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Genomic insights into antibiotic-resistance and virulence genes of *Enterococcus faecium* strains from the gut of *Apis mellifera*

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Abstract

Enterococcus faecium is a lactic acid bacterium that confers beneficial health effects in humans. However, lately, a number of *E. faecium* strains have been linked to the spread of nosocomial infections in the hospital environment. Therefore, any potential commercial usage of *E. faecium* isolates should be preceded by an assessment of infection risk. In the current study, the genomes of two novel *E. faecium* strains Am1 (larval isolate) and Bee9 (adult bee isolate) isolated from the gut of *Apis mellifera* L. (honeybee) were sequenced to allow evaluation of their safety. In particular, their genomes were screened for antibiotic-resistance and virulence genes. In addition, their potential to spread resistance in the environment was evaluated. The analysis revealed that Am1 and Bee9 possess 2832 and 2844 protein-encoding genes, respectively. In each case, the genome size was 2.7 Mb with a G+C content of 37.9 mol%. Comparative analysis with probiotic, non-pathogenic and pathogenic enterococci revealed that there are variations between the two bee *E. faecium* isolates and pathogenic genomes. They were, however, closely linked to the probiotic comparison strains. Phenotypically, the Am1 and Bee9 strains were susceptible to most antibiotics tested, but showed intermediate sensitivity towards erythromycin, linezolid and trimethoprim/sulfamethoxazole. Notably, no genes associated with antibiotic resistance in clinical isolates (e.g. vancomycin resistance: *vanA*, *vanB*, *vanS*, *vanX* and *vanY*) were present. In addition, the insertion sequences (IS*16*, IS*Efa11* and IS*Efa5*), acting as molecular pathogenicity markers in clinically relevant *E. faecium* strains, were also absent. Moreover, the analysis revealed the absence of three key pathogenicity-associated genes (*acm*, *sgrA*, *ecbA*) in the Am1 and Bee9 strains that are found in the prominent clinical isolates DO, V1836, Aus0004 and Aus0085. Overall, the findings of this investigation suggest that the *E. faecium* isolates from the bee gut have not suffered any recent clinically relevant antibiotic exposure. It also suggests that *E. faecium* Am1 and Bee9 are safe potential probiotic strains, because they lack the phenotypic and genetic features associated with strains eliciting nosocomial infections.

DATA SUMMARY

For *Enterococcus faecium* Am1, the whole-genome shotgun project has been deposited at GenBank/ENA/DDBJ under the accession number JAHLTJ000000000. The version described in this paper is version JAHLTJ010000000. For *E. faecium* Bee9, this whole-genome shotgun project has been deposited at GenBank/ENA/DDBJ under the accession number JAHLXG000000000. The version described in this paper is version JAHLXG010000000. The raw sequencing data are available in the Sequence Read Archive (SRA) database under the accession numbers SRR15238610 and SRR15245957, respectively.

INTRODUCTION

Enterococci are facultative anaerobic, non-spore-forming, Gram-positive lactic acid bacteria (LAB). They are characterized by the different relationships that they may establish with their hosts, where they may act as commensals, probiotics or opportunistic

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Keywords: *Apis mellifera*; antibiotic resistance; comparative genomics; *Enterococcus faecium*; lactic acid bacteria (LAB); virulence.

Abbreviations: ARG, antimicrobial-resistance gene; AST, antimicrobial-susceptibility testing; CDS, coding sequence; CFS, cell-free supernatant; CLSI, Clinical and Laboratory Standards Institute; CRISPR, clustered regularly interspaced short palindromic repeat; GI, genomic island; HGT, horizontal gene transfer; ICE, integrative and conjugative element; LAB, lactic acid bacteria; MIC, minimum inhibitory concentration; NCBI, National Center for Biotechnology Information; PATRIC, Pathosystems Resource Integration Center; SEM, scanning electron microscopy; WGS, whole-genome sequencing.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. One supplementary figure and seven supplementary tables are available with the online version of this article.

Impact Statement

Enterococcus species are widespread indigenous bacteria that inhabit the gastrointestinal tract of humans, insects and animals. Although *Enterococcus* species have not yet obtained generally regarded as safe (GRAS) status from the US Food and Drug Administration (FDA), probiotic strains have been made commercially available. Thus, comprehensive assessment of their safety characteristics is necessary to distinguish between beneficial and pathogenic *Enterococcus* strains. Whole-genome sequencing (WGS) provides a valuable tool for assessing the safety of *Enterococcus* strains. Thus, we have used WGS to provide insight into the virulence and antibiotic-resistance properties of two *Enterococcus faecium* strains (Am1 and Bee9) isolated from honeybee gut. Our combined WGS, *in silico* and phenotypic analyses indicates that the two *E. faecium* isolates are stable and safe, and of low microbial risk.

pathogens. Therefore, enterococci are one of the most controversial groups of LAB. The majority of enterococci have been isolated from humans, animals, plants and insects as commensal LAB. For example, some *Enterococcus faecium* strains have beneficial probiotic qualities. They also have an impact on intestinal flora balance and modulate the immune system of humans [1]. However, other strains have emerged as opportunistic human pathogens with a high propensity for spreading antibiotic-resistance and virulence genes via horizontal gene transfer (HGT) [2]. Given the paucity of treatment alternatives, vancomycin-resistant *Enterococcus*, in particular, is considered a persistent global problem affecting both developed and developing nations [3].

Before considering application of any *Enterococcus* strain as a safe starter culture in the food or feed sectors, a thorough examination is required to ensure that such strains do not represent any risk to health. For instance, one of the main challenges is determining potential *E. faecium* strains that are most likely to have generally recognized as safe (GRAS) status while avoiding pathogenic ones. Adopting next-generation sequencing (NGS) technology to analyse the bacterial genomes of *Enterococcus* sp. strains, with special emphasis on antibiotic-resistance and virulence genes, is one of the currently recommended techniques [4]. Whole-genome sequencing (WGS) is becoming the method of choice for identifying and characterizing bacterial genomes. A recent survey conducted on enterococci isolated from different environments confirmed that conventional molecular approaches are less effective than WGS in providing a detailed analysis of antimicrobial-resistance genes (ARGs) and virulence genes [5].

The gut microbiota of social insects, *Apis mellifera* L. (honeybee), is one of the *E. faecium* habitats that has recently been explored. A broad range of bacteria, including many LAB, may be found in the gut of bees [6]. By competing with pathogens for resources and production of organic acids, antimicrobial peptides and bacteriocins, the gut microbiota of *A. mellifera* protect their hosts from pathogenic attacks [6, 7].

Therefore, in the current study, we adopted WGS technology to identify the antibiotic-resistance and virulence genes encoded by two honeybee *E. faecium* strains (Am1 and Bee9). Furthermore, we conducted a comparative study of their genomes versus other *E. faecium* strains isolated from hospitalized patients, milk and commercial probiotics. Overall, the findings of this study support the safe use of *E. faecium* Am1 and Bee9 as these strains lack phenotypic and genotypic characteristics associated with the emergence of nosocomial infections.

METHODS

LAB isolation from bee gut

LAB were isolated from *A. mellifera* larvae and adults. The larvae were provided by the Department of Applied Entomology and Zoology, Faculty of Agriculture, Alexandria University, Egypt. The adult bees were collected from Kafr El-Dawar governorate (Egypt) in early Summer 2019. Briefly, every bee was surface sterilized using 96% ethanol and dissected on the surface of a sterilized wax plate or glass slide to separate the insect's gut. The separated gut was kept in De Man–Rogosa–Sharpe (MRS; HiMedia) broth for enrichment at 37 °C, for 24 h, under microaerophilic conditions generated by a candle jar. For purification, each liquid culture was serially diluted and plated on the surface of MRS agar under the same incubation conditions for 24–48 h. Finally, individual phenotypically unique colonies were picked for a further two rounds of purification using the streak-plate method. Pure cultures were stored at −20 °C in MRS broth supplemented with 50% (v/v) glycerol for further investigation.

Morphological and biochemical characterization

Two bacterial isolates, one isolated from the *A. mellifera* larval stage (Am1) and one from the adult stage (Bee9), were morphologically and biochemically characterized. The two bacterial isolates' morphology was checked using scanning electron microscopy (SEM; JSM-IT 200; JEOL), at the EM Unit, Alexandria University, Egypt. The bacterial isolates were examined for Gram staining and catalase reaction, followed by full biochemical characterization using the VITEK 2 GP ID card (bioMérieux; https://www.biomerieux.com). The haemolytic activity was evaluated according to procedures described elsewhere [8]. *E. faecium* cultures were streaked onto 5% (v/v) blood agar plates and incubated for 24 h at 37 °C.

Genomic DNA extraction

Genomic DNA was isolated from 1.5 ml of 18 h overnight grown bacterial culture in MRS broth at 37 °C using a GeneJET genomic DNA purification kit (Thermo Fisher Scientific), following the manufacturer's instructions for Gram-positive bacterial DNA isolation. In the final step, the DNA was eluted in nuclease-free water (Thermo Fisher Scientific), instead of an elution buffer, for subsequent library preparation. The concentration and purity of the extracted DNA were checked using a NanoDropND-2000 spectrophotometer (Thermo Fisher Scientific) and 1% (w/v) agarose gel electrophoresis.

Genome sequencing, assembly and quality assurance

Genomic DNA libraries were prepared using the Nextera XT library prep kit (Illumina), following the manufacturer's protocol. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz). Pooled libraries were quantified using the Kapa Biosystems library quantification kit for Illumina. WGS was performed by MicrobesNG (http://microbesng.uk) on a HiSeq platform (Illumina), using a 250 bp paired-end protocol with 30× sequence coverage. The adapters were trimmed using Trimmomatic (version 0.30) with a sliding cut-off of Q15 [9]. *De novo* assembly was performed with SPAdes software (version 3.7.0) [10]. The quality of the genome assemblies was assessed using the Quality Assessment Tool for Genome Assemblies (QUAST) [11].

Genome annotation

Genome annotation was performed using Prokka software (version 1.11) [12], the Pathosystems Resource Integration Center (PATRIC; version 3.6.12) server (https://www.bv-brc.org/) [13] and the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP) [14]. The contigs were recorded and reoriented relative to the reference genome based on a MUMmer whole-genome alignment [15]. Total numbers of coding sequences (CDSs), tRNA and rRNA genes from the genomes were predicted using PATRIC. Subsystem categories and features distribution of all *E. faecium* genomes included in this study was assessed using Rapid Annotations using Subsystems Technology (annotation scheme: RASTtk) [16].

Phylogenetic analysis using 16S rRNA gene sequences

For phylogenetic analysis of the two *E. faecium* isolates, the 16S rRNA sequences of *Enterococcus* type strains were downloaded from the NCBI database, followed by use of ClustalW 2.1 for alignment, Gblocks 0.91b for alignment refinement, phylogeny using two programs (PhyML 3.1/3.0 aLRT and MrBayes) and tree rendering by TreeDyn 198.3. The previously listed programs were used through phylogeny.fr [17]. Finally, FigTree (version 1.4.4) (available through http://tree.bio.ed.ac.uk/software/figtree/) was used as a tree graphical viewer.

Comparative genomic analyses

A comparative genomic analysis of *E. faecium* Am1 and Bee9 WGS assemblies was performed to reveal significant similarities and differences between these two bee isolates and other published *E. faecium* strains, namely: SM21 (bee gut isolate); DO, V1836, Aus0004, Aus0085 (clinical isolates); T110, FS86 (commercial probiotic); and NRRL_B-2354 (milk and dairy utensils isolate). These comparator strains were selected as representative examples of probiotic, non-pathogenic and pathogenic isolates. The chromosomal genome sequences were downloaded from the GenBank database and accession numbers are listed in Table 1. Command-line search tools such as BLAST were used for comparison of sequences locally through the web server. BLAST Atlas was generated to align query genomes using *E. faecium* DO as a reference. GView server (https://server.gview.ca) [18] was used with a cut-off value 1×10^{-10} and percentage identity cut-off values of 80%.

Identification of ARGs and antimicrobial-susceptibility testing (AST)

ARGs were identified in the *E. faecium* genomes using the Genome Annotation Service in PATRIC by deploying the *k*-mer-based ARG detection method, which use PATRIC's curated collection of representative antibiotic-resistance variants [19] and assigns to each ARG functional annotation, broad mechanism of antibiotic resistance, drug class and specific antibiotic it confers resistance to.

ARG screening results were validated using minimum inhibitory concentration (MIC) and antibiotic-sensitivity tests. Bacterial suspensions equivalent to 0.5 McFarland turbidity were prepared and 3 ml suspension equivalent to 10^7 c.f.u. ml^{-1} was subjected to AST. The test was performed using the GP susceptibility card AST-P592 (bioMérieux) and the VITEK 2 system (version 9.02), according to the manufacturer's guidelines. MIC interpretation was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). The antibiotic-sensitivity test was carried out on Muller Hinton agar (MHA; HiMedia) plates containing antibiotic discs. The plates were incubated at 37 °C for 24 h and the results were expressed in millimetres of inhibition. The isolates were classified as susceptible (S), intermediate (I) or resistant (R) based on CLSI recommendations [20].

Table 1. General genome features of *E. faecium* strains Am1 and Bee9 against other selected *E. faecium* genomes

Strain	Source	Geographical location	Accession no.*	Genome length (bp)	Total number of genes (CDSs)	tRNA	rRNA	G+C (mol%)	Reference
Am1	Bee gut – larvae	Egypt	JAHLTJ000000000	2732750	2832	62	6	37.99	This study
Bee9	Bee gut – adult	Egypt	JAHLXG000000000	2734007	2844	56	6	37.97	This study
SM21	Bee gut	Argentina	NZSDXT00000000	2544244	2477	60	10	38.10	[7]
NRRL_B-2354	Milk and dairy utensils	USA	CP004063	2635572	2832	48	18	37.84	[48]
T110	Commercial probiotic product	India	CP006030	2693877	2522	66	18	38.47	[61]
FS86	Commercial probiotic product	Russia	CP053704	2685395	2634	57	16	38.40	[62]
DO	Clinical isolate	USA	CP003583	2698137	2788	31	3	37.8	[63]
V1836	Clinical isolate	Denmark	CP044264	2884831	2885	67	18	37.95	[64]
Aus0004	Clinical isolate	Australia	CP003351	2955294	3006	47	18	38.36	[65]
Aus0085	Clinical isolate	Australia	CP006620	2994661	3089	76	18	38.32	[65]

*All accession numbers are from GenBank.

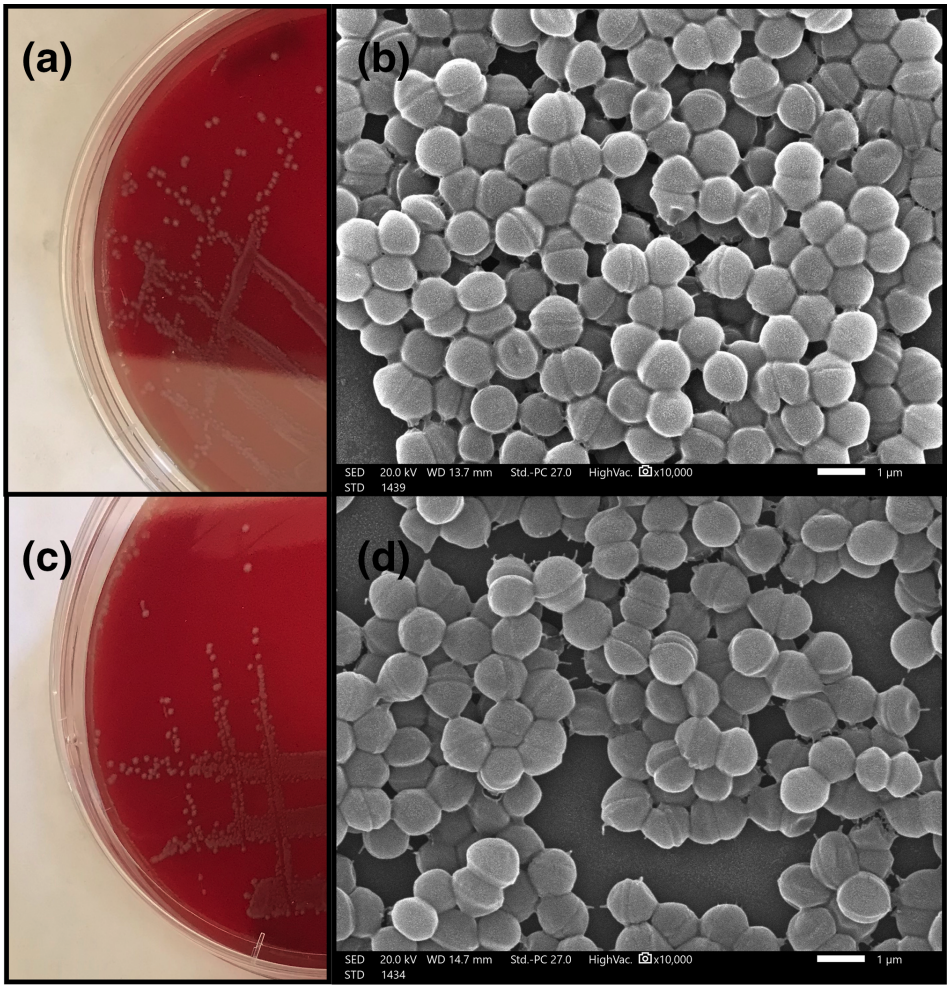

Fig. 1. Growth of *E. faecium* Am1 (a) and Bee9 (c) on the surface of blood agar after 24 h incubation at 37 °C. (b) and (d) demonstrate SEM examinations of Am1 and Bee9 cells, respectively.

Screening for virulence genes

The VFanalyzer platform (https://www.mgc.ac.cn), available through the Virulence Factor Database (VFDB), was used to detect virulence factors in the genomes under investigation. *E. faecium* DO was specified as a representative genome for this analysis. The DO strain was selected for this analysis because it has a complete genome sequence and has been used in multiple pathogenesis studies [21, 22]. The statistics (as of 4th August 2021) of the genus *Enterococcus* in the VFDB are the following: number of species and strains is 2 and 13, respectively. Moreover, the number of virulence factors and genes is 25 and 113, respectively. For validation of virulence-gene screening results, the presence/absence of two virulence genes (collagen-binding, *ace* [23], and surface protein, *esp* [8]) was examined using conventional PCR. *Enterococcus faecalis* 24FS (NCBI accession no. PGCH01000000) was used as a positive control.

Detection of genetic mobile elements and antimicrobial peptide genes

Genes recently acquired by HGT were predicted with IslandViewer 4 (https://www.pathogenomics.sfu.ca/islandviewer/) using the IslandPath-DIMOB method [24] and *E. faecium* DO as a reference. Insertion sequences (ISs) and transposons were predicted with the ISfinder server (https://www-is.biotoul.fr) [25] using BLASTN (version 2.2.31+). Integrative and conjugative elements (ICEs) were identified by the ICEfinder web-based tool [26], with an *E* value of 1×10^{-150}. Plasmids were detected by the PlasmidFinder (version 2.0) online tool [27] provided by the Center for Genomic Epidemiology (https://www.genomicepidemiology.org). Clustered regularly interspaced short palindromic repeats (CRISPRs) were predicted with the CRISPRFinder tool [28]. Prophage sequences were identified using the PHAge Search Tool Enhanced Release web server (PHASTER) (https://phaster.ca) [29]. The FASTA sequence format of the *E. faecium* Am1 and Bee9 genome contigs were mined for bacteriocin-encoding genes using the BAGEL4 server (http://bagel4.molgenrug.nl) [30].

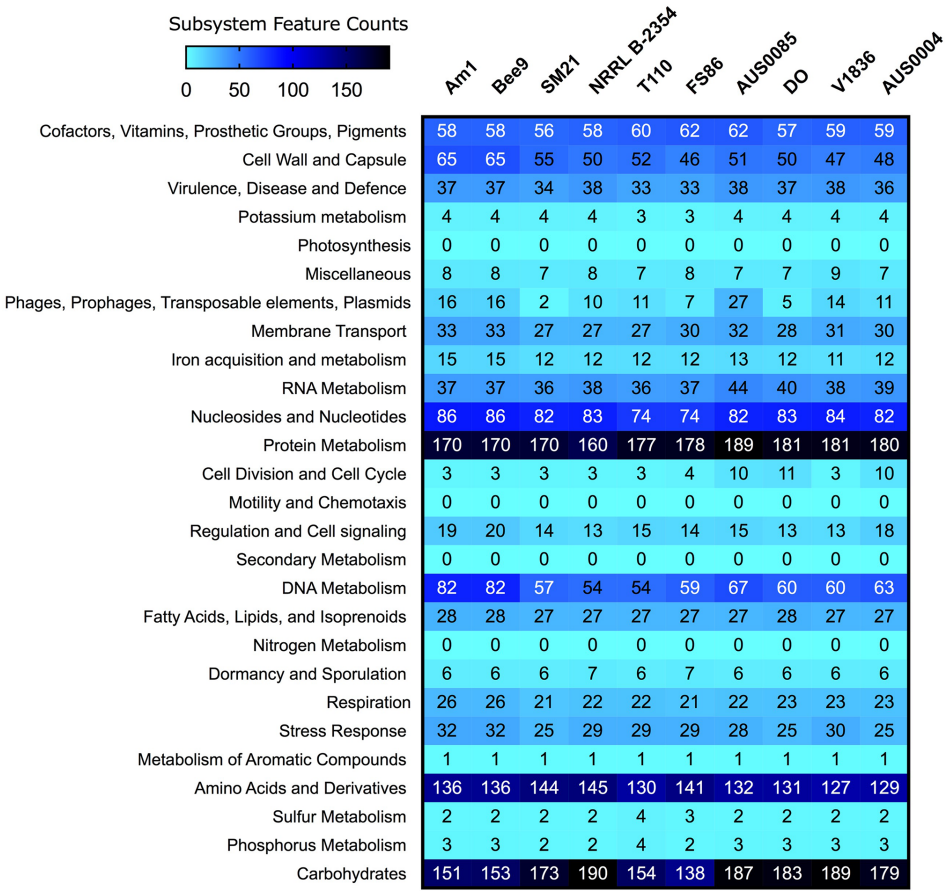

Fig. 2. Subsystem categories and features distribution of the *E. faecium* genomes based on the RASTk annotation server. For each *E. faecium* strain, the number of genes identified in each category is displayed. The colour scale represents the number of genes found in each category; the deeper the colour, the more genes were detected in that category.

Examination of LAB characteristics

The antagonistic activity of Am1 and Bee9 neutralized cell-free supernatant (CFS) was determined by the agar well diffusion assay [31] against Gram-negative bacteria, *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853), and Gram-positive bacteria, *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923). Am1 and Bee9 were also investigated for auto-aggregation properties and *in vitro* cell surface hydrophobicity [32, 33]. *Lactiplantibacillus plantarum* 10CH (NCBI accession no. CP023728) was used as the control strain.

RESULTS

Morphological and biochemical characterization using the VITEK 2 system

Two stable Gram-positive and catalase-negative bacterial isolates, namely Am1 and Bee9, were identified by 99% probability as being *E. faecium* species using the VITEK 2 system. Furthermore, the VITEK 2 biochemical characterization revealed that the two isolates were able to grow on different carbon sources and had the ability to tolerate a high concentration of NaCl (6.5%). The detailed results of this biochemical characterization are reported in Table S1 (available with the online version of this article). Neither isolate displayed blood haemolysis activity on the surface of blood agar and, thus, were identified as γ-haemolytic bacteria. SEM revealed that the two strains possess a coccoidal cell morphology (Fig. 1).

Genome assembly features of *E. faecium* Am1 and Bee9

WGS of Am1 and Bee9 yielded 595821 and 407492 reads, respectively. *De novo* assembly was performed using SPAdes yielding 81 and 76 contigs of over 1000 bp (Table S2). The genomes had 2832 and 2844 predicted protein CDSs, respectively. The general genomic features of Am1 and Bee9 indicated total genome sizes of 2.7 Mb and a G+C content of 37.9mol%, as predicted using the PATRIC server.

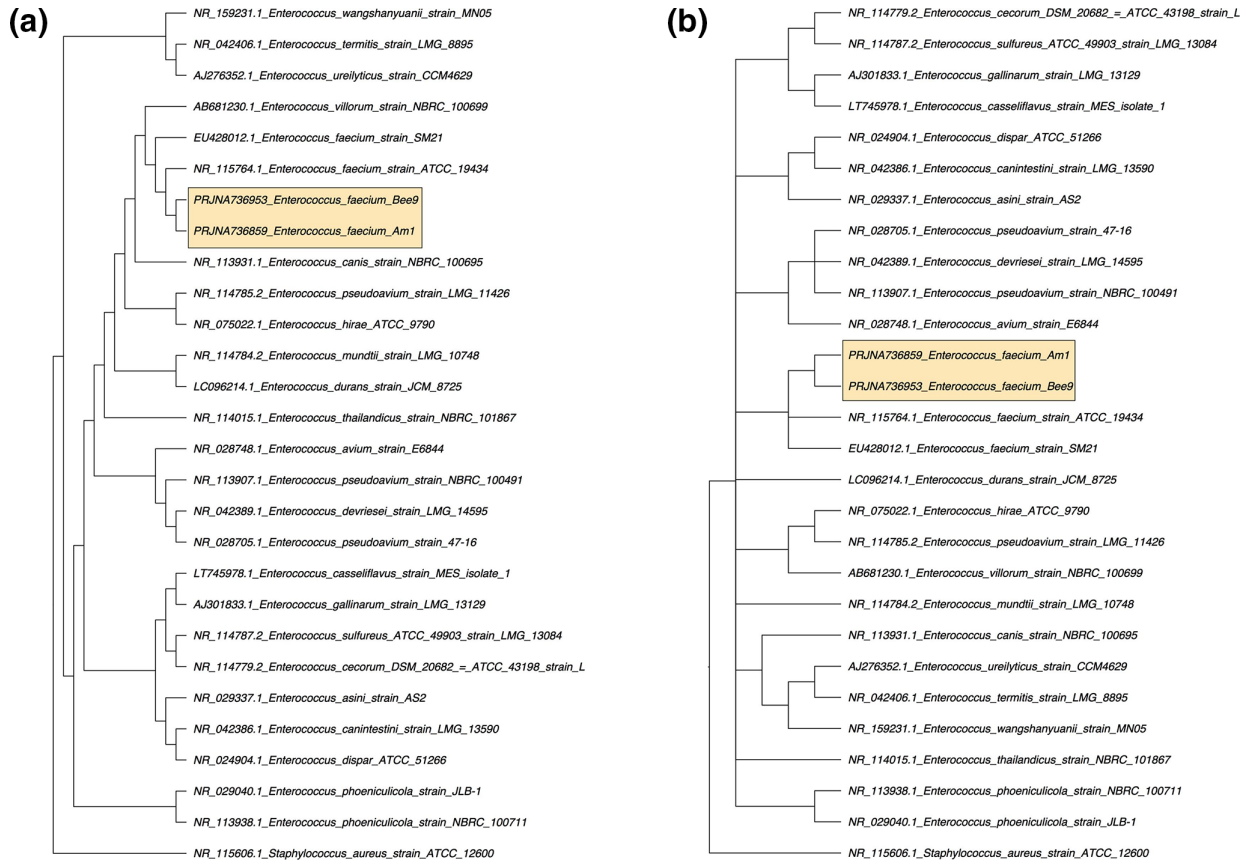

Fig. 3. Phylogenetic analyses of *E. faecium* Am1 and Bee9 with closely related homologues inferred using the maximum-likelihood method (a) and Bayesian inference (b). The evolutionary analyses were carried out in phylogeny.fr (available at https://www.phylogeny.fr/), with tree building using PhyML and MrBayes programs, respectively. The evolutionary trees were graphically displayed using the FigTree program (available at http://tree.bio.ed.ac.uk/software/figtree/).

Subsystem analysis

The genome annotations obtained using the RASTk server were used to generate an overview of the subsystem categories and feature distribution of genomes under investigation. The distribution of Gene Ontology (GO) categories was similar among the tested *E. faecium* isolates, including Am1 and Bee9. For metabolism categories, genes for carbohydrate and protein metabolism were the most abundant, followed by genes for DNA and RNA metabolism. Furthermore, genes related to cell wall and capsule biogenesis were among the most abundant, especially in Am1 and Bee9. The genomes also revealed genes involved in biosynthesis of essential amino acids (glycine, valine, methionine, isoleucine, arginine, serine, lysin and tryptophan) and vitamins (B6, biotin, thiamine, riboflavin and folate). An overview of the genomes subsystem annotations is provided in Fig. 2.

Phylogenetic analysis

Phylogenetic analysis based on the 16S rRNA gene sequence using two different methods (Fig. 3) revealed the evolutionary relationships for the two bee-gut isolates with respect to other closely related species of *Enterococcus*. The two isolates, regardless of the phylogenetic analysis method used, were grouped together in the same clade with *E. faecium* strain ATCC 19434 (NCBI accession no. NR_115764) and *E. faecium* strain SM21 (NCBI accession no. EU428012.1). The SM21 strain was isolated from the intestinal tract of *A. mellifera*.

Comparative genome analysis

The BLAST Atlas presentation of the gene content revealed common characteristics between the two bee *E. faecium* isolates and the aligned genomes. Regions of significant similarity were identified among the two strains (Am1 and Bee9), non-clinical *E. faecium* (NRRL B-2354) and probiotic strains (T110 and FS86), illustrating their similarity in genomic content (Fig. 4). However, two pathogenic islands including most of the virulence and antibiotic-resistance genes predicted in clinical strains (DO, V1836, Aus0004 and Aus0085) were absent in both probiotic strains and the two bee isolates.

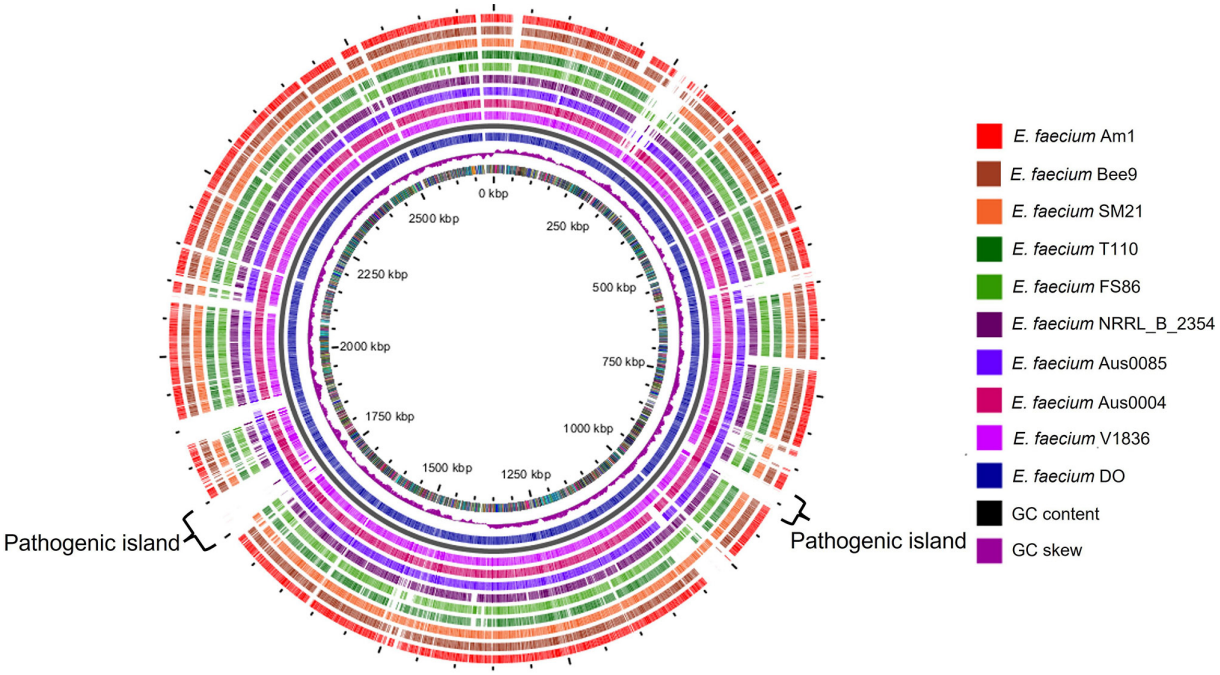

Fig. 4. BLAST Atlas overview of *E. faecium* (Am1 and Bee9) and selected genomes from the NCBI database based on BLASTN. The outermost circles demonstrate the two *E. faecium*, Am1 (red) and Bee9 (brown), and the genomes of strains used in comparison: SM21 (orange), T110 (dark green), FS86 (light green), NRRL B-2354 (purple), Aus0085 (blue), Aus0004 (red violet), V1836 (light purple). Strain *E. faecium* DO (navy blue) was used as a reference genome. The innermost circles represent the G+C content (black), G+C skew curve (violet) and Clusters of Orthologous Genes (COG) categories. The genome sequences were analysed using the GView server using both alignment length and percent identity cut-off values of 80%.

ARG analysis and AST

ARG analysis of the two bee isolates' genomes revealed genes conferring resistance to low-level aminoglycosides [aac(6')-li], fusidane (ef-G, ef-TU; translation elongation factor), peptides (*liaR*, *liaS*; transcriptional regulatory protein), (*efrB*; ABC multidrug efflux pump), erythromycin and other macrolide antibiotics (*msrC*; macrolide-resistance gene), (*efmA*; multidrug efflux pump), (*dfrE*; dihydrofolate reductase); (*adeC*; efflux pump) (*clsA*; cardiolipin synthase A). The genomes did not contain the genes for vancomycin resistance (*vanA*, *vanB*, *vanS*, *vanX* and *vanY*), which are responsible for vancomycin-resistant *Enterococcus* infections in the human population (Fig. 5).

AST profiles, determined over a ~12 h period by VITEK 2 analysis, provided MIC values for clinically relevant antibiotics (Table 2). Both AST profile and the disc diffusion method (Table S3) revealed that the isolates were susceptible to the following antibiotics: ampicillin, imipenem, gentamicin, streptomycin, ciprofloxacin, teicoplanin, vancomycin, tetracycline and tigecycline. Isolate Am1 showed intermediate sensitivity to erythromycin and linezolid, while isolate Bee9 was susceptible to linezolid in the AST profile. Furthermore, the disc diffusion assay revealed that the two isolates were resistant to penicillin G, cefoxitin, azithromycin and colistin sulphate.

Virulence-gene analysis

Screening for virulence factors using the VFanalyzer platform revealed that the two bacterial genomes possess six adherence-associated genes (*ebpA*, *ebpB*, *ebpC*, *strC*, *efaA* and *scm*), three specifying antiphagocytosis (*cpsA/uppS*, *cpsB/cdsA* and *cpsC*), one biofilm gene (*bopD*) and four immune evasion-associated factors. Interestingly, the comparative analysis with the other *E. faecium* strains (SM21, NRRL B-2354, T110, FS86, Aus0004, Aus0085, DO and V1836) included in our analyses revealed that two of the immune evasion factors (*eps3* and *wchJ*) were unique to *E. faecium* Am1 and Bee9 (Fig. 5). The protein products of *eps3* and *wchJ* share similarity with exopolysaccharide biosynthesis protein and UDP-*N*-acetylglucosamine-LPS *N*-acetylglucosamine transferase, respectively. Interestingly, three virulence genes (*acm*, *sgrA*, *ecbA*) were uniquely absent in Am1 and Bee9 strains in comparison to the clinical isolates (DO, V1836, Aus0004 and Aus0085). However, three genes were detected in all *E. faecium* strains regardless the source of isolation, namely *bopD*, *cpsA/upps* and *cpsB/cdsA*. PCR validation confirmed the absence of *ace* and *esp* virulence genes (Fig. S1).

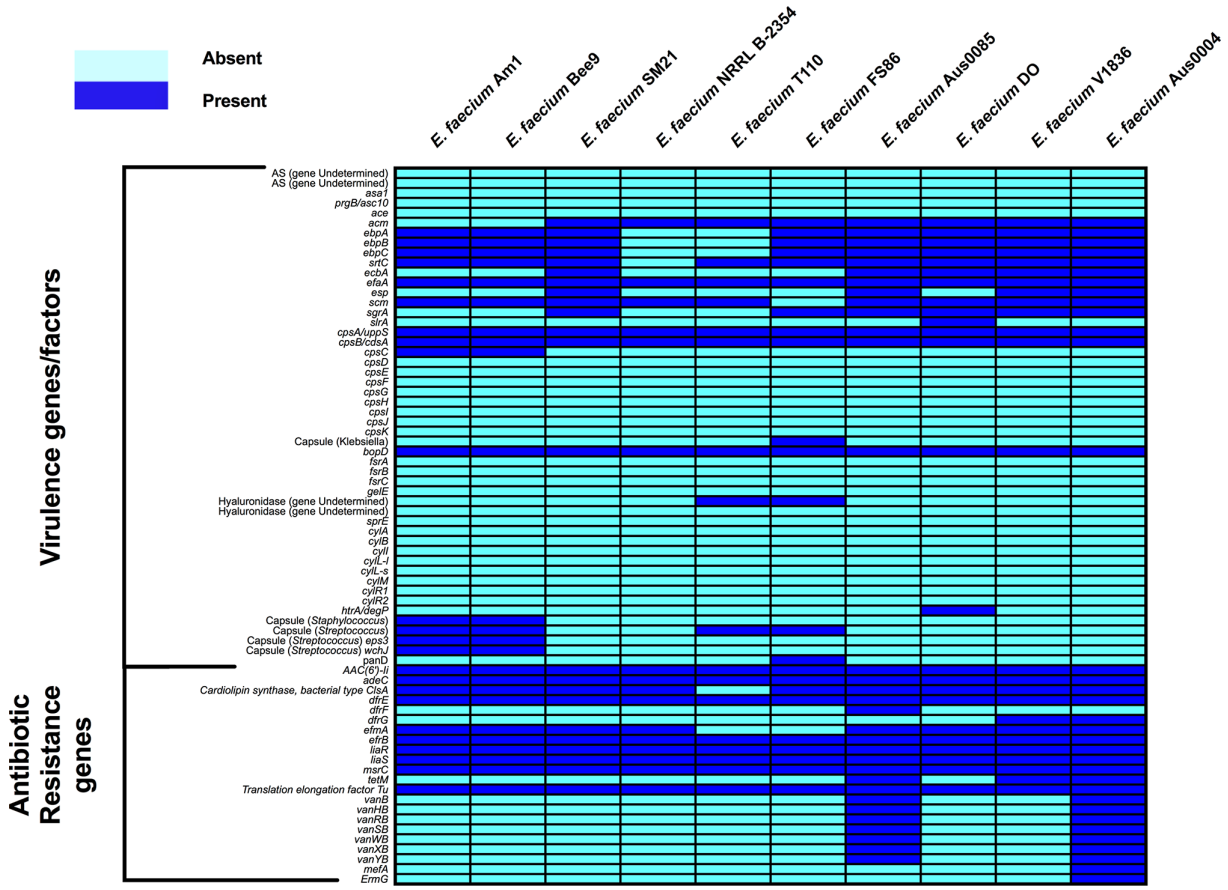

Fig. 5. Heat map representation of virulence genes/factors and antibiotic-resistance genes identified in *E. faecium* Am1 and Bee9 using the VFanalyzer platform and the *k*-mer-based ARG detection method available through PATRIC, respectively. The dark and light blue colours represent present and absent genes, respectively.

Genomic islands (GIs)

The analysis of the Am1 and Bee9 genomes revealed 9 and 12 GIs, respectively, based on the IslandPath prediction method (Fig. 6). The GIs were well distributed over the genome, and each GI encoded proteins of both hypothetical and known function (Tables S4 and S5). GIs included ribosomal proteins, transposable elements (IS*3*, IS*4*, IS*6*, IS*30*, IS*256*, IS*200*/IS*605*, IS*630*), prophage integrase (phiRv2), tyrosine recombinase (*xerC*, *xerD*) and conserved virulence factor B (*cvfB*).

Genetic mobile elements (insertion sequences, ICEs, plasmids, CRISPRs)

Insertion sequence elements, major genetic mobile elements in *E. faecium* isolates, were predicted with ISfinder and BLASTN analysis. Seventy-two IS elements were predicted in *E. faecium* Am1, while in *E. faecium* Bee9 48 IS elements were identified. Most IS elements in Am1 and Bee9 belong to the IS*3* family (Tables S6 and S7). The isolates showed an absence of IS*16*, IS*Efa11* and IS*Efa5* insertion sequences, known as molecular pathogenicity markers in clinically relevant *E. faecium* strains [34–36]. Although several IS elements were found and pathogenicity related IS elements were absent in the genomes of Am1 and Bee9, further analysis using different approaches would support this *in silico* finding.

ICEs were detected with the ICEfinder web-based tool. In *E. faecium* Am1, two putative ICE elements (IMEs; integrative and mobilizable elements) of 25368 and 38627 bp length were detected that contained integrase and relaxase genes. The *E. faecium* Bee9 genome also revealed the presence of two putative IMEs, of 34956 and 27880 bp. Larval isolate Am1 contained a 214319 bp plasmid (pNB2354_1) with a repUS15 replicon (accession no. CP004064) and 8347 bp plasmid (pGL) with a rep29 replicon (accession no. HQ696461). Isolate Bee9 contained only one plasmid (pNB2354_1). The detected plasmids lack the critical virulence factors present in pathogenic *E. faecium* strains. The CRISPR finder tool identified one confirmed CRISPR element (199 bp) in isolate Bee9 with four direct repeats of 24 bp and three spacers, with one associated with a cas-family gene.

Table 2. MIC (μg ml^{-1}) interpretation of *E. faecium* Am1 and Bee9 detected by VITEK 2 as per CLSI guidelines

Antimicrobial	MIC value (interpretation*)	
	Am1	Bee9
Ampicillin	≤ 2 (S)	≤ 2 (S)
Imipenem	≤ 1 (S)	≤ 1 (S)
Gentamicin high level (synergy)	SYN-S (S)	SYN-S (S)
Streptomycin high level (synergy)	SYN-S (S)	SYN-S (S)
Ciprofloxacin	≤ 0.5 (S)	≤ 0.5 (S)
Erythromycin	1 (I)	1 (I)
Linezolid	4 (I)	2 (S)
Teicoplanin	≤ 0.5 (S)	≤ 0.5 (S)
Vancomycin	≤ 0.5 (S)	≤ 0.5 (S)
Tetracycline	≤ 1 (S)	≤ 1 (S)
Tigecycline	≤ 0.12 (S)	≤ 0.12 (S)

*Interpretation of MIC values S (susceptible) and I (intermediate) according to the CLSI [20].

Prophages

The PHASTER tool identified one intact (PHAGE_Entero_IME_EFm5, 17.3 kb) and three incomplete (PHAGE_Escher_RCS47, 15.3 kb; PHAGE_Entero_phiFL3A, 15.3 kb; and PHAGE_Lister_LP_030_3, 9 kb) prophage genomes in *E. faecium* Am1. The Bee9 isolate carried one intact (PHAGE_Entero_IME_EFm5, 23.9 kb) and three incomplete prophage regions (PHAGE_Entero_phiFL3A, 18 kb; PHAGE_Staphy_SPbeta_like, 15.1 kb; and PHAGE_Lister_A118, 10.1 kb).

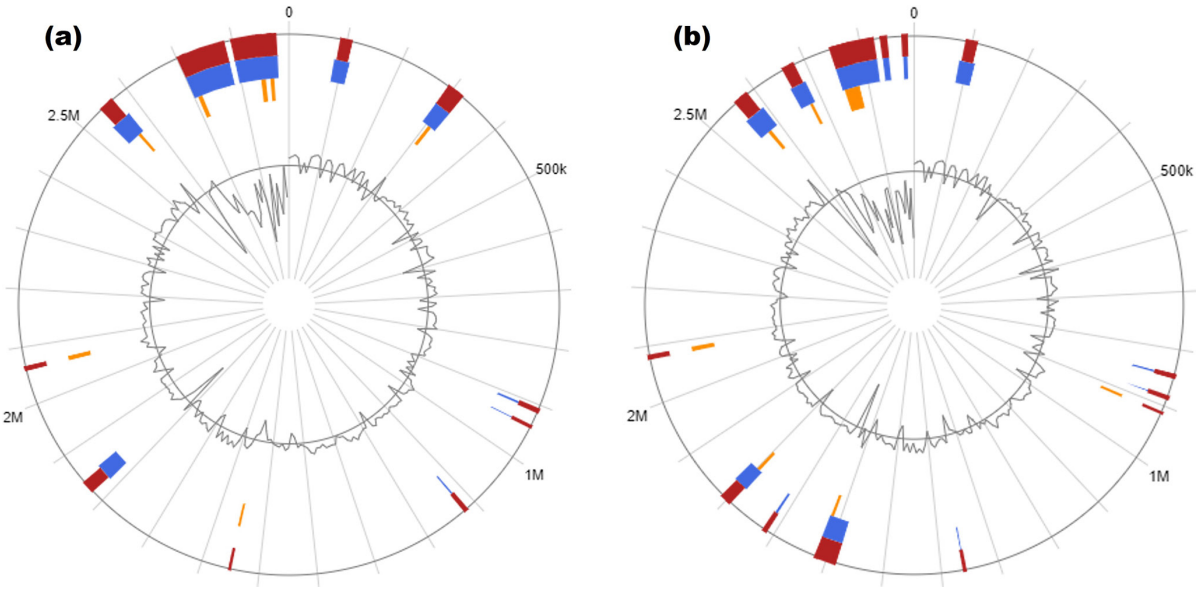

Fig. 6. Analysis of the GIs of *E. faecium* Am1 (a) and Bee9 (b) predicted by IslandViewer web server. The predicted GIs are shown in different colours within the circular image based on the tools used: SIGI-HMM, which predicts GIs based on a hidden Markov model (orange); IslandPath-DIMOB, which predicts GIs based on features associated with GIs (blue); and an integration of three methods (red).

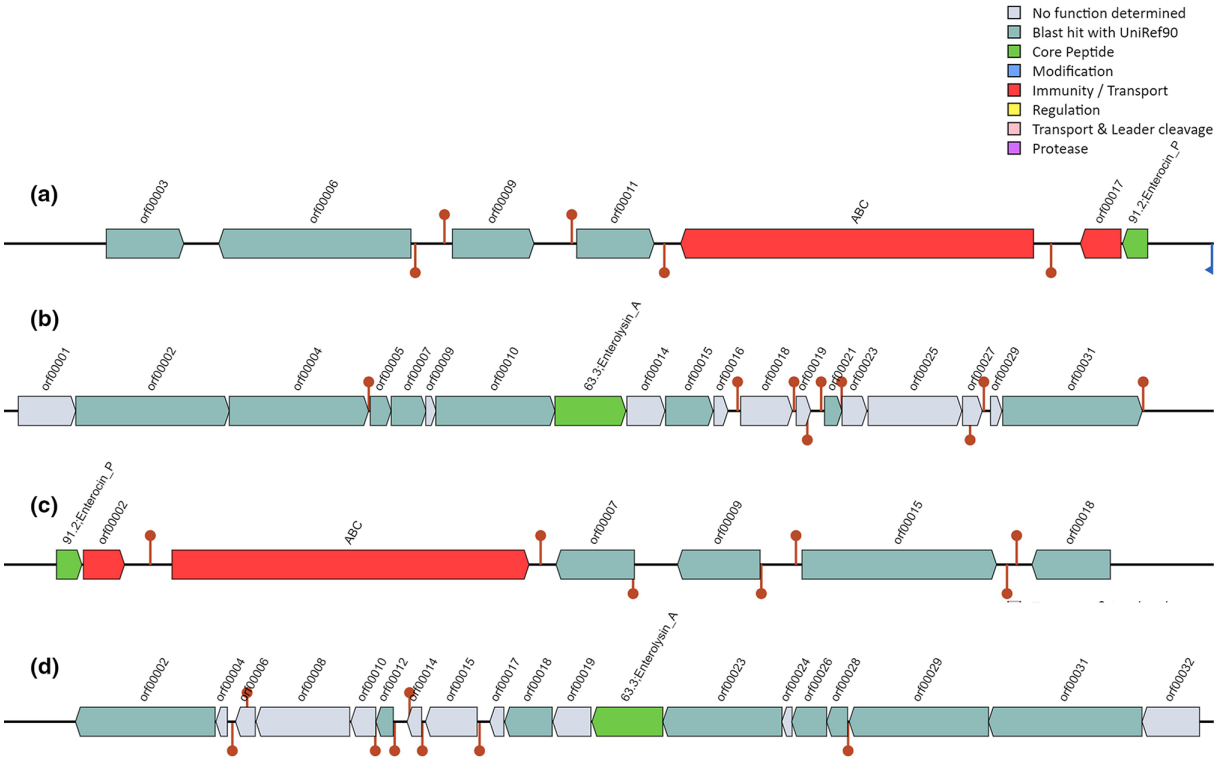

Fig. 7. Putative bacteriocin-related gene clusters using the BAGEL4 web server. (a, b) Predicted enterocin P (EntP) and enterolysin A (EnlA) in *E. faecium* Am1 genome; (c, d) Predicted EntP and EnlA in *E. faecium* Bee9 genome.

Bacteriocin genes

The bacteriocinogenic potential of Am1 and Bee9 was assessed using the BAGEL4 server. The data revealed that the predicted bacteriocins corresponded to a class II (small heat-stable non-lantibiotic peptides) bacteriocins designated enterocin P (EntP; 5735 Da) and a class III (large thermolabile bacteriolysins) bacteriocin designated enterolysin A (EnlA; 43155 Da) (Fig. 7).

Hydrophobicity, autoaggregation and antagonistic characteristics

The affinity of Am1 and Bee9 toward the hydrophobic agent xylene was investigated and the results revealed 54.78 and 54.58 % hydrophobicity, respectively. The ability of the two isolates to form autoaggregates was determined as 45.44 and 44.65 % for Am1 and Bee9, respectively. Am1 and Bee9 neutralized CFS revealed antagonistic activity against *S. aureus* (ATCC 25923) and *Escherichia coli* (ATCC 8739) (Fig. 8).

DISCUSSION

In the current study, we utilized WGS technology to assess two *E. faecium* isolates, Am1 and Bee9, originally isolated from *A. mellifera* larval and adult guts, respectively, in terms of their antibiotic and virulence-gene profiles. Moreover, a comparative genomic study was conducted to identify unique and common characteristics between these two isolates and other probiotic, non-pathogenic and pathogenic *E. faecium* strains.

The analysis of antibiotic-resistance genes revealed the absence of vancomycin- and teicoplanin-associated-resistance genes in Am1 and Bee9 isolates. This result was further confirmed using the VITEK 2 system and the disc diffusion method, which indicated a sensitive (S) status for these clinically relevant antibiotics. Sensitivity to vancomycin is essential for the safe application of *Enterococcus* strains intended for food or feed consumption. The reason for this is that vancomycin is regarded as a 'drug of last resort' commonly used in the treatment of enterococcal infections [37].

The unique absence of three key virulence genes (*acm*, *sgrA*, *ecbA*) in Am1 and Bee9, in contrast to clinical isolates, indicates the safety of these bee-gut isolates. These three genes were reported from previous studies to be associated with enhancing the virulence of clinical isolates. For instance, *sgrA* and *ecbA* along with *esp* were reported as important markers enriched in hospital-associated *E. faecium* isolates and ~70–100 % of *E. faecium* hospital-associated isolates possess *sgrA* and *ecbA* genes.

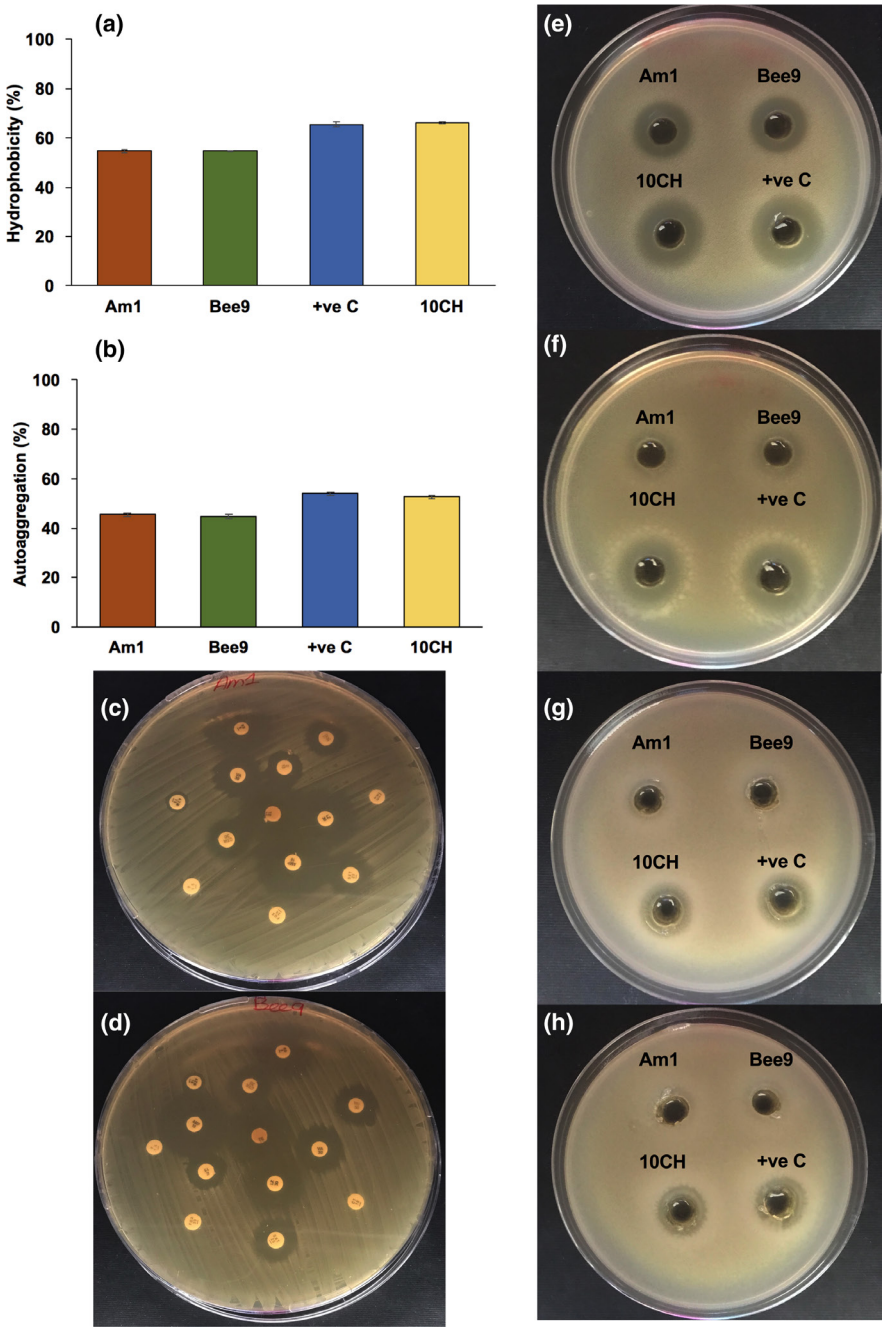

Fig. 8. Examination of some LAB characteristics. Histograms in (a) and (b) show the hydrophobicity and autoaggregation percentage achieved by *E. faecium* Am1 and Bee9. The histogram shows the percentages obtained by *L. plantarum* strain 10CH and *L. plantarum* laboratory isolate (+ve C). The results of the antibiotic disc diffusion method for Am1 and Bee9 are shown in (c) and (d), respectively. The (e), (f), (g) and (h) plates demonstrate the antagonistic activity of Am1 and Bee9 neutralized CFS against *S. aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739), *P. aeruginosa* (ATCC 27853) and *B. subtilis* (ATCC 6633), respectively.

Furthermore, the presence of *esp* was found to enhance the ability of *E. faecium* and *E. faecalis* to form biofilms that facilitate the colonization of medical devices [38–40]. Therefore, these genes may play an important role in adhesion, colonization, biofilm formation and pathogenesis. Hence, it is highly recommended to include hospital-associated *E. faecium* strains in comparative genomic studies against commensal ones as it pinpoints important genes [41, 42]. However, the two bee isolates were found to have two distinct Exopolysaccharide (EPS)-related genes, *eps3* and *wchJ*. Although the production of exopolysaccharide (EPS) may be seen as virulent, it may also aid in the mobility of these non-motile bacteria. It also improves their capacity to endure stressful conditions such as high pH, osmolarity and temperature. It might also explain

the two isolates' antagonistic properties toward other bacterial species, since EPS can accumulate metabolites that are toxic to other bacteria [1].

When ingesting a meal or feed contaminated with glycopeptide-antibiotic-resistant or virulent enterococci, there are two main dangers. First, there is the risk of the resistant bacteria being established in the host gut. Second, by conjugation, resistance or virulence genes from glycopeptide-resistant enterococci may transfer to other gut bacteria [37, 43, 44]. It is confirmed that enterococci can pass on their resistance towards glycopeptide antibiotics to other enterococci [45] and even other Gram-positive bacteria [46]. Therefore, it is critical to ensure the absence of these transferable resistances before use of any new *Enterococcus* strain in food or feed applications. However, the presence of glycopeptide-resistance or virulence genes does not imply that these genes will be easily transferred to other bacteria. As a result, SCAN (Scientific Committee on Animal Nutrition) advises checking whether these genes are transferable [43]. Screening of Am1 and Bee9 genomes for IS elements revealed that they harboured different numbers of IS families. IS elements are considered the smallest transposable elements that play an important role in shaping bacterial genomes [47]. Notably, both strains revealed the absence of IS*16* insertion sequences, which are used as markers for hospital-acquired multidrug-resistant (MDR) *E. faecium* strains [35]. The Am1 and Bee9 genomes also lacked the IS*Efa5* and IS*Efa11* insertion sequences that are associated with the vancomycin-resistance genes *vanS* (sensor histidine kinase), *vanX* (dipeptidase) and *vanY* (carboxypeptidase) [34]. The number of IS elements was significantly higher in the adult (Bee9) than the larval bee (Am1) isolate. This might be related to the exposure of adult bees to different ecological niches as they move freely without restrictions. Further investigations are required to verify these findings.

Additionally, the Am1 and Bee9 genomes were mined for other mobile genetic elements, including plasmids, transposons, CRISPR-cas and prophages. Mega plasmid pNB2354_1 was predicted in both bee isolates. This plasmid was previously reported in the non-clinical *E. faecium* NRRL B-2354 strain [48]. ICE and transposon gene induction leads to the potential transfer of DNA to appropriate recipients through processes of HGT [49]. Unlike clinical strains, the genomes of Am1 and Bee9 lacked transposons Tn*1547*, Tn*1549* and Tn*5382*, which are responsible for the transfer of *vanB* resistance alleles [50–52]. CRISPR functions in defence against bacteriophage and foreign DNA [47]. According to genomic data, the larval isolate Am1 has no CRISPR elements, but the adult bee isolate (Bee9) has one CRISPR locus that may offer phage defence in the adult bee gut. The biotic and abiotic complexity of the adult insect's gut ecosystem, where multiple bacterial species and individuals coexist, as well as variation in phage and plasmids, may explain how this CRISPR element evolved and was maintained [53].

The RAST-based annotation suggested an abundance of carbohydrate metabolism subsystems. This apparent high capacity for carbohydrate utilization agrees with the ability of *E. faecium* strains to utilize a wide variety of saccharides [54]. The ability to utilize various saccharides plays a role in biofilm formation by Gram-positive bacteria [55]. Gut colonization through biofilm formation is required for the interaction of bacterial cells with the host and supports resistance to gastrointestinal tract conditions [56]. The metabolic diversity in bee-gut associated *E. faecium* is vital in the adaptation of these strains to the host bee gut under various environmental and stress responses. Overall, enterococci often require rich and complex nutrients to support growth as a result of their fastidious nature [57].

Am1 and Bee9 strains were found to possess two bacteriocin gene clusters specifying enterocin P (*entP*) and enterolysin A (*enlA*). Enterocin P is a class II bacteriocin that displays a strong inhibitory activity against food pathogenic *Listeria monocytogenes* by permeabilizing the membrane, causing an ionic imbalance and leakage of inorganic ions [58]. Enterolysin A is a class III bacteriocin that inhibits growth of bacteria by the cleavage of the peptide bonds within the stem peptide, as well as in the interpeptide bridge, of Gram-positive bacterial cell walls [59]. The presence of bacteriocin genes in bee-gut-associated *E. faecium* genomes suggests a protective activity for these bacterial strains against host pathogens that would support their colonization of the gastro-intestinal tract. Finally, Am1 and Bee9 isolates revealed hydrophobicity and autoaggregation potential, which are beneficial characteristics for LAB. These characters are necessary for adhesion to intestinal cells and to form a barrier that prevents the colonization by pathogens [60].

Conclusion

The genomes of Am1 and Bee9 isolated from larval and adult bee gut were sequenced and followed by their incorporation in a comparative genome analysis. The assembled genomes have provided insights into the virulence and antibiotic resistance in bee-gut *E. faecium* and the data from this study would support the use of bee-gut *E. faecium* as a safe and of low microbial risk source of LAB.

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Author contributions

H.A.H.Z. and N.M.E. contributed significantly to design experimental work, data acquisition analysis and interpretation in all aspects. Furthermore, both authors took part in drafting, revising and critically reviewing the article and gave final approval of this version.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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