Original Article

CHROMOSOMAL AND PLASMID-ASSOCIATED GENE PROFILES OF *ENTEROCOCCUS FAECALIS*: ADAPTIVE PATTERNS ACROSS ENDODONTIC ENVIRONMENTS

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ABSTRACT

Enterococcus faecalis is a microorganism frequently implicated in endodontic infections, capable of colonising both biological tissues and inert surfaces. However, the relationship between its chromosomal and plasmid gene repertoires and the type of endodontic environment it inhabits remains poorly elucidated. To analyse the distribution of *E. faecalis* virulence genes and their association with the ecological origin of biotic and abiotic isolates. Seventy-five E. faecalis isolates obtained from biotic and abiotic samples collected between 2018 and 2022 were analysed. Taxonomic identification was performed using a MALDI-TOF MS system, and virulence genes were detected by conventional PCR. Gene profiles and their association with the ecological origin of the isolates were evaluated using Factor Analysis of Mixed Data (FAMD), followed by Ward's hierarchical clustering and K-means optimisation. Three genetic clusters of *E. faecalis* were identified and differentiated by their gene repertoires: chromosomal (ace, efaA, gelE), plasmid (asa, asa373, cylA), and mixed (esp). Chromosomal repertoires predominated in biotic isolates, whereas plasmid repertoires were more frequent in abiotic isolates, indicating a significant genetic structure according to ecological origin ($p < 0.05$). The gene repertoires of *E. faecalis* tend to cluster according to their chromosomal or plasmid nature, reflecting distinct patterns of ecological adaptation between biotic and abiotic endodontic environments.

Key words: *Enterococcus faecalis*, Virulence factors, Plasmids, Chromosomes, Bacterial, Endodontics.

Introduction

Enterococcus faecalis is a key pathogen in the aetiology of persistent endodontic infections and root canal treatment failure [1]; its genetic plasticity confers a remarkable ability to adapt to changing conditions in the endodontic environment, allowing it to resist antimicrobial agents and survive under conditions of nutritional or environmental stress. This versatility, its tolerance to alkaline pH, and its ability to form biofilms contribute to the persistence of the microorganism in root canal systems, even after disinfection and sealing procedures [2-6].

Beyond these survival mechanisms, *E. faecalis* possesses an ample repertoire of virulence genes that favour its adaptation to endodontic environments. Among the most studied are the genes for enterococcal surface protein (esp), collagen adhesin (ace), endocardial adhesion factor (efaA), gelatinase (gelE), cytolysin (cylA), aggregation protein (asa) and its variant (asa373), which are involved in adhesion, invasion, tissue degradation, and immune evasion processes, enhancing the microorganism's colonization capacity [7-9]. Complementarily, biofilm has been identified as an essential component of their persistence, as it promotes antimicrobial tolerance, protects against the host immune response, and prolongs bacterial survival. This structure is closely related to the expression of the esp gene and other genes associated with adhesion and tissue degradation, such as *ace, efa*A and *gel*E [10]. However, at the molecular level, there is still no clear consensus on how these factors are integrated into the genetic plasticity of the species, nor on their association with plasmid or chromosomal elements, or their possible modulation by the nature of the environment, whether biotic (of tissue origin) or abiotic (of material origin) [7]. Understanding these relationships would clarify the ecological organization of the *E. faecalis* gene repertoire and its potential for dissemination in the endodontic ecosystem [1].

In abiotic environments, various authors have reported viable isolates of *E. faecalis* in dental materials and instruments, with frequencies ranging from 10 to 40%, depending on the type of surface, including K files, gutta-percha, apex cones, and dentin debris [1, 3]. These results support the possibility that endodontic materials act as persistent microenvironments or extracellular reservoirs, facilitating the horizontal transfer of plasmid genes and the spread of virulence and resistance determinants [11, 12]. However, no adaptive genetic pattern is known linking the coexistence plasmid-borne and chromosomal genes with the

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ecological behavior of *E. faecalis* in these niches.

Recent studies have identified mixed virulence genotypes, characterized by the coexistence of chromosomal genes (*gelE, efaA, ace*) and plasmid genes (*asa, asa373, cylA*), often accompanied by the *esp* gene, whose dual location reinforces its role in bacterial adaptation [7, 8, 13, 14]. These combined profiles, such as esp^+_$gelE^+$_asa^+ or ace^+_$efaA^+$_asa^+, illustrate the interplay between attachment mechanisms, enzymatic tissue degradation, and genetic transfer, which would explain their ability to persist in both biological tissues and inert dental surfaces. However, most studies have not differentiated the relative contribution of plasmid and chromosomal genes, even though this distinction is crucial for understanding the genetic basis of persistence and virulence, and for identifying the horizontal dissemination routes between clinical and environmental strains. This aspect has been featured in genomic studies on conjugative plasmids and adaptive plasticity of *E. faecalis* [3, 7, 15]. This differentiation is particularly relevant in the field of endodontics, where biotic and abiotic environments impose different selective pressures that can modulate the expression and maintenance of mobile genetic elements, especially plasmids, which are capable of being transferred between strains. For example, the plasmid pAD1, which carries both the *asa* and *cyl*A genes, has been described, as have plasmids that co-transfer antimicrobial resistance determinants, such as the *cfr* gene (linezolid resistance), together with virulence factors such as cytolysin and hemolysin (*cln* and *hln*) on the same plasmid [16-18].

In this context, the present study aimed to compare the distribution and coexistence of plasmid and chromosomal virulence genes in *E. faecalis* isolates from biotic and abiotic endodontic environments, and to identify genetic clustering patterns associated with their ecological origin.

Materials and Methods

Isolates and study samples
Seventy-five *Enterococcus faecalis* isolates collected between 2018 and 2022 were analyzed, originating from abiotic samples (n = 50; gutta-percha, K files, paper cones, and ejectors) and biotic samples (n = 25; cheek mucosa, palate, tongue, jugal groove, and gums).

The clinical samples corresponded to patients who attended endodontic consultations, had no history of antibiotic treatment in the previous three months, no systemic diseases, and provided informed consent to participate in the study.

The isolates were initially identified as *E. faecalis* using the MicroScan Pos ID PC34 panel (Dade Behring Inc., West Sacramento, CA, USA) and cryopreserved in BHI broth with 15% glycerol and oxacillin until processing [19].

Microbiological identification
Taxonomic confirmation followed a recognized methodology previously reported, which agrees with the CLSI standards in force at the time of analysis.

Cryopreserved samples were reactivated by controlled gradual thawing (−80 °C → 4 °C → room temperature), ensuring bacterial viability [8, 17].

After material reactivation, samples streaked onto Chromocult®Enterococci Agar (Merck, Darmstadt, Germany) Petri dishes, incubated for primary isolation, from which viable and pure colonies were isolated. Following, the colonies identified to genus and species level using a MALDI-TOF MS Biotyper (Bruker Daltonics, Germany), by accessing the Bruker Taxonomy Database v3.3.1 and BioExplorer v1.0 software [3, 12]. All tests were in duplicate and included *E. faecalis* strains ATCC 29212 and ATCC 700802 as reference controls [20].

Gene detection
Molecular detection of virulence genes was carried out by conventional PCR, following the manufacturer's recommendations and protocols reported in the literature. Genomic DNA was extracted using the DNeasy Blood & Tissue 69504 kit (Qiagen, Santa Clarita, CA, USA). The presence of the asa1, asa373, ace, esp, cylA, gelE, and efaA genes was determined using primers and amplification conditions previously described [3, 21-23]. The amplified products were separated on 1% (w/v) agarose gels in 1X TBE buffer, stained with Sybr Green, and visualized on a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc., USA). *E. faecalis* ATCC 12030 served as the positive control.

Virulence profiles
To evaluate the individual distribution of each gene according to sample type, absolute and relative frequencies were calculated for both groups and compared using Fisher's exact test ($p < 0.05$). Once the most representative combined genotypes for each group identification occurred (those shared by biotic and abiotic sources), (those shared by biotic and abiotic sources), the predominant gene repertoires were described.

Multivariate analysis (FAMD–Ward–K-means)
The virulence gene profiles of E. faecalis were analysed using Factor Analysis of Mixed Data (FAMD) to integrate categorical and quantitative variables, reduce data dimensionality, and visualise patterns of association among the isolates [18, 24]. Based on the factorial coordinates obtained, Ward's hierarchical clustering was applied, followed by K-means optimization, to define genetic clusters according to the similarity of virulence gene repertoires. Finally, the relationship between clusters and the isolates' ecological origin (biotic or abiotic) was assessed using Fisher's exact test ($p < 0.05$).

All analyses were performed in R v4.3.1, using the

FactoMineR and factoextra packages.

Results and Discussion

Identificación microbiológica

The 75 isolates analyzed were confirmed as *Enterococcus faecalis* based on MALDI-TOF MS Biotyper analysis, with scores ≥ 2.0, a value that corresponds to reliable identification at the species level according to the manufacturer's criteria.

Frequency of virulence genes

The distribution of isolates according to sample type showed that biotic environments presented a higher number of virulence genes, while abiotic isolates exhibited lower genetic diversity. Statistically significant differences ($p < 0.05$) were observed for the *asa*, *esp*, *gel*E, *ace*, and *efa*A genes, all of which were more frequent in biotic strains **(Table 1)**. In addition, the coexistence of two contrasting gene repertoires were identified: chromosomal genes (*ace*, *efa*A, *gel*E) were more frequent in biotic isolates, while plasmid genes (*asa*, *asa*373, *cyl*A) tended to be more prevalent in abiotic isolates.

Table 1. Frequency of *Enterococcus faecalis* virulence genes according to ecological origin (biotic or abiotic)

Genes	Biotic (%) (*n*=25)	Abiotic (%) (*n*=50)	*p-valor* (Fisher)
***asa*1**	100 (25/25)	54 (27/50)	< 0.0001
***asa*373**	0 (0/25)	4 (2/50)	0.5495
esp	92 (23/25)	30 (15/50)	< 0.0001
***cyl*A**	52 (13/25)	52 (26/50)	1.0000
***efa*A**	88 (22/25)	100 (50/50)	0.0341
ace	100 (25/25)	82 (41/50)	0.0251
***gel*E**	84 (21/25)	32 (16/50)	< 0.0001

Virulence profiles

Combined gene analysis allowed the identification of predominant genotypes differentiated by ecological origin **(Table 2)**. In biotic isolates, the pattern asa^+ esp^+ $cylA^-$ $efaA^+$ ace^+ $asa373^-$ $gelE^+$ stood out, being observed in 40% (10/25) of strains, while in abiotic isolates, asa^+ esp^- $cylA^+$ $efaA^+$ ace^+ $asa373^-$ $gelE^-$ predominated, being present in 18% (9/50). Likewise, genotypes shared between both origins were in low frequency, being the most representative asa^+ esp^- $cylA^+$ $efaA^+$ ace^+ $asa373^-$ $gelE^+$, found in 8% (2/25) of biotic isolates and 16% (8/50) of abiotic isolates.

The total number of genotypes identified per group, as well as other combinations and their frequencies, are summarized in **Table 2**.

Table 2. Most frequent virulence genotypes detected in *E. faecalis* according to the ecological origin of the isolates

Origin	Number of genotypes	Most common genotypes	Frequency % (*n*)
Biotics	7	asa^+ esp^+ $cylA^-$ $efaA^+$ ace^+ $asa373^-$ $gelE^+$	40 (10/25)
		asa^+ esp^+ $cylA^+$ $efaA^+$ ace^+ $asa373^-$ $gelE^+$	32.0 (8/25)
		asa^+ esp^+ $cylA^-$ $efaA^-$ ace^+ $asa373^-$ $gelE^-$	8.0 (2/25)
		Other combinations	20 (5/25)
Abiotics	7	asa^+ esp^- $cylA^+$ $efaA^+$ ace^+ $asa373^-$ $gelE^-$	18.0 (9/50)
		asa^+ esp^- $cylA^+$ $efaA^+$ ace^+ $asa373^-$ $gelE^+$	16.0 (8/50)
		asa^- esp^+ $cylA^-$ $efaA^+$ ace^+ $asa373^-$ $gelE^-$	14.0 (7/50)
		Other combinations	52 (26/50)
Common	2	asa^+ esp^- $cylA^+$ $efaA^+$ ace^+ $asa373^-$ $gelE^+$	Biotics 8.0 (2/25) Abiotics 16 (8/50)

Multivariate analysis (FAMD–Ward–K-means)

Multivariate analysis revealed three distinct genetic clusters, based on the repertoire of predominant virulence genes.

Cluster 1, composed mainly of the *ace*, *efa*A, and *gel*E genes, c represented a chromosomal profile, while Cluster 2, composed of *asa*, *asa*373, and *cyl*A, reflected a plasmid repertoire. Cluster 3 showed a mixed or transitional pattern, characterized by the presence of the *esp* gene together with partial combinations of both repertoires.

Figure 1 illustrates the distribution of isolates in the factorial space, where the ellipses represent the 95% covariance regions. Biotic isolates were mainly grouped in Cluster 1, while those isolates from abiotic origin distribution were in Clusters 2 and 3. A partial overlap was observed between Clusters 1 and 2, suggesting a transitional zone combinig chromosomal and plasmid gene repertoires. In contrast, In contrast, Cluster 3 Cluster 3 displayed an independent arrangement, representing a more homogeneous abiotic set of genes.

Taken together, these results indicate that the genetic structure of virulence repertoires was significantly associated with the type of endodontic environment, evidencing distinct adaptive gradients between biotic and abiotic niches ($p < 0.05$).

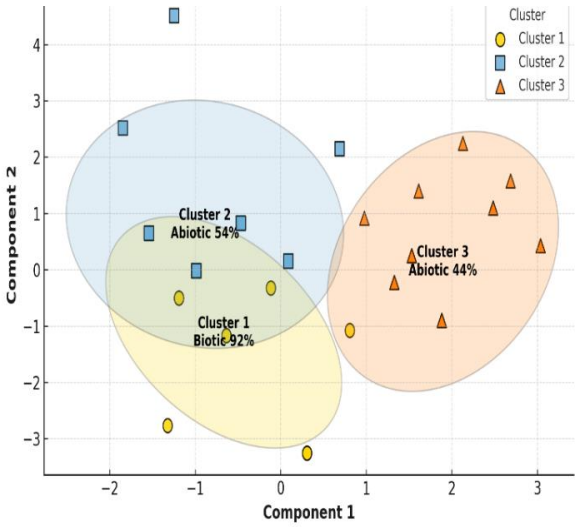

Figure 1. Factor Analysis of Mixed Data (FAMD, PCA approach) by Ward's hierarchical clustering and K-means optimisation of *Enterococcus* isolates

As a complementary analysis, an exploratory index of chromosomal and plasmid gene load per isolate confirmed the trend observed in the FAMD. The differences between biotic and abiotic origins, evaluated using the Mann–Whitney U test showed significantly higher chromosomal load ($p = 0.00016$) and slight but significantly lower plasmid loads ($p = 0.048$) in abiotic osilates. The effect size (r) was 0.21 for plasmid load and 0.40 for chromosomal load, indicating a small and moderate effect, respectively.

Table 3. Comparison of chromosomal and plasmid gene load between biotic and abiotic isolates of *E. faecalis*

Index	Biotic (average ± SD)	Abiotic (average ± SD)	Median Abiotic	Median Biotic	p-value	Effect size (r)
Plasmid load	1.52 ± 0.51	1.10 ± 0.89	2	1	0.048	0.21
Chromosome load	2.72 ± 0.61	2.14 ± 0.64	3	2	0.00016	0.40

E. faecalis, isolated from both biotic and abiotic samples, has previously been reported by various authors [8, 25, 26]. The present study obtained comparable findings, detecting it on abiotic surfaces such as gutta-percha, K-files, paper cones, and ejectors, as well as in biotic samples such as cheek mucosa, palate, tongue, jugal groove, and gums. Its detection in these endodontic samples confirms its association with therapeutic failure, its remarkable genetic plasticity for adapting to different niches, and the relevance of the oral cavity as a reservoir of microorganisms with pathogenic and virulent potential [1, 3, 25, 27].

The localisation of E. faecalis in various biotic and abiotic niches is associated with its capacity to withstand adverse environmental conditions, its high genetic plasticity, and the coordinated expression of virulence genes that promote persistence and colonisation [2, 27-29]. Numerous studies have characterized virulence genes such as *esp*, *gel*E, *asa*, *ace*, *cyl*A, *hyl*, and *efa*A, describing their roles in adhesion, biofilm formation, and immune evasion [10, 30]. However, aspects such as the role of the oral cavity as a reservoir for virulence genes, plasmid or chromosomal transfer, and the relationship between genetic repertoires and ecological niches are only beginning to be explored, and the available data at the oral level remain scarce [31].

In this study, the most frequently identified genes were *asa*, *esp*, gelE, *ace*, and *efa*A, with greater genetic diversity observed in biotic isolates than in abiotic isolates ($p < 0.05$) **(Table 1)**. This distribution may be attributed to biological surfaces providing more stable and nutrient-rich conditions that favour the expression of genes associated with adhesion and virulence (*ace, efaA, gelE*); conversely, abiotic environments may select for strains carrying mobile or plasmid elements (*asa, cylA*), facilitating survival under stressful conditions [30, 32].

This dual pattern, characterized by chromosomal genes linked to colonization and virulence, and plasmid genes associated with aggregation and genetic transfer, is consistent with previous demonstrating a differential distribution of these determinants depending on the isolation environment and their contribution to the persistence of *E. faecalis* [33-35]. The results suggest that environmental conditions influence the expression and conservation of virulence factors, reinforcing the need for comparative genotypic studies to clarify the ecological and evolutionary adaptations of *E. faecalis* [2, 28, 36, 37].

The combined gene analysis revealed differentiated genotypic profiles according to ecological origin, with a predominance of combinations including *asa, esp, ace, efaA*, and *gelE* in biotic isolates, a higher representation of *asa* and *cylA* in abiotic isolates, and a low proportion of genotypes shared between both origins **(Table 2)**. This distribution of genes agrees with that reported by other authors, who have associated these genes with adhesion, biofilm formation, and resistance to stress conditions — all fundamental aspects for the persistence and virulence of *E. faecalis* [25, 33].

In this study, the observed distribution suggests the existence of two functional gene repertoires: one plasmidic (*asa, cylA*), associated with aggregation and genetic transfer, and another chromosomal (*ace, efaA, gelE*), linked to adhesion and tissue colonisation. The presence of a third repertoire shared between both origins suggests gene flow between reservoirs or lineages with a broad ecological spectrum, a phenomenon that could explain the capacity of *E. faecalis* to adapt, persist, and disseminate across intra- and extra-host environments [2, 28, 33, 38].

To analyse genotypes and their distribution, isolate clustering was performed using the FAMD-Ward–K-means methodology, based on the guidelines described by Le *et al*. [24], and the K-means consolidation previously outlined [39]. This approach integrated ecological and genetic variables and, in this case, enabled a more refined delimitation of virulence clusters, facilitating the biological interpretation of relationships between isolates and their ecological origin

The analysis identified three distinct clusters. Cluster 1 displayed a chromosomal repertoire dominated by genes associated with adhesion and colonisation; Cluster 2 grouped isolates with a predominance of plasmid genes related to aggregation and genetic transfer; and Cluster 3 showed a transitional profile, combining features of both repertoires **(Figure 1)**. These differences were statistically significant ($p < 0.05$) and suggest that the virulence structure of *E. faecalis* may be modulated by environmental type and by interactions between chromosomal and plasmid genes, supporting the hypothesis of ecological plasticity mediated by complementary functional repertoires.

Based on the cluster patterns observed, a chromosomal and plasmid load index was applied to quantify the relative contribution of each gene repertoire to the adaptation and ecological persistence of *E. faecalis.* The analysis revealed a higher chromosomal load in biotic isolates and a slightly higher plasmid load in abiotic isolates, indicating functional differentiation according to environmental conditions **(Table 3)**. These results corroborate the overall trend, where chromosomal repertoires confer stability and colonisation capacity, whereas plasmid repertoires contribute adaptive flexibility and transfer potential [2, 28, 33].

Although this study has certain limitations, such as the lack of more advanced molecular techniques to establish causal associations, the methodology employed enabled the identification of consistent trends between sample type and the chromosomal and plasmid repertoires of *E. faecalis*. These findings reinforce the proposed ecological model, suggesting that the virulence structure responds to distinct selective pressures.

Conclusion

E. faecalis exhibited a differential genetic structure associated with the endodontic environment: chromosomal persistence genes predominated in biotic isolates, whereas plasmid genes were prevalent in abiotic isolates. Multivariate analysis confirmed this segregation, supporting an adaptive model in which E. faecalis adjusts its gene repertoire in response to varying ecological pressures.

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Ethics statement: All research procedures complied with the ethical standards of the institutional research committee, the 1964 Declaration of Helsinki and its later amendments. The study protocol was reviewed and approved by the Ethics Committee of the Faculties of Dentistry and Sciences, Pontificia Universidad Javeriana, Bogotá D.C., Colombia (Act. SIAP00007699).

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