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Genotypic and phenotypic diversity in *Enterococcus faecalis*: is agar invasion a pathogenicity score?

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ABSTRACT

Objectives. The main objective of the present study is to analyze different genotypic and phenotypic traits related to virulence in *Enterococcus faecalis*, as well as evaluated the agar invasion phenotype in a collection of isolates with different clinical origins.

Material and methods. Seventy-nine *E. faecalis* isolates, with invasive and non-invasive clinical origins, have been used in this work. Presence of cytolysin activator (*cylA*), gelatinase (*gelE*), surface protein (*esp*), aggregation substance (*asa1*), endocarditis antigen (*efaA*), and collagen-binding protein (*ace*) have been analyzed by PCR. Phenotypic characterization included gelatinase activity, haemolysin production, biofilm formation and agar invasion.

Results. All the isolates tested harboured at least one of the virulence determinants. The 95.5% of isolates from haematologic samples were positive for agar invasion test, significantly higher than isolates from non-invasive diseases. A significant reduction in relative invasion area was observed in three selected agar-invasive strains after 15 serial passages.

Conclusions. It has been observed a significant high prevalence of agar-invasion positive isolates among strains belonged to haematological samples. Agar invasiveness is reduced after adaptation of clinical isolates to laboratory conditions, showing that agar invasion phenotype can be modulate by culture conditions as other virulence factors observed in different bacterial species.

Key words: *Enterococcus faecalis,* **virulence, agar invasion.**

Diversidad genotípica y fenotípica en *Enterococcus faecalis*: ¿Es la invasión en agar un marcador de patogenicidad?

RESUMEN

Objetivos. El principal objetivo de este trabajo es la caracterización de determinantes de virulencia genotípicos y fenotípicos relacionados con patogenicidad en *Enterococcus faecalis*, evaluando además el fenotipo de invasión en agar en una colección de aislados clínicos de diversa procedencia.

Material y métodos. Se han analizado 79 cepas de *E. faecalis* aisladas en infecciones invasivas y no invasivas. La detección de los principales determinantes asociados a la virulencia (*cylA*, *gelE*, *esp*, *asa1*, *efaA* y *ace*) se ha realizado mediante PCR. La caracterización fenotípica incluyó la detección de actividad gelatinasa, hemólisis, formación de biofilm y el test de invasión en agar.

Resultados. Todos los aislados presentaron, al menos, un determinante de virulencia. El 95,5% de las cepas provenientes de hemocultivos resultaron positivas para el test de invasión en agar, significativamente superior a lo observado en cepas de origen clínico no invasivo. En tres cepas seleccionadas, positivas para el test de invasión en agar, se observó una reducción significativa del área relativa de invasión tras 15 pases seriados.

Conclusiones. Se ha observado una alta prevalencia de cepas con alto grado de invasión en agar en los aislados hematológicos. Dicho grado de invasión disminuye significativamente al adaptar tres cepas al crecimiento en condiciones de laboratorio, sugiriendo una modulación en función de las condiciones de cultivo tal y como ocurre con otros determinantes asociados a virulencia en diferentes especies bacterianas.

Palabras clave: *Enterococcus faecalis*, **virulencia, invasión en agar.**

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INTRODUCTION

Enterococcus faecalis is an aerobic gram positive coccus commonly found in the normal human microflora and historically considered as non pathogenic. Since 1970s-1980s this bacterial specie has become one of the major nosocomial pathogens, and nowadays is the second pathogen associated with urine tract infection (UTI) and infected surgical wounds, and the third most common cause of bacteraemia in USA[1].

Even though it has been demonstrated that given the required conditions of intestinal overgrowth *E. faecalis* is able to establish a systemic infection in mice[2], some genetic factors have been associated with virulence in clinical strains. These genes encode secreted proteolytic enzymes (*gelE, cylA*) and adhesion proteins to eukaryotic cells and surfaces (*esp, asa1, efaA, ace*) which help the pathology process. These genes can be found in conjugative plasmids, such as pAD1[3] or organized in complex pathogenic island[4], allowing the horizontal intra- and inter-species transmission of these factors[5,6].

In addition to the presence of different genes, phenotypic characteristics, such as haemolysis, gelatinase activity or biofilm formation have been recognized as critical in the *in vivo* bacterial growth[7]. Recently, a new quantitative bacterial agar invasion test has been developed[8]. Agar invasion has been described as a strain-specific phenotypic character, and it has been hypothesized that the expression of this phenotype could be associated with an increased pathogenicity, as it occurs in yeasts[8,9]. The main objective of this work is analyzing different genotypic and phenotypic traits related to virulence as well as evaluated the agar invasion phenotype in a collection of clinical isolates of *E. faecalis* with different clinical origins.

MATERIAL AND METHODS

Strains. Seventy-nine *E. faecalis* isolates from the strain collection of the Department of Microbiology, School of Medicine, Complutense University of Madrid have been analyzed. Samples were identified as EFx (where x takes values from x=1 to x=79). Strains were isolated from bacteraemia (EF1-EF22, n=22), urinary tract infections (EF23-EF48, n=26), exudates (EF49-EF63, n=15) and faeces (EF64-EF79, n=16). All assays were realized from stock bacterial suspensions in tryptone soy broth (TSB, Biomedics S.L., Madrid, Spain) adjusted to an OD_{600} = 0.5.

Genotypic characterization. The presence of virulence-associated genes that encode for cytolysin activator (*cylA*), gelatinase (*gelE*), surface protein (*esp*), aggregation substance (*asa1*), endocarditis antigen (*efaA*), and collagen-binding protein (*ace*) were investigated by PCR using primers and conditions previously described (table 1).

Hemolysin production. Haemolytic activity was investigated plating bacterial suspensions onto agar Müeller-Hinton supplemented with 5% of human blood[15]. The presence of clear zone around the colonies (β haemolysis) was reported as a positive result.

Table 1 Primers used in this study for detection of virulence genes by PCR.

Gene	Primer	Sequence (5'-3')	Reference
cylA	CYT I	ACTCGGGGATTGATAGGC	10
	CYT IIb	GCTGCTAAAGCTGCGCTT	
esp	ESP 14F	AGATTTCATCTTTGATTCTTGG	11
	ESP 12R	AATTGATTCTTTAGCATCTGG	
asa1	ASA 11	GCACGCTATTACGAACTATGA	12
	ASA 12	TAAGAAAGAACATCACCACGA	
gelE	GEL 11	TATGACAATGCTTTTTGGGAT	12
	GEL 12	AGATGCACCCGAAATAATATA	
efaA	TE5	GACAGACCCTCACGAATA	13
	TE6	AGTTCATCATGCTGCTGTAGTA	
ace	ACE1	AAAGTAGAATTAGATCCACAC	14
	ACE2	TCTATCACATTCGGTTGCG	

Gelatinase production. Gelatinase activity was detected using Gelatinase medium (BD Difco, Franklin Lakes, NJ). Nutrient gelatin tubes were inoculated with bacterial suspensions, incubated at 35ºC for up to 15 days, and checked every day for gelatin liquefaction. A strain was considered positive when gelatinase medium remained liquid after exposure to 4ºC for 30 minutes.

Biofilm production. Biofilm formation was performed on microtiter plates following the spectrophotometric methodology described by Stepanovic et al[16]. Briefly, six wells of polystyrene microtiter plates (Materlab S.L., Madrid, Spain) were inoculated with 200 µl of each bacterial suspension and incubated at 35ºC for 24 h. Formed biofilm was stained with crystal violet and then dye retained in the biofilm was solubilized with 200 µl of 95% ethanol. Finally, OD_{575} of each well was measured with a spectrophotometer (Cintra 10, GBC Scientific Equipment, Braeside, Australia). OD_{575} average value of the six wells was used for comparisons. Sterile TSB was used as negative control. In order to classify strains into categories (non-producer, weak-producer, moderate-producer, high-producer) three cut-off values were established:

Cut-off 1 = OD_{575} average value of negative control + 3 standard deviation (SD)

Cut-off 2 = Cut-off 1 x 2

Cut-off 3 = Cut-off 1 x 4

The algorithm used to classify strains based on their OD_{575} average values is as follows:

Non-producer ≤ cut-off 1 < weak-producer ≤ cut-off 2 < moderate-producer ≤ cut-off 3 < high producer

Agar invasion test. Agar invasiveness of different strains was analyzed using a recently described agar invasion test[9]. For this purpose stock bacterial suspensions were diluted 1/1000 in sterile TBS and 5 µl of each dilution (1x10^5 cfu/ml approx-

imately) were spotted onto the surface of standard Müeller-Hinton medium in triplicate (three spots in a row). Plates were then incubated for 5 days at 35ºC under aerobic conditions and constant humidity to allow colony/biofilm development. After incubation period, the area of each colony/biofilm was measured from images taken by stereo microscopy. Subsequently, plates were gently washed with sterile water to remove colonies/biofilms from the agar surface and flooded with 0.5% methylene blue for 5 minutes to allow dye penetration into the agar. Plates were then examined at 50x with a light microscope (Leica DM5000B) to detect bacterial grown within culture medium thickness. Digital images of bacterial invasions were analyzed with image analysis software (Leica QWin) and total area of each invasion was measured. Finally, relative invasion area (in percentage) of each invasion footprint respect to the total area occupied by colony/biofilm onto the agar surface was calculated.

All strains were classified into categories based on invasion relative area according to the following algorithm:

No-invasiveness ≤ 0.2% < weak-invasiveness ≤ 3.0% < moderate-invasiveness ≤ 6.0% < high-invasiveness.

In order to determine if agar invasion phenotype could be modified by bacterial adaptation to laboratory conditions, three invasive strains (EF43, EF46 and EF75) were subjected to 15 serial passages on blood agar. Then the three derivate strains were subjected again to agar invasion test and the results compared with those of the parental strains.

Table 2 **Incidence (number of strains and percentage) of genotypic virulence traits according to clinical origin of *E. faecalis* isolates.**

Origin	Nº strains	*gelE*	*asa1*	*cylA*	*esp*	*efaA*	*ace*
Bacteraemia	22	18 (81.8%)	16 (72.7%)	8 (36.4%)	7 (31.8%)	16 (72.7%)	13 (59.1%)
UTI	26	18 (69.2%)	20 (76.9%)	13 (50.0%)	20 (76.9%)	25 (96.2%)	12 (46.2%)
Exudates	15	13 (86.7%)	12 (80.0%)	5 (33.3%)	11 (73.3%)	15 (100%)	8 (53.3%)
Faeces	16	13 (81.3%)	14 (87.5%)	11 (68.8%)	7 (43.8%)	15 (93.8%)	12 (75.0%)
Total	**79**	**62 (78.5%)**	**62 (78.5%)**	**37 (46.8%)**	**45 (57.0%)**	**71 (89.9%)**	**45 (57.0%)**

Table 3 **Incidence (number of strains and percentage) of phenotypic virulence determinants according to clinical origin of *E. faecalis* isolates.**

Origin	Nº strains	Haemolysis	Gelatinase	Biofilm	Agar invasion
Bacteraemia	22	4 (18.2%)	13 (59.1%)	18 (81.8%)	21 (95.5%)
UTI	26	11 (42.3%)	7 (26.9%)	24 (92.3%)	19 (73.1%)
Exudates	15	4 (26.7%)	10 (66.7%)	13 (86.7%)	11 (73.3%)
Faeces	16	9 (56.3%)	4 (25.0%)	15 (93.8%)	11 (68.8%)
Total	**79**	**28 (35.4%)**	**34 (43.0%)**	**70 (88.6%)**	**62 (78.5%)**

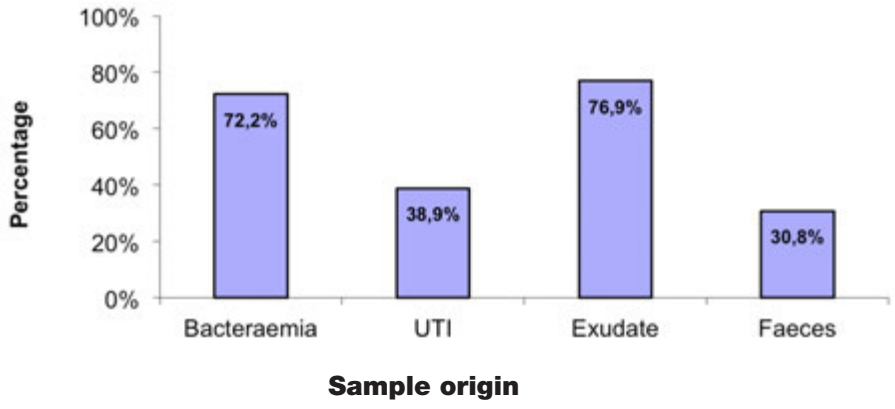

Figure 1 **Frequency of *gelE* gene expression as percentage of strains carrying the gene which show *in vitro* gelatinase activity on the basis of its clinical origin.**

RESULTS AND DISCUSSION

In this work different virulence factors have been analyzed in a large collection of *E. faecalis* strains isolated from invasive (hematologic isolates) and non-invasive diseases (urinary tract infections and exudates) and from faeces of healthy individuals. We have studied the presence of different genes associated with virulent strains as well as phenotypic traits such as biofilm formation, gelatinase activity, haemolysis and agar invasion. All the isolates tested harboured at least one of the virulence determinants. Only two strains (EF16 and EF70) showed negative amplification of genetic determinants, as well as negative phenotypic detection of gelatinase activity or haemolysis. Nevertheless, both strains were positive for biofilm formation, and one of them (EF16) positive for agar invasion.

Gelatinase is an extracellular zinc endopeptidase encoded by *gelE* gene that hydrolyzes gelatin, collagen and small peptides[17]. This gene has been commonly found in clinical isolates of *E. faecalis*[18,19]. In this work, *gelE* was detected in the

78.5 % of total strains, in accordance with previous studies[18,19]. *GelE* prevalence was lower in strains from UTI (69.2%) than isolates from other sources (above 80%), although not significant differences were observed on the basis of the sample origin (table 2). A discrepancy between *gelE* presence and its phenotypic expression was observed frequently[14]. In this study, only 54.8% of *gelE*-positive strains showed *in vitro* gelatinase activity (table 3). *GelE* expression was significantly more frequent (p=0.022) among bacteraemia and exudate isolates (above 72%) when compared to UTI and faeces isolates (below 39%) (figure 1).

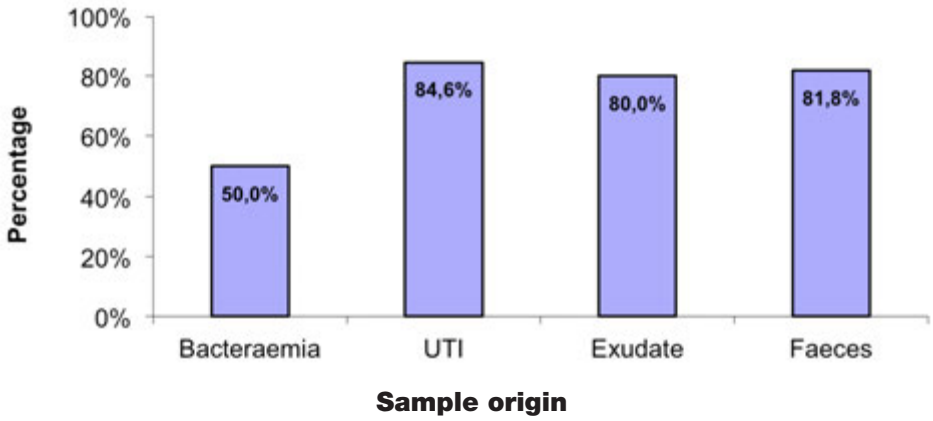

Figure 2 Percentage of strains carrying *cylA* gene which show beta-haemolysis on the basis of its clinical origin.

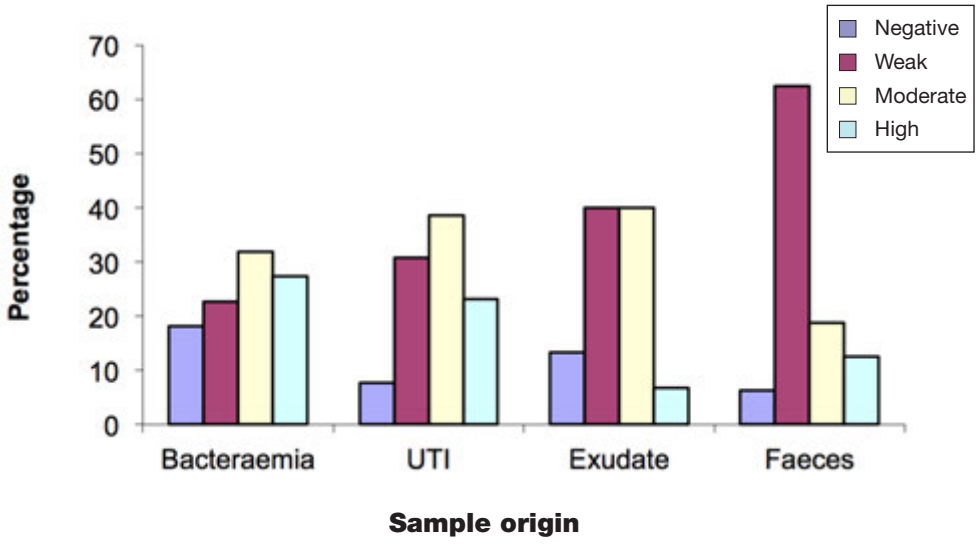

Figure 3 Strains distribution according to the intensity of biofilm production and origin of the isolate.

The enterococcal cytolysin (haemolysin) is a secreted virulence factor with bactericidal properties and beta-haemolytic effects in human erythrocytes, and its activity contributes to the disease severity[20]. Haemolysin is a plasmid-borne operon composed by non regulatory genes (*cylL*), and the activator gene (*cylA*), which is essential for the haemolytic activity[21]. In this work *cylA* gene was detected in 46.8 % of strains tested (table 2). The prevalence was significantly higher in strains from faeces compared with exudates (68.7% vs 33.3%, p=0.041) or haematological samples (68.7% vs 36.4%, p=0.044), similar to the observed in other studies[14,15]. However, the presence of *cylA* gene not perfectly correlated with haemolysis detection. Thus, only 28 out of 37 (75.7%) *cylA* positive strains caused beta-haemolysis (table 3). Interestingly, bacteraemia isolates showed both the lowest percentage of haemolytic strains (18.2%) as the lowest prevalence of haemolysis among *cylA* positive isolates (50%) (figure 2).

The enterococcal surface protein (*esp*) is a cell-wall-associated protein related to adhesion to eukaryotic cells, immune system evasion and biofilm formation. It has also been associated with colonization of urinary tract epithelium[22,23]. In our work, this gene was found in 57.0% of total isolates (table 2), showing a significantly higher prevalence among strains from UTI and exudates than other clinical origin (p<0.01).

The endocarditis antigen *efaA* gene has been strongly associated with clinical isolates, especially from endocarditis, reaching a prevalence of 100% in different studies[18,19]. In accordance with these studies, the prevalence in the strains studied here was 89.9% (table 2). However, we have observed a significant lower prevalence of *efaA* gen among hematologic isolates comparing with UTI (72.7% vs 96.1%, p=0.022) or exudates samples (72.7% vs 100%, p=0.027), but not with respect of faeces samples (72.7% vs 93.7%, p=0.099).

Aggregation substance, encoded by *asa1* gene is a pheromone-inducible protein involved in conjugation transfer and associated with virulence trough the adhesion to epithelial cells[24,25]. ACE is a collagen binding protein, associated with

adhesion to collagen (types I and IV) and laminin. *Asa1* and *ace* genes were found in the 78.5% and 57.0% of total isolates respectively (table 2). In both cases, faecal isolates showed the highest proportion of these genes (87.5% and 75.0% respectively).

Table 4 **Incidence (number of strains and percentage) of *E. faecalis* genes associated to virulence in relation to biofilm production degree.**

Biofilm production	Nº strains	*gelE*	*asa1*	*cylA*	*esp*	*efaA*	*ace*
Non producer	9	5(55.6%)	6 (66.7%)	3 (33.3%)	6 (66.7%)	9 (100%)	3 (33.3%)
Weak	29	27 (93.1%)	25 (86.2%)	18 (65.5%)	18 (62.1%)	26 (89.7%)	18 (62.1%)
Moderate	26	22 (84.6%)	21 (80.8%)	10 (38.5%)	13 (50.0%)	24 (92.3%)	14 (53.8%)
High	15	8 (53.3%)	10 (66.7%)	5 (33.3%)	8 (53.3%)	12 (80.0%)	10 (66.7%)
Total	**79**	**62 (78.5%)**	**62 (78.5%)**	**37 (46.8%)**	**45 (57.0%)**	**71 (89.87)**	**45 (57.0%)**

Biofilm production has been recognized as an important factor in the in vivo growth of bacterial species[7]. In our work, 70 strains (88.6%) were positive for biofilm formation (table 3). We observed a higher number of biofilm producers than other study[23], but similar to the observed by Mohamed et al. when analyzed non-endocarditis strains[26]. Among all studied strains, 29 (36.7%) were classified as weak biofilm producers, 26 (32.9%) moderate producers, and 15 (19.0%) showed high biofilm production. No significant difference (p=0.349) was observed between intensity of biofilm formation and sample origin (figure 3). Table 4 shows the relationship between the presence of the studied determinants and the degree of biofilm production. In this sense, biofilm has been related to the presence of *esp* gene[19], although it has been also demonstrated the *esp*-independent biofilm production and the impact of *esp* on the degree of biofilm formation rather than the absence/presence of this phenomenon[27]. In our work no association between *esp* gene presence and biofilm was observed (p=0.53), although a strong tendency was detected between biofilm production and *gelE* presence (p= 0.075), a relationship previously described[27].

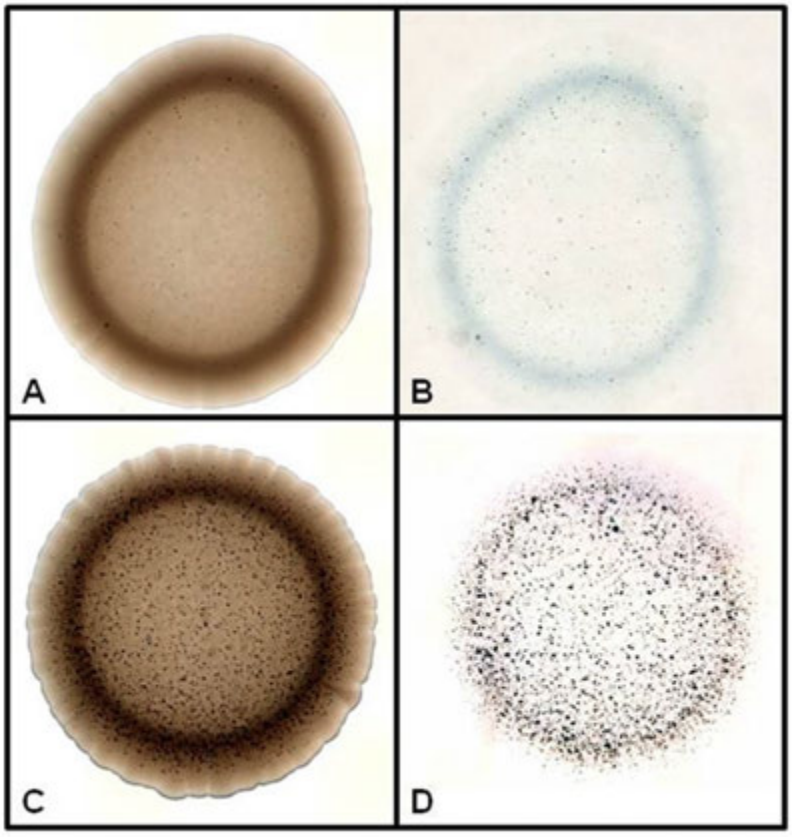

Figure 4 **Agar invasion test. *E. faecalis* colonies/biofilms were grown onto Müeller-Hinton agar and visualized with a stereomicroscope (A, C). Then plates were washed and stained with methylene blue, and invasion footprint were analyzed under light microscope at 50x (B, D). A-B: EF2 strain (weak invasiveness); C-D: EF24 strain (high invasiveness).**

Since none of the virulence determinants studied (except gelatinase activity) was significantly associated with isolates from invasive diseases rather than with strains from non-invasive diseases, or commensals strains from healthy individuals, we focused our attention on a novel bacterial phenotypic character[8]. To our knowledge, this is the first study that investigates the occurrence of agar invasion as putative virulence marker in clinical and commensal strains. Agar invasion test was found positive in 62 (78.5%) isolates (table 3), where 21 (26.6%) showed weak, 14 (17.7%) moderate, and 27 (34.2%) high invasiveness level (figure 4). A significant difference

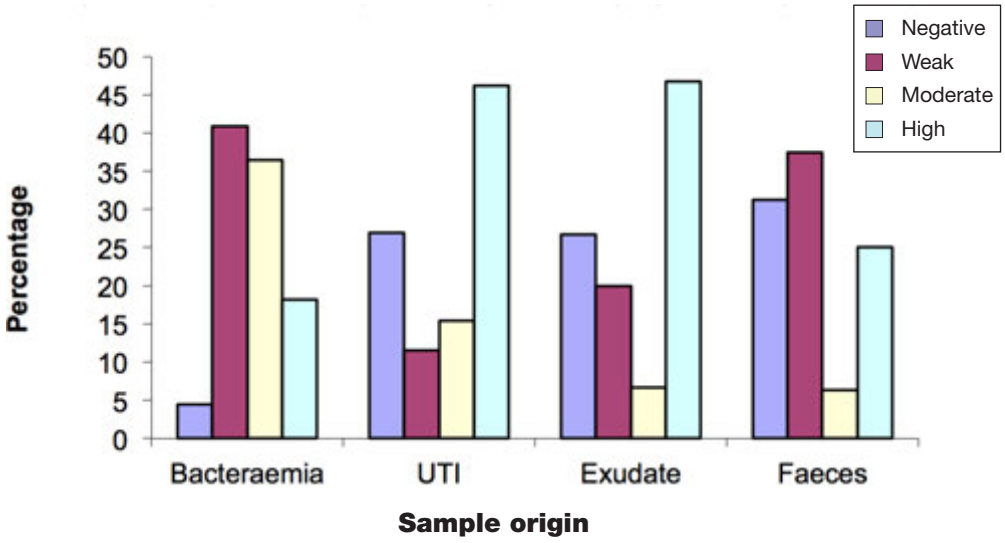

Figure 5 Strains distribution according to the intensity of agar invasion and origin of the isolate.

Table 5 Incidence (number of strains and percentage) of *E. faecalis* genes associated to virulence in relation to agar invasion.

Agar Invasion	Nº strains	*gelE*	*asa1*	*cylA*	*esp*	*efaA*	*ace*
No invasion	17	13 (76.5%)	14 (82.4%)	9 (52.9%)	12 (70.6%)	15 (88.2%)	10 (58.8%)
Weak	21	19 (90.5%)	17 (81.0%)	12 (57.1%)	9(42.9%)	19 (90.5%)	13 (61.9%)
Medium	14	10 (71.4%)	10 (71.4%)	6 (42.9%)	5 (35.7%)	12 (85.7%)	5 (35.7%)
High	27	20 (74.1%)	21 (77.8%)	10 (37.0%)	19 (70.4%)	25 (92.6%)	17 (63.0%)
Total	**79**	**62 (78.5%)**	**62 (78.5%)**	**37 (46.8%)**	**45 (57.0%)**	**71 (89.9%)**	**45 (57.0%)**

(p=0.021) was observed regarding the clinical origin and the agar invasion results (figure 5). 95.5% of isolates from invasive disease (hematologic samples) were positive for this test, higher than isolates from non-invasive diseases (73.1% UTI isolates, p<0.01; 73.3% strains from exudates, p=0.018) or commensal strains (68.7% faeces samples, p=0.047). None of the other genotypic and phenotypic virulence factors analyzed in this work showed statistical relation with agar invasion (table 5).

It is known that bacterial adaptation to laboratory conditions can modify the phenotype of different bacterial species[28]. One of the characteristics which can be lost by standardized growth conditions is the bacterial virulence, which has been largely studied in the development of attenuated vaccine. Thus, the *in vitro* growth in absence of the host selective pressure can modify factors related to "*in vivo* growth". It has been described that the virulence phenotype could be lost by serial passages[29], being enough fifteen passages to observe significant changes in virulence expression patterns[28,30,31].

In order to determine if agar invasion phenotype could act as virulence factor, we generated three derivative strains adapted to laboratory conditions obtained after fifteen serial passages of three parental agar-invasion positive strains.

Table 6 Comparison of results observed with agar invasion test in three *E. faecalis* parental strains and their derivative strains obtained after fifteen serial passages.

Strain		Invasion relative area (%) (mean value ± SD)	P value
EF43	Parental	1.4 ± 0.44	0.039
	Derivative	1.0 ± 0.41	
EF46	Parental	14.6 ± 3.56	0.032
	Derivative	7.4 ± 0.96	
EF75	Parental	15.7 ± 1.57	0.006
	Derivative	8.4 ± 0.26	

Quantifying agar invasion of parental and derivative strains could determine whether the ability to invade the agar represents a selectable marker present in clinical strains that may be lost due to adaptation to laboratory conditions. Results

showed a significant reduction in the invasion area comparing with the respective parental strains (table 6), showing that agar invasion phenotype can be modulate by culture conditions as other virulence factors observed in other bacterial species[29,30].

Although it has been observed the existence of distinctive virulence patterns depending on the clinical origin of *E. faecalis* strains[18], the presence of these determinants in samples from environment or healthy carriers[18,19,32] shows that virulence characterization is insufficient taking into account that other factors, such as host status or competition with other bacterial species, could be essential in the pathology. Thus, further studies are necessary on *E. faecalis* virulent strains in order to cluster phenotypes to define colonization and/or host adaptation index[33] and the relevance of these determinants on the disease development.

Taken together, our data show that agar invasion may provide an ecological marker which could increase the probability of population adaptation in different environments and points out directions for future research although we are only at the beginning of their exploration. Even these results showed the potential relevance of this test in the study of enterococcal virulence, in order to shedding light on agar invasion further studies are necessary to determine the *in vivo* relevance of this finding and the potential correlation between agar invasion and tissue invasion in *E. faecalis*.

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