

ENTEROCOCCUS FAECALIS BIOFILM. FORMATION AND DEVELOPMENT IN VITRO OBSERVED BY SCANNING ELECTRON MICROSCOPY

María de los Á. Bulacio¹, Lucas R. Galván¹, Cristina Gaudio², Rosa Cangemi², Marta I. Erimbaue¹

¹ Department of Endodontics, School of Dentistry, National University of Tucumán, Tucumán, Argentina..

² Department of Microbiology, School of Biochemistry, National University of Tucumán, Tucumán, Argentina.

ABSTRACT

Biofilm produced by *Enterococcus faecalis* isolated from root canals was detected by growing it on microplates and using 10% crystal violet stain, elution with alcohol and three procedures: no fixation, heat fixation and 10% formaldehyde fixation. The biofilm was evaluated using a Versamax Microplate Reader (USA).

Twenty sterile root portions were incubated in TS broth with *E. faecalis* (10⁸) for 48 hours, 4, 7, 14 and 30 days, after which they were processed and observed by scanning electron microscopy (SEM).

Significantly more biofilm was found on the microplates for formaldehyde fixation than for heat fixation or no fixation (ANOVA $p < 0.0001$). SEM showed *E. faecalis* growth at all times and biofilm development as from 14 days' incubation.

Fixation with 10% formaldehyde was the most appropriate technique for detecting *E. faecalis* biofilm development on microplates. SEM confirmed biofilm formation after 14 days incubation.

Key words: biofilm; *Enterococcus faecalis*; scanning electron microscopy.

BIOFILM DE ENTEROCOCCUS FAECALIS. PRODUCCIÓN IN VITRO Y DESARROLLO OBSERVADO CON MICROSCOPIA ELECTRÓNICA DE BARRIDO

RESUMEN

Se realizó la detección de biofilm de *Enterococcus faecalis* aislados de conductos radiculares, desarrollándolos en microcubetas realizando tinción con Cristal Violeta 10 %, elución con alcohol y tres procedimientos: sin fijar, fijación con calor y fijación con formaldehído 10%. Se evaluó el biofilm formado realizando la lectura con lector de microplaca Versamax-Microplate-Reader (USA).

Se incubaron 20 porciones radiculares estériles en el medio TS caldo con *E. faecalis* (10⁸) durante 48 hs, 4, 7, 14 y 30 días. Transcurrido cada tiempo experimental se procesaron y observaron al microscopio electrónico de barrido (MEB).

Se evidenció significativamente mayor formación de biofilm en microplacas cuando se realizó fijación con formaldehído, que al fijar con calor y sin fijar (ANOVA $p < 0,0001$). Al MEB se observó crecimiento del *E. faecalis* en todos los tiempos y desarrollo de biofilm a partir de 14 días de incubación.

La fijación con formaldehído 10% fue la técnica más apropiada para detectar biofilm de *E. faecalis* desarrollado en microplacas. Mediante el MEB se confirmó la formación de biofilm después de 14 días de incubación.

Palabras clave: biofilm; *Enterococcus faecalis*; microscopía electrónica de barrido.

INTRODUCTION

Enterococci are natural inhabitants of the oral cavity and normal intestinal flora, and are recognized as opportunistic pathogens. *Enterococcus faecalis* is the most common species, and is responsible for 80 - 90% of human infections¹.

Research has shown that areas in the canal which are inaccessible to instruments and irrigants may remain contaminated after biomechanical preparation and be responsible for persistent infection².

Cases of apical periodontitis resistant to conventional treatment³ are attributed to the presence of

biofilm inside the canal and at the apical end of teeth with pulp necrosis and chronic periapical lesion⁴.

SEM observations of *E. faecalis* have shown that it adheres to collagen structures, colonizes dentin surfaces and may progress towards dentin tubules and form mature biofilm⁵.

The aim of this study was to compare detection on microplates of biofilm formed by *Enterococcus faecalis* isolated from root canals, in Tryptone Soya (TS) broth supplemented with 1% glucose and 10% human serum, using three different techniques: no

fixation, heat fixation and 10% formaldehyde fixation, and observing the development of biofilm on root portions under scanning electron microscopy (SEM) at 48 hours, 4, 7, 14 and 30 days.

MATERIALS AND METHODS

The study included 10 patients who attend the Endodontics Department at the School of Dentistry at the National University of Tucumán and provided informed consent, with indication for retreatment of teeth due to the persistence of chronic-type periapical lesion.

After clinical-radiographic diagnosis, the patients rinsed their mouths with 0.12% chlorhexidine solution (Plac Out. Microsules Bernabó, Argentina). The area was completely isolated and the operative field disinfected with 1% chlorhexidine solution. Caries was eliminated using hand instruments and irrigation with 1% chlorhexidine. The access cavity was prepared with water-cooled high-speed diamond burs. The operative field was disinfected and the canal filling material was removed with H-files, followed by irrigation with 1 mL distilled water. Samples were taken with two standard N° 20 paper points placed inside the root canal for one minute, and then placed individually in test tubes containing TS broth and transferred immediately to the laboratory.

In the laboratory we performed a semi-quantitative study by dilution in the following media: Azide agar for Enterococcus and Staphylococcus, MacConkey agar for Gram-negative bacilli, Blood agar for Streptococcus. The different colonies were counted and *E. faecalis* was identified according to morphological and physiological features⁶.

The three strains with greatest development on plates were selected and their ability to produce biofilm was studied.

Biofilm formation on microplates

The culture medium used was Trypticase Soya (TS) broth supplemented with 1% glucose and 10% human serum. Three *E. faecalis* strains isolated from the canals were selected and called strains 1, 2 and 3.

Microplates were loaded with 200 µl. of the medium with inoculum at a concentration of 10⁸, incubated at 37°C for 24 hours, without stirring, in triplicate. To detect biofilm production, the microplates were stained with 10% crystal violet

(C.V.), washed with 95% ethanol to remove excess dye and subject to one of three different procedures:

- Without fixation
- Heat fixation
- Formaldehyde fixation

The biofilm formed was evaluated by measuring absorbance using a Versamax Microplate Reader (USA).

Biofilm development observed with scanning electron microscope (SEM)

Twenty disks were cut from the middle third of the roots of recently extracted upper central incisors using a water-cooled, high-speed N° 1090 cylindrical bur (Sorensen, Brazil). The disks were sterilized and placed individually in TS broth enriched with 1% glucose and 10% human serum, and inoculated with *E. faecalis* at a concentration of 10⁸. The media were refreshed every 72 hours with the same inoculum concentration. Five experimental incubation times were used: 48 hs, 4, 7, 14 and 30 days, after which the samples were removed from the medium and placed individually in a solution of 8% paraformaldehyde, phosphate buffer and 16% glutaraldehyde and fixed for 48 hours at 4°C. They were washed 3 times for 10 minutes with phosphate buffer. A 1/1 osmium/phosphate buffer solution (2% OsO₄) was added and left overnight. The osmium was discarded and the samples were washed twice for 10 minutes with 30% ethanol. The samples were dehydrated by passing through a series of alcohol concentrations: ethanol 50%, 70%, 80%, 90%, and 3 times 100%, with 10 minutes for each step. The 100% ethanol was discarded and 100% acetone added for 1 hour⁷. The samples were mounted on an aluminum specimen holder, sputter-coated with gold and viewed under high vacuum with a JEOL JSM-35CF microscope (Tokyo, Japan). Microphotographs were taken at 10000 X.

RESULTS

Biofilm forming assay on microplates

Strain 2 had the greatest development in TS broth supplemented with 1% glucose and 10% human serum for all three techniques used (Table 1), so we selected it for the SEM study. The control value (0.0360) was taken from the reading for the microplate without biofilm.

Table 1: Analysis of Variance. Absorbance reading on biofilms in nm for three *Enterococcus faecalis* strains using three techniques. The mean is the average of the readings for the four wells seeded with the same strain.

Treatment	Strain	Mean	95% Confidence Interval		ANOVA
			Lower Limit	Upper Limit	
No fixation	1	0.1463	0.14	0.1525	p < 0.0001
	2	0.1613	0.1446	0.178	
	3	0.1263	0.1211	0.1315	
Heat	1	0.1288	0.1225	0.135	p = 0.0017
	2	0.1393	0.1372	0.1414	
	3	0.1289	0.1237	0.134	
Formaldehyde	1	0.2587	0.216	0.3014	p < 0.0001
	2	0.7303	0.5714	0.8892	
	3	0.1681	0.1573	0.1788	

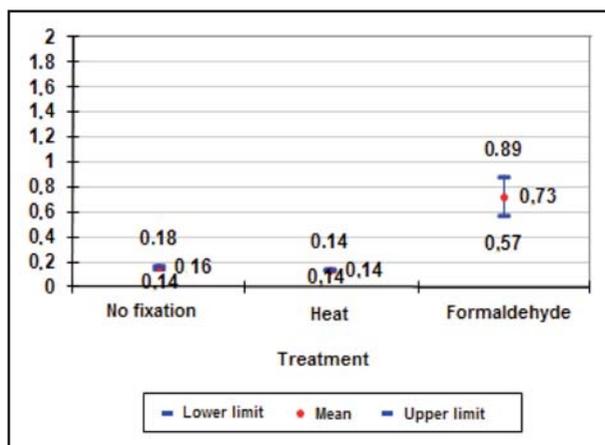


Fig. 1: Biofilm obtained. Absorbance (nm) measurements of *E. faecalis* strain 2, with three different fixation procedures.

The values obtained with strain No. 2 for the formaldehyde fixation treatment were significantly higher than for the other treatments (ANOVA, $p < 0.0001$), Fig. 1.

Biofilm development observed with scanning electron microscope

Figures 2 to 6 show the development of *Enterococcus faecalis* under SEM at the different times. Biofilm is apparent as from 14 days.

DISCUSSION

This study used the ability of *E. faecalis* to form biofilm on polystyrene⁶, which occurs due to the presence of a surface protein, the Esp-encoding gene

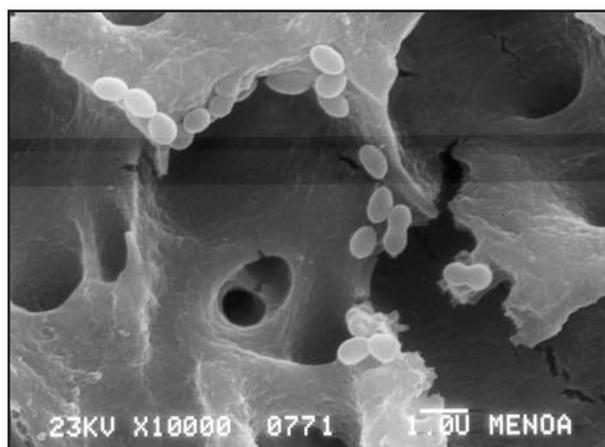


Fig. 2: SEM. *E. faecalis* development at 48 hs. The reference line corresponds to 1 μ m.

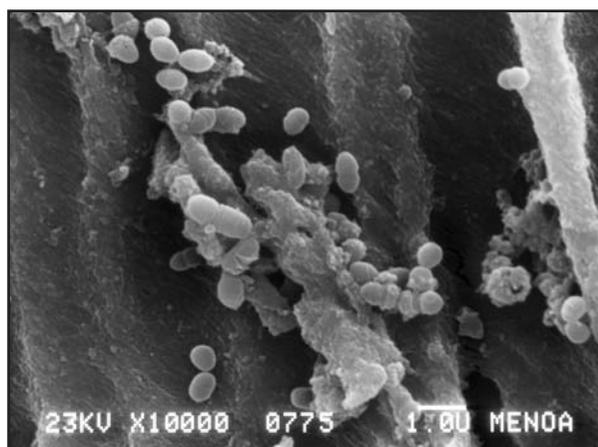


Fig. 3: SEM. *E. faecalis* development at 4 days. The reference line corresponds to 1 μ m

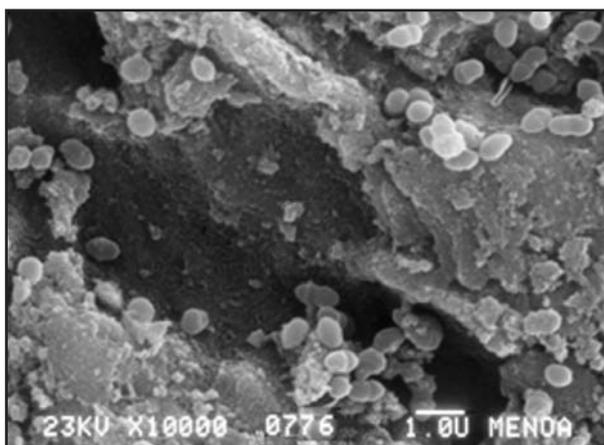


Fig. 4: SEM. *E. faecalis* development at 7 days. The reference line corresponds to 1 μ m.

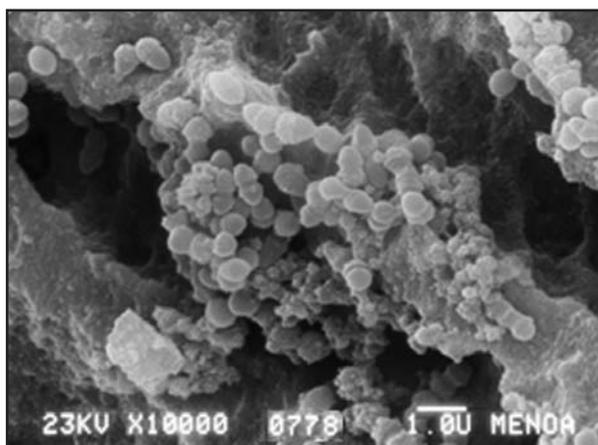


Fig. 5: SEM. *E. faecalis* biofilm at 14 days. The reference line corresponds to 1 μ m.

(*esp*)⁸, although Kristich et al.⁹ claim that *E. faecalis* is able to form biofilms in absence of this gene.

Biofilm production often depends on factors such as cell density and glucose concentration¹⁰, with several authors having proved that glucose supplement increases biofilm formation by *E. faecalis*⁹⁻¹¹ and other papers reporting that biofilms are denser in presence of glucose¹². Our study took this factor into account.

Not all strains have the same biofilm-producing capacity. Our study used 3 *E. faecalis* strains isolated from root canals with endodontic failure. We selected the strain with the greatest capacity to produce biofilm on microplates, and used it to contaminate root dentin and observe biofilm development under SEM.

Duggan and Sedgley¹³ found no statistically significant difference in the formation of biofilm by *E. faecalis* obtained from root canals, oral cavity and other parts of the body, using a similar culture medium and evaluating the optical density of the microplates, suggesting that the origin of the strain did not influence the result of their study.

Zheng et al.¹⁴ found that 75.47% of the *E. faecalis* strains isolated from root canals with endodontic failure were able to develop biofilm.

Wang et al.¹⁵ also found high biofilm forming rates by *E. faecalis* isolated from root canals with endodontic failure, employing growth on microplates and SEM observations.

Wilcox¹⁶ report that early formaldehyde fixation may help show biofilm formation, and this technique was employed successfully in our study.

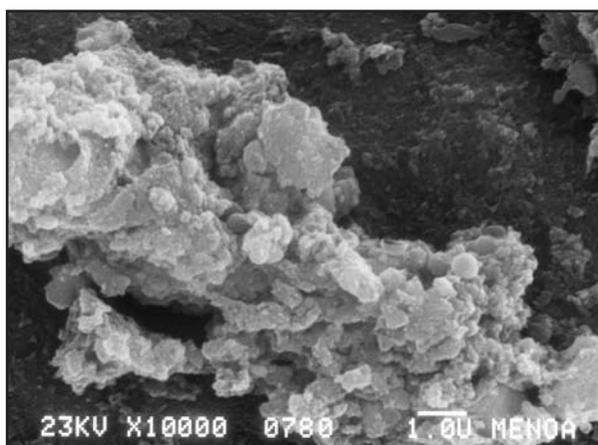


Fig. 6: SEM. Mature *E. faecalis* biofilm at 30 days. The reference line corresponds to 1 μ m.

Our study found that *E. faecalis* biofilm was observable at 14 days and mature biofilm at 30 days. Guerreiro-Tanomaru et al.¹⁷ report similar results. They used different substrates such as bovine dentin and bone, hydroxyapatite and gutta-percha, and reported biofilm development at 14 and 21 days, which they observed by using staining methods and confocal microscopy, finding the greatest development on hydroxyapatite. Wu et al.¹⁸ inoculated *E. faecalis* on human teeth for 4 weeks to obtain biofilm. Somayaji et al.¹⁹ used SEM and found a high percentage of biofilm development 45 days. Du et al.²⁰ made observations under confocal microscope on pieces of dentin incubated with *E. faecalis* for 1 and 21 days, finding young and a mature biofilm, respectively, at those times. Our study shows

that under the experimental conditions applied, it is possible to generate mature *E. faecalis* biofilm at 30 days' incubation on the surface of human root dentin.

CONCLUSIONS

Enterococcus faecalis isolated from root canals was able to produce biofilm on microplates,

which was best seen using 10% crystal violet stain, elution with alcohol and fixation with 10% formaldehyde.

Mature *E. faecalis* biofilm formation was confirmed by SEM after 30 days' incubation, using pieces of root dentin in TS broth supplemented with 10% human serum and 1% glucose.

ACKNOWLEDGMENTS

Partially supported by CIUNT, Tucuman, Argentina. We thank Dr. Marcela D'Urso for the statistical analysis.

CORRESPONDENCE

Dr. María de los Ángeles Bulacio.
Crisóstomo Álvarez 1487. CP 4000
San Miguel de Tucumán, Tucumán. Argentina
maritabulacio@hotmail.com

REFERENCES

1. Ruoff KL, de la Maza L, Murtagh MJ, Spargo JD, Ferraro MJ. Species identities of enterococci isolated from clinical specimen. *J Clin Microbiol* 1990;28:435-437.
2. Nair PNR. Biology and pathology of apical periodontitis. In: Estrela C. *Endodontic Science*. 2nd ed. São Paulo: Artes Médicas 2009 p. 285-347
3. Ferreira FB, Ferreira AL, Gomes BP, Souza-Filho FJ. Resolution of persistent periapical infection by endodontic surgery. *Int Endod J* 2004; 37:61-69.
4. Leonardo MR. Endodoncia. Tratamiento de Conductos Radiculares. Principios Técnicos y Mecánicos. Editora Artes Médicas, Argentina 2005: p437.
5. Shehab ED, Soha AE. Development of an intracanal mature *Enterococcus faecalis* biofilm and its susceptibility to some antimicrobial intracanal medications; an in vitro study. *Eur J Dent* 2012;6:43-50.
6. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST, editors. *Bergey's Manual of Determinative Bacteriology*. Baltimore: Williams and Wilkins; 1994 p. 538.
7. Karnovsky MJ. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J Cell Biol* 1965;27:137-8A.
8. Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, Amorena B, Leiva Penade JR, Lasa I. The Enterococcal Surface Protein, Esp, Is Involved in *Enterococcus faecalis* Biofilm Formation. *Appl Environ Microbiol*. 2001; 67:4538-4545.
9. Kristich CJ, Li YH, Cvitkovitch DG, Dunny GM. Esp-independent biofilm formation by *Enterococcus faecalis*. *J Bacteriol* 2004; 186:154-163.
10. Pillai SK, Sakoulas G, Eliopoulos GM, Moellering RC Jr, Murray BE, Inouye RT. Effects of glucose on fsr-mediated biofilm formation in *Enterococcus faecalis*. *J Infect Dis* 2004; 190:967-97011.
11. Seneviratne CJ, Yip JW, Chang JW, Zhang CF, Samaranyake LP. Effect of culture media and nutrients on biofilm growth kinetics of laboratory and clinical strains of *Enterococcus faecalis*. *Arch Oral Biol*. 2013; 58:1327-1334.
12. Cerca N, Pier GB, Vilanova M, Oliveira R, Azeredo J. Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of *Staphylococcus epidermidis*. *Res Microbiol* 2005;156:506-514.
13. Duggan JM, Sedgley CM. Biofilm formation of oral and endodontic *Enterococcus faecalis*. *J Endod*. 2007; 33:815-818.
14. Zheng J, Wang L, Song Q, Li J, W Niu . The correlation analysis on *Enterococcus faecalis* biofilm and clinical manifestation in retreatment of root canal. *Hua Xi Kou Qiang Yi Xue Za Zhi* 2012; 65-67, 72.
15. Wang L, Dong M, Zheng J, Song Q, Yin W, Li J, Niu W. Relationship of biofilm formation and gelE gene expression in *Enterococcus faecalis* recovered from root canals in patients requiring endodontic retreatment. *J Endod* 2011; 37:631-636.
16. Wilcox MH. Comparison of formalin and Bouin's reagent for fixation of coagulase negative staphylococcal biofilm. *J Clin Pathol* 1994; 47:1044-1046.
17. Guerreiro-Tanomaru JM, de Faria-Júnior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. Comparative analysis of *Enterococcus faecalis* biofilm formation on different substrates. *J Endod* 2013;39: 346-350.
18. Wu D , Fan W , Kishen A , Gutmann JL, Fan B. Evaluation of the antibacterial efficacy of silver nanoparticles against *Enterococcus faecalis* biofilm. *J Endod* 2014;40: 285-290.
19. Somayaji K, Acharya SR, Bairy I, Prakash PY, Rao MS, Ballal NV. In vitro scanning electron microscopic study on the effect of doxycycline and vancomycin on enterococcal induced biofilm. *Iran Endod J* 2010;5: 53-5820.
20. Du T, Wang Z , Shen Y, Ma J, Cao Y, Haapasalo M. Effect of Long-term Exposure to endodontic disinfecting solutions on young and old *Enterococcus faecalis* biofilms in dentin canals. *J Endod* 2014; 40:509-514.