Microbiological Research 293 (2025) 128057

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Transcriptomic analysis of sub-MIC Eugenol exposition on antibiotic resistance profile in Multidrug Resistant *Enterococcus faecalis* E9.8

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ARTICLE INFO

Keywords:
Enterococcus
Multidrug resistance
Antibiotic resistance
Eugenol
Essential oils, transcriptomic analysis

ABSTRACT

The spread of multidrug-resistant (MDR) bacteria and their resistance genes along the food chain and the environment has become a global threat aggravated by incorrect disinfection strategies. This study analysed the effect of induction by sub-inhibitory concentrations of eugenol – a major ingredient in clove essential oil commonly used in disinfectant agents – on the phenotypic and genotypic response of MDR *Enterococcus faecalis* E9.8 strain, selected based on the phenotypic response of other enterococci. Eugenol treatment irreversibly reduced several antibiotics' minimum inhibitory concentration (MIC), confirmed by kinetic studies for kanamycin, erythromycin, and tetracycline. Furthermore, transcriptomic analysis indicated the reversion of antibiotic resistance through direct and indirect measures, such as down-regulation of genes coding for proteins involved in antibiotic resistance, toxin resistance and virulence factors. Regarding antibiotic resistance genes (ARGs), ten differentially expressed genes (five down-regulated and five up-regulated genes) were related to the main transporter families, which present key targets in antibiotic resistance reversion. Our study thus highlights the importance of considering indirectly related genes as targets for antibiotic resistance reversion besides ARGs *sensu stricto*. These results allow us to propose using eugenol as an antibiotic resistance reversing agent to be included in disinfectant solutions as an excellent alternative to limit the spread of MDR bacteria and their ARGs in the food chain and the environment.

1. Introduction

The spread of multidrug-resistant (MDR) bacteria and associated resistance genes along the environment has become a global threat (Caballero Gómez et al., 2022). Every year, five million people succumb to infections by MDR bacteria due to the ability of many bacterial species to resist antibiotic treatment (Łapińska et al., 2022). Animals are an important reservoir for MDR bacteria, which could spread to the environment, e.g., from the slaughterhouse higher up throughout the food chain (Sobsey et al., 2006). MDR bacteria (such as enterococci) can be transferred during different processing steps in the slaughterhouse and significantly contribute to increasing risk for food safety and consumer health (Zhu et al., 2013; Lavilla Lerma et al., 2013, 2014a, 2014b; Campos Calero et al., 2018, 2020). Enterococci are a dominant bacterial group in the gut microbiota of humans and animals and emerged as important nosocomial pathogens over the last three decades with acquired resistance to many antimicrobials such as antibiotics and biocides (Bourgeois-Nicolaos et al., 2005; Sobhanipoor et al., 2021). To tackle this challenge, it is crucial to understand underlying cellular control mechanisms for antimicrobial adaptation/fitness. The mechanisms of how bacteria gain resistance through disinfectant treatment remain elusive and reports on its characterisation are scarce. Therefore, understanding the factors affecting the emergence of disinfectant resistance could ultimately help us to improve their efficiency (Tong et al., 2021). At the same time, new sustainable strategies need to be developed as alternatives to currently used disinfection methods to counteract bacterial adaptation and antibiotic cross-resistance.

Here, the benefits of essential oil compounds (EOCs) from plant extracts as ingredients of novel disinfectants have been demonstrated (Hu et al., 2018). In particular, eugenol (EOCs derived from clove oil) has

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https://doi.org/10.1016/j.micres.2025.128057
Received 30 September 2024; Received in revised form 25 December 2024; Accepted 9 January 2025
Available online 10 January 2025

shown promising results, including inhibitory effects on the migration, adhesion, virulence factor expression and biofilm formation, the ability to denature proteins, reaction with cell membrane phospholipids and inhibition of Gram-negative and Gram-positive bacteria as well as different types of yeasts (Walsh et al., 2003; Chaieb et al., 2007; Nuñez, D'Aquino, 2012; Martins et al., 2016; Hu et al., 2018, Jeyakumar and Lawrence, 2021; Caballero Gómez et al., 2022). Furthermore, eugenol can increase bacterial sensitivity to disinfection treatment by reducing efflux pump gene expression (Tong et al., 2021). However, further in-depth research is required before these inhibitors can be exploited. Each new disinfection strategy needs to be carefully evaluated since resistance usually emerges when genetic changes provide the microorganism with an advantage against antimicrobials or when the microorganism performs the biochemical reactions necessary for survival at a slower pace (Łapińska et al., 2022). As the resistome is influenced by the influx and efflux of genes and the environmental pressure the bacteria are exposed to, special attention should be paid to the expression of existing genes under different stress conditions to better understand the development of AMR (Alonso García et al., 2023). In this regard, the sensitised strain method can identify specific cellular targets, and transcriptional profiling can provide insights into the inhibited pathways and the physiological responses to antibiotic stress (Nonejuie et al., 2013). Here, we report the impact of the exposure to sublethal eugenol concentrations on MDR *E. faecalis* E9.8 bacteria isolated from goat and lamb slaughterhouses. This strain/EOC was selected based on the promising results of biofilm inhibition and the synergistic effects of different antimicrobials (Lavilla Lerma et al., 2013; Caballero Gómez et al., 2023). We notably investigated whether eugenol induction of *E. faecalis* E9.8 strain may influence its phenotypic and genotypic responses to antibiotics, laying the groundwork for understanding how sublethal exposure to eugenol may affect the fitness of MDR enterococcal strains.

2. Material and methods

2.1. Bacterial strains and growth conditions

Strains used in this study were the MDR *Enterococcus faecalis* E7.10, E9.8 and E30.12 (Table 1) (Lavilla Lerma et al., 2013; Caballero Gómez et al., 2023). Eugenol (EU, Sigma-Aldrich Spain) was used as an EOC to reverse antibiotic resistance in enterococci strains. The selection of the *Enterococcus* strains and the EOC was based on promising results obtained previously regarding biofilm inhibition and the synergistic effects with different antimicrobials (Caballero Gómez et al., 2022).

All strains were routinely cultivated in Tryptone Soy Broth (TSB; Scharlab, Barcelona, Spain) at 37ºC for 24 h and stored in TSB containing 20 % glycerol at −20 or −80°C.

2.2. Strain identification

Total DNA extractions were done using the Zymo BIOMICS DNA Miniprep Kit (Zymo Research, California, United States) according to the manufacturer's instructions. For strain identification, amplification by PCR of the 16S rRNA gene and subsequent sequencing was performed using the primer pair 27f-YM/1492r (Table S1, Supplementary Material) as described by Zicca et al. (2020) and Manetsberger et al. (2023). The partial sequences obtained were assembled with the A plasmid editor (ApE) software (Davis and Jorgensen, 2022) and the homology was searched with the BLASTN algorithm (National Center for Biotechnology Information [NCBI], USA) based on the highest alignment score and the percentage of identity.

Table 1
Enterococcus spp.* strains used in this study.

Enterococcus spp. strains	Source	16S rRNA gene identity (%)
E. faecalis E7.10	Sacrifice room	99
E. faecalis E9.8	Sacrifice room	100
E. faecalis E30.12	White room	99

* Strains were isolated throughout meat chain production in a lamb and goat slaughterhouse of Jaén (Lavilla Lerma et al., 2013).

2.3. Effect of eugenol on the reversion of antibiotic resistance in MDR Enterococcus spp. strains

To determine *in vitro* whether eugenol induced the reversion of antibiotic resistance in the MDR pathogenic *Enterococcus* spp. strains, each bacterium was separately inoculated at 0.5 McFarland turbidity units with sub-inhibitory concentrations of 0.5 "Minimum Inhibitory Concentration" (MIC) of eugenol in TSB medium (total volume of 2 ml). This re-inoculation was repeated six times (Ilin et al., 2017) and the induced strain was stored at −80ºC until use (revival in fresh TSB medium without eugenol).

The broth microdilution method determined the antibiotic MICs in eugenol-induced and non-induced *Enterococcus* spp. strains (Clinical and Laboratory Standards Institute CLSI, 2020). Briefly, overnight bacterial cultures grown at 37ºC for 24 h were diluted 1/10 (V/V) in fresh Mueller Hinton (MH) broth corresponding to an inoculum density of 0.5 McFarland, and 20 µl were added to each well of a 96-well microtiter plate. Then, 180 µl of MH broth supplemented with different concentrations of antibiotics were added prior to incubation for 24 h under aerobic conditions. The following thirteen antibiotics belonging to different classes (all Sigma-Aldrich, Spain) were tested: ampicillin (AMP), amoxicillin (AMX), gentamicin (CN), ciprofloxacin (CIP), tetracycline (TE), rifampicin (RD), erythromycin (ER), nitrofurantoin (F), chloramphenicol (CL), imipenem (IPM), cefuroxime (CX), kanamycin (KN), and streptomycin (SP). After overnight incubation at 37ºC, the MIC was determined, corresponding to the lowest concentration of each antimicrobial agent that inhibited the visible growth of the strain. Each experiment was carried out in triplicate, and results were considered reliable if at least two of the three replicates showed similar results.

2.4. Monitoring of the effect of eugenol induction on antibiotic resistance reversion by kinetic studies in the selected MDR E. faecalis E9.8 strain

E. faecalis E9.8 strain was selected as a representative of MDR *Enterococcus* spp. strains, based on the determined MIC reductions after induction with eugenol. Bacterial growth kinetics were studied in 96 well Microtiter plates at 37ºC ± 0.3ºC for a period of 72 h. The optical density (OD) at 580 nm was determined for each well using a Tecan Infinite M200 multimode microplate reader equipped with monochromator optics (Tecan Group Ltd., Männedorf, Switzerland). Before each measurement, orbital shaking conditions were selected (4 mm amplitude and 15 s shaking cycles), and measurements were taken every 60 min using the multiple-reads-per-well mode (filled-circle alignment, 3 ×3 spots, five reads per well, border 2000 mm). Each experiment was performed in triplicate.

2.5. Molecular response of eugenol-induced E. faecalis E9.8 strain

2.5.1. RNA extraction

RNA was extracted from the non-induced and eugenol-induced *E. faecalis* E9.8 strain using the RNase Micro Kit (Qiagen) according to the manufacturer's instructions and as described by Alonso García et al. (2023). Briefly, RNA was extracted by addition of RNA Protect bacteria reagent (Qiagen), 500 µl of TESL (25 mM Tris, 10 mM EDTA, 20 % sucrose, and 20 mg/ml lysozyme; all from Sigma) and 20 µl mutanolysin (20 U) to cell pellets (from 2 ml overnight culture), followed by incubation at 37ºC for 60 min with gentle shaking. Total RNA was isolated from three biological replicates. DNaseI digestion was performed according to the manufacturer's instructions (Panreac). RNA quantification and quality assessment were done using a NanoDrop 2000

spectrophotometer (Thermo Scientific), and samples were frozen at –80ºC until required.

2.5.2. Transcriptomic analysis of E. faecalis E9.8 strain in response to eugenol induction

The RNA library preparation, sequencing and bioinformatic analysis were performed at Biopolis S.L. (Valencia, Spain) as described by Alonso García et al. (2023). The Pathofact program (de Nies et al., 2021) detected toxins, virulence factors and antibiotic-resistance genes. Gene expression in each sample was quantified with the Salmon v.1.5.1 program (Patro et al., 2017). These genes were annotated with the eggNOG-mapper tool (Huerta-Cepas et al., 2017). Once the gene expression matrix was obtained, a gene filter was applied, eliminating those genes with no or only one count in all samples (reducing potential noise). A Principal Component Analysis (PCA) was applied to this filtered matrix to visualise group separation. The matrix for the PCA was normalised using the 'vst' normalisation. Finally, differential expression analysis was calculated with the DESeq2 R package (Love et al., 2014). All graphics were generated with ggplot (Wickham, 2016), ggpubr (Kassambara, 2023) and ComplexHeatmap (Gu, 2022) R packages, except for the circus plot, which was generated using Circos software (Krzywinski et al., 2009).

The raw data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB76247.

2.6. Validation of differentially expressed genes (DEGs) by qRT-PCR

RT-PCR experiments were performed to validate the differentially expressed genes (DEGs) obtained by transcriptomic analysis using total RNA extracted previously (Section 2.4.) and the primers described in Table S1 (Supplementary Material). Quantitative real-time PCR (qRT-PCR) of the selected genes was done as described by Alonso García et al. (2023) using the following conditions: initial denaturation at 95ºC for 2 min, followed by 40 cycles of denaturation at 95ºC for 30 s, annealing at variable temperature for 30 s (Table S1, Supplementary Material) and extension at 72ºC for 30 s; followed by final extension for 2 min. Fluorescence signals were collected during annealing and extension, while melting-curve analysis included 95ºC for 10 s, 65ºC for 5 s, and 95ºC for 50 s.

2.7. Statistical analysis

Statistical analyses were conducted using Excel 2016 (Microsoft Corporation, Redmond, WA, United States) to determine averages and standard deviations in growth kinetic studies. All analyses were performed in triplicate.

3. Results

3.1. Identification of MDR enterococci

16S rRNA sequencing and BLAST homology search identified the *Enterococcus* spp. strains previously isolated from a goat and lamb slaughterhouse surface in Jaén (Spain) (Lavilla Lerma et al., 2013; Caballero Gómez et al., 2023) as *E. faecalis* (Table 1).

3.2. Phenotypic response of Enterococcus spp. strains to eugenol induction

3.2.1. Effect of eugenol on antibiotic resistance reversion in MDR Enterococcus spp. strains

The phenotypic response of *Enterococcus* spp. strains to eugenol induction was heterogeneous depending on the analysed strain and respective antibiotic (Table 2). Overall, MICs of amoxicillin, ciprofloxacin, cefuroxime, imipenem, nitrofurantoin and rifampicin exhibited no changes regardless of eugenol treatment, whereas a 62,5-fold increase was observed in the case of ampicillin after eugenol induction of *E. faecalis* E9.8. On the other hand, MICs values of tetracycline, gentamycin, streptomycin and kanamycin (two of three strains), and chloramphenicol and erythromycin (one of three strains) decreased in the range of 4-> 400 fold in the eugenol-induced *E. faecalis* strains, reverting the bacteria's original resistance and making strains sensitive to these antibiotics in the majority of cases (Table 2). Based on these results the MDR *E. faecalis* E9.8 strain was selected for further studies to represent MDR enterococci present in the sacrifice room of slaughterhouses.

3.2.2. Growth kinetic and antibiotic resistance reversion in MDR E. faecalis E9.8 strain

Different effective exposure times and concentration-dependent effects on the growth dynamics of the *E. faecalis* E9.8 strain became evident when analysing growth kinetics and the effect of eugenol induction on antibiotic MIC reduction over 72 h. MIC reductions were observed for kanamycin, erythromycin and tetracycline (Fig. 1)

3.3. Molecular response of E. faecalis E9.8 strain to eugenol induction

3.3.1. Global transcriptomic analysis of the eugenol-induced E. faecalis E9.8 strain

The assembly of the RNA sequences, mapping and expression quantification yielded 3111 genes (Table S2, Supplementary Material). A filtering of genes was performed, eliminating those with no counts or those with only one count in all samples (thus reducing possible noise). A Principal Component Analysis (PCA) was applied to the filtered matrix containing 3020 genes as an exploratory analysis. PCA was applied to all six samples (eugenol-induced and non-induced samples) (Fig. 2), and the matrix was normalised using the 'vst' normalisation, recommended in the DESeq2 package (Love et al., 2014). Principal component analysis

Table 2
The effect of eugenol induction on the antibiotic susceptibility of *Enterococcus faecalis* strains isolated from a local goat and lamb slaughterhouse in Jaen (Spain).

Enterococcus spp. strains	MIC of antimicrobials* (µg/ml)												
	AMP	AMX	CIP	CL	CN	CX	ER	F	IMP	KN	RD	SP	TE
***E. faecalis* E9.8**													
Wild strain	40	5	3.125	866	1500 **(R)**	> 1050	3200 **(R)**	92.5	25	1112 **(R)**	400	2900 **(R)**	237.5 **(R)**
Eugenol-induced strain	> 2500	5	3.125	866	< 375 **(S)**	> 1050	< 8 **(S)**	92.5	25	85 **(S)**	400	< 125 **(S)**	< 3.7 **(S)**
***E. faecalis* E7.10**													
Wild strain	2500	5	6.25	100	1500 **(R)**	> 1050	3200	9	0.5	> 4450 **(R)**	< 100	2900 **(R)**	237 **(R)**
Eugenol-induced strain	2500	5	6.25	100	< 375 **(S)**	> 1050	< 400	9	0.5	< 278 **(S)**	< 100	< 363 **(S)**	< 3.7 **(S)**
***E. faecalis* E30.12**													
Wild strain	300	5	6.25	500	> 1500	4000	3200	200	5	4450	50	> 3000	237
Eugenol-induced strain	300	5	6.25	100	> 1500	4000	3200	200	5	4450	50	> 3000	237

R, resistant; S, sensitive according to CLSI and EUCAST cut-offs.

* : AMX, amoxicillin; AMP, ampicillin; CIP, ciprofloxacin; CL, chloramphenicol, CN, gentamicin; CX, cefuroxime; ER, erythromycin; F, nitrofurantoin; IPM, imipenem; KN, kanamycin; RD, rifampicin; SP, streptomycin; TE, tetracycline.

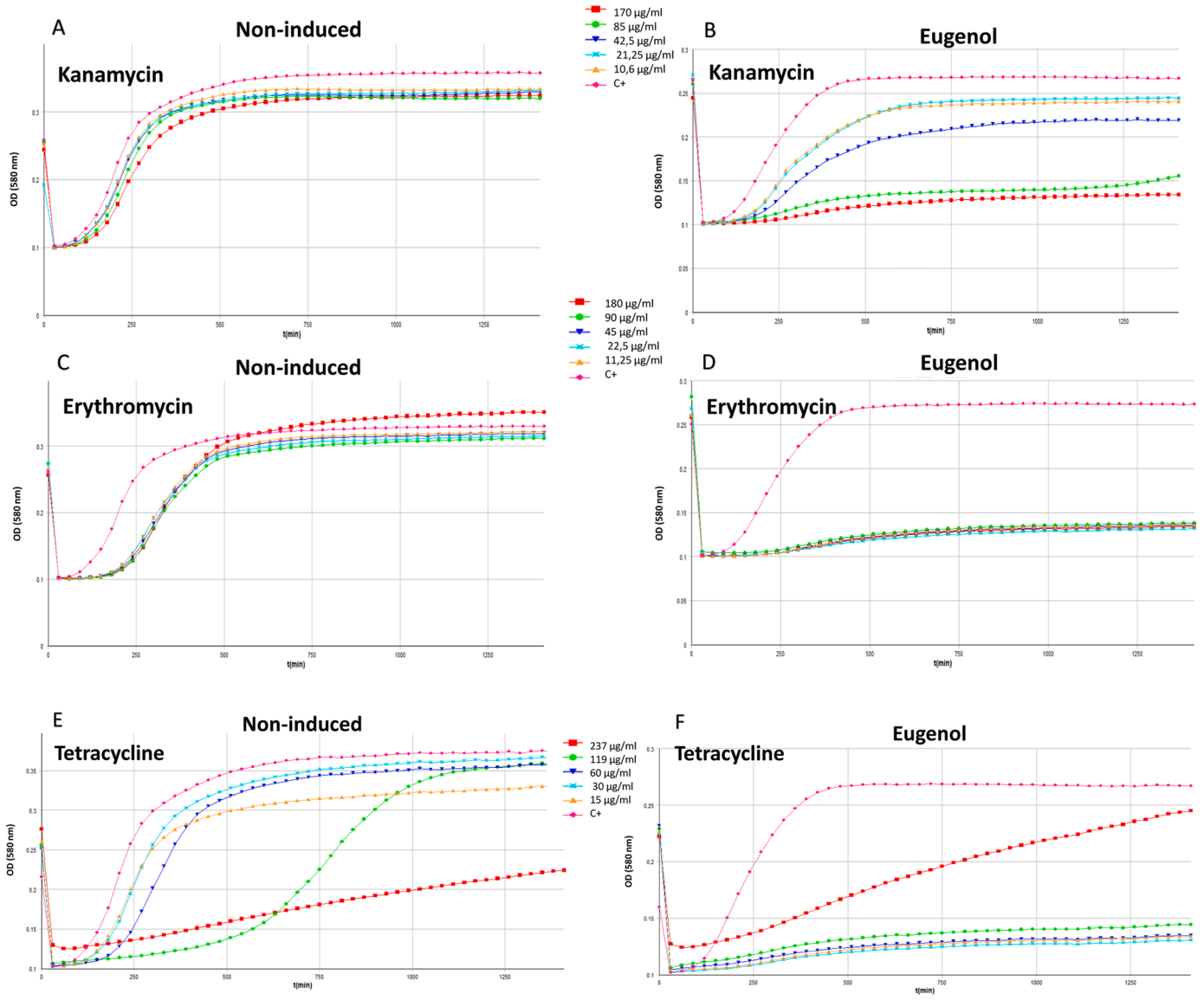

Fig. 1. Effect of Antibiotics Kanamycin (A-B), Erythromycin (C-D) and Tetracycline (E-F) at different concentrations on the growth dynamics of non-induced (A-C-E) and eugenol-induced (B-D-F) *E. faecalis* E9.8 strain. Each data point (time resolution 72 h: 1 h) represents mean values of triplicate cultivations, normalised with data from identical incubations without bacterial cells (negative controls).

yielded two components: the first principal component (PC1) explained 96 % of the variability between samples, and the second principal component (PC2) explained 3 %. Two separate experimental groups (non-induced "C" and eugenol-induced "Eu") could be identified in a clear-cut way (Fig. 2).

GO terms analysis was used to decipher the biological processes involved in the *E. faecalis* E9.8 strain induction with eugenol (Eu). Overall, we found 878 genes were significantly up-regulated and 806 genes significantly down-regulated (adjusted $p < 0{,}05$) when comparing the Eu condition versus the control (C) (Table S2, Supplementary Material). Eugenol induction notably up-regulated genes whose putative functions were classified as a response to stimulus and macromolecule metabolic process (Fig. 3). On the other hand, we generally observed that down-regulated genes were involved in cellular functions. In particular, down-regulation was apparent for genes related to metabolic processes of small molecules, oxoacid, organic acid and carboxylic acid, organic substances, organic cyclic compounds, cellular and overall biosynthesis processes (Fig. 3).

3.3.2. *Identification of DEGs involved in toxin, virulence factors and antibiotic resistance in the eugenol-induced E. faecalis E9.8 strain*

Concerning toxins, the eugenol-induced *E. faecalis* E9.8 strain showed differential expression of 29 genes ($p < 0{,}05$) versus the control, with 19 of them differentially up-regulated and 10 genes differentially down-regulated (Fig. 4; Table S3, Supplementary Material). All down-regulated genes encoded non-secreted toxins except the *hlyx* gene coding for the hemolysin transporter associated domain responsible for HlyX hemolysin secretion. Other down-regulated genes were related to hemolysins (*hlyIII*); pathogenicity (adherence to the host, lysis/dissemination of microorganisms); metabolism (*cls*, *galE* and *ytmP* genes) or processing/transport (*ydgI* gene) (Fig. 4; Table S3, Supplementary Material). On the other hand, 19 up-regulated genes (encoding non-secreted toxins) included five genes coding for glyoxalase system (*XK27_00215*, *ykcA*, *mhqA_2, gloA* and *catE* genes), 7 genes related to DNA or RNA regulation (*lexA* gene, k127_481_2, k127_324_4, k127_498_3, k127_301_2, k127_373_9, k127_450_10), and three genes involved in nitrogen metabolism (*ylbA* and *XK27_02070* genes, and k127_478_2) (Fig. 4; Table S3, Supplementary Material).

Regarding virulence factors, 311 genes in the eugenol-induced

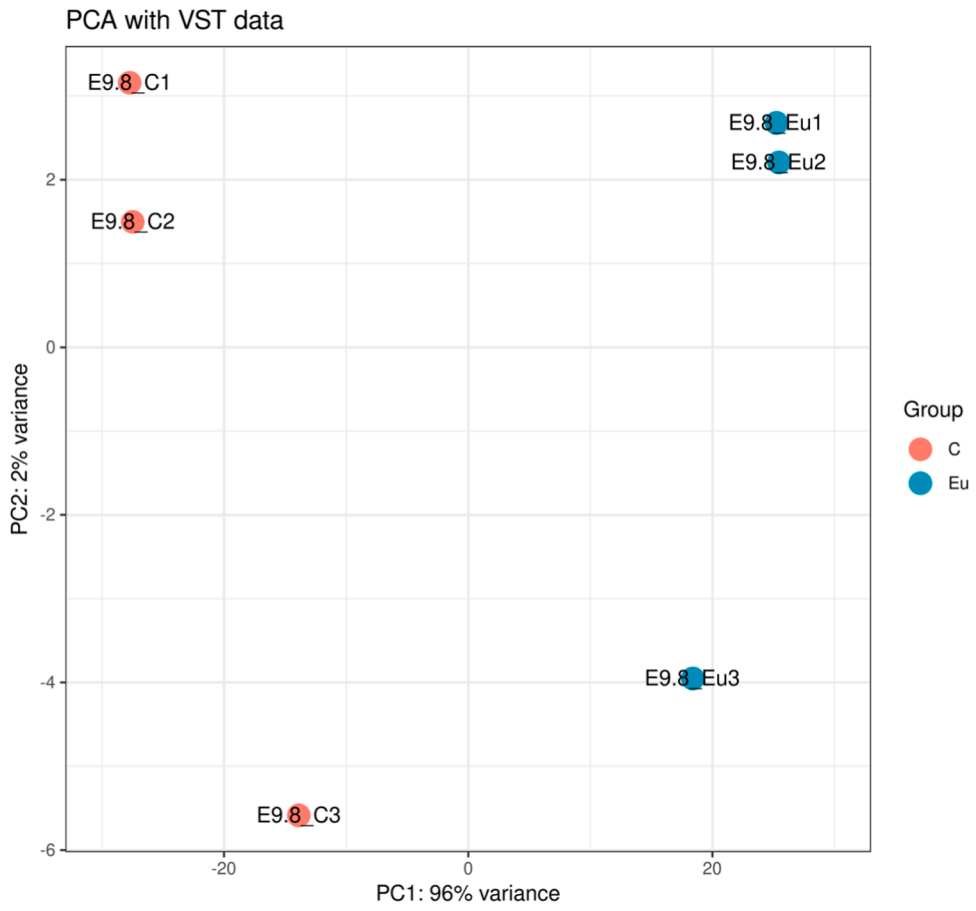

Fig. 2. Principal component analysis (PCA) plot; red plots represent control strain (*Enterococcus faecalis* E9.8), and blue plots corresponded to eugenol induced strain.

E. faecalis E9.8 strain were differentially expressed ($p < 0,05$), with 143 of these genes up-regulated (33 of them were secreted virulence factors and 110 were non-secreted virulence factors) and 168 genes down-regulated (51 were secreted virulence factors and 117 were non-secreted virulence factors) (Figure S1; Table S3, Supplementary Material). Concerning down-regulated genes, *potD3* gene coding for bacterial extracellular solute-binding protein stood out (-3.88 Log_2FC in Eu versus C), followed by *oadG* gene (coding for biotin-requiring enzyme), *rcfB* gene (coding for bacterial regulatory proteins, crp family), and other coding genes for different functions (DnaD domain protein, glycosyl transferase family group 2, cell septum assembly, male sterility protein, group 2 family protein, cell envelope-related transcriptional attenuator domain, O-Antigen ligase, LysR substrate-binding domain, prophage endopeptidase tail) which were down-regulated in the range between −2 and −2,7 Log_2FC in Eu versus C (Figure S1; Table S3, Supplementary Material). On the other hand, the most prevalent genes which were differentially up-regulated ($p < 0,05$), in the range between 5,9 and 5 Log_2FC in Eu versus C, were mainly genes coding for IstB-like ATP binding protein; prophage endopeptidase tail; peptidoglycan catabolic process; cell adhesion, Maj_tail_phi13 phage major tail, phi13 family protein; Helix-turn-helix XRE-family like proteins (Figure S1; Table S3, Supplementary Material).

Modification in antimicrobial resistance of *E. faecalis* E9.8 strain after eugenol induction was supported by the up-regulation and down-regulation of ten DEGs (Fig. 5). Five genes were up-regulated coding for transmembrane ABC transporter (k127_519_2) involved in resistance to macrolides, lincosamides, streptogramines (MLS); multidrug ABC transporter (k127_18_2, *bmr3_1* gene) implicated in sugar transport (amongst others) and also in resistance to fluoroquinolones; Major Facilitator Superfamily (MFS-1 family) with resistance to fosfomycin (k127_569_1, *pmrA* gene); multidrug ABC transporter (k127_82_7, *yjcA* gene) and MFS multidrug associated with resistance to acridine dye (k127_355_9, *blt* gene) (Fig. 5A; Table S3, Supplementary Material). However, the five down-regulated genes were k127_90_2 (*msbA_1* gene) and k127_644_8 (*ydiC1* gene) coding for ABC and sugar transporters, respectively, being involved in multidrug resistance. Moreover, k127_514_2 (*vanR* gene) involved in glycopeptide resistance, k127_333_2 coding for MFS with resistance to fluoroquinolones and k127_604_5 (*csrR* gene) coding for transcriptional regulatory protein, C terminal were identified (Fig. 5A; Table S3, Supplementary Material).

Circos' plot analysis also showed that MDR was the most prevalent resistance mechanism under both conditions (control and eugenol-induced strain) (Fig. 5B). MDR was highly expressed in eugenol-induced *E. faecalis* E9.8 strain (87 % for Eu versus 81 % for C). Other genes that less expressed but up-regulated in eugenol-induced *E. faecalis* E9.8 strain were identified as fosfomycin (3,6 % for Eu versus 2.5 % for C) and MLS (1 % for Eu versus 0,8 % for C). On the other hand, genes involved in resistance to other antibiotics were under-expressed in eugenol-induced *E. faecalis* E9.8 strain versus control, such as glycopeptide (2,8 % in eugenol-induced strain versus 7,4 % in control) and fluoroquinolone (2,7 % in eugenol-induced strain versus 4,5 % in control). ARGs coding for resistance to acridine dye, diaminopyrimidine, beta-lactam and tetracycline showed slight or no differences in their expression after eugenol induction (Fig. 5B).

3.3.3. *Validation of differential expression by quantitative real-time PCR*

The results obtained by transcriptomic analysis were validated by qRT-PCR of four selected genes (two up-regulated and two down-regulated genes). The up-regulated genes *k127_82_7* (coding for multidrug ABC transporter) and *k127_355_9* (coding for MFS multidrug

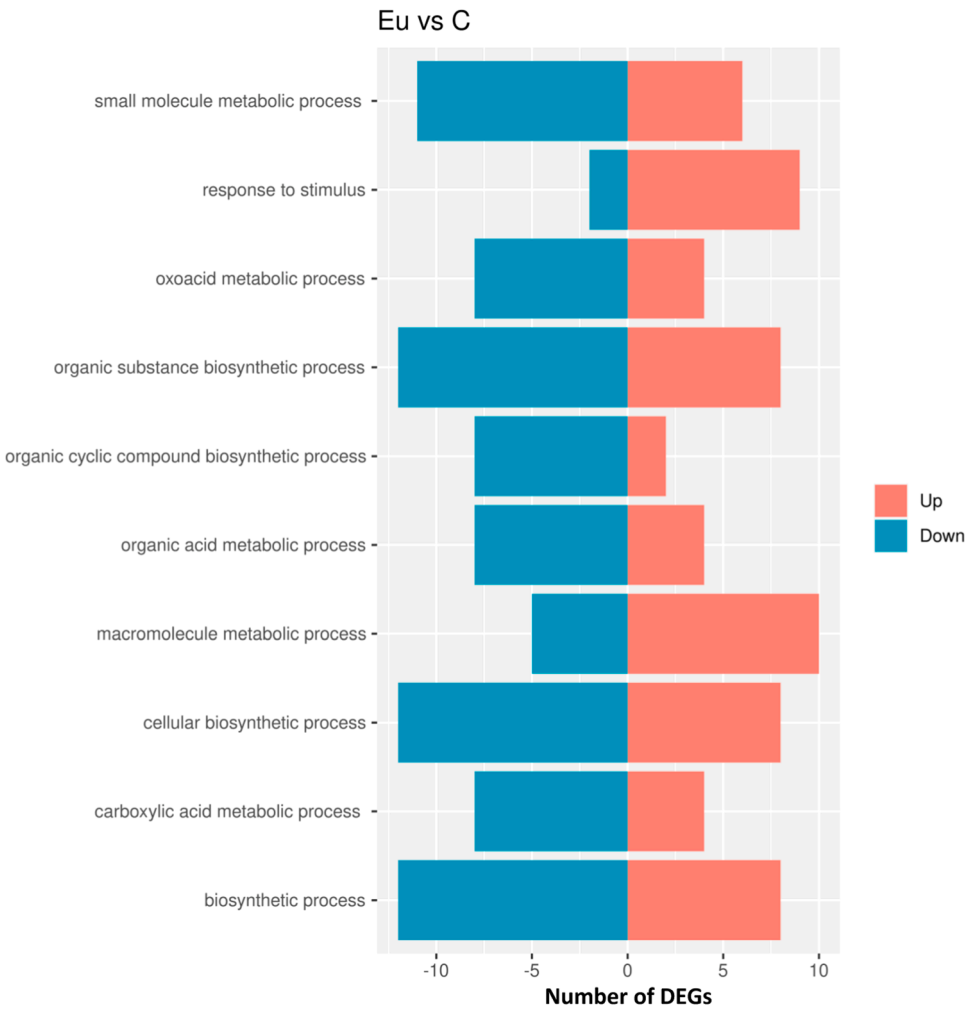

Fig. 3. GO classification of significantly differentially expressed genes (DEGs) in eugenol-induced *E. faecalis* E9.8 strain. Significantly up-regulated (brick bars) and down-regulated (turquoise bars) DEGs (log_2 fold change >1.5) between control (C) and eugenol-induced (Eu) *E. faecalis* E9.8 strain.

associated with resistance to acridine) detected in the transcriptome of eugenol-induced *E. faecalis* E9.8 strain also showed over-expression in qRT-PCR analysis under the same conditions of induction (Table S4, Supplementary Material). Furthermore, the down-regulated genes *k127_106_15* (pathogenicity) and *k127_380_1* (metabolism) showed a reduction in both RNA-Seq and qRT-PCR studies (Table S4, Supplementary Material). Thus, qRT-PCR validated the differential expression obtained with RNA-Seq, confirming that the transcriptomics results were representative.

4. Discussion

To cope with antibiotic-resistant bacteria (ARB) and their antibiotic-resistant genes (ARGs) in different environments, several novel strategies have been explored to eradicate these bacteria more efficiently both in planktonic and sessile (biofilm) states (Caballero Gómez et al., 2022). However, only a few strategies have proven successful, especially considering that the novel antimicrobial agent should be both sustainable and environmentally friendly. In this sense, the use of EOCs as antimicrobial agents has been considered an excellent antimicrobial alternative due to their ability to decrease or inhibit pathogen growth and biofilm formation (Sharma et al., 2016; El-Tarabily et al., 2021; Millezi et al., 2016; Reda et al., 2020; Caballero Gómez et al., 2022). Therefore, the application of clove oil and its principal component eugenol ($C_{10}H_{12}O_2$, CAS No.: 97–53–0), in the food industry as an antiseptic has received much attention recently (Hu et al., 2016; Patel, 2015; Jessica Elizabeth et al., 2017). While numerous studies demonstrate the antimicrobial efficacy of eugenol (see above), the consequences of prolonged exposure to sub-inhibitory concentrations need to be explored further. Despite EOCs' promising effects, it is essential to take into account that some oils (e.g., manuka, marjoram and oregano) at sub-inhibitory concentrations can also induce antibiotic resistance (Turchi et al., 2019). The diluted and remaining disinfectants in the environment could thus increase bacterial tolerance through phenotypic adaptation, gene mutation, and horizontal gene transfer (HGT) (Cloete, 2003).

Our study, therefore, aims to address the implications of exposure to sublethal concentrations of eugenol in the MDR *E. faecalis* strains, using the adaptation protocol described by Il et al. (2016) with the objective to simulate conditions of repeated exposure to the disinfectant that closely resemble real-life conditions, for example in a clinical or food manufacturing facility. We first analysed the effect of eugenol induction on the strain's phenotypic responses. *E. faecalis* E7.10 and E9.8 showed mostly the same response to MICs reductions, except to ampicillin. However, for strain E30.12, this behaviour was not observed, displaying differences at the strain level. It is noteworthy that strain E30.12 has been isolated from a slaughterhouse white room (which receives comparatively a different disinfection treatment than other areas). It is well known that selective pressure increases in environments continuously facing disinfection treatments (such as slaughterhouse surfaces), resulting in changes in antimicrobial resistance (Nordholt et al., 2021). In this context Harrison et al. (2020) evidenced significantly ascended

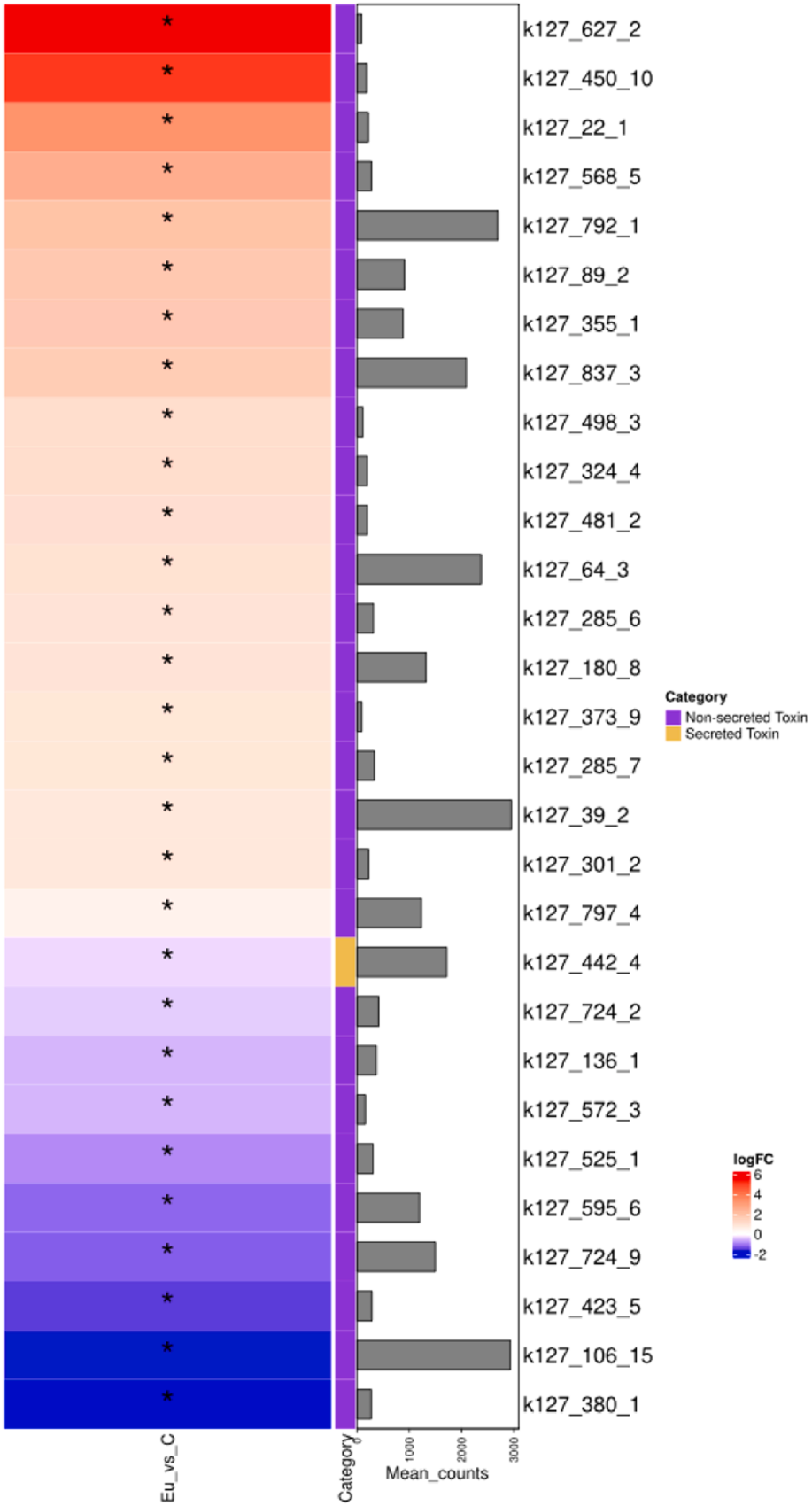

Fig. 4. Heatmap of the 'log$_2$ fold change' (Log$_2$FC) of the expression of genes coding for toxins in eugenol-induced *E. faecalis* E9.8 strain. Pathofact shows significant differences between eugenol-induced (Eu) and control (C) groups. The colour category indicates whether the toxins are secreted (yellow) or not secreted (violet). * denotes significant differences between control (C) and eugenol-induced (Eu) strain ($p < 0.05$).

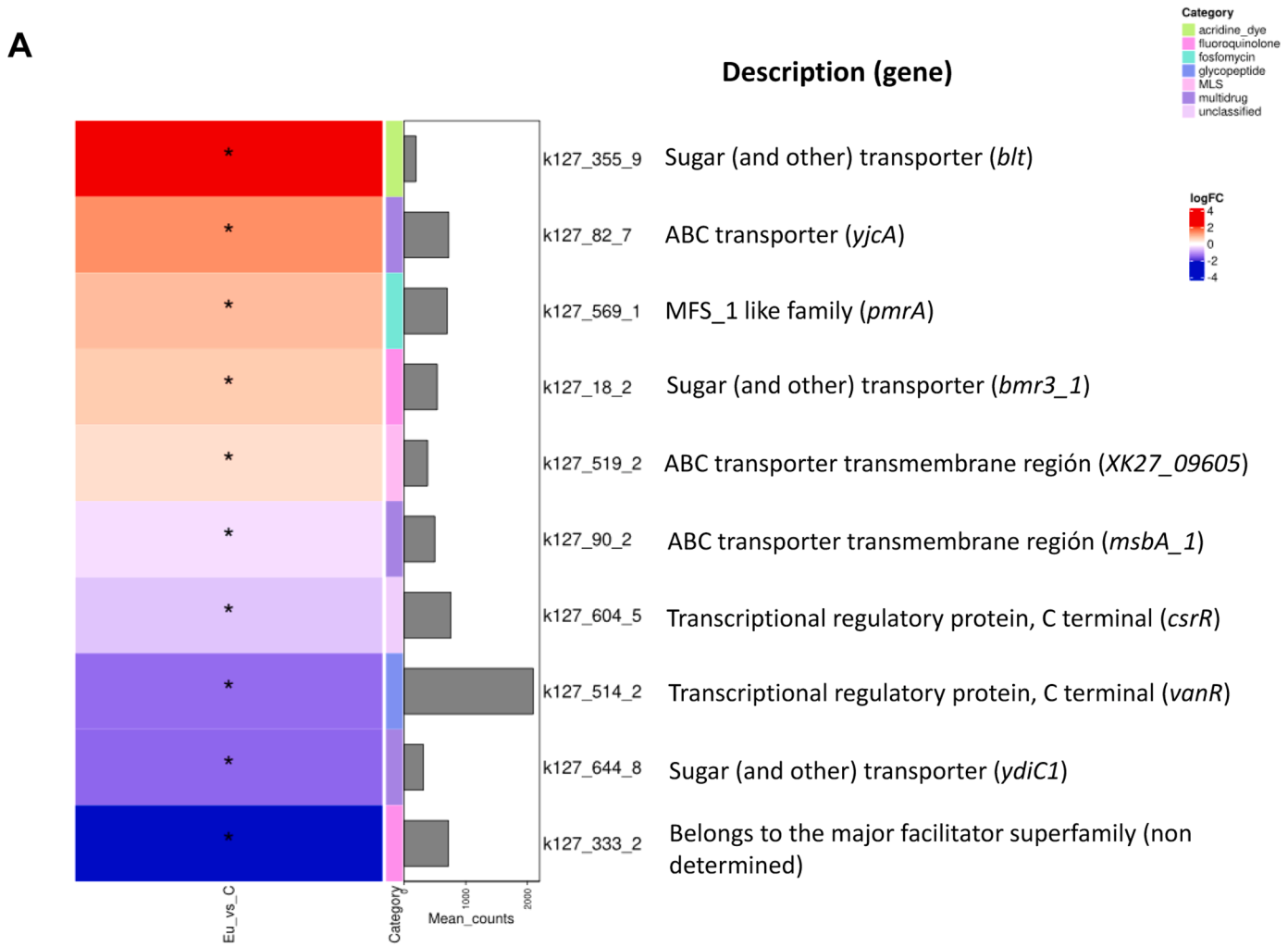

Fig. 5. Differentially expressed genes (DEGs) were expressed in eugenol-induced *E. faecalis* E9.8 strain. **A,** Heatmap of the '$\log_2$ fold change' (Log_2FC) of the expression of genes coding for antibiotic resistance genes (ARGs). Pathofact analysis shows significant differences between eugenol-induced (Eu) and control (C) groups. The colour category indicates resistance types: 'acridine_dye' (green), 'fluoroquinolone' (fuchsia), 'fosfomycin' (turquoise), 'glycopeptide' (blue), 'MLS' (pink), 'multidrug' (violet) and 'unclassified' (lilac). * denotes significant differences between control (C) and eugenol-induced (Eu) strain ($p < 0.05$). **B,** Circos plot showing the abundance of differentially expressed genes (DEGs) related to antibiotic resistance in eugenol-induced *E. faecalis* E9.8 strain (Eu) versus control (C). The outer circle represents the relative values (in percentage), and the inner circle represents the absolute values from the count table. A specific ribbon color represents each ARG, and the width of each ribbon demonstrates the abundance of each differentially expressed ARG. MLS: Macrolides, Lincosamides, Streptogramins. The annexed table showed DEGs counts of different antimicrobial resistance (AMR) categories.

sul1 and *blaTEM* gene abundance in microbial community by 5,4 and 19, 2 times under QAC exposure (Harrison et al., 2020). This environmental pressure differences between *E. faecalis* E30.12 and the others two strains could explain the different behaviour to eugenol exposures. However, further studies on the environmental influence and different behaviours towards sub-lethal eugenol exposures are needed.

The present study showed that eugenol induction of *E. faecalis* E9.8 strain caused a 62,5-fold increase in ampicillin MIC. This however was the only case of increased MIC for all tested strains. These results are in line with previous studies demonstrating that physicochemical stresses, including antimicrobials, chemicals and UV light, can change susceptibility patterns to antibiotics for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, while decreasing the MIC for clindamycin, erythromycin, streptomycin and trimethoprim in most *L. pentosus* strains isolated from Aloreña table olives (Casado Muñoz et al., 2016; Alonso García et al., 2023). Nonetheless, in most cases, eugenol induction caused a reversion of antibiotic resistance (tetracycline, gentamicin, erythromycin, kanamycin and streptomycin). These changes of behaviour towards different antibiotics highlights the importance of investigating the mechanisms involved in each of the processes observed in future studies. Based on these results, *E. faecalis* E9.8 strain was chosen for further analysis.

Comparative transcriptomic analysis of *E. faecalis* E9.8 strain was conducted under eugenol induction and non-induction conditions, considering the far-reaching implications of the molecular mechanisms behind the changes in antibiotic resistance. Results revealed that eugenol induced significant changes in *E. faecalis* E9.8 strain. We generally observed many down-regulated genes involved in cellular functions comparing eugenol-induced versus non-induced *E. faecalis* E9.8 strain. These results suggest that the continued presence of sub-lethal doses of eugenol may cause a large-scale energy-sparing state by reducing most cellular functions. Among the down regulated genes, we found a $-1{,}9$ $\log_2$ fold change of *rpoE* gene expression in *E. faecalis* E9.8 (eugenol-induced). Interestingly, Xie et al. (2016) found that the *rpoE* mutant of the human pathogen *Salmonella enteric* serovar Typhi (*S.* Typhi) had an increased resistance to various antimicrobial agents, especially ampicillin when compared to the wild-type. The study hence suggested a role for *rpoE* as a potential antimicrobial regulator in *S.*

B

Sample	AMR_category	Counts	%
C	acridine_dye	73	0,17
C	beta-lactam	6	0,01
C	diaminopyrimidine	540	1,25
C	fluoroquinolone	1936	4,50
C	fosfomycin	1060	2,46
C	glycopeptide	3191	7,42
C	MLS	327	0,76
C	multidrug	34945	81,22
C	tetracycline	10	0,02
C	unclassified	938	2,18
Total C		**43026**	**100**
Eu	acridine_dye	312	0,73
Eu	beta-lactam	2	0,00
Eu	diaminopyrimidine	476	1,12
Eu	fluoroquinolone	1164	2,73
Eu	fosfomycin	1542	3,62
Eu	glycopeptide	1186	2,78
Eu	MLS	433	1,01
Eu	multidrug	36939	86,62
Eu	tetracycline	11	0,03
Eu	unclassified	580	1,36
Total Eu		**42643**	**100**

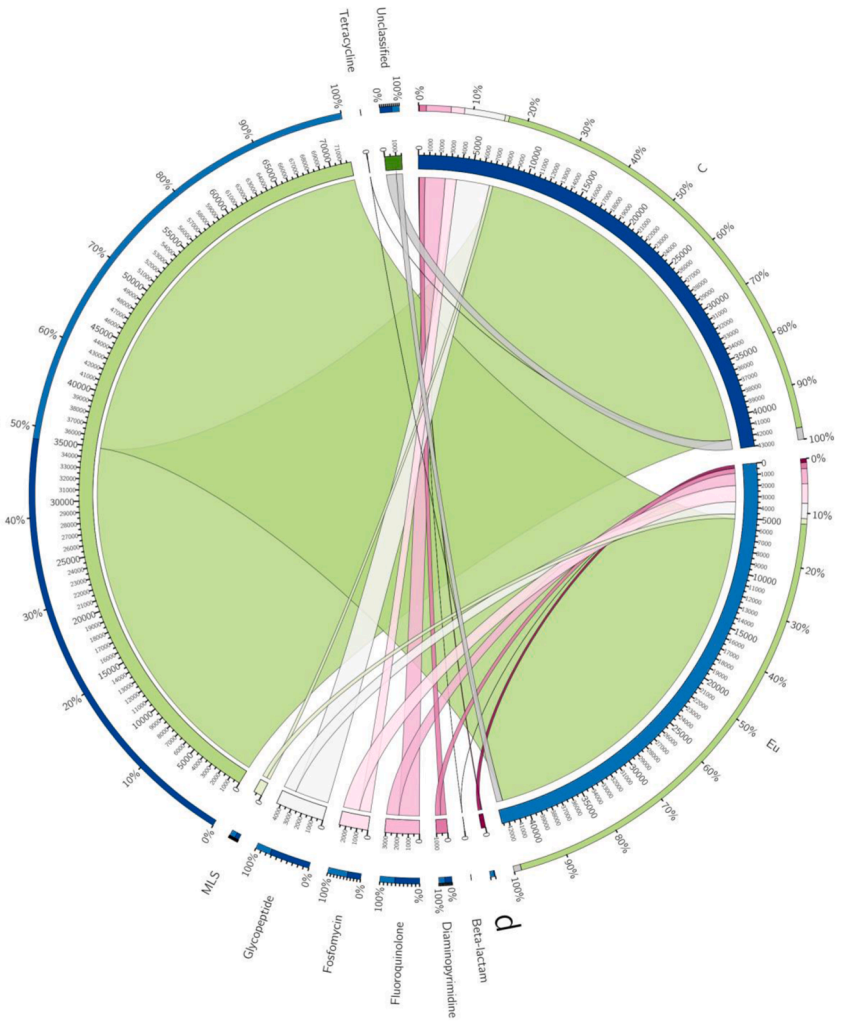

Fig. 5. (*continued*).

Typhi, controlling both the down-regulation of the OMP genes and up-regulating the efflux system. Our results are aligned with these findings, confirming that this mechanism linked to ampicillin resistance is not extensive to antibiotics for which we observed a reduction of MIC after eugenol induction.

Intending further to investigate the response behind the reversion of antibiotic resistance, we identified DEGs coding for pathogenic factors -related to antibiotic resistance or associated with the same mobile genetic elements- such as toxins and virulence factors, besides antibiotic resistance *sensu stricto*. Concerning toxins, among the down-regulated genes, all of them encoded non-secreted toxins except the *hlyx* gene coding for the hemolysin transporter-associated domain responsible for HlyX hemolysin secretion. Other down-regulated hemolysin genes were *hlyIII, cls*, *gal*E, *ytm*P and *ydg*I related to toxins, pathogenicity (adherence to the host, lysis/dissemination of microorganisms) or processing/transport. Confirming our study, the escalation of drug resistance is often linked to overexpressed virulence factors, some of which are implicated in biofilm formation in *Enterococcus* infections (Hyderi et al., 2023). In addition, El-Far et al. (2021) reported that eugenol inhibited biofilm formation and established biofilms of methicillin-resistant *S. aureus* clinical isolates. These results are thus aligned with the data presented in our study. Interestingly, subinhibitory concentrations of antimicrobial agents like antibiotics often increase biofilm formation (Hoffman et al., 2005), however, exposure to sublethal concentrations of eugenol caused the opposite behaviour. Nostro et al. (2007) showed that the exposure of *S. aureus* to non-biocidal concentrations of carvacrol compromised the initial attachment phase to polystyrene microtiter plates. These findings suggest that antibiotic resistance reversion may be linked to the down-regulation of toxins in biofilm formation since this feature was reduced in eugenol-induced *E. faecalis* E9.8. On the other hand, 19 up-regulated genes included five genes coding for the glyoxalase system, which performs an essential metabolic function in cells by detoxifying methylglyoxal (MG) and other endogenous harmful metabolites into non-toxic D-lactate (He et al., 2020) highlighting the induction of protective mechanisms against oxidative stress.

Regarding virulence factors, the transcriptional analysis showed that most differentially expressed genes were related to non-secreted virulence factors. Concerning down-regulated genes, the *PotD3* gene (coding for bacterial extracellular solute-binding protein stood out), followed by proteins related to envelope structure and functions, *rlrG* gene coding for LysR substrate binding domain; *ywtF_2* gene coding for cell envelope-related transcriptional attenuator domain; O-Antigen ligase and cell septum assembly. Thus, these genes are related to bacterial virulence and may play an important role in antibiotic resistance. Nevertheless, we highlight the genes coding for IstB-like ATP binding protein and peptidoglycan catabolic process among the up-regulated genes. In this context, He et al. (2020) suggested that the binding of antibacterial agents was reduced physically by altering the target through mutations or enzymatic modification at or near the binding site.

Overall, the down regulated genes coding for toxins and virulence factors in eugenol-induced *E. faecalis* strains can reduce the ability of the bacteria to cause infections, since often infection is mediated by toxin

production and virulence factors. By down-regulating toxin genes, bacteria become less toxic and less likely to cause severe illness by damaging host cells and tissues. Furthermore, the down-regulation of the coding genes for virulence factors (such as adhesins, enzymes, etc.) weakens the bacteria's ability to colonize, spread, and resist the host's defenses. Thus, the overall impact of the down-regulation of toxins and virulence factor genes is the reduction of bacterial pathogenesis as well as infection control. Furthermore, even if infection occurs, its severity is reduced and the immune system response is enhanced. In addition to the reduction of expression of virulence genes and pathogenesis of Eugenol-induced *E. faecalis*, the down-regulation of ARGs coding for efflux pumps may decrease the susceptibility to different antibiotics leading to the accumulation of these agents in the cells.

Antibiotic resistance has been extensively studied to explore the changes in antimicrobial targets (Munir et al., 2020), which could explain the down-regulation of the *PotD3* gene. On the other hand, Machado et al. (2013) indicated that the adaptive resistance of *P. aeruginosa* to benzalkonium chloride mainly occurs at the bacterial outer membrane, attributed to the decreased expression of porins (OprF and OprG) and lipoproteins (OprL and OprI) involved in the maintenance of cell shape and membrane fluidity. Hence, the down-regulation of genes related to quorum sensing proteins such as LysR substrate binding domain and O-Antigen ligase, as well as those related to the cell wall structure like cell envelope-related transcriptional attenuator domain and cell septum assembly and the up-regulated genes related with peptidoglycan catabolic process are directly related to the susceptibility to antibiotics and thus resistance reversion. In this context, we observed a clear overproduction (5,7 fold-$\log_2$ change) of k127_841, a membrane-bound lytic murein transglycosylase A (MltA). MltA belongs to the lytic transglycosylase (LT) enzyme family, a group of peptidoglycan-remodelling enzymes that are involved in the cleavage of glycosidic bonds of peptidoglycan in order to promote appropriate cell division and create spaces within the peptidoglycan to facilitate the assembly of membrane bacterial molecular machines, including flagella, pili and secretion systems (Scheurwater et al., 2008; Lee et al., 2013).

Finally, analysing the expression of genes related to antibiotic resistance, we found ten differentially expressed genes (ARGs), five of them down-regulated and five up-regulated. Both cases involved genes related to the main transporter families, providing evidence that the balance between the expression of these transporter family genes could be critical in antibiotic resistance reversion. In this regard, chemicals such as eugenol can increase bacterial sensitivity to antimicrobial compounds by reducing the expression of efflux pumps, providing essential insights into the mechanisms of anti-disinfectant resistance (Tong et al., 2021) and developing novel disinfection strategies.

5. Conclusions

The results obtained in the present study showed that sub-MIC exposure to eugenol induced changes in antibiotic MICs patterns. Interestingly, our results suggest that these changes may be influenced by the strain and the isolation source. Bacterial response to eugenol induction highlights the complex interplay between environmental factors and bacterial physiology. However, further studies on environmental effects and different behaviours towards sub-lethal eugenol exposures are needed. On the other hand, *E. faecalis* E9.8 ampicillin MIC increase could be due to *rpo*E gene under-expression in accordance with previous studies, although these results do not extend to other tested antibiotics.

We conclude that further research is needed to investigate the reversion of antibiotic resistance in MDR bacteria, while special attention should be paid also to other genes, such as those coding for toxins and virulence factors (notably those involved in biofilm formation). These could yield critical novel targets for antibiotic resistance reversion besides ARGs *sensu stricto*.

CRediT authorship contribution statement

Julia Manetsberger: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Sonia Castillo-Gutiérrez:** Validation, Data curation. **Charles W. Knapp:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Natacha Caballero Gómez:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Nabil Benomar:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Data curation, Conceptualization. **Hikmate Abriouel:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hikmate Abriouel reports financial support was provided by Government of Andalusia.

Acknowledgements

This work was funded by a Research Project Grant awarded by FEDER (project P20_00983, Modalidad Frontera, Convocatoria PAIDI 2020). Natacha Caballero Gómez is the recipient of a Grant for temporarily incorporating postdoctoral research staff (Acción 7, Universidad de Jaén). Julia Manetsberger is the recipient of a Marie Sklodowska-Curie Individual Postdoctoral Fellowship (grant agreement No 101029930).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2025.128057.

Data availability

Data will be made available on request.

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