Biosynthesis and FPLC purification of antibacterial peptide from the biotherapeutic agent *Enterococcus faecium*

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Background
Probiotics are microorganisms that play an essential role in microbial intestinal balance and in health care.
Objective
To isolate a probiotic that can be used to produce antimicrobial peptides potentially used as inhibitors against pathogenic bacteria.
Materials and methods
The research protocol was carried out through isolation of samples from different dairy product and screening for the most potent probiotic exhibiting antimicrobial activity against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739 according to the guidelines of Clinical and Laboratory Standards Institute using the disc diffusion method. The molecular identification of this probiotic strain was done by 16S ribosomal DNA sequencing, and the phylogenetic tree was obtained. The purification process and characterization of the antibacterial peptide were done by $(NH_4)_2SO_4$ and performing fast protein liquid chromatography.
Results and discussion
Bacterial probiotic strains obtained from different samples were screened for the best antimicrobial activity, where isolate number 9 from 18 isolates showed the highest antibacterial activity against *S. aureus* and *E. coli*. Therefore, it was chosen for molecular identification. The molecular identification process revealed that isolate number 9 was *Enterococcus faecium*. Results of antibiotics sensitivity indicated that *S. aureus* is more sensitive to antibiotics than *E. coli.* The fast protein liquid chromatography purification and characterization process of the peptide produced from the probiotic *E. faecium* showed that the active fraction was precipitated at 60% saturation of $(NH_4)_2SO_4$. Moreover, single absorbance peaks confirmed the presence of the peptide 'enterocin.'

Keywords:
16S ribosomal DNA, antibacterial, biotherapeutic agent, *Enterococcus faecium*, fast protein liquid chromatography, probiotic

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Introduction

Probiotics are microorganisms that participate in the microbial intestinal balance and play an essential role in good health maintenance. Probiotics are defined as 'living microorganisms which, when administrated in adequate numbers, confer a health benefit to the host' according to FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food (London, Ontario, Canada, April 30 and May 1, World Health Organization) as described by Mani-Lopez *et al.* [1].

Probiotics have many benefits for the health of the human organs in addition to their benefit in reducing serum cholesterol concentration [2,3]. The use of exopolysaccharides from probiotic *Lactobacillus brevis* as an anticolon cancer remedy was recommended [4]

Bacteriocins produced by lactic acid bacteria (LAB) are used as natural food preservatives. They have gained increased attention because of their inhibitory effect against food-borne pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, and Bacillus cereus* [5,6].

Bacteriocins are proteins or short polypeptides that have antibacterial activity. Bacteriocins perform this antibacterial effect by destroying pathogenic cells with specific surface receptors [7]. In a microbial community, cells can be bacteriocinogenic (produce bacteriocin), sensitive, or resistant to bacteriocin. When all three cell types are present and compete for limited resources, only a small percentage of bacteriocinogenic cells will be induced to produce and release bacteriocin [8]. Some sensitive cells are

immediately destroyed, whereas others have mutations to increase their resistance. Resistant cells can quickly replace producing cells [9].

The bacteriocins of LAB have been classified by Klaenhammer [10] into four classes based mainly on structural characteristics. Most of the bacteriocins isolated so far belong to class I or II. Class I bacteriocins named as lantibiotics are small (<5 kDa) membrane-active peptides that contain amino acid residues like lanthionine. Nisin is the best-studied and well-known lantibiotic [11]. Class II bacteriocins are small, heat-stable, nonlanthionine-containing peptides. Class III consists of large heat-labile bacteriocins (>10 kDa), whereas Class IV complex bacteriocins consist of a protein moiety with one or more other chemical moieties (e.g. carbohydrate and lipid).

Recent studies have shown that it is common for LAB to produce more than one type of bacteriocin, for example, the production of two synergistically acting bacteriocins, enterocin L50A and B [12], plantaricin NC8α and β, and plantaricin Wα and β [13]. The potential of bacteriocins for different applications is determined by their biochemical properties such as their stability and mode of antimicrobial action. Many bacteriocins from LAB have been reported to be produced from probiotics but only a few have been completely purified and characterized [14].

The aim of the current search focuses on the role of probiotics as biotherapeutic agents, as well as screening and identification of a probiotic strain that exhibits an antibacterial effect. Moreover, we focus on the promising antimicrobial activity of this probiotic producing peptide antibacterial agents that have a broad spectrum against gram +ve and gram –ve bacteria.

Materials and methods

Screening for probiotic isolates with antibacterial activity

Isolation of LAB has been carried out from different dairy sources: milk, cheese, and fermented milk. A culture of 18 probiotic bacterial isolates was propagated using growth medium De Man, Rogosa and Sharpe (MRS) broth medium, containing (g/l) peptone, 10.0; meat extract, 8.0; yeast extract, 4.0; D(+) glucose, 20.0; dipotassium hydrogen phosphate, 2.0; sodium acetate trihydrate, 5.0; triammonium citrate, 2.0; magnesium sulfate heptahydrate, 0.2; and magnesium sulfate tetrahydrate, 0.05, with a pH 6.2 [15]. The evaluation process of probiotic properties was carried out. A total of 18 isolates of probiotic bacteria were screened to detect the potency of their antibacterial activity, and further biosynthesis of antibacterial agent was carried out by using MRS broth medium at 30°C under static cultivation conditions. After 24 h of incubation, the bacterial cells were separated from the culture broth by centrifugation, and then the cell-free supernatant was assayed as antibacterial activity against *S. aureus* ATCC 6538 and *E. coli* ATCC 8739.

Antibacterial assay

The antibacterial activity was assayed using agar well-diffusion method as follows: 40.0 ml of nutrient agar medium incubated at 55–60°C was inoculated with 200.0 µl of the pathogenic bacteria cell suspensions under test separately and poured into 150.0-mm diameter Petri dishes, mixed well, and allowed to solidify. After solidification, holes of 5.0-mm diameter were made in the agar plate with the aid of a sterile cork borer. For each sample, duplicate holes were made, and then 100.0 µl of the culture filtrate was poured in the prepared holes using an automatic micropipette. The Petri dishes were kept in the refrigerator for one hour at 4–5°C to permit homogenous diffusion of the antimicrobial agent before growth of the tested probiotics, and then the plates were incubated at 37°C for 24 h for gram +ve and gram –ve bacteria. The antimicrobial activities of the isolates under study were determined by measuring the diameter of inhibition zone [16].

Molecular identification

PCR was used to amplify the 16S ribosomal DNA gene of selected isolate. The 16S ribosomal DNA sequence was determined by direct sequencing. Total DNA was isolated using Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Primers were used for PCR and DNA sequencing. The PCR amplification was performed with the primer pair SPO/SP6 targeted against regions of 16S ribosomal DNA, where StrepF was 5-AAGAGTTTGATCCTGGCTCAG-3 and StrepR was 5-CTACGGCTACCTTGTTACGA-3. Amplification of DNA was performed in a Mastercycler personal thermal cycler (Eppendorf). PCR conditions included a hot start at 96°C (5 min), 35 cycles consisting of hybridization at 50°C (1 min), polymerization at 72°C (2 min), denaturation at 96°C (1 min), and a final extension at 72°C (2 min). PCR products were resolved by electrophoresis in 1% (w/v) agarose gel and visualized by ethidium bromide (1 µl/10 ml) staining. 16S ribosomal DNA PCR applicants were purified

following the microcon YM-100 kit (Microcon centrifuge devices, Bedford, MA, USA) and sequenced using the Big Dye Terminator V3.0 kit as specified by the supplier with primers while automated sequencing of both strands of the PCR products gene sequencer (ABI, Forster City, California, USA). The sequences obtained (500–750 bp) were then assembled in silico (Vector NTI) using overlapping zones between the various sequences to form the contiguous sequence. Phylogenetic analysis was realized by an alignment of sequence consensus of the 16S ribosomal DNA genes collected in an international database (Gene bank). The results were then expressed in percentage of homology between the submitted sequence and the sequences resulting from the database [17].

Antibiotic sensitivity

The antibiotic resistance test of *S. aureus* and *E. coli* ATCC 8739 strains was carried out according to the guidelines of the Clinical and Laboratory Standards Institute and was examined using the disc diffusion method [18,19]. The antibiotics tested covered the main four antibiotic categories based on their mechanism of action including cell wall damage and interference with protein, DNA, and nucleic acid biosynthesis mechanism. Tested plates were incubated at 37°C for 24 h, and the results were read and reported based on the inhibition zone diameter (mm) as sensitive or resistant [20].

Biosynthesis and precipitation of the antibacterial peptide agent from *Enterococcus faecium*

Enterococcus faecium was propagated on 1 l MRS broth that was inoculated with 10 ml of bacterial culture. Turbidity of bacterial growth was calibrated using 0.5 McFarland standard where a hundred microliter (1×10^7 cells/ml) of bacterial growth was used as a standard inoculum and it was incubated for 48 h under static conditions at 30°C. The cell-free supernatant was obtained by centrifugation at 10 000 rpm for 20 min, at 4°C, and freeze-dried using lyophilizer overnight. The precipitation process of the polypeptide was carried out using solid $(NH_4)_2SO_4$ added slowly to crude extract solution until 60% saturation. The mixture was stirred for 2 h at 4°C and later centrifuged at 10 000 rpm for 1 h at 4°C. A dialysis bag was used for 24 h against distilled water with repeated changes of water [21].

Purification and fast protein liquid chromatography characterization of antibacterial peptide

The fast protein liquid chromatography (FPLC) system (ÄKTA Avant 150, GE Healthcare Life Sciences, Staten Island, New york, 10314 USA) process was carried out at the Central Laboratories Network, National Research Centre, Cairo, Egypt, for the purification and characterization of the peptide produced by the most potent probiotic. The antibacterial peptide sample was resuspended in 20 mm of sodium dihydrogen orthophosphate buffer (pH 6.0), and then subjected to FPLC-cation exchange chromatography on a HiPrep SP HP 16/10 column (GE Healthcare) using an AKTA Purifier (GE Healthcare). The absorbed material was eluted with a linear gradient of 0–1 M NaCl in 20 mm sodium dihydrogen orthophosphate buffer (pH 6.0) at a flow rate of 3.0 ml/min. The elution process was monitored by a UV detector at 215 nm [22].

Results and discussion

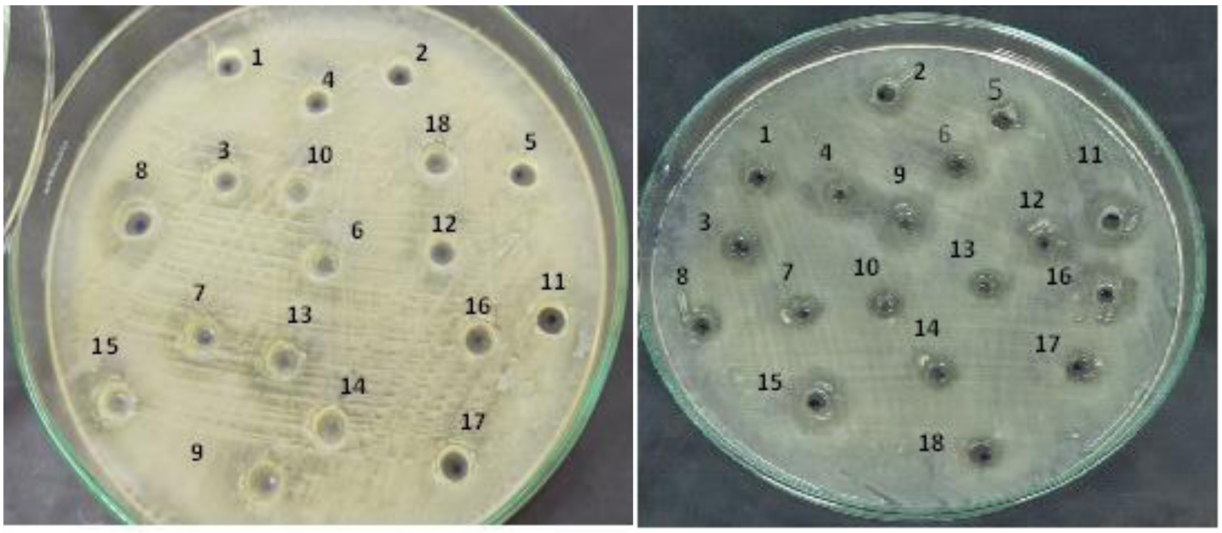
Isolation and screening of probiotics with antibacterial activity

The screening for antibacterial activity of 18 probiotic isolates propagated from different dairy samples was carried out against *S. aureus* and *E. coli*. Results are listed in Table 1 and Fig. 1. Results revealed that five isolates were active against *S. aureus* (numbers 7, 8, 9, 13, and 15), whereas seven isolates were active against *E. coli* (numbers 4, 6, 9, 11, 12, 15, and 16). The broad spectrum was recorded on two isolates: numbers 9 and 15. Results shown clearly demonstrate that probiotic isolate number 15 gave better antibacterial activity with inhibition zones of 21 and 17 mm against *S. aureus* and *E. coli,* respectively.

Table 1 Screening of antibacterial activity for probiotic isolates

	Inhibition zone diameter (mm)	
	Test organisms	
Isolate numbers	*Staphylococcus aureus*	*Escherichia coli*
1	0.0	0.0
2	0.0	0.0
3	0.0	0.0
4	0.0	15.0
5	0.0	0.0
6	0.0	16.0
7	15.0	0.0
8	16.0	0.0
9	11.0	18.0
10	0.0	0.0
11	0.0	19.0
12	0.0	19.0
13	20.0	0.0
14	0.0	0.0
15	21.0	17.0
16	0.0	17.0
17	0.0	0.0
18	0.0	0.0

Figure 1



Staphylococcus aureus ***Escherichia coli***

Screening for antibacterial activity of probiotic isolates against *Staphylococcus aureus* and *Escherichia coli*.

Antimicrobial activity is one of the most crucial selection precedents for effective probiotics [23]. Reported data proved that probiotics are known to be inhibitory to the growth of a wide range of intestinal pathogens in human. In addition to the favorable effects against disease caused by an imbalance of the gut microflora, several experimental observations have revealed a potential protective effect of probiotic bacteria against the development of colon tumors [24].

In the study of Osuntoki *et al.* [25] *Lactobacillus spp.* isolated from fermented dairy products showed antibacterial activity against some clinically important pathogens such as Enterotoxigenic *E. coli* (4.2 mm), *Salmonella typhimurium* (4.3 mm) and *L. monocytogenes* (5.0 mm). Isolates of the present study have better antimicrobial capability than this *Lactobacillus spp.* isolates.

Molecular identification of the most potent isolate

The most potent isolate number 15 was gram +ve, creamy white in color, had round, smooth colonies, was catalase negative, and had antibacterial activity toward both gram +ve and gram –ve bacteria. It was identified using 16S ribosomal DNA gene sequence analysis. According to the sequence and phylogenetic analysis, strain number 15 was identified as *E. faecium* and showed a similarity of 99.8%.

The phylogenetic tree is demonstrated in Fig. 2.

The most potent isolate number 15 was identified as *E. faecium*. These results are in harmony with the results of Saeedi *et al.* [26], proving that 16S ribosomal DNA gene sequencing is a reliable identification method of bacterial strains isolated from various environmental sources and fermentation specimens. In a similar research, *E. faecium* KU-B5 produced enterocin X, which had an antimicrobial activity against different pathogens, as reported by Hu *et al.* [27].

Antibiotic sensitivity of test organisms

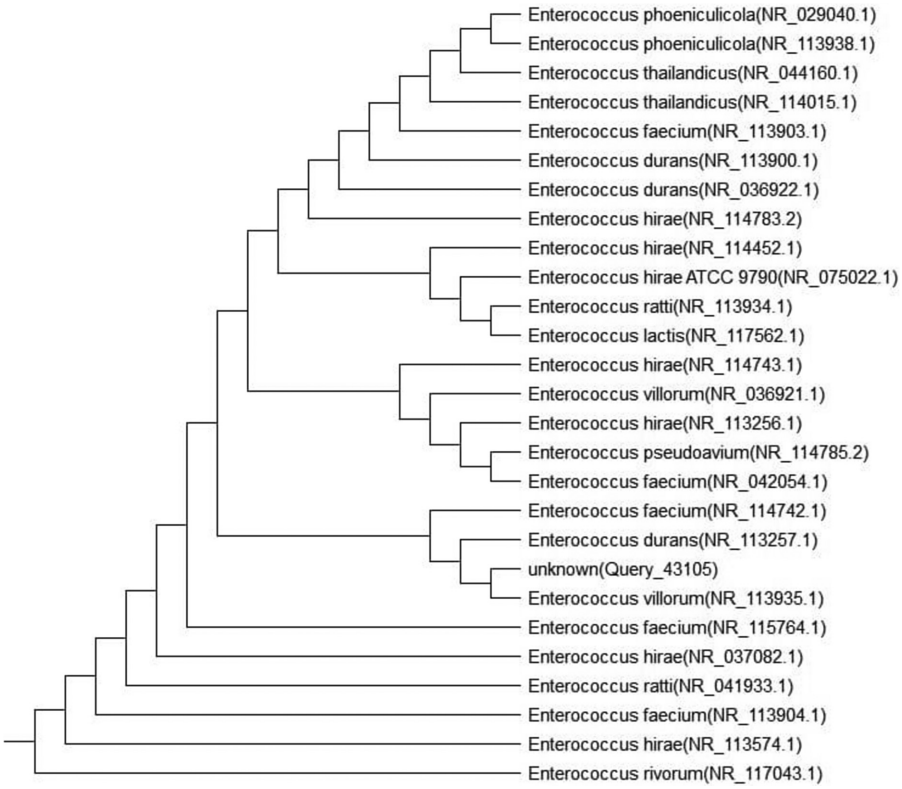
A total of 18 antibiotics were used for the antibiotic sensitivity test of *S. aureus* and *E. coli*. Data in Table 2 and Fig. 3 indicated that *S. aureus* was sensitive to 10 types of antibiotics and resistant to the eight other types. However, *E. coli* showed more resistance to antibiotics. It was sensitive to four types of antibiotics only and resistant to the 14 types.

Biosynthesis, purification, and fast protein liquid chromatography characterization of antibacterial peptide from *E. faecium*

The antibacterial active fraction was precipitated from the filtrate of centrifuged cell suspension by 60% ammonium sulfate saturation. The FPLC was used for purification and characterization of the active peptide as demonstrated in Fig. 4. The precipitated fractions were subjected to FPLC, where a single absorbance peak confirmed the homogeneity of purified enterocin. Enterocin was detected as the major peak, whereas plantaricin was also detected. The separated peaks were designated as enterocin and plantaricin, respectively.

In similar research, *E. faecium* produced enterocin X, which was observed to variably enhance or reduce

Figure 2



Phylogenetic tree based on 16S ribosomal DNA sequences of *Enterococcus faecium*.

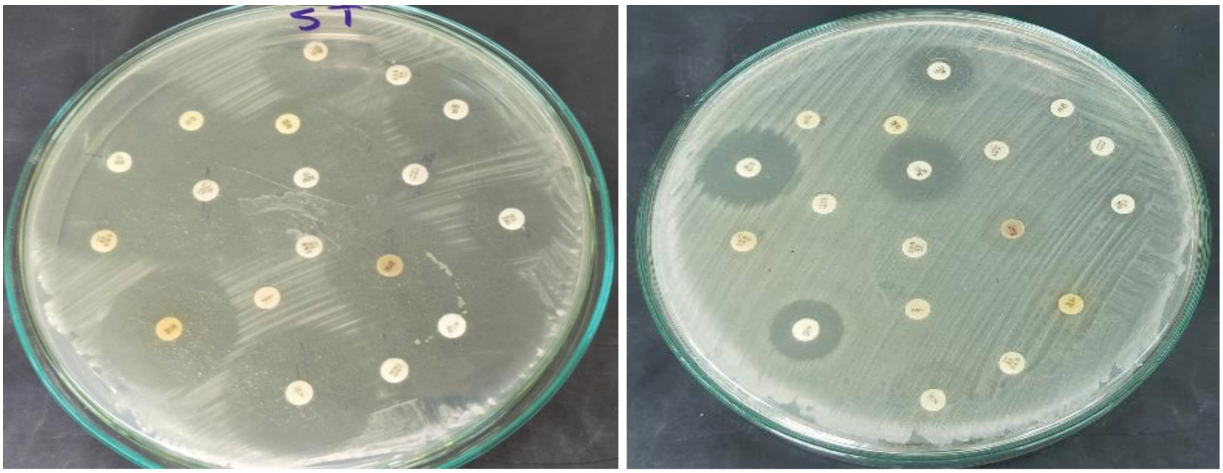
Table 2 Antibiotics sensitivity for test organisms

Antibiotics	Symbol	Inhibition zone diameter (mm)			
		Staphylococcus aureus		*Escherichia coli*	
Tobramycin	TOB (10)	29.0	Sensitive	16.0	Sensitive
Cefotaxime	CTX (30)	0.0	Resist	0.0	Resist
Novobiocin	NV (30)	31.0	Sensitive	0.0	Resist
Ampiclox	AX (25)	00.0	Resist	0.0	Resist
Cephalothin	KF (30)	0.0	Resist	0.0	Resist
Imipenem	IPM (10)	38.0	Sensitive	25.0	Sensitive
Linezolid	LZD (30)	42.0	Sensitive	0.0	Resist
Azithromycin	AZM (15)	30.0	Sensitive	22.0	Sensitive
Cefadroxil	CFR (30)	0.0	Resist	0.0	Resist
Tetracycline	TE (30)	30.0	Sensitive	0.0	Resist
Lincomycin	L (2)	0.0	Resist	0.0	Resist
Ceftriaxone	CRO (30)	0.0	Resist	0.0	Resist
Rifamycin	RF (30)	32.0	Sensitive	0.0	Resist
Cephalaxin	CL (30)	0.0	Resist	0.0	Resist
Piperacillin/tazobactam	TZP (110)	23.0	Sensitive	0.0	Resist
Cephradine	CE (30)	0.0	Resist	0.0	Resist
Kanamycin	K (30)	34.0	Sensitive	0.0	Resist
Streptomycin	S (10)	30.0	Sensitive	17.0	Sensitive

antibacterial activity toward a panel of indicators, as reported by Hu *et al.* [27].

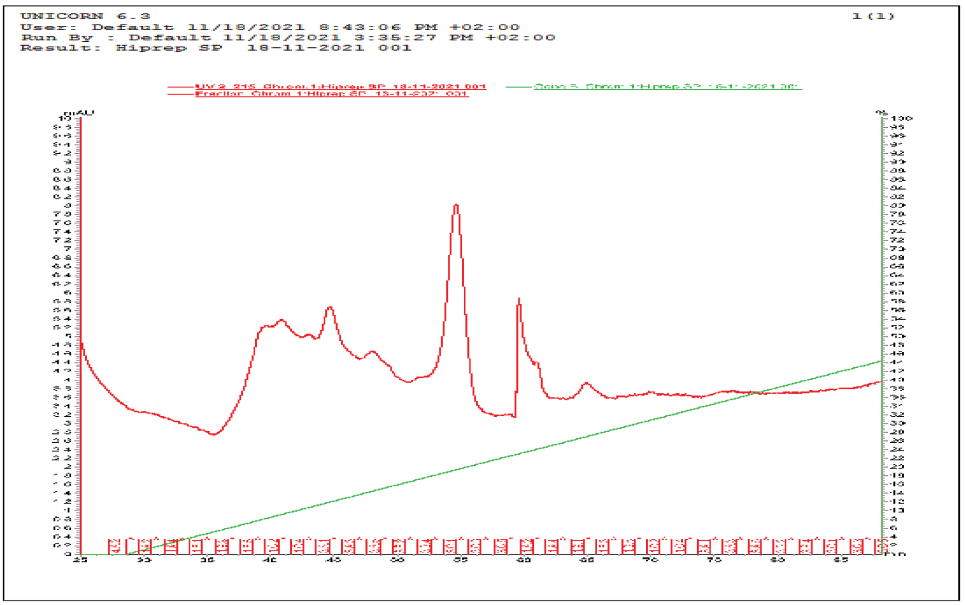
As stated by Savadogo *et al.* [23], antimicrobial effects produced by probiotic isolates were caused by low-molecular-weight antimicrobial substances and bacteriocins. Bacteriocins are peptides with bactericidal or bacteriostatic activities and are found in both gram +ve and gram –ve bacteria. Moreover, enterocins produced by *Enterococci* were found to

Figure 3



Antibiotics sensitivity test for *Staphylococcus aureus* and *Escherichia coli.*

Figure 4



FPLC analysis of *Enterococcus faecium* active peptide. FPLC, fast protein liquid chromatography

exhibit an antimicrobial effect against some human pathogenic bacteria [28].

The mode of action of bacteriocin as bactericidal substance could be attributed to making holes in the cell membrane, making it leaky [29]. It was reported that enterocin and plantaricin acted as complementary to each other in performing the antibacterial effect. This may suggest that it is a two-peptide bacteriocin. However, plantaricin 35d and pediocin PD-1 acted in the same manner [30,31]. On the contrary, some bacteriocins exhibit a bacteriostatic effect like the antimicrobial substance produced by *L*actobacillus *delbrueckii* strain 1043, which demonstrated a bacteriostatic effect [32]. Some bacteriocins, such as thoeniicin 447, are reported to have both modes of action where they are bactericidal for some strains and bacteriostatic for others [33].

Among the reported enterocins, we found enterocin A, which showed antibacterial activity against a wide range of gram +ve bacteria and some gram −ve bacteria [34,35]. Enterocin kills the targeted pathogenic bacteria by making holes in the cytoplasmic membrane [36]. In a study by Ankaiah *et al.* [36], the antibacterial and antibiofilm activities of

a peptide-complex formed of enterocins A and B gave better results than the inhibitory effect of enterocin B alone.

Conclusion

The probiotic *E. faecium* can be used as a promising biotherapeutic agent and it has a broad spectrum against gram +ve and gram −ve bacteria; therefore, it can be tested for further applications in the treatment of pathogenic bacterial infections.

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Conflicts of interest

There are no conflicts of interest.

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