalis	Viridans streptococci
17	63	Periodontitis apicalis	Viridans streptococci
18	39	Dentoalveolar abscess‡	Viridans streptococci
19	77	Dentoalveolar abscess‡	Viridans streptococci
20	51	Periodontitis apicalis	Viridans streptococci
21	28	Periodontitis apicalis	Negative
22	33	Periodontitis apicalis	*Candida albicans*
23	36	Dentoalveolar abscess‡	*Staphylococcus* sp. cogulase-negative
24	40	Periodontitis apicalis	Viridans streptococci
25	28	Periodontitis apicalis	Negative
26	10	Periodontitis apicalis	Negative
27	17	Periodontitis apicalis	Viridans streptococci
28	62	Periodontitis apicalis	Viridans streptococci
29	23	Periodontitis apicalis	*E. faecalis*, viridans streptococci
30	24	Periodontitis apicalis	*E. faecalis*, viridans streptococci
31	27	Periodontitis apicalis	Viridans streptococci
32	45	Periodontitis apicalis	*E. faecalis*, viridans streptococci

Cultivation and identification of E. faecalis strains

Cultivation of the samples was performed in the broth for 24 hours at 35 şC in ambient atmosphere and subcultured on blood agar under at the same conditions. Suspected enterococcal strains were preliminary identified according to the standard diagnostical

methods (19), and their species-level identification was performed using the commercial biochemical identification ENCOCCUS test (Erba Lachema, Czech Republic)

The tested E. faecalis strains

Four "fresh" clinical *E. faecalis* isolates were selected for the further testing. Additionally, two lab-collected *E. faecalis* strains isolated from deep dental infections and the CCM 4224 *E. faecalis* strain were included in the study, as well.

Antimicrobial susceptibility testing

Susceptibilty of the enterococcal strains to ampicillin, amoxicillin-clavulanic acid, erythromycin, azithromycin, ciprofloxacin, moxifloxacin, tetracycline, gentamicin, chloramphenicol, and vancomycin was determined by performing by the disk-diffusion method according to the CLSI guidelines (20, 21).

The tested antimicrobial irrigant agents

Four antimicrobial irrigants were selected for their antimicrobial effect evaluation: sodium hypochlorite (NaClO; pH ≅ 12) in 1 %, 2,5 %, 5,25 %, and 10 % concentration; chlorhexidine digluconate (CHX; pH ≅ 6,5) in 1 %, 2,5 %, and 5 % concentration; 17 % ethylene-diamine-tetraacetic acid disodium salt (Na_2EDTA; pH ≅ 9).

The solutions were prepared in the pharmacy of the St. Elisabeth´s Hospital in Bratislava. The fourth antimicrobial agent was octenidine hydrochloride (pH ≅ 7), in the commercial preparation Octenisept® (Schulke-Meyer, Germany). Sterile physiological solution was used as a negative control.

In vitro antimicrobial activity testing of irrigant solutions

The inhibitory antibacterial activity testing of the selected agents was accomplished by a disk diffusion technique according to the CLSI guidelines (20, 21) with modifications described by Sharma et al (15). Briefly, 24-hours´ cultures of the tested bacterial strains were adjusted to suspensions responding to Mc Farland 0.5 (approximately 10^8 CFU.ml^{-1}) and spread on the Mueller-Hinton blood agar (OXOID, United Kingdom). The tested solutions were applied in the volumes of 15 µl on the sterile cellulose disks with 6 mm diameter (OXOID, United Kingdom) placed on the surface of the inoculated plates, and left at room temperature for 20 minutes. The plates were then cultivated for 24 hours at 35 °C in an ambient atmosphere, and the inhibition zones were measured at the end of the cultivation.

The bactericidal activity was detected by three various methods. The first of them proceeded after the inhibitory activity reading in the disk-diffusion test. The paper disks soaked with tested agents were removed from the agar plate by sterile forceps, and the side in contact with bacterial inoculum was blotted to the surface of blood agar medium free of any antimicrobial agents. After overnight cultivation at 35 °C in ambient atmosphere, the blotted areas were observed for any signs of bacterial growth.

The second method used 24-hours´ cultures of the tested bacterial strains grown on blood agar, adjusted to the working concentration of 10^6 CFU.ml^{-1} in sterile physiological solution. Ten µl volumes were then inoculated into 1 ml volumes of the particular tested solutions (in order to obtain a final inoculum concentration of 10^4 CFU.ml^{-1}), and immediately vortexed. After vortexing, 10 µl volumes were inoculated onto blood agar plates at time intervals of 0.5, 5, 10, and 20 minutes. The inoculated plates were cultivated overnight at 35 °C in ambient atmosphere, and the bactericidal effect was evaluated at the end of the cultivation.

The third method detected the bactericidal activity with a higher bacterial inoculum (1.10^7 CFU.ml^{-1}) in 500 µl volumes of the tested agents. After 24-hours´ cultivation at 35 °C in ambient atmosphere, 1500 µl of Brain-heart Infusion (OXIOD, United Kingdom) was added to the test vial. The viability of bacteria was detected after additional 24-hours´ cultivation of the vials, inoculation of 10 µl volumes onto blood agar and subsequent 24-hours´ cultivation at 35 °C in ambient atmosphere. Sterile physiological solution (pH 7.2) was used as a negative control in every one of the test modifications. The tests were performed in triplicate.

Results

Aerobic cultivation of 32 dental samples (11 from male and 21 from female patients) yielded only four *Enterococcus faecalis* strains (12.5 %), two from middle-aged males, and two from young females, suffering from periodontitis apicalis (Table 1). All four currently isolated clinical *E. faecalis* strains, two deep dental caries *E. faecalis* strains from the laboratory collection of bacteria, and one reference strain (*E. faecalis* ATCC 29212) were subjected to the further analysis. Although anaerobic bacteria, naturally inhabiting the oral cavity, play important role in the dental infections, no anaerobic cultivation was performed, as the aim of cultivation was focused on the isolation of enterococcal strains.

All *E. faecalis* clinical isolates were susceptible to ampicillin, which is the drug of choice for patients with non-life-threatening enterococcal infections. Two clinical isolates were resistant to tetracycline, two to erythromycin and azithromycin (additional 4 had intermediate susceptibility), and one strain had intermediate susceptibility to ciprofloxacine and moxifloxacine. There was detected neither vancomycine, nor high level-gentamicin resistant strain was detected (Tab. 2).

Antibacterial inhibitory effect of the investigated agents was measured by a disk-diffusion method; the results are shown in the Table 3.

Table 2. Antimicrobial susceptibility of *E. faecalis* strains, tested by disk-diffusion test.
(S – susceptible; R – resistant, I – intermediately susceptible, HL – high level).

The tested drug	*E. faecalis* strains						
	1	2	3	4	5	6	CCM 4224
Ampicillin	S	S	S	S	S	S	S
Co-amoxicillin	S	S	S	S	S	S	S
Tetracycline	S	S	S	R	S	R	R
Chloramphenicol	S	S	S	S	S	S	S
Erythromycin	I	I	I	R	I	R	R
Azithromycin	I	I	I	R	I	R	R
Ciprofloxacin	S	S	S	S	S	I	I
Moxifloxacin	S	S	S	S	S	I	I
Gentamicin (HL)	S	S	S	S	S	S	S
Vancomycin	S	S	S	S	S	S	S

Table 3. Effect of the irrigants on *E. faecalis* strains, tested by the disk-diffusion method.
All tested agents produced concentration-dependent inhibitory zones. The antimicrobially not active physiological solution had no inhibitory effect (6 mms respond to the diameter of the cellulose disks used in the test).

Irrigant	**E. faecalis strains / average inhibitory zones** [mm] ± **SD**						
	1	2	3	4	5	6	CCM 4224
NaOCl 1%	8.4 ± 0.49	9.1 ± 0.53	9.4 ± 0.53	9.1 ± 0.56	8.9 ± 0.46	8.8 ± 0.67	8.6 ± 0.42
NaOCl 2.5 %	9.2 ± 0.44	9.9 ± 0.70	10 ± 0.50	9.7 ± 0.75	9.5 ± 0.43	9.3 ± 0.79	9.4 ± 0.55
NaOCl 5.25 %	10.6 ± 0.39	10.4 ± 0.42	10.7 ± 0.71	11.1 ± 0.55	10.5 ± 0.71	10.5 ± 0.50	10.7 ± 0.43
NaOCl 10 %	16.7 ± 2.27	13.9 ± 0.74	13.6 ± 1.39	16.1 ± 3.27	14.4 ± 1.14	14.7 ± 1.39	15.4 ± 2.04
CHX 1 %	16.3 ± 1.12	14.7 ± 1.23	14.9 ± 0.17	14.4 ± 0.55	15.3 ± 0.66	15.3 ± 0.70	15.7 ± 0.44
CHX 2.5 %	18.0 ± 1.39	16.8 ± 1.20	16.9 ± 0.17	16.4 ± 0.30	17.1 ± 0.63	17.3 ± 1.25	17.4 ± 0.78
CHX 5 %	18.8 ± 0.86	18.3 ± 1.27	18.1 ± 0,42	17.9 ± 0.30	17.2 ± 0.57	18.5 ± 1.80	19 ± 0.97
Na_2EDTA	19.9 ± 0.85	20.6 ± 0.96	21.5 ± 1.37	20.1 ± 0.74	20.4 ± 0.53	18.9 ± 2.32	19.8 ± 0.90
OCT	12,4 ± 0.95	12.6 ± 0,82	12.4 ± 0.46	12.6 ± 1.19	12.1 ± 0.74	12.9 ± 0.72	12.8 ± 0.25
PS	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00

The bactericidal activity of the antimicrobial irrigant solutions was tested by three methods. The first method detected the viability of bacteria growing on blood agar medium after overnight exposition to the tested antimicrobial irrigants applied on the cellulose disks during the disk-diffusion test. After the reading of the disk-diffusion test results, the paper disks were blotted on the antimicrobials-free blood agar.

Chlorhexidine in all concentrations, octenidine, and NaClO in 10% concentration inactivated all tested enterococcal strains, when tested by this "disk-blotting" assay. No evident bactericidal effect was observed in the case of 1 % and 2.5 % NaClO and 17% Na2EDTA. Two strains survived exposition to 5.25 % NaClO, as well. Results are shown in the Table 4.

Table 4. Viability of *E. faecalis* strains detected by the "blotting" assay after an overnight exposition to the tested agents during the disk diffusion test.
(NaClO – sodium hypochlorite; CHX – chlorhexidine digluconate; Na_2EDTA - ethylene-diamine-tetraacetic acid disodium salt; OCT – octenidine hydrochloride; PS – physiological solution; + detectable bacterial growth; - inactivation of the tested bacteria).

Irrigant	*Enterococcus faecalis* strains						
	1	2	3	4	5	6	CCM 4224
NaClO 1%	+	+	+	+	+	+	+
NaClO 2.5 %	+	+	+	+	+	+	+
NaClO 5.25 %	+	+	-	-	-	-	-
NaClO 10 %	-	-	-	-	-	-	-
CHX 1 %	-	-	-	-	-	-	-
CHX 2.5 %	-	-	-	-	-	-	-
CHX 5 %	-	-	-	-	-	-	-
Na_2EDTA 17%	+	+	+	+	+	+	+
OCT	-	-	-	-	-	-	-
PS	+	+	+	+	+	+	+

The second method detected the bactericidal activity of antimicrobial irrigants with a lighter bacterial inoculum (104 CFU.ml^{-1}) in a time-dependent assay after 0.5, 5, 10, and 20 minutes˘ exposure. Except to 17 % Na_2EDTA, all the other tested irrigants inactivated the bacteria in 30 seconds. The time interval necessary for inactivation of bacteria in the case of Na_2EDTA was 5 minutes, and one strain was inactivated just after 10 minutes exposure (Tab. 5).

Table 5. Time to inactivation of *E. faecalis* strains in the inoculum size of 10^4 CFU.ml^{-1} tested directly in the antimicrobial irrigants solution.

Irrigant	***E. faecalis* strains / time to bacterial inactivation** [minutes]						
	1	2	3	4	5	6	CCM 4224
NaClO 1 %	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaClO 2.5 %	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaClO 5.25 %	0.5	0.5	0.5	0.5	0.5	0.5	0.5
CHX 1%	0.5	0.5	0.5	0.5	0.5	0.5	0.5
CHX 2.5 %	0.5	0.5	0.5	0.5	0.5	0.5	0.5
CHX 5 %	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Na_2EDTA 17%	5	5	5	10	5	5	5
OCT	0.5	0.5	0.5	0.5	0.5	0.5	0.5
PS	-	-	-	-	-	-	-

The third method evaluated the bactericidal activity of 1%, 2.5 %, and 5.25 % NaClO, 1 %, 2.5 %, and 5 % CHX, 17 % Na2EDTA, and octenidine hydrochloride with heavy bacterial inoculum (107 CFU.ml-1) after 24-hours´ exposition. All tested agents with exception of Na2EDTA inactivated all of the tested enterococcal strains. Na2EDTA inactivated 5 strains, and in 2 strains

only suppressed their multiplication (Tab. 6). No bactericidal activity was observed with the physiological solution, independently on the test procedure.

Table 6. *E. faecalis* strains survival after 24 hours exposition of the 10^7 CFU.ml^{-1} inoculum size to the tested antimicrobial irrigants solutions.
(NaClO – sodium hypochlorite; CHX – chlorhexidine digluconate; Na_2EDTA - ethylene-diamine-tetraacetic acid disodium salt; OCT – octenidine hydrochloride; PS – physiological solution; - no bacterial survival).

Irrigant	*E. faecalis* strains / Number of surviving bacteria (CFU.ml^{-1})						
	1	2	3	4	5	6	CCM 4224
NaClO 1 %	-	-	-	-	-	-	-
NaClO 2.5 %	-	-	-	-	-	-	-
NaClO 5.25 %	-	-	-	-	-	-	-
CHX 1%	-	-	-	-	-	-	-
CHX 2.5 %	-	-	-	-	-	-	-
CHX 5%	-	-	-	-	-	-	-
Na_2EDTA 17 %	-	-	10^5	-	10^6	-	-
OCT	-	-	-	-	-	-	-
PS	> 10^7	> 10^7	> 10^7	> 10^7	> 10^7	> 10^7	> 10^7

Discussion

Several microorganisms from the oral cavity may contribute to endodontic infections (1). However, only few of them have a remarkable potential to survive adverse conditions during root canal treatment, with eventual treatment failure. Since *Enterococcus faecalis* has such potential, it guided us to select clinical *E. faecalis* strains obtained from endodontic infections for detection of their susceptibility to several antimicrobial irrigants used in dentistry. To date, various in vitro methods of antimicrobial agents' activity detection has been published worldwide. They differ in the media used in the test, in the exposition time, inoculum size of the tested microbes, and whether an organic challenge (sach as blood, mucus, or albumin) is included. Some authors implemented even highly sophisticated dental root models (15, 16, 18, 22, 23, 24, 25, 26). However, they still cannot simulate all the conditions in the living organism in their complexity. Despite the limitations of the in vitro tests, they can provide useful information about the antimicrobial effect of the tested agents, and can compare their activity in various test modifications.

Four assays measuring in vitro antimicrobial activity of the tested irrigant solutions have been employed in this study. The first one, a disk-diffusion test, is able to detect only the bacteriostatic activity of the tested agents, without respect to their bactericidal effect. Moreover, the inhibitory zones are related to the ability of the tested agents to diffuse into the agar medium. Consequently, it is not possible to make comparative conclusions on antimicrobial activity of the particular agents, and the results can give only general information about the presence or absence of antimicrobial activity. As the best example supporting this argument may serve results from our study, obtained with Na_2EDTA. It produced the largest inhibitory zones in the disk-diffusion test, but revealed insufficient bactericidal effect.

Considering the fact, that the goal of the dental root irrigation during dental treatment is to kill all microbes present in the dental root space, assays assessing microbicidal activity are superior in the testing. Three microbicidal assays were employed in this study. The first method detected the survival of bacteria growing on the inoculated Mueller-Hinton agar under the cellulose disk, onto which the tested agent was poured during the disk-diffusion test. The two other assays tested the bactericidal activity directly in the irrigants solutions. They differed in the inoculum size (10^4 CFU.ml^{-1}, versus 10^7 CFU.ml^{-1}), the exposition time (0.5, 5, 10, and 20 minutes versus 24 hrs), and the mode of viable count detection (direct inoculation onto antimicrobials-free blood agar medium versus a previous 24-hrs cultivation in a broth medium). The cellulose disk blotting assay seemed to be the most susceptible one. To some extent, it simulated the organic environment due to the content of blood and other organic molecules in the blood agar.

Four antimicrobial irrigants used in endodontics were selected for the study to reveal their activity against clinical isolates of *E. faecalis*: sodium hypochlorite, chlorhexidine digluconate, ethylene-diamine-tetraacetic acid disodium salt, and octenidine hydrochloride.

Sodium hypochlorite (NaClO) in concentrations ranging from 0.5 % to 5.25 %, is one of the most commonly used antibacterial irrigant solutions, with a broad spectrum of antimicrobial activity (15, 17). NaClO was tested in our study at 1 %, 2.5 %, 5.25 % and 10 % concentrations. The highest activity against enterococci was reached with 5.25 % and 10 % NaClO concentration, in accordance with some other research results (15, 25). However, NaClO at 5.25 % concentration was not able to inactivate two *E. faecalis* clinical isolates in the "disk-blotting" assay. Considering the potential tissue toxicity of NaClO, the highest concentration recommended in the endodontic therapy is 5.25 % (14). As this agent is usually used in combination with 17 % Na_2EDTA (14), their synergistic killing effect on bacteria may be assumed (14, 27).

To the frequently used irrigants in endodontic treatment belongs also chlorhexidine digluconate (CHX), a synthetic cationic bis-guanide. It is known to exert excellent antimicrobial activities, mediated by several mechanisms. The positively charged molecules of chlorhexidine can adsorb into dentine and prevent microbial colonization for a longer period due to its substantive antimicrobial property (22, 13), which may last for up to 12 weeks (28). Gomez et al. (25) detected a superior antimicrobial activity of CHX to that of NaClO against the reference *E. faecalis* strain, even in the presence of blood, mucus or albumine (22). Chlorhexidine was one of the most efficient antimicrobial agents also in our study. It exerted a bactericidal activity against all

tested *E. faecalis* strains in all used assays even at the lowest tested concentration (1%).

Ethylene-diamine-tetraacetic acid disodium salt (Na_2EDTA) is used in root canal treatment as a dentine softener. It is highly effective in smear layer removal, important for effective root canal disinfection. However, Na_2EDTA itself is not a powerful bactericide, but may potentiate the activity of chemically unrelated antibacterial compounds against Gram-negative bacteria (16). Despite the inferior activity of Na_2EDTA against Gram-positive bacteria, this antimicrobial irrigant produced the largest inhibitory zone in the disk-diffusion test in this study. Nevertheless, the bactericidal effect was much less prominent – it inactivated the tested strains only in a lower inoculum size ($10^4.ml^{-1}$) after 5 to 10 minutes of exposure. As the antibiofilm activity of Na_2EDTA has already been demonstrated by in vitro studies (27, 29), it may be useful in combination with some other, antimicrobially more effective agents, especially in chronic dental root infections, where E. faecalis biofilm production is highly suspected.

The fourth antimicrobial solution tested was 0.1 % octenidine hydrochloride, contained together with 2% phenoxyethanol in the Octenisept® preparation, used as antiseptic for skin burns, wound disinfection and mouth rinses. Octenidine hydrochloride [N,N′-(1,10 decanediyldi-1[4H]-pyridinyl-4-ylidene)bis(1-octanamine) dihydrochloride] belongs to the bipyridines carrying two cationic active centres per molecule and demonstrates broad spectrum antimicrobial effects covering both Gram-positive and Gram-negative bacteria, fungi and several viral species. The mode of action is microbicidal by interfering with cell walls and membranes (18). Although several in vitro studies demonstrated an excellent antimicrobial activity of octenidine, this study is the first to demonstrates its powerful activity against clinical *E. faecalis* strains, isolated from endodontic infections. Octenidine killed all the tested strains regardless of the employed method, and did so in the shortest detection time intervals.

Conclusions

Numerous factors may have impact on the final antimicrobial effect of irrigant solutions during endodontic treatment, such as pH, the content of organic molecules in the environment, exposition time, the bacterial inoculum size, the metabolic activity of bacteria, or their capacity to form biofilms (15, 22, 29). However, without respect to these factors, the in vitro tests may help to select agents with the highest antimicrobial activity and the presumable most beneficial effect on the treated or disinfected areas of human body. Based on the results obtained in the present study, chlorhexidine digluconate and octenidine hydrochloride showed the most effective antibacterial activity against the tested enterococcal clinical isolates – they had bacteriostatic, as well as bactericidal activity in every assay employed. Some enterococcal isolates included in the study showed individual susceptibility to 5.25 % NaClO, and 17% Na_2EDTA. Considering all results obtained in this study, the most appropriate irrigants for inactivation of enterococci during root canal treatment are the traditionally used chlorhexidine digluconate and the newer antiseptic agent octenidine hydrochloride. The antimicrobial activity of Octenidine was proved also against clinical *E. faecalis* strains from endodontic infections.*

Compliance with Ethics Requirements: The authors declare, that all the procedures and experiments of this research respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008 (5), as well as the national law.

Conflict of interest: The authors declare no conflict of interest.

References

1. PINHEIRO ET, CANDEIRO GT, TEIXEIRA SR, SHIN RC, PRADO LC, GAVINI G, MAYER MP. RNA-based assay demonstrated *Enterococcus faecalis* metabolic activity after chemomechanical procedures. J Endod 2015, 41 (9): 1441-1444.
2. SIQUEIRA JF, Jr, RÔÇAS IN. Present status and future directions: Microbiology of endodontic infections. Int Endod J 2022, 55 (Suppl. 3): 512-530.
3. SANCHEZ-SANHUEZA G, BELLO-TOLEDO H, GONZALEZ-ROCHA G, GONCALVES AT, VALENZUELA V, GALLARDO-ESCARATE C. Metagenomic study of bacterial microbiota in persistentendodontic infections using next-generation sequencing. Int Endod J 2018, 51 (12): 1336-1348.
4. AL-AHMAD A, MAIER J, FOLLO M, SPITZMÜLLER B, WITTMER A, HELLWIG E, HÜBNER J, JONAS D. Food-borne enterococci integrate into oral biofilm: an in vivo study. J Endod 2010, 36 (11): 1812-1819.
5. RAZAVI A, GMÜR R, IMFELD T, ZEHNDER M. Recovery of *Enterococcus faecalis* from cheese in the oral cavity of healthy subjects. Oral Microbiol Immunol 2007, 22 (4): 248-251.
6. VIDANA R, SULLIVAN A, BILLSTRÖM H, AHLQUIST M, LUND B. *Enterococcus faecalis* infection in root canals - host-derived or exogenous source? Lett Appl Microbiol 2011, 52:109-115.
7. LOVE RM. *Enterococcus faecalis* – a mechanism for its role in endodontic failure. Int Endod J 2001, 34 (5): 399-405.
8. SKUCAITE N, PECIULIENE V, VITKAUSKIENE A, MACHIULSKIENE V. Susceptibility of endodontic pathogens to antibiotics in patients with symptomatic apical periodontitis. J Endod 2010, 36 (10): 1611-1616.
9. KOUIDHI B, ZMANTAR T, MAHDOUANI K, HENTATI H, BAKHROUF A. Antibiotic resistance and adhesion properties of oral Enterococci associated to dental caries. BMC Microbiol 2011, 11 (1): 155.
10. KAYAOGLU G, ŘRSTAVIK D. Virulence factors of *Enterococcus faecalis*: Relationship to endodontic disease. Crit Rev Oral Biol Med 2004, 15 (5): 308-320.
11. ZOLETTI GO, PEREIRA EM, SCHUENCK RP, TEIXEIRA LM, SIQUIWRA JF, JR, SANTOS KR. Characterization of virulence factors and clonal diversity of *Enterococcus faecalis* isolates from treated dental root canals. Res Microbiol 2011, 162 (2): 151-158.
12. GAO Y, JIANG X, LIN D, CHEN Y, TONG Z. The Starvation Resistance and Biofilm Formation of *Enterococcus faecalis* in Coexistence with *Candida albicans*, *Streptococcus gordonii*, *Actinomyces viscosus*, or *Lactobacillus acidophilus*. J Endod 2016, 42 (8): 1233-1238.

13. TEJA KV, JANANI K, SRIVASTAVA KC, SHRIVASTAVA D, NATOLI V, DI BLASIO M, CICCIU M, MINERVINI G. Comparative evaluation of antimicrobial efficacy of different combinations of calcium hydroxide against *Enterococcus faecalis*. BMC Oral Health 2023, 23 (1): 849.
14. KOVAC J, KOVAC D. Effect of irrigating solutions in endodontic therapy. Bratisl Lek Listy 2011, 112 (7): 410-415.
15. SHARMA R, PANADAN J, SHARMA M, TANWEER SA. Antimicrobial Effectiveness of Different Root Canal Irrigants on Viablility of Root Canal Flora. JCDR 2023, 17 (2): 37-41.
16. PINHEIRO ET, KARYGIANNI L, ATTIN T, THURNHEER T. Antibacterial Effect of Sodium Hypochlorite and EDTA in Combination with High-Purity Nisin on an Endodontic-like Biofilm Model. Antibiotics (Basel) 2021, 10 (9): 1141.
17. GOMES BPFA, AVEIRO E, KISHEN A. Irrigants and irrigation activation systems in Endodontics. Braz Dent J 2023, 34 (4): 1-33.
18. CASSIANO AFB, COAGUILA-LLERENA H, SANTOS CS, DA SILVA LR, NOGUEIRA LFB, CIANCAGLINI P, FARIA G. The Effect of Octenidine on Proliferation, Migration, and Osteogenic Differentiation of Human Dental Pulp and Apical Papilla Stem Cells. J Endod 2022, 48 (12): 1502-1510.
19. CAROLL KC, PFALLER MA, KARLOWSKY JA, LANDRY ML, MCADAM AJ, PATEL R, PRITT BS. Manual of Clinical Microbiology, Multi-Volume. 13th edition. Washington, DC: American Society for Microbiology, 2023.
20. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disc Susceptibility Tests; Approved Standard; Twelfth Edition. Clinical and Laboratory Standards Document M02-A12. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute, 2015.
21. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Tests; 13th Edition. Clinical and Laboratory Standards Document M02. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute, 2018.
22. LUDDIN N, AHMED HMA. The antibacterial activity of sodium hypochlorite and chlorhexidine against *Enterococcus faecalis*: A review on agar diffusion and direct contact methods. JCD 2013, 16 (1): 9-16.
23. KRANZ S, GUELLMAR A, BRAEUTIGAM F, TONNDORF-MARTINI S, HEYDER M, REISE M, SIGUSCH B. Antibacterial effect of endodontic disinfections on *Enterococcus faecalis* in dental root canals – An in-vitro model study. Materials 2021, 14 (9): 2427.
24. ZOU X, ZHENG X, LIANG Y, *et al.* Expert consensus on irrigation and intracanal medication in root canal therapy. Int J Oral Sci 2024, 16: 23.
25. GOMES BPFA, FERRAZ CCR, VIANNA ME, BERBER VB, TEIXEIRA FB, SOUZA-FILHO FJ. In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. Int Endod J 2001, 34 (6): 424-428.
26. GENC O, ERDEMIR A. The effectiveness of three different irrigant activation methods in the elimination of *Enterococcus faecalis* from root canals. Photobiomodul Photomed Laser Surg 2024, 42 (10): 628-633.
27. XU J, HE J, SHEN Y, ZHOU X, HUANG D, GAO Y, HAAPASALO M. Influence of Endodontic Procedure on the Adherence of *Enterococcus faecalis*. J Endod 2019, 45 (7): 943-949.
28. PALAZZI F, DEL FABBRO M, TASCHIERI F, MOHAMMADI Z, ASGARY S, BUKIET F. Comparison of antimicrobial substantivity of six root canal irrigants against *Enterococcus faecalis*. Iran Endod J 2018, 13 (4): 446-452.
29. GOMES BPFA, AVEIRO E, KISHEN A. Irrigants and irrigation activation systems in Endodontics. Braz Dent J 2023, 34 (4): 1-33.

Accepted for publication 10.3.2025.

Address for correspondence:
MUDr. Mgr. Ján Kováč, PhD., MPH
Department of Stomatology and Maxillofacial Surgery of FM CU
and St. Elisabeth´s Hospital
Heydukova 10
812 50 Bratislava
Slovak Republic
E-mail: *mudr.jan.kovac@gmail.com*