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Probiotic potential of *Enterococcus* strains with multiple enterocin-encoding genes

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Abstract

In this work, we sought to identify and characterize three strains of *Enterococcus* isolated from camel milk and fermented wheat, based on the capacity to produce bacteriocins and the probiotic potential. Polymerase chain reaction analyses were used to identify bacterial isolates and structural genes of bacteriocins, and also to detect potential enterococcal virulence genes (*cyl*A, *esp*, *gel*E, *efa*Afs, *hyl*, *ace*, *asa*1, *van*A, and *van*B). The antimicrobial activity of the strains was investigated in solid media by the agar spot method against several pathogenic bacteria. The probiotic potential of the strains was also analysed using low pH (pH 3.0), bile salt resistance, DNAase, and antibiotic susceptibility assays. The strains were identified using rRNA16S sequencing gene, showing their belonging to *E. faecium* species. Based on PCR results, *E. faecium* CM9 and CM18 strains included in the genome the structural gene of enterocin A, enterocin B, and enterocin P, while *E. faecium* H3 possessed enterocin MR10A/B structural gene. The sequence analysis revealed that the H3 strain included in the genome the structural gene of enterocin L50A/B. All the pathogenic bacteria (*Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes*, *E. coli*, and *Pseudomonas aeruginosa*) were inhibited by the three strains *E. faecium* CM9, CM18, and H3. In the well diffusion test, the supernatants of the three strains exhibited inhibitory activity against *Listeria monocytogenes* CECT 4032. The strains showed high tolerance to low pH and bile salts, did not possess DNase, were susceptible to the majority of antibiotics assayed, and did not possess any of the virulence genes analyzed in this study. A promising candidate strains were identified as potential probiotics with anti-bacterial action against pathogenic bacteria.

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Keywords: enterocins; *Enterococcus*; lactic acid bacteria; probiotics; antibiotic resistance; virulence factors

Introduction

Enterococci, which belong to the group of lactic acid bacteria (LAB), have received increasing attention in recent years for various reasons (Kavitake *et al*., 2023; Rodríguez-Lucas and Ladero, 2023). Enterococci are important in environmental, food and clinical microbiology, and are ubiquitous microorganisms due to their predominant character (Moreno *et al*., 2006). Enterococci confer a technological advantage in the production of various fermented foods such as sausages and cheeses, either when they are used as starter cultures or when their presence results from environmental contamination. Some strains are successfully used as probiotics. However, the genus *Enterococcus* has not obtained the status generally recognized as safe (GRAS) (Suvorov, 2020). Nevertheless, due to functional properties such as tolerance to bile salts and acidity, absence of antibiotic resistance genes, *in vitro* adhesion capabilities, some *Enterococcus* strains are candidate as starter cultures in fermentation processes and are potential probiotics (Strompfová and Lauková, 2007; Javed *et al*., 2011; Zommiti *et al*., 2018; Zaghloul *et al*., 2023).

Numerous and extensive research have been carried out in recent years for the detection of virulence factors in food-borne and clinical enterococci isolates. Two virulence factors have been isolated and characterized: i) surface factors that affect the colonization of host cells and ii) agents secreted by enterococci that damage the tissues (Sava *et al*., 2010). Several studies have shown the difference in the presence of virulence factors between the two origins and have noted the absence of the virulence factors in food-borne isolates (Suvorov, 2020; Wang *et al*., 2020).

It is known that several enterococci can produce broad-spectrum bacteriocins, collectively named enterocins, which makes them interesting candidates for the food industry (Moreno *et al*., 2006). Bacteriocins are ribosomal-synthesized peptides and are able to inhibit the growth of pathogenic bacteria (Kasimin *et al*., 2022). At present, the latest and most authoritative three-category classification method of bacteriocins has replaced the previous four-category classification scheme. Class I bacteriocins refer to small-molecule (<10 kDa) thermostable bacteriocins with extensive post-translational modifications. Class II bacteriocins refer to unmodified small-molecule (<10 kDa) thermostable bacteriocins after translation, which do not involve the formation of uncommon amino acids. Class III bacteriocins include unmodified macromolecular heat-labile bacteriocins with molecular weights greater than 10 kDa (Wu *et al*., 2022).

Most of the characterized enterocins belong to the class II bacteriocins. Class II is an interesting class for food applications (Nes *et al*., 2014). Enterocin A is a representative bacteriocin of enterocins and is the first enterocins of class II.a (pediocin-like bacteriocins) to be isolated, purified, and relatively well-studied from *E. faecium*. Enterocin X is a class II.b (two-peptide) bacteriocin derived from *E. faecium* KU-B5 and is composed of two antibacterial peptides (Xα and Xβ). Class II.c enterocins are composed of homologous peptides (high homology of more than 70% in amino acid sequences). Enterocin L50 is composed of enterocins L50A and L50B. Both of the two enterocins have antibacterial activity on their own, with entL50A being the most active. Enterocin B belongs to class II.d and shows strong homology to carnobacteriocin A (Wu *et al*., 2022).

Enterococcus can possess in their genome more than one enterocin gene cluster, but it does not mean that their biosynthesis occurs simultaneously, it depends on various regulatory pathways (Cintas *et al*., 2000; De Vuyst *et al*., 2003; Perez *et al*., 2012).

The purpose of this work is to identify and characterize three bacteriocinogenic *Enterococcus* strains isolated previously from camel milk and fermented wheat, that can be used as potential probiotics. A series of assays are usually carried out in order to identify and characterize potential probiotic strains. These assays

include evaluating the ability to survive under harsh conditions, e.g., tolerance to bile salts, the presence of antibacterial activity, resistance to antibiotics, and lacking virulence genes.

Materials and Methods

Bacteria and growth media

The bacterial strains used in this study were *Enterococcus* sp CM9, *Enterococcus* sp CM18 and *Enterococcus* sp H3, and were provided from the Laboratory of Micro-organisms Biology and Biotechnology, University of Oran, Algeria. These strains were previously isolated from camel milk originated from Mauritania and fermented wheat originated from Algeria, and produced bacteriocins (Benmouna *et al*., 2018, 2020).

The three strains were grown in MRS broth (de Man, Rogosa and Sharpe) (Lab M, Bury, UK) at 30 °C for 24 h, whereas the other strains were grown in Tryptone Soy Broth (Lab M) at 37 °C for 24 h.

Identification of strains

Phenotypic methods

Phenotypic identification of *Enterococcus* species was based upon phenotypic characteristics, including the ability to grow at 10 °C and 45 °C in the presence of 6.5% (w/v) NaCl. Fermentative metabolism was examined according to Facklam and Collins (1989). Afterward, the API 50 CHL identification system (bioMérieux, Lyon, France) was used for species identification.

16S rRNA sequencing gene

Total cellular DNA was extracted using the genomic DNA purification kit (Xtrem biotech, SL. Granada, Spain) in accordance with the instructions of the manufacturer. DNA samples were maintained at -20 °C until use.

PCR reactions were performed in a total volume of 25 µL, containing 12.5 µL of Taq Master Mix 2× (IBIAN Technologies, Saragossa, Spain), 2 µL of forward and reverse primer (27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-ACGGCTACCTTGTTACGACTT-3′), 6.5 µL of PCR water, and 2 µL of template DNA. As a negative control, 2 µL of PCR water was used. Amplifications were made in a thermocycler (Mastercycler, Eppendorf, France) (Sonsa-Ard *et al*., 2015).

The amplification program consisted of an initial denaturation step at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min for denaturation, annealing, and elongation, respectively. The size of the PCR product was 1500 bp. This product was verified using 1% (w/v) agarose gel electrophoresis, which was then visualized using ethidium bromide (Sonsa-Ard *et al*., 2015).

Bacterial 16S rRNA genes obtained by PCR amplification were purified using FavorPrep Gel/PCR Purification Kit (Favorgen Biotech. Corp., Taiwan) according to the manufacturer's protocol and sent to the DNA Sequencing Service (CIC, University of Granada, Spain). The resulting sequences were compared through a local alignment search of the GenBank database using the BLAST version 2.2.9 program of the National Center for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Study of the antimicrobial activity of the isolates

Antimicrobial activity of Enterococcus strains

The antimicrobial activity of the identified bacterial strains was measured using agar spot test as described by Fleming *et al*. (1975). Briefly, 5 µL of an overnight culture of the bacteriocin producer strains was spotted onto buffered (KH_2PO_4-Na_2HPO_4, 0.2 M, pH 7.0) MRS plates. After incubation at 30 °C for 18 h, the plates were overlaid with 7 mL of brain heart infusion (BHI) (1.8% BHI, 0.8% agar) inoculated (2%) with an overnight culture of the indicator strains: *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes*,

E. coli, and *Pseudomonas aeruginosa* (Table 3). After that, the plates were incubated at 30 °C for 24 h. The antimicrobial activity was visually detected by observing clear inhibition zones around the strain spot. The diameters of clear halos around the spotted cultures were measured.

Antibacterial activity of the cell free supernatant (CFS) of *Enterococcus* strains

To prepare CFS, the strains were grown overnight in buffered MRS broth at 30 °C (Benmouna *et al*., 2018). CFS were collected by centrifugation at 6000 × g for 20 min at 4 °C in a Thermo-Scientific centrifuge and filtered through a sterile 0.22-mm filter (Millipore Corp., Bedford, MA, USA). CFSs were stored at 4 °C until required.

The antibacterial activity of the CFSs was determined using the agar well diffusion method as described by Schillinger and Lücke (1989). MRS agar plates were overlaid with 0.7% BHI soft agar or trypticase soy agar inoculated with 0.5% of the actively growing test strains (0.5 NTU - McFarland scale). Wells with 5 mm diameter were cut in the agar plate using a cork borer and 100 µL of CFSs was seeded. After diffusion (at 4 °C for overnight), the plates were incubated at 37 °C for 24 h.

PCR detection of enterocin-encoding genes

The primers coding for enterocin genes belonging to diverse bacteriocin groups were used (Table 1). PCR-amplifications were performed in 25 µL reaction mixtures, containing 2 µL of DNA sample, 2.5 µL of each primer, 12.5 µL of Taq Master Mix 2× (IBIAN Technologies, Saragossa, Spain), and 5.5 µL of PCR water. Samples were subjected to an initial cycle of denaturation (94 °C for 3 min), followed by 30 cycles of denaturation (94 °C for 1 min), annealing (43 °C for 1 min) and elongation (72 °C for 1 min), and ending with a final extension step at 72 °C for 5 min (Ogaki *et al*., 2016).

Table 1. PCR primers used for analysis of enterocin genes

Bacteriocins	Genes	Primers	References
Enterocin A	*ent*A	P9: 5′-GAGATTTATCTCCATAATCT-3′ P10: 5′-GTACCACTCATAGTGGAA-3′	Aymerich *et al*., 1996
Enterocin B	*ent*B	EntB(r): 5′-GTTGCATTTAGAGTATACATTTG-3′ EntB(f): 5′-GAAAATGATCACAGAATGCCTA-3′	Du Toit *et al*., 2000
Enterocin P	*ent*P	EntP1: 5′-ATGAGAAAAAAATTATTTAGTTT-3′ EntP2: 5′-TTAATGTCCCATACCTGCCAAACC-3′	Gutiérrez *et al*., 2002
Enterocin AS-48	*ent*AS-48	AS-48-1: 5′-AATAAACTACATGGGT-3′ AS-48-5: 5′-CCAAGCAATAACTGCTCTTT-3′	Martínez-Bueno *et al*., 1998
Enterocin MR10A	*ent*MR10A	LICIJ1: 5′-ATGGGAGCAATCGCAAAA-3′ LICJ2A: 5′-TTAAATATGTTTTTTAATCCA-3′	Cintas *et al*., 1998
Bacteriocin 31	*bac*31	Bac31f: 5'-CCTACGTATTACGGAAATGGT-3' Bac31r: 5'-GCCATGTTGTACCCAACCATT-3'	De Vuyst *et al*., 2003

The PCR-generated fragments were purified by a FavorPrep Gel/PCR Purification Kit according to the manufacturer's protocol and sent to the DNA Sequencing Service. The resulting sequences were compared through a local alignment search of the GenBank database using the BLAST version 2.2.9 program of the NCBI.

Assessment of probiotic potential of strains

Bile tolerance

Tolerance to bile salts was analyzed according to the method described by Saavedra *et al*. (2003) using bile salt agar (4% of bile salt) (Scharlau Microbiology, Barcelona, Spain). Five microliters of overnight culture of bacteria were spotted onto bile salt agar. The bile salt hydrolase activity was indicated by the formation of

opaque halos of precipitated deconjugated bile acids around the colonies grown in bile salt-containing MRS agar.

Acid tolerance

For the acid tolerance assay, MRS broth was used to simulate the acidity of the gastrointestinal tract after adjusting to pH 3.0 with 1 N HCl. An overnight culture of bacteria was used to inoculate MRS broth at 1% level. Samples of 100 µL were taken at 0 h and after 1, 2, and 3 h of incubation at 37 °C, simulating the time spent in the human stomach. The number of bacterial cells was then enumerated by the pour plate method of all samples using decimal serial dilutions prepared in physiological water (Zommiti *et al*., 2018).

DNase activity

DNase activity was determined by spotting 5 µL of overnight cultures of bacteria onto DNase agar plates (Scharlau); after incubation at 37 °C for 48 h, plates were overlain with HCl 1 N for 5 min. The presence of DNase activity was detected by the formation of opacity around the colonies (Cebrián *et al*., 2012).

Antibiotic resistance

Antibiotic susceptibility test of *Enterococcus* strains was carried out using the method described by Nami *et al*. (2019). Twenty-seven antibiotics were tested: penicillin G (10 µg), ampicillin (10 µg), oxacillin (1 µg), ticarcillin (75 µg), amoxicillin-clavulanic acid (20 µg/10 µg), cefazolin (30 µg), cefalexin (10 µg), cefuroxim (30 µg), cefotaxim (30 µg), cefixim (5 µg), ceftazidim (30 µg), fosfomycin (50 µg), amikacin (30 µg), streptomycin (10 µg), azithromycin (15 µg), erythromycin (15 µg), clindamycin (2 µg), lincomycin (2 µg), fusidic acid (10 µg), trimethoprim-sulfamethoxazol (1.25 µg/23.75 µg), rifampicin (30 µg), nitroxolin (30 µg), amoxicillin (30 µg), cefoxitin (30 µg), kanamycin (30 µg), chloramphenicol (30 µg), and tetracyclin (30 µg). Strains were classified as sensitive or resistant depending on the inhibition clear zone diameters according to the recommendations published by the Antibiogram Committee of the French Microbiology Society (CA-SFM, 2010).

Detection of virulence factors

Detection of the presence of virulence factor genes (Table 2) involved in the cytolysin activator (*cyl*A), enterococcal surface protein (*esp*), gelatinase (*gel*E), endocarditis antigen (*efa*Afs), hyaluronidase (*hyl*), adhesion collagen protein (*ace*), aggregation substance (*asa*1), and vancomycin resistance (*van*A and *van*B), was checked for all strains according to Ben Braïek *et al*. (2017).

Table 2. PCR primers used for detection of virulence determinant genes

Virulence factors	Genes	Primers	Product Sizes (bp)	References
Cytolysin activator	*cyl*A	CYTI: 5'-ACTCGGGGATTGATAGGC-3' CYTIIB: 5'-GCTGCTAAAGCTGCGCTT-3'	688	Ben Braïek *et al*., 2017
Enterococcal surface protein	*esp*	ESP 14F: 5'-AGATTTCATCTTTGATTCTTGG-3' ESP 12R: 5'-AATTGATTCTTTAGCATCTGG-3'	510	Martín-Platero *et al*., 2009
Gelatinase	*gel*E	GEL 11: 5'-TATGACAATGCTTTTTGGGAT-3' GEL 12: 5'-AGATGCACCCGAAATAATATA-3'	213	Martín-Platero *et al*., 2009
Endocarditis antigen	*efa*Afs	EFA-AF: 5'-GCCAATTGGGACAGACCCTC-3'	688	Cebrián *et al*., 2012

		EFA-AR: 5'-CGCCTTCTGTTCCTTCTTTGGC-3'		
Hyaluronidase	*hyl*	HYL n1: 5'-ACAGAAGAGCTGCAGGAAATG-3' HYL n2: 5'-GACTGACGTCCAAGTTTCCAA-3'	276	Martín-Platero *et al*., 2009
Adhesion collagen protein	*ace*	ACE-F: 5'-GAATTGAGCAAAAGTTCAATCG-3' ACE-R: 5'-GTCTGTCTTTTCACTTGTTTC-3'	1008	Ben Braïek *et al*., 2017
Aggregation substance	*asa*1	ASA11: 5'-GCACGCTATTACGAACTATGA-3' ASA12: 5'-TAAGAAAGAACATCACCACGA-3'	375	Ben Braïek *et al*., 2017
Vancomycin resistance	*van*A	VAN-AF: 5'-TCTGCAATAGAGATAGCCGC-3' VAN-AR: 5'-GGAGTAGCTATCCCAGCATT -3'	377	Cebrián *et al*., 2012
	*van*B	VAN-BF: 5'-GCTCCGCAGCCTGCATGGACA-3' VAN-BR: 5'-ACGATGCCGCCATCCTCCTGC-3'	529	Cebrián *et al*., 2012

Results

Identification of the bacteriocin-producing strains

According to the phenotypic characteristics: homofermentative metabolism, ability to grow at 10 °C and 45 °C and in the presence of 6.5% NaCl, and to the metabolic profile obtained using the API 50 CHL identification system, the strains CM9, CM18, and H3 were assigned to the species *E. faecium*.

The results obtained with the primers 27F and 1492R designed to amplify 1500 bp fragment of the 16S rDNA, which was further sequenced, have confirmed that the CM9, CM18, and H3 strains belong to the species *E. faecium*, with a similarity of 99%.

Inhibitory activity

The antimicrobial activity of the *E. faecium* strains was investigated in solid media by the agar spot method against several pathogenic bacteria. As shown in Table 3, all pathogenic bacteria were inhibited by the three strains *E. faecium* CM9, CM18, and H3.

In the well diffusion test, the supernatants of the *E. faecium* strains have exhibited inhibitory activity against *Listeria monocytogenes* CECT 4032. However, no activity was observed against the other pathogenic bacteria.

Table 3. Bacterial strains used as indicator in this study

Pathogenic bacteria	Gram stain	Origins	Spot agar test			Well diffusion test		
			CM9	CM18	H3	CM9	CM18	H3
Staphylococcus aureus CECT 976	Gram-positive	Spanish Type Culture Collection	++	++	+	-	-	-
Salmonella enterica Serovar. Derby CTC 1022	Gram-negative		+/-	+/-	+/-	-	-	-
Listeria monocytogenes CECT 4032	Gram-positive		+++	+++	++	+++	+++	++
E. coli ATCC 23922	Gram-negative	American Type Culture Collection	+++	+++	ND	-	-	-
Staphylococcus aureus ATCC 25923	Gram-positive		+	+	ND	-	-	-
Pseudomonas aeruginosa ATCC 27853	Gram-negative		+++	+++	ND	-	-	-

ND: not determined, -: no inhibition, +: diameter inhibition zone ≤ 5 mm, ++: diameter inhibition zone ≤ 10, +++: diameter inhibition zone > 10 mm, +/-: not clear inhibition

PCR detection of enterocin genes

The genes *ent*A, *ent*B, *ent*P, *ent*AS-48, *ent*MR10, and *bac*31, which encode enterocins A, B, P, AS-48, MR10, and bacteriocin 31, respectively, were investigated in *E. faecium* CM9, CM18, and H3 strains. The PCR products obtained with primers deduced from structural genes of known enterocins were purified and the nucleotide sequence of the amplicon was sequenced. As shown in Figure 1, the PCR results revealed the presence of *Ent*A, *Ent*B, and *Ent*P genes in CM9 and CM18 strains. However, only the *Ent*MR10 gene was detected in H3 strain. The analysis of enterocins A, B, and P gene sequences of CM9 and CM18 strains showed high homology with those of other enterocin genes (A, B, and P, respectively) found in the GenBank database using the BLAST tool.

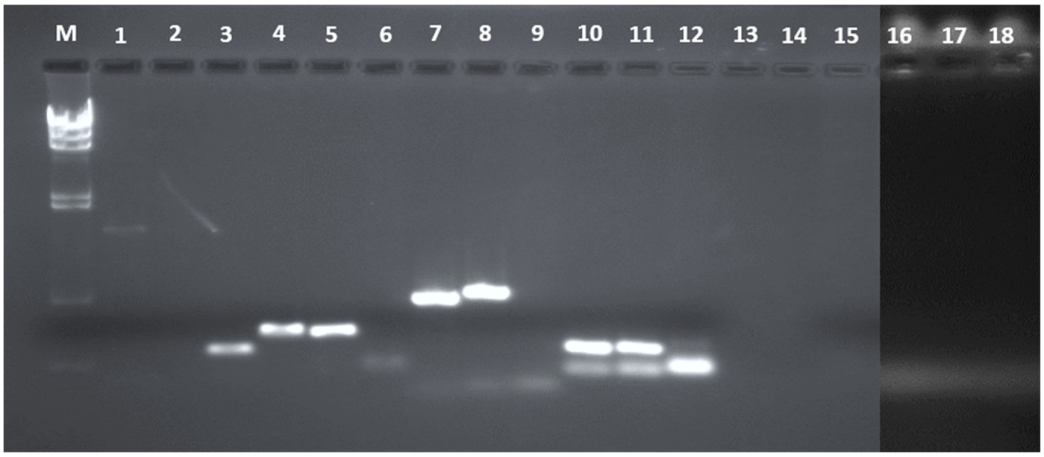

Figure 1. Enterocin gene screening. Electrophoretic analysis of PCR products with different enterocin primers of the strains *E. faecium* CM9, CM18, and H3 using 1.5% agarose gels

Lane M: molecular marker (λ/HindIII); lane 1: CM9 (*ent*MR10A); lane 2: CM18 (*ent*MR10A); lane 3: H3 (*ent*MR10A); lane 4: CM9 (*ent*P); lane 5: CM18 (*ent*P); lane 6: H3 (*ent*P); lane 7: CM9 (*ent*A); lane 8: CM18 (*ent*A); lane 9: H3 (*ent*A); lane 10: CM9 (*ent*B); lane 11: CM18 (*ent*B); lane 12: H3 (*ent*B); lane 13: CM9 (*bac*31); lane 14: CM18 (*bac*31); lane 15: H3 (*bac*31); lane 16: CM9 (*ent*AS-48); lane 17: CM18 (*ent*AS-48); lane 18: H3 (*ent*AS-48)

The result of enterocin MR10A gene sequence analysis of H3 strain showed high homology with enterocin L50A gene. The result showed also that the amplified enterocin B gene of the H3 strain has a molecular weight smaller than that of enterocin B gene, which suggests that the amplification was nonspecific.

Probiotic characteristics of Enterococcus strains

The three strains were further examined for probiotic properties including acid and bile salt tolerance as described by Zommiti *et al*. (2018). The results showed that the three strains have a good tolerance to acidity after exposure to MRS at pH 3.0 (Table 4) and have also tolerance to bile salts at concentration of 40 gL^{-1}, since all of them produced opaque halos around the colonies grown in bile salt-containing MRS agar.

Table 4. Survival of *E. faecium* strains in the medium at pH 3.0

Strains	Viable count (Log10 $CFUmL^{-1}$)			
	Time of exposure (h)			
	0 h	1 h	2 h	3 h
CM9	5.15	5.25	5.48	5.56
CM18	5.20	5.49	5.58	5.23
H3	5.36	5.75	5.53	ND

ND: not determined

The DNase activity was not detected in any of the strains examined in this study.

Table 5 illustrates the antibiotic resistance of the studied strains of *E. faecium* against twenty-seven antibiotics. Strains were commonly susceptible to most clinically relevant antibiotics: penicillin G, ampicillin, oxacillin, ticarcillin, amoxicillin, amoxicillin/clavulanic acid, cefazolin, cefoxitin, cefotaxim, ceftazidim, fosfomycin, clindamycin, lincomycin, fusidic acid, tetracyclin, rifampicin, and chloramphenicol. Also, all strains were resistant against cefalexin, cefixim, amikacin, kanamycin, streptomycin, azithromycin, trimethoprim/sulfamethoxazole, and nitroxolin. However, CM9 and CM18 strains were found to be cefuroxime-resistant and erythromycin-susceptible. Conversely, H3 strain was susceptible against cefuroxime and resistant against erythromycin.

Table 5. Antibiotic susceptibility profile of *E. faecium* strains

Antibiotics	CM9	CM18	H3
Penicillin G, ampicillin, oxacillin, ticarcillin, amoxicillin, amoxicillin/clavulanic acid, cefazolin, cefoxitin, cefotaxim, ceftazidim, fosfomycin, clindamycin, lincomycin, fusidic acid, tetracyclin, rifampicin, chloramphenicol	S	S	S
Cefalexin, cefixime, amikacin, kanamycin, streptomycin, azithromycin, trimethoprim/sulfamethoxazol, nitroxolin	R	R	R
Cefuroxim	R	R	S
Erythromycin	S	S	R

S: sensitive; R: resistant

According to the PCR performed with several primers encoding for some critical virulence factors and vancomycin resistance determinants, none of the studied genes was found in CM9, CM18, and H3 strains (Figure 2).

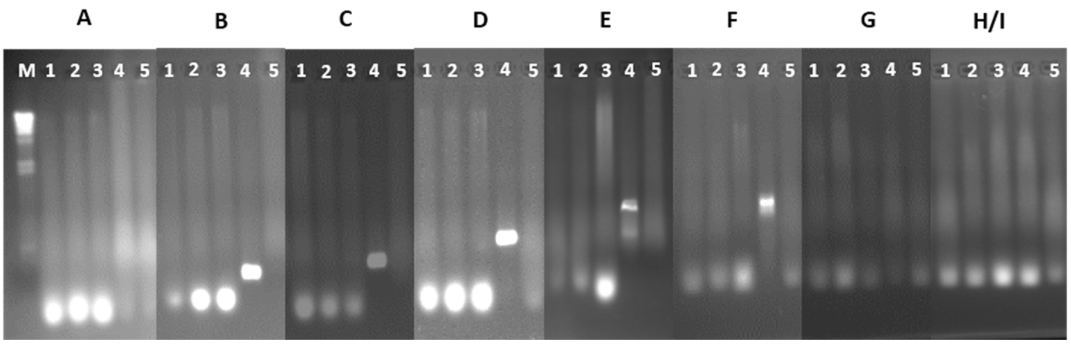

Figure 2. PCR detection of potential enterococcal virulence genes in *E. faecium* CM9, CM18, and H3 strains

A: *cyl*A; B: *esp*; C: *gel*E; D: *efa*Afs; E: *hyl*; F: *ace*; G: *asa*1; H/I: *van*B/*van*A. Lane M: molecular marker (λ/HindIII); lane 1: CM9 strain; lane 2: CM18 strain; lane 3: H3 strain; lane 4: *E. faecalis* 9091 (positive control); lane 5: negative control (no DNA added)

Discussion

The *Enterococcus* genus is naturally present in many Mediterranean foods, and consequently it may be assumed that the local population has consumed these LAB for centuries. Interestingly, enterococci contribute to flavor and aroma and the ripening of foods and, they are used as probiotics (Martín-Platero *et al*., 2009). The present work revealed the comprehensive status of the genetic diversity, antimicrobial profile, and probiotic potential of three bacteriocinogenic *Enterococcus* strains isolated previously from camel milk, and fermented wheat. The strains CM9, CM18, and H3 were classified as belonging to *E. faecium* species on the basis of their morphological, biochemical, and genetic characteristics, together with the sugar fermentation patterns, which are the reliable criteria for distinguishing among the *Enterococcus* species (Martín-Platero *et al*., 2009; Achemchem *et al*., 2012).

The obtained findings revealed that all pathogenic bacteria were inhibited by the identified strains. Similarly, Shehata *et al*. (2016) have reported the ability of LAB among the *Enterococcus* genus to inhibit both Gram-positive and Gram-negative bacteria. *Enterococcus* genus has gained attention due to their ability to produce bacteriocins recognized for their wide-range effectiveness on pathogenic and spoilage bacteria (Hanchi *et al*., 2018). In addition, our previous studies have shown that the inhibitory activity of three strains CM9, CM18, and H3 was due to the production of bacteriocins. This was confirmed using buffered medium and catalase to overcome the inhibitory effects of organic acids and hydrogen peroxide, respectively, and using proteases also completely abolished the activity (Benmouna *et al*., 2018, 2020).

In the well diffusion test, only *Listeria monocytogenes* CECT 4032 was inhibited. This result can be explained by the low concentration of bacteriocins in the CFS able to inhibit pathogenic bacteria or by the resistance of pathogenic bacteria towards bacteriocins. In effect, Achemchem *et al*. (2012) have observed the resistance of *Staphylococcus* sp. and *E. coli* against bacteriocins produced by *E. hirae* F420 isolated from Moroccan raw goat milk. In addition, Ghomrassi *et al*. (2016) have observed that supernatant of six strains of *E. faecium* did not exhibit inhibition against several Gram-negative bacteria, despite the fact that these strains harboring in their genome the enterocin A and B genes.

The ability to produce multiple bacteriocins is a common feature among bacteriocinogenic LAB (Nes *et al*., 2007). Moreover, the production of these enterocins by *E. faecium* was reported previously by Sonsa-Ard *et al*. (2015). The obtained results are in accordance with the literature and, in fact, the enterocin P gene has been always found together with the enterocin A and B genes (Abriouel *et al*., 2006; Rehaiem *et al*., 2014). Both

enterocins are grouped in class II.a bacteriocins, which are very effective against pathogenic bacteria (Karpiński and Szkaradkiewicz, 2013) and some fungal species (Ben Braïek *et al*., 2017). Interestingly, the presence of more than one enterocin gene cluster is due to the remarkable ability of enterococci to disseminate and receive genetic material between strains but also among bacteria (Rehaiem *et al*., 2014). Ogaki *et al*. (2016) have observed that 30% of *E. faecium* strains harbored a combination of the genes *Ent*A and *Ent*P. In another work, Özdemir *et al*. (2011) have noted that the structural genes of enterocins A and B were the most frequent in enterococcal isolates of different sources.

The production of enterocin MR10 was frequently detected in *E. faecalis* strains (Martín-Platero *et al*., 2006; Ruiz-Rodríguez *et al*., 2012). It belongs to the class II.b bacteriocins, a type of N-terminal leaderless peptide. The result of enterocin MR10A gene sequence analysis of H3 strain is in agreement with the literature and, in fact, the enterocin MR10A/B has been detected in the genome of *E. faecalis* (Martín-Platero *et al*., 2006; Nes *et al*., 2007). Interestingly, in previous work the upstream region of the enterocin X locus was found similar to that of the enterocin B locus for *E. faecium* KU-B5. These sequence-similar regions contribute to the formation of almost-identical leader sequences on enterocin B and enterocin Xα prepeptides (Hu *et al*., 2010).

The probiotic potential of bacteria depends on their ability to survive the passageway through the upper digestive tract to the intestine, where their beneficial effect is anticipated, and this represents a crucial condition (Marteau *et al*., 1997; Tuomola *et al*., 2001). According to Maragkoudakis *et al*. (2006), the pH inside the human stomach ranges from 1 (during fasting) to 4.5 (after a meal) and the food digestion can take up to 3 h (Zommiti *et al*., 2018). The three strains have shown good tolerance to acidity and bile salts. Similar results were observed by several researchers (Ben Braïek *et al*., 2018; Zommiti *et al*., 2018). The bile tolerance of strains is subsequently required in order to increase the intestinal survival. This criterion is important to qualify the strains as probiotics (Bustos *et al*., 2012). In the study of Singhal *et al*. (2019), *E. faecium* has shown a remarkable capability to assimilate cholesterol, which was further increased in the presence of bile salts.

None of the *E. faecium* strains showed DNase activity, indicating their safety in potential probiotic preparations. The absence of this enzyme was reported in the majority of *Enterococcus* strains tested in different studies (Cebrián *et al*., 2012; Singhal *et al*., 2019).

The FAO/WHO (2002) recommended that, as a safety measure, the antibiotic resistance profile of a proposed probiotic should also be evaluated. Antibiotic susceptibility testing showed sensitivity of *E. faecium* isolates to most clinically relevant antibiotics. The susceptibility of strains to ampicillin and penicillin is clinically very interesting, since they cure infections with multiple antibiotic-resistant enterococci strains (Favaro *et al*., 2014). In the same test, CM9 and CM18 strains were resistant to cefuroxime and sensitive to erythromycin, which is natural resistance (Lukášová and Šustáčková, 2003).

In order to confer health benefits, probiotics should be lacking pathogenic and transferable antibiotic resistance genes (Fernández *et al*., 2015). Some *E. faecium* strains may harbor an intrinsic antibiotic resistance gene (Özmen Toğay *et al*., 2010; Wardal *et al*., 2014) to adapt to their environment (García-Solache and Rice, 2019). Enterococci survive in environmental conditions that destroy other microorganisms of sanitary significance. Enterococci have been known to be resistant to antibiotics used in clinical practice. They are naturally resistant to cephalosporins, aminoglycosides and clindamycin, and may also be resistant to tetracycline and erythromycin. They are intermediate sensitive to penicillin, ampicillin, and glycopeptides (Lukášová and Šustáčková, 2003). Since these characteristics are strain specific, each strain should be evaluated individually for the safety assessment of potential probiotics (Vankerckhoven *et al*., 2008; Hanchi *et al*., 2018).

The use of molecular procedures to verify the absence of virulence factors in *Enterococcus* sp. is needful due to the risk of genetic transfer, since these genes are usually located in conjugative plasmids (Anagnostopoulos *et al*., 2018). None of the strains harbored genes for virulence factors and, thus, may be considered as safe probiotic candidates (Singhal *et al*., 2019). These results are in agreement with those reported by Ben Braïek *et al*. (2018) for enterococcal strains. Conversely, Chajęcka-Wierzchowska *et al*. (2016) have

reported the presence of *esp*, *gel*E, *efa*, and *ace* genes in the genome of *E. faecium* strains isolated from raw shrimps. The same authors have also reported that none of the *E. faecium* strains isolated from breast milk carried virulence factors. Also, similar results were reported by Franz *et al*. (2001) for food isolates.

The presence of virulence genes is responsible for the persistence of enterococci in the environment, such as, for example, the colonization (*esp*, *efa*, and *ace*), the destruction of host's tissues and the severity of enterococcal infections (*cyl*A, *gel*E, and *hyl*) (Reviriego *et al*., 2005). The absence of virulence factors in *E. faecium* strains could be explained by food origin of the strains studied in this study (Benmouna *et al*., 2020). Remarkably, the difference between clinical enterococcal strains and food enterococcal isolates used as probiotics was more than significant (Suvorov, 2020).

The results concerning the probiotic potential are in accordance with data published by other authors for *Enterococcus* sp. isolated from food ecosystems (Hanchi *et al*., 2018). In a previous study by Benmouna *et al*. (2020), the studied strains have formed biofilm and inhibited biofilm formation of pathogenic bacteria, this feature makes them also interesting candidates as potential tools to control biofilm formation of pathogenic bacteria.

Conclusions

The results of this study indicated that *E. faecium* CM9, CM18, and H3 strains are safe probiotic strains. These strains fulfil several criteria, such as remarkable probiotic potential, resistance to low pH and bile salts, absence of virulence genes, potential production of more than one enterocin, and susceptibility to the majority of antibiotics. The isolates could be used in functional foods since they adapt to the conditions and could provide protection against pathogens. However, further studies will be needed to evaluate probiotic and technological characteristics such as auto- and co-aggregation, absence of cytotoxicity, and cholesterol assimilation.

Authors' Contributions

Conceptualization: Z.B., F.D., and M.M.-B.; formal analysis: Z.B., E.V., and M.M.-L.; funding acquisition: H.Z.-K., N.-E.K., and M.M.-B.; investigation: Z.B., E.V., M.M.-L., and F.D.; methodology: All authors; resources: H.Z.-K., and M.M.-B.; supervision: F.D., and M.M.-B.; validation: Z.B., E.V., M.M.-L., F.D., and M.M.-B.; writing-original draft: Z.B., and M.M.-B.; writing-review & editing: Z.B., and M.M.-B. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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