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# Screening of the Enterocin-Encoding Genes and Their Genetic Determinism in the Bacteriocinogenic *Enterococcus faecium* GHB21

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## Abstract

Enterococci are well-known for their ability to produce a variety of antimicrobial peptides called enterocins. Most of these enterocins withstand extreme conditions and are very effective against a broad spectrum of undesirable bacteria including some Gram-negative bacteria. The same enterococci strain can produce multiple enterocins simultaneously. The genetic determinants of these bacteriocins can either be located on plasmids or on bacterial chromosome. Digestion of *Enterococcus faecium* GHB21 plasmids with various restriction endonucleases suggests the presence of two plasmids named pGHB-21.1 and pGHB-21.2 whose respective sizes are ~ 10.0 kb and ~ 3.3 kb. The screening of enterocin-encoding genes among *E. faecium* GHB21 genome by PCR followed by amplicon sequencing indicated the presence of three different enterocin structural genes similar to *ent*A, *ent*B, and *ent*P genes previously detected in other *E. faecium* strains. These enterocin genes were, subsequently, localized on the bacterial chromosome based on PCR-targeted screening using total DNA and plasmids of *E. faecium* GHB21 as separate templates.

**Keywords** *E. faecium* GHB21 · Plasmids · PCR · Enterocin genes · Genetic determinism

## Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides that eliminate closely related bacteria [1–3]. However, some bacteriocins have a broader activity spectrum against more distant species [4, 5]. The bacteriocin-producing genes are commonly plasmid-borne, but they can be located on bacterial chromosome or on mobile genetic elements [1, 6, 7]. When several class II bacteriocins are produced by the same strain, their genetic determinants might be located on bacterial chromosome, on one or different plasmids, or on both chromosome and plasmid [8].

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The genus *Enterococcus* produces a large variety of bacteriocins called enterocins; they are considered as good candidates to be used as food biopreservatives, and could represent, thus, a great alternative to chemical additives which can be harmful to consumer health. In the last decade, many enterocins have been isolated and characterized from different enterococci species and particularly from *E. faecium* and *E. faecalis* [5, 9–13]. The abundance and the notable features of these enterocins have encouraged researchers to suggest specific classification schemes for them [14–16]. Franz et al. [14] have classified enterocins based on their peptide sequence as well as their post-translational modifications. This classification includes four distinct classes: (i) enterocins of class I (lantibiotic enterocins), (ii) enterocins of class II (non-lantibiotic small peptides), (iii) enterocins of class III (cyclic enterocins), and (iv) enterocins of class IV (large proteins). The class II enterocins subdivided itself into two subclasses which are subclass II.1 (pediocin-like enterocins) and subclass II.2 (leaderless enterocins). Lately, Nes et al. [16] proposed a new classification scheme of enterocins including two main classes, based on their structural and their chemical characteristics. In this classification, almost all enterocins belong to class II of bacteriocins except for the two-peptide enterocins, cytolysin ($Cyl_L$ and $Cyl_S$) and enterocin W (Wα, Wβ) produced by *E. faecalis* species [15–17]. Most of

characterized enterocins were isolated from *E. faecium* and *E. faecalis* from different sources, however, they have also been isolated from other species such as *Enterococcus mundtii*, *Enterococcus avium*, *Enterococcus hirae*, and *Enterococcus durans* [5, 15, 16]. Many enterococci strains have been shown to produce more than a single enterocin. In fact, most of *E. faecium* strains harbor at least one enterocin structural gene and more often the two enterocin structural genes *ent*A and *ent*B [18–22]. Moreover, multi-enterocin-producing strains have also been reported by many researchers [11, 12, 23, 24]. The present study provides evidence about the occurrence of multiple enterocin-encoding genes in *E. faecium* GHB21 as well as their chromosomal location.

## Materials and Methods

### Strain and Growth Conditions

The bacteriocinogenic strain *E. faecium* GHB21 isolated from Algerian paste of dates "ghars" was used in this study. This strain has been selected during the previous study due to its remarkable antimicrobial activity against Gram-positive bacteria as well as some Gram-negative bacteria [25]. It was grown at 30 °C, in liquid or solid (1.5% (*w*/*v*) agar) MRS medium [26] (Fluka, Saint Louis, USA), and it was conserved at room temperature after freeze-drying or by storage at − 80 °C either in skimmed milk 10% or in MRS supplemented with 40% glycerol.

### Screening of the Enterocin-Encoding Genes by PCR

In order to investigate the occurrence of enterocin structural genes among *E. faecium* GHB21, we performed a screening using 11 sets of primers specific to 11 well-known enterocin genes. The amplification of the *Adenylate kinase* gene was used as a positive control of PCR. All sets of primers used were synthesized by the Eurofins Genomics (Munich, Germany). They are listed in Table 1.

Chromosomal and plasmid DNA were first extracted from *E. faecium* GHB21 using, respectively, GenElute Bacterial Genomic Kit (Sigma-Aldrich, Saint Louis, USA) and QIAprep Spin Miniprep Kit (Qiagen, UK) as specified by the manufacturers. Several attempts were carried out to confirm the results.

### PCR and DNA Sequencing

The PCR was carried out into a gradient Thermal Cycler (Applied Biosystems, Waltham, MA, USA) in order to identify the best annealing temperature required for an optimal amplification for each set of primers.

The PCR mixture was prepared according to the manufacturer's recommendations for the Kit Taq DNA polymerase (with W-1) (Invitrogen, CA, USA). All obtained amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, UK) and then sequenced at Sanger Sequencing Service, Source BioScience (Glasgow, Scotland, UK). The obtained sequences were subsequently assembled with ChromasPro (version 1.34) and then aligned using both the online tool BLASTn and CLC Genomics Workbench software (version 7.5) in order to confirm their belonging to the group of enterocins.

### Genetic Determinism of Enterocin Structural Genes

In order to find out whether the genetic determinants of enterocins are carried on bacterial chromosome or on plasmids, we performed an enterocin genes-targeted PCR using, both, total DNA and plasmids as separate templates.

### Diagnostic Restriction Digests of Plasmids

In fact, the same plasmid can migrate at different points on the agarose gel due to its conformation. The supercoiled or the covalently bonded circular forms are tightly packed and they run faster in an agarose gel than the nicked-circular form. However, all these forms can be gathered to a single form by plasmid linearization. The linear form of plasmid can be obtained simply by making a single double-stranded cut with a restriction endonuclease. In this contrast, an enzymatic digestion of the plasmid extract was performed in order to know the exact number and sizes of plasmids contained within *E. faecium* GHB21. Plasmids were digested with five unique enzymes which are EcoRI, BamHI, XmaI, SalI, and NdeI (BioLabs, New England, UK). A volume of 0.5 μl of each endonuclease was added separately to an aliquot containing 2.5 μl of Cutsmart buffer (10×), 10 μl of plasmid extract, and 12 μl of sterile distilled water and then incubated for 2 h at 37 °C. After agarose gel electrophoresis, plasmids were recovered from the gel using the Qiaquick Gel Extraction Kit, and sample concentrations were measured using the NanoDrop-2000 (UV-Vis Spectrophotometer, Waltham, MA, USA). Electrophoresis was carried out according to Sambrook and Russell [32]. Total and plasmid DNA were separated using 0.8% (*w*/*v*) agarose while amplification successes were monitored by 1.2% agarose gel electrophoresis.

## Results

### Screening of the Enterocin-Encoding Genes

Plasmid profile analysis of *E. faecium* GHB21 revealed the presence of three distinct plasmid bands whose respective apparent sizes are ~ 8.3 kb, ~ 6.9 kb, and ~ 3.3 kb (Fig. 1).

**Table 1** Primer sets used for the screening of enterocin structural genes

| Peptide | Target | Primers 5′ → 3′ | Size (bp) | References |
|---|---|---|---|---|
| Enterocin A | *ent*A | F GGTACCACTCATAGTGGAAA<br>R CCCTGGAATTGCTCCACCTAA | 138 | [24] |
| Enterocin B | *ent*B | F CAAAATGTAAAAGAATTAAGTACG<br>R AGAGTATACATTTGCTAACCC | 211 | [20] |
| Enterocin L50A | *ent*L50A | F ATGGGAGCAATCGCAAAATTA<br>R TTTGTTAATTGCCCATCCTTC | 135 | [27] |
| Enterocin L50B | *ent*L50B | F ATGGGAGCAATCGCAAAATTA<br>R TAGCCATTTTTCAATTTGATC | 135 | [27] |
| Enterocin P | *ent*P | F ATGAGAAAAAAATTATTTAGTTTAGCTCTTATTGG<br>R TTAATGTCCCATACCTGCCAAACCAG | 216 | [28] |
| Enterocin Q | *ent*Q | F GGAATAAGAGTAGTAGTGGAATACTGATATGAGAC<br>R AAAGACTGCTCTTCCGAGCAGCC | 653 | [29] |
| Enterocin AS-48 | *ent*AS-48 | F GAGGAGTATCATGGTTAAAGA<br>R ATATTGTTAAATTACCAA | 339 | [27] |
| Enterocin 31 | *ent*31 | F CCTACGTATTACGGAAATGGT<br>R GCCATGTTGTACCCAACCATT | 130 | [20] |
| Cytolysin | *cyl* | F GGCGGTATTTTTACTGGAGT<br>R CCTACTCCTAAGCCTATGGTA | 248 | [27] |
| Enterocin CRL35 | *ent*CRL35 | F GCAAACCGATAAGAATGTGGGAT<br>R TATACATTGTCCCCACAACC | 3128 | [30] |
| Mundticin KS | *mun*KS | F TGAGAGAAGGTTTAAGTTTTGAAGAA<br>R TCCACTGAAATCCATGAATGA | 380 | [31] |
| Adenylate kinase | *ad*K | F TTTTTCGTCCCGTCTAAGC<br>R GGGGAAAGGGACACAAGC | 570 | https://pubmlst.org/ |

*F* forward, *R* reverse, *bp* base pairs

In order to detect the enterocin structural genes in *E. faecium* GHB21, we performed a specific amplification using 11 primer sets specific to 11 different enterocins using total DNA as a template (Fig. 2). After gel analysis, five amplification bands have been detected; they correspond respectively to the genes *ent*A, *ent*B, *ent*L50A, *ent*P, and the control gene *adk*. No specific bands were observed for the other primers which indicate the absence of the other enterocin-encoding genes.

The best amplification specificity was observed at 54 °C for primers used. Although, good amplifications have been detected at 52 °C and 56 °C, but with a less intensity for the enterocin P band (data not shown). The obtained amplicon sizes match perfectly the expected sizes, except for the amplicon obtained by the primer sets of the *ent*L50A gene which did not correspond to the expected size.

In order to confirm the presence of the enterocin structural genes, the obtained amplicons were purified using the Clean-up PCR kit (Qiagen, UK) and then sequenced. Sequence alignment analysis with reference sequences using CLC Genomics Workbench software (version 7.5) showed a significant identity degree with the enterocin A, enterocin B, and enterocin P. However, the amplicon obtained with the primer sets of the *ent*L50A gene did not match the expected gene; it corresponds to a small portion from the 23S rRNA gene of *E. faecium* GHB21.

The obtained results indicate the simultaneous presence of the three enterocin-encoding genes: *ent*A, *ent*B, and *ent*P encoding respectively enterocin A, enterocin B, and enterocin P. *E. faecium* GHB21 is therefore a potential multi-bacteriocin-producing strain, which could explain the pronounced activity as well as the broad activity spectrum previously recorded by Merzoug et al. [25]. This activity could be, thus, due to the synergistic effect between these enterocins.

## Genetic Determinism of Enterocin Structural Genes

In order to locate the enterocin structural genes found in GHB21 strain, we performed another specific amplification using total DNA and plasmids as separate templates. We have first determined the exact number of plasmids harbored by GHB21 strain in order to look for the enterocin genes separately in each plasmid. According to the resulting restriction profiles (Fig. 3), the enzymes BamHI, XmaI, and SalI did not cut the plasmids. However, when plasmid extract was digested with either EcoRI or NdeI (lanes 3 and 7), a clear restriction profile change was observed.

The first enzyme EcoRI has probably cut once within the largest plasmid and led to its linearization. We have noticed, thus, the presence of only two distinct bands (~ 10 kb and ~ 3.3 kb). This result suggests that the two bands (~ 8.3 kb, ~ 6.9 kb) previously detected on the top might correspond to a single circular plasmid which adopted two different forms. On the other hand, the action of NdeI seems to be larger since we noticed a complete change of plasmid restriction profile after treatment. Besides, NdeI has probably two cleavage sites on both plasmids; we detected a large band on the top close to

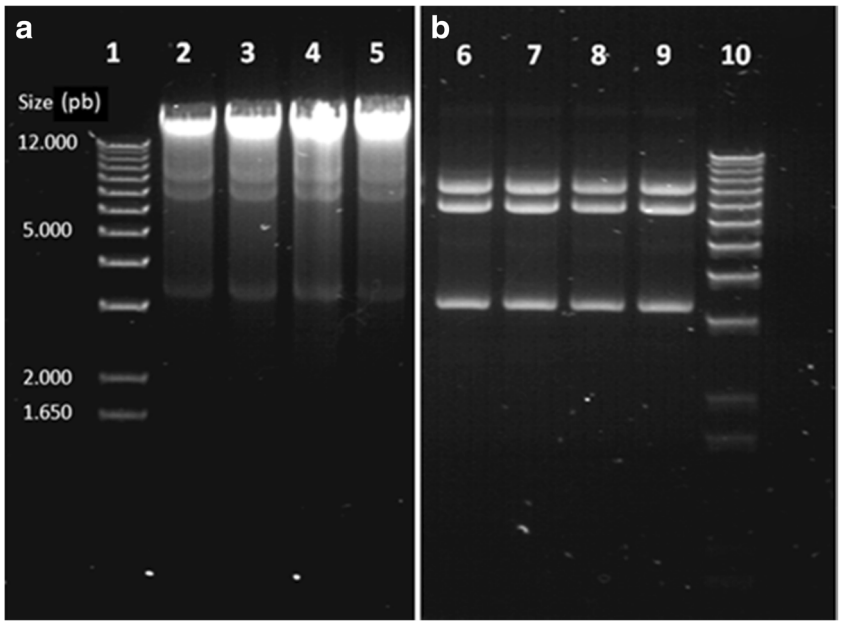


**Fig. 1** Total DNA (**a**) and plasmid profile (**b**) of *E. faecium* GHB21. 1 and 10, DNA marker (1 kb plus DNA Ladder). 2, total DNA (attempt 1). 3, total DNA (attempt 2). 4, total DNA (attempt 3). 5, total DNA (attempt 4). 6, plasmid DNA (attempt 1). 7, plasmid DNA (attempt 2). 8, plasmid DNA (attempt 3). 9, plasmid DNA (attempt 4)

10 kb similar to the previously detected band obtained after linearization of the larger plasmid, and this band corresponds probably to the residual amount of the larger plasmid after partial digestion. In fact, we have recorded the presence of two closely sized bands in the 5-kb area which probably correspond to products of the partial digestion. NdeI has probably cut twice the plasmid at two opposite restriction sites. For the smaller plasmid, two suggestions could be done according to the subsequent profile; NdeI has probably multiple cleavage sites on this plasmid which probably generated several undetectable small restriction fragments, or maybe it has cut once the supercoiled form which resulted in its linearization and led to a 4.7-kb band. These results suggest the presence of only two plasmids in *E. faecium* GHB21, named hence, pGHB-21.1 (~ 10 kb) and pGHB-21.2 (~ 3.3 kb). These two plasmids were then recovered from agarose gel and used separately as templates for the PCR-based localization of the enterocin genes.

According to the PCR-based screening, the three enterocin structural genes are located on the chromosome of *E. faecium* GHB21. No amplification was recorded when using plasmids as templates (see Fig. 4).

## Discussion

Plasmids with similar apparent sizes to those harbored by *E. faecium* GHB21 have already been found in other enterococci, such as the rolling circle replicating plasmids pJS42 (4.1 kb) in *E. faecium* JH95 [33] and pNJAKD (3.8 kb) in *E. faecium* [34]; and also the theta-replicating plasmids pGL (8.3 kb) in *E. durans* 41D [35], pAMα1 (9.8 kb) in *E. faecalis* DS5 [36], and pJS33 (3.1 kb) in *E. faecium* JH95 [35].

Enterocins similar to those detected in *E. faecium* GHB21 have already been isolated and characterized by other researchers, all of which have demonstrated their effectiveness against various microbes [1, 5, 37]. In fact, enterocin A is a subclass IIa bacteriocin belonging to the pediocin-like group; it was first identified in *E. faecium* CTC492 by Aymerich et al.

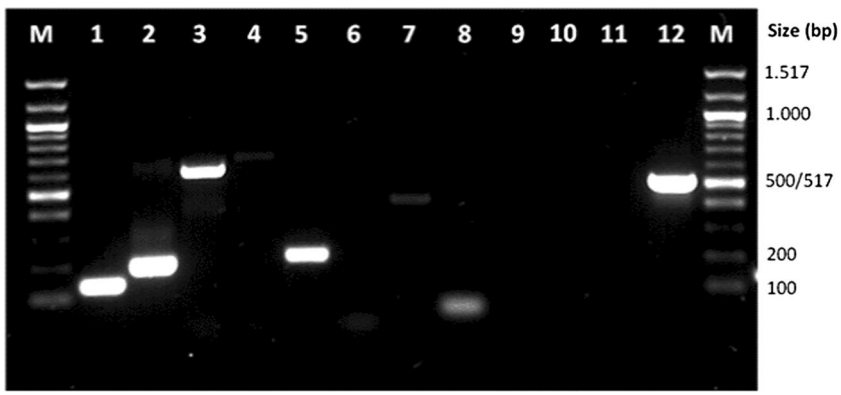


**Fig. 2** Screening of enterocin structural genes in *E. faecium* GHB21 genome. M, DNA marker (100 pb DNA Ladder). 1, *ent*A. 2, *ent*B. 3, *ent*L50A. 4, *ent*L50B. 5, *ent*P. 6, *ent*Q. 7, *ent*AS-48.; 8, *ent*31. 9, *cyl.* 10, *ent*CRL35. 11, *mun*KS. 12, *ad*K (control)

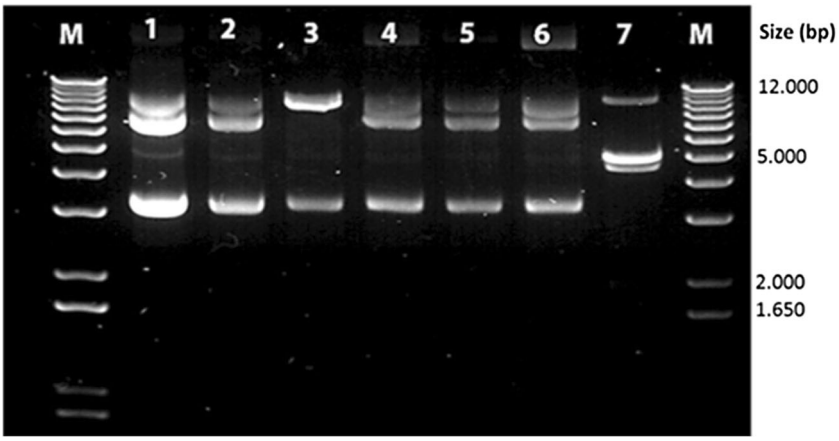


**Fig. 3** Restriction profile of the plasmid extract from *E. faecium* GHB21. M, DNA marker (1 kb plus DNA Ladder). 1, untreated control. 2, control treated at 37 °C/2 h. 3, plasmids + EcoRI. 4, plasmids + BamHI. 5, plasmids + XmaI. 6, plasmids + SalI. 7, plasmids + NdeI

[38] and then found in a wide variety of *E. faecium* species, is a subclass IId bacteriocin according to Franz et al. [14], but due to its unique structural characteristics that do not correspond to any of the subclasses proposed so far, it has been reclassified into a separate group of class II bacteriocins by Nes et al. [16]; enterocin B was first identified by Casaus et al. [18] in *E. faecium* T136. Finally, enterocin P is a subclass IIa bacteriocin belonging to the pediocin-like group and it was identified at the first time by Cintas et al. [39] in *E. faecium* P13.

Some multi-producing bacteriocin strains have already been reported in earlier research; it is the case of *E. faecium* JCM 5804T [24], *E. faecium* B1 [23], and *E. faecium* [11] which, like *E. faecium* GHB21, have been shown to carry the three enterocin structural genes, *ent*A, *ent*B, and *ent*P. In addition, among 13 enterococci isolated by Mojsova et al. [12], 4 were found to be holders of three different enterocin genes: *E. faecalis* 4 (*ent*P, *ent*B, and *cyl*), *E. faecium* 15 (*ent*A, *ent*B, and *ent*P), *E. faecium* 29 (*ent*A, *ent*B, and *ent*P), and *E. hirae* 36 (*ent*A, *ent*P, and *ent*EJ97). Likewise, 45% of *E. faecium* strains isolated by Brandão et al. [40] contain three enterocin structural genes: 9.1% (*ent*P, *ent*A, and *ent*B), 4.5% (*ent*P, *ent*A, and *hir*JM79), and 27.3% (*ent*P, *ent*L50A, and *ent*L50B). On the other hand, enterococci strains isolated by Javaherzadeh et al. [41] carry at least two enterocin structural genes. Similarly, García de Fernando [10] noted the presence of one or a combination of these enterocin structural genes, *ent*A, *ent*B, *ent*P, *ent*Q, *ent*AS-48, *ent*L50A/B, *bac*31, and *cyl* in some enterococci strains.

According to Izquierdo et al. [42], the genus *Enterococcus* is one of the most LAB genera known for its ability to produce several bacteriocins by the same strain. However, the occurrence of strains harboring three enterocin genes at the same time remains lower than that harboring one or two enterocin-encoding genes. Some strains carrying two enterocin genes at a time have also been cited before; it is the case of *E. faecium* T136 [18], *E. faecium* WHE 81 [19], *E. faecium* LMG 11423T, *E. faecium* RZS C5, *E. faecium* RZS C13 [20], *E. faecium* FAIR-E 406 [22], and *E. faecium* CN-25 [21]. All these strains contain within their genomes the two enterocin-encoding genes *ent*A and *ent*B. In fact, these two enterocins are usually identified together in the same isolate and are well-known for their synergistic activity against a wide range of Gram-positive bacteria including *Listeria*, *Clostridium*, *Staphylococcus*, *Propionibacterium*, and also against some LAB species [18, 21, 37]; this may explain the strong inhibitory activity exerted by the partially purified bac-ghb21 against *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 noticed by Merzoug et al. in a previous study [25]. In fact, the presence of *ent*B gene was always associated with the presence of

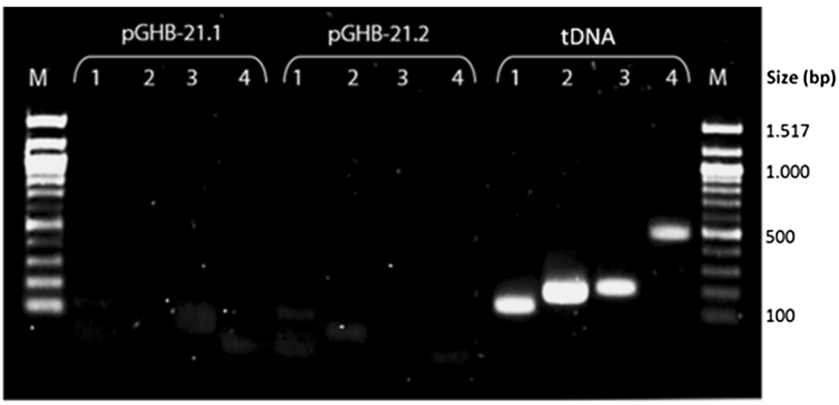


**Fig. 4** PCR-based genetic localization. M, DNA marker (100 pb DNA Ladder). 1, *ent*A. 2, *ent*B. 3, *ent*P. 4, *adk* (control). pGHB-21.1 and pGHB-21.2, plasmides of GHB21. tDNA, total DNA

the *ent*A gene; this may be especially due to the absence of the genes encoding the ATP-binding cassette (ABC) transporter and the accessory protein in the *ent*B operon [27, 40, 41, 43–45].

All the enterocin-encoding genes previously detected by PCR-targeted amplification were localized on *E. faecium* GHB21 chromosome. This result seems to be in agreement with those obtained by Aymerich et al. [38] who demonstrated the chromosomal location of *entA* gene cluster, Franz et al. [14] who detected the *ent*B structural gene on the chromosome of *E. faecium* BFE 900, and Cintas et al. [39] who demonstrated the chromosomal location of *ent*P gene in *E. faecium* L50. In addition, Hu et al. [46] have localized the enterocin structural genes *ent*A and *ent*B on *E. faecium* KU-B5 genome. Likewise, genome analysis of *E. faecium* CRL 1879 indicated the presence of several enterocin-encoding genes which are *ent*A, *ent*B, *ent*P, *ent*SE-K4-like, and *ent*X [9]. However, Achemchem et al. have localized *ent*P structural gene on 65-kb plasmid of *E. hirae* F420 [47].

## Conclusion

*E. faecium* GHB21 is a potential multi-bacteriocin-producing strain previously characterized and its antimicrobial activity was demonstrated against a wide range of Gram-positive and Gram-negative bacteria. Diagnostic restriction digests of *E. faecium* GHB21 plasmids with various restriction endonucleases suggest the presence of two plasmids within this strain named pGHB-21.1 (~ 10.0 kb) and pGHB-21.2 (~ 3.3 kb). The search for enterocin-encoding genes among *E. faecium* GHB21 genome by PCR indicated the presence of three enterocin structural genes, *ent*A, *ent*B, and *ent*P encoding, respectively, enterocin A, enterocin B, and enterocin P. Furthermore, genetic determinism study indicated a chromosomal location of the three detected enterocin genes according to PCR-targeted amplification from both chromosomal and plasmid templates. The chromosomal location of the enterocin structural genes could be the reason of the stable antimicrobial activity of *E. faecium* GHB21. This strain could, thus, represent an excellent candidate to be used as a bacteriocin-producing starter culture without a risk of losing its activity unlike some bacteriocinogenic strains whose bacteriocin structural genes are plasmid-borne.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

For this type of study, formal consent is not required.

This manuscript has not been published and is not under consideration for publication elsewhere.

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