Physiological and molecular insights of bacteriocin production by *Enterococcus hirae* ST57ACC from Brazilian artisanal cheese

Valéria Quintana Cavicchioli¹ · Svetoslav Dimitrov Todorov^{1,2} · Ilia Iliev³ · Iskra Ivanova⁴ · Djamel Drider⁵ · Luís Augusto Nero¹

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Abstract

The bacteriocinogenic *Enterococcus hirae* ST57ACC recently isolated from a Brazilian artisanal cheese was subjected here to additional analyses in order to evaluate its bacteriocin production and the potential influence of ABC transporter system in its expression. Besides these physiological and molecular aspects, the bacteriocin was evaluated for its cytotoxicity against HT-29. Differences in the inoculum size had no impact on the growth of *E. hirae* ST57ACC; however, the bacteriocin was only produced after 9 h of growth when the strain was inoculated at 5% or 10% (v/v), with similar levels of bacteriocin production obtained by both conventional growth and batch fermentation. Furthermore, potential expression of ABC transporters corresponding to the bacteriocin transport and sugar metabolism was identified. In terms of adverse effects, when a semi-purified fraction of the bacteriocin and the cell-free supernatant were tested against HT-29, total cell viability was similar to observed on untreated cells, indicating the absence of cytotoxic effect. Based on the obtained results, *E. hirae* ST57ACC can produce its bacteriocin at industrial level by using bioreactors, its bacteriocin expression is potentially influenced by the ABC transporter system, and no cytotoxic effects were observed on HT-29 cells, indicating its potential use as a bio-preservative.

Keywords *Enterococcus hirae* · bacteriocin · ABC transporter · cytotoxicity

Introduction

Enterococcus spp. are known to be largely distributed in the environment as they have been isolated from humans, animals, soil, surface waters, plants and vegetables [1, 2]. At

medical level, *Enterococcus* is known as an important opportunistic pathogen, especially *E. faecalis* and *E. faecium*, causing a wide variety of infections. However, *Enterococcus* are associated with the development of sensory characteristics in fermented food, delineating rich and diverse metabolic pathways [1–3]. These microorganisms are also endowed with antagonistic properties attributed to their abilities to produce a variety of antimicrobial compounds, such as organic acids and bacteriocins [4].

Bacteriocins are characterized as antimicrobial peptides with 2 to 10 kDa, ribosomally synthesized by bacteria with activity against closely related bacterial targets [5]. These peptides are usually classified in two major groups: class I, the most extensively studied and including peptides that present lanthionine and β -methyllanthionine, and class II, called “unmodified” bacteriocins [5, 6]. The bacteriocins produced *Enterococcus* spp. are usually named as enterocins, and they are being increasingly studied for applications in both human and veterinary medicine [4, 7, 8]. Most of known enterocins are produced by *E. faecium* and *E. faecalis* [4], but only a few studies describe the enterocins produced by *E. hirae* [9–13]. In general, these enterocins belong to class II [4] and some of

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✉ Luís Augusto Nero
nero@ufv.br

¹ Departamento de Veterinária, Universidade Federal de Viçosa, Campus UFV, Viçosa, MG 36570-900, Brazil

² Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, São Paulo, SP 05508-000, Brazil

³ Department of Biochemistry and Microbiology, Faculty of Biology, Plovdiv University, 4 Tzar Asen Str, 4000 Plovdiv, Bulgaria

⁴ Department of General and Applied Microbiology, Faculty of Biology, Sofia University “St. Kliment Ohridski”, 8 Dragan Tzankov Blvd., 1164 Sofia, Bulgaria

⁵ Université de Lille, INRA, Université d’Artois, Université du Littoral-Côte d’Opale, EA 7394-ICV-Institut Charles Viollette, F-59000 Lille, France

them are described as possessing a high inhibitory potential against Gram positive and negative bacteria [10, 12, 13].

E. hirae ST57CC has been recently isolated from a Brazilian artisanal cheese and characterized as a bacteriocin-producing strain [14]. Based on its virulome and resistome, *E. hirae* ST57CC can be considered as a safe bacteriocinogenic strain [15]. Considering the inhibitory potential and safety of *E. hirae* ST57CC, this study aimed to assess the interference of some industrial conditions for bacteriocin production, the potential role of ABC transporter system in bacteriocin expression and the cytotoxicity of the purified peptide against a human cell line.

Material and Methods

E. hirae ST57ACC: bacteriocin producer strain

E. hirae ST57ACC was previously isolated from a Brazilian artisanal cheese [14] and maintained in de Man, Rogosa and Sharpe broth (MRS, BD, Franklin Lakes, NJ, USA) supplemented with glycerol 20% (v/v) and stored at -20°C . Before use, an aliquot of 100 μL of the stored culture was transferred to 5 mL of MRS broth and incubated at 37°C for 18 h.

Interference of industrial conditions for bacteriocin production

MRS broth (100 mL, BD) was inoculated with *E. hirae* ST57ACC at 1%, 2%, 5%, and 10% (v/v) and incubated at 37°C . Samples were collected at different time intervals (0, 3, 6, 9, 18, and 24 h) and subjected to pH measurement (Hanna Instruments, Vöhringen, Germany) and spectrophotometry at 600 nm in order to determine the optical density (Beckman Coulter®, Brea, CA, USA). Bacteriocin production was detected using a qualitative assay previously described by Todorov [16]. In summary, *E. hirae* ST57ACC was centrifuged (12,000 $\times$ g, 10 min, 4°C) and the pH of the cell-free supernatant (CFS) was adjusted to 6.5 with 1 M NaOH. The supernatant was heat treated (10 min at 80°C), and then 10 μL of the treated CFS was spotted onto the surface of a brain heart infusion (BHI) agar plate supplemented with 1% agar (BD), previously inoculated with a culture of the target *E. faecium* ATCC 19443 strain (at 10^6 colony forming units per mL, CFU/mL). The plates were then incubated at 37°C for 24 h. Inhibition halos larger than 2 mm were considered as indication of bacteriocin production. This experiment was conducted in duplicate, in two independent repetitions.

MRS broth (BD) was inoculated with *E. hirae* ST57ACC at 5% (v/v), transferred to sterile flasks (500 mL) for conventional growth and to a bioreactor (3,000 mL, Infors-HT, Bottmingen, Switzerland) for batch fermentation. Conventional growth was performed at 37°C ; batch

fermentation was performed at 37°C with agitation (50 rpm), with constant adding of 1 M NaOH to keep pH stable at 5.5. For both growth conditions, samples were taken after 0, 6, 12, and 24 h of incubation and subjected to spectrophotometry and pH measuring, as described above. Bacteriocin production was measured using a quantitative assay, as described by Campos et al. [17] and Todorov [16]. In brief, CFS was obtained and treated, as described above, and then twofold diluted in 10 mM phosphate buffered saline (PBS) at pH 6.5. Aliquots (10 μL) of the diluted CFS were spotted onto the surface of BHI agar (BD), previously plated with the target strain *E. faecium* ATCC 19443 (at 10^6 CFU/mL). The plates were then incubated at 37°C for 24 h. Bacteriocin activity was expressed as arbitrary units per mL (AU/mL), corresponding to the reciprocal of the highest dilution that presented a detectable halo of inhibition (higher than 2 mm). This experiment was conducted in duplicate, in two independent repetitions.

Preliminary screening for expression of ABC-transporter-related genes

Based on a study focused on expression of ABC-transporter-related genes by *Lactobacillus* [18], the bacteriocinogenic *E. hirae* ST57ACC was subjected to a similar protocol in order to conduct a preliminary assay regarding the relevance of this system in the expression of its bacteriocin. A culture of *E. hirae* ST57ACC was subjected to RNA extraction using the GeneMATRIX Universal RNA Purification Kit (EURx Ltd., Gdansk, Poland) according to manufacturer's instructions. The obtained RNA was used for measuring the expression of the genes associated with ABC-transporter system, using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Brea, CA, USA), as previously described [18]. The housekeeping gene *kanR* was used as an external control. Primers were designed based on the complete genome of *L. plantarum* subsp. *plantarum* ST-III (CP002222.1); primers sequences, gene functions and length of products are presented in Table 1.

Reverse transcriptase (RT) reactions were prepared with a final volume of 20 μL , containing 3 μL of DNase/RNase free water, 4 μL of $5\times$ RT buffer, 5 μL *kanR* RNA, 1 μL RT enzyme (GenomeLab™ GeXP Start Kit, Beckman Coulter), 2 μL of primers (10 pmol/mL), and 5 μL of RNA. Then, reactions were conducted based on the following protocols: 1 min at 48°C , 60 min at 42°C , 5 min at 95°C , and held at 4°C (Thermal Cycler, VWR, Radnor, PA, USA). Each experiment included a RT-negative and a no-template control (NTC).

PCR samples were prepared to a final volume of 20 μL , containing 4 μL $5\times$ PCR buffer, 4 μL 25 mM MgCl_2 , 0.7 μL Thermo-Start DNA polymerase (GenomeLab™ GeXP Start Kit, Beckman Coulter), 2 μL of primers plex (10 pmol/mL),

Table 1 Primers used in the study expression of ABC transporters related genes by *Enterococcus hirae* ST57ACC

Primer	Sequence	Function	Length of the product (bp)	Genome region*	Protein**
zj316_2428 F	AGGTGACACTATAGAAATACGGTTCGTCGAACCTAACA	Efflux ABC transporter,	347	2195262..2197025	ADN99272.1
zj316_2428 R	ATAAGCGGTGTTCAGGCGAAGTACGACTCACTATAGGGA	ATP-binding and permease protein			
LBP_eg0987 F	AGGTGACACTATAGAAATACCGGCAACGAGTAGCTT	Sugar ABC transporter,	564	1179411..1180517	ADN98288.1
LBP_eg0987 R	TGGACCTGACAGATTGTGCGTAGCACTCACTATAGGGA	ATP-binding protein			
dhL1 F	AGGTGACACTATAGAAATACCTGCGGCAAGTACCCCAAT	Malate/lactate dehydrogenase	251	1907270..1908202	ADN98990.1
dhL1 R	GCCGGATTATTCGCAAGCAGGTACGACTCACTATAGGGA				
msmK1 F	AGGTGACACTATAGAAATACCGGTGCAATTCGGAAGAC	Multiple sugar ABC transporter,	413	3198900..3200036	ADO00175.1
msmK1 R	GTACGGATGCACCGAATCTTGACGACTCACTATAGGGA	ATP-binding protein			
plnG F	AGGTGACACTATAGAAATATGCCCCTTTCTTGTGACCCG	Bacteriocin ABC-transporter,	226	368888..371038	ADN97575.1
plnG R	CCCACCCTGCCAATGACTGTACGACTCACTATAGGGA	ATP-binding and permease protein			
JDM1_2227 F	AGGTGACACTATAGAAATACGGTCCAAATTTGTGCGGT	ABC transporter ATP-binding protein	373	2433365..2434108	ADN99496.1
JDM1_2227 R	TTAGGGATGGAGGCTGTGGAGTACGACTCACTATAGGGA				
gluc F	AGGTGACACTATAGAAATATCTTTGGCGGTACTGGTGAC	Glucose-6-phosphate 1-dehydrogenase	358	2345864..2347348	ADN99419.1
gluc R	TGTTGGCAATCGTACCGAAGTAGCACTCACTATAGGGA				
Aldeh F	AGGTGACACTATAGAAATACAAATGGCTCGGCCATTACG	Alcohol dehydrogenase	429	1451918..1452961	ADN98542.1
Aldeh R	CCATTGTGCTGCCGATCTTCGGTACGACTCACTATAGGGA				

*Primers designed based on full genome of *Lactobacillus plantarum* subsp. *plantarum* ST-III (CP002222.1)

**Access number, GenBank (NCBI, Bethesda MD, USA)

and 9.3 µL of cDNA samples from the RT plate. PCRs were conducted in a thermal cycler (VWR) under the following conditions: 10 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 68 °C, and then the reactions were held at 4 °C.

Then, the GeXP fragment and data analyses followed the manufacturer instructions (Beckman Coulter). Aliquots of 1 µL from the PCR products were added to the appropriate wells of a 96 well microplate. DNA size standard 400 (0.5 mL, GenomeLab™ GeXP Start Kit, Beckman Coulter) was added to 38.5 µL of sample loading solution, thoroughly mixed and added to the 96 well microplate. The PCR product was separated based on the fragment size by capillary gel electrophoresis (GenomeLab™ GeXP Genetic Analysis System, Beckman Coulter). The strength of the dye signal was measured after normalization to *kanR* RNA, using the GeXP express profiler software (Beckman Coulter). Results were expressed as either absence (–) or different degrees of gene expression (+ weak, < 100 units; ++ medium, 100–300 units; +++ high, > 300 units).

Bacteriocin purification

E. hirae ST57ACC was grown in BHI broth (BD) at 37 °C for 18 h. The culture was centrifuged at 4 °C and 10,000×g, the pH of the CFS corrected with 3 M NaOH to pH 6.5 and then heated to 80 °C for 10 min. For gel filtration (GF), the resin CM Sephadex® C-25 (GE Healthcare Life Sciences, Milwaukee, WI, USA) was washed with 3 column volumes (20 mL) of distilled water and then charged with 40 mL of CFS; the resin-bound bacteriocin was eluted with 2 column volumes in a gradient of 0.5 M, 1.5 M, and 4 M NaCl until complete elution. Active fractions were further subjected to solid phase extraction (SPE) on C-18 silica-based columns (45 µm, 60 Å, Sigma-Aldrich, St. Louis, MI, USA), using 20%, 60%, and 80% acetonitrile (ACN) solutions (v/v) containing 0.1% (v/v) trifluoroacetic acid (TFA). The samples were then evaporated in a Speed-Vac (Savant, Thermo Fisher Scientific, Waltham, MA, USA).

Further purification of the bacteriocin was performed using reversed-phase high-performance liquid chromatography (RP-HPLC), on a C-18 silica-based column (5 µm, 300 Å, VWR Corp., Radnor, PA, USA). Bacteriocin was eluted using the following linear gradient: ACN, 0.1% TFA and water (at 0 min 80% water, 20% [ACN/0.1% TFA] to 40 min 20% water, 80% [ACN/0.1% TFA]). After each purification step, the protein concentration was measured using a bicinchoninic acid assay (Sigma-Aldrich) and the antibacterial activity measured using the quantitative assay as described above; the target strain was *Listeria monocytogenes* 162, previously obtained from food [19].

Bacteriocin cytotoxicity

A cytotoxicity test was performed on HT-29 cells, grown in 96 well tissue culture plates for 48–72 h, with 5% CO₂, and in Dulbecco's Modified Eagle Medium (DMEM). The active fraction obtained by solid phase extraction (corresponding to the fraction eluted by 60% ACN) and the CFS were tested. Non-diluted aliquots of samples were added to the cell culture monolayer and incubated for 24 h at 37 °C. After incubation, the supernatants were removed, and the HT-29 monolayer was washed twice with DMEM medium containing gentamicin (8 µg/mL) and fetal bovine serum. In addition, the CCK-8 assay (Dojindo Molecular Technologies, Rockville, MD, USA), based on the reduction of the tetrazolium salt by active mitochondria, was used to assess cell viability of the treated HT-29 cells. Plates were then read at 450 nm in a microplate reader spectrophotometer (Xenius, Safas, Monaco). 0.1% Triton X-100 was used as a cytotoxic control and non-treated cells were used as negative control.

Results

Interference of industrial conditions for bacteriocin production

The growth of *E. hirae* ST57ACC with different inoculum concentrations is presented in Fig. 1. During the first 9 h of growth, the optical density (OD) appeared proportional to the inoculum size (Fig. 1). Then, from 9 to 24 h, the registered OD values were similar, discarding the effect of the initial inoculum (Fig. 1). Changes in pH were overall similar throughout the experiment, decreasing from 6 to 4, after 24 h of growth (Fig. 1). Yet, bacteriocin production was detected only after 9 h of growth when *E. hirae* ST57ACC was inoculated at 5% or 10%. The production of bacteriocin remained detectable in such conditions up to 24 h of growth (Fig. 1). Usually, the inoculum size has no effect on bacterial growth; however, bacteriocin production could be affected. Regarding these findings, the initial inoculum of 5% of the bacteriocin-producing strain was then standardized in this study.

To gain more insights on the bacteriocin production by *E. hirae* ST57ACC, the growth dynamics of this bacteriocinogenic strain was established (Fig. 2), upon its conventional growth in MRS broth, as well as under fermentation in a bioreactor. Usually, bacteriocin production is associated to cell density and the obtained data indicated that this occurred especially during exponential phase, under both growth conditions (Fig. 2). It should be pointed out that *E. hirae* ST57ACC, cultivated in the bioreactor after 24 h of fermentation at a controlled pH of 5.5, presented similar levels of bacteriocin production to those obtained from cultivation in flasks (Fig. 2).

Preliminary screening for expression of ABC-transporter-related genes

Based on the protocol and primers designed for *Lactobacillus*, it was possible to evaluate the potential role of ABC-transporter system in the expression of bacteriocin(s) by the bacteriocinogenic strain *E. hirae* ST5ACC. Except for the sugar ABC transporter ATP-binding protein and alcohol dehydrogenase, all tested genes were expressed, including the bacteriocin ABC-transporter (Table 2). Based on the results, glucose-6-phosphate 1-dehydrogenase was expressed at high levels (> 300 units), while malate/lactate dehydrogenase, bacteriocin ABC-transporter ATP-binding and permease protein, and ABC transporter ATP-binding protein were expressed at levels between 100 and 300 units (Table 2).

Bacteriocin purification and cytotoxicity

The bacteriocin produced by *E. hirae* ST57ACC was purified based on a similar protocol adopted for enterocin DD14 [20], consisting in the removal of the overnight incubation of the CFS and the Sephadex matrix. Also, as MRS broth interferes on bacteriocin purification [21], *E. hirae* ST57ACC was grown on BHI. Higher bacteriocin activity was obtained in the elution with 1.5 M NaCl when compared to other used eluents (Table 3). The use of SPE increased the specific activity of the bacteriocin by 15-fold when compared to the CFS, and almost tenfold when compared to GF (Table 3). However, the specific activity after RP-HPLC was reduced (Table 2). Three major peaks were observed in the RP-HPLC analysis with retention times of 8.5, 10.5, and 18.5 min. These samples were individually collected to evaluate their antimicrobial activity. An inhibition zone was only observed around the spot corresponding to the 8.5 min retention time, suggesting that the bacteriocin produced by *E. hirae* ST57ACC was eluted in a single peak.

Regarding cytotoxic effects, the semi-purified fraction obtained by elution with 60% ACN during SPE and the CFS were tested, and presented cell viability similar to the observed for the negative control (data not shown).

Discussion

In our study, the growth of *E. hirae* ST57ACC was not influenced by the size of the inoculum, however, bacteriocin production was observed only when the strain was inoculated at 5 and 10% (v/v, Fig. 1). Still, similar OD values were observed for conventional and batch fermentation, while for batch fermentation, where pH was controlled, the lag phase was longer (Fig. 2). Additionally, similar levels of bacteriocin production were observed when *E. hirae* ST57ACC was cultivated by conventional growth and in batch fermentation (Fig. 2),

Fig. 1 Variation of optical density (black points) and pH (gray points) values of *Enterococcus hirae* ST57ACC inoculated in de Man, Rogosa and Sharpe broth (BD) at 1 (A), 2 (B), 5 (C), and 10 (D) % and incubated

at 37 °C for 24 h. Mean values and standard errors. The asterisks correspond to bacteriocin production

indicating that this approach could be applied in industrial scale with no losses. Bacteriocin production and cell-density in LAB are substantially influenced by different factors including, pH, temperature, and media composition [21, 22]. Lv et al. [23] reported that bacteriocin production in LAB follows a scheme of primary metabolite growth-associated kinetics, occurring during the exponential growth phase and ceases once the stationary phase is reached. These data indicate that bacteriocin production is, indeed, dependent on the total biomass [23]. Nevertheless, the relationship between bacteriocin production and growth also depends on the strain used. In fact, a correlation between the peptide production and cell-density is evident in some cases, whereas in other cases the bacteriocin production starts only when stationary phase had been reached [24]. As observed in this study, it should be mentioned that high cell density does not necessarily lead to high bacteriocin production (Fig. 2).

There is a complex relationship between the environmental conditions and bacteriocin production. For *E. mundtii* CRL1656, it was reported that the biomass and level of

agitation did not affect the bacteriocin production or the strain growth, although 5% inoculum significantly reduced the length of the lag phase [25]. Furthermore, Herranz et al. [26] observed that the production of enterocin P was strongly dependent on pH during continuous cultivation of *E. faecium* P13. Moreover, bacteriocins production was optimally produced at a pH of 6.0, lower than that for optimal growth of the producer (pH 7.0) [26]. Therefore, optimization of the culture conditions can lead to a more effective recovery of bacteriocins. This strategy could be adopted for a large-scale production of bacteriocin and industrial applications.

ABC transporters form one of the largest protein super-families, being well conserved and common to a diversity of bacterial groups. They present uptake of different compounds across the membrane which request the energy from ATP hydrolysis [27]. Considering that LAB generate their primary energy via glycolysis, upregulation of ABC transporters linked to sugar metabolism is an essential feature [28]. ABC transporters are also related to the release of different peptides,

Fig. 2 Variation of biomass and pH (black and gray points, respectively, in graphs A and B) values and bacteriocin production (gray bars, graphs C and D) of *Enterococcus hirae* ST57ACC inoculated in de Man, Rogosa

and Sharpe broth (BD) at 5% and incubated at 37 °C for 24 h by conventional culture (A, C) and in bioreactor (B, D). Mean values and standard errors

Table 2 Expression of genes related to ABC transport system by the bacteriocinogenic strain *Enterococcus hirae* ST57ACC

ABC transport system function	Expression*
Efflux ABC transporter, ATP-binding and permease protein	+
Sugar ABC transporter, ATP-binding protein	–
Malate/lactate dehydrogenase	++
Multiple sugar ABC transporter, ATP-binding protein	+
Bacteriocin ABC-transporter, ATP-binding and permease protein	++
ABC transporter ATP-binding protein	++
Glucose-6-phosphate 1-dehydrogenase	+++
Alcohol dehydrogenase	–

*Expression levels measured by using the GenomeLab™ GeXP genetic analysis system (Beckman Coulter, Brea, CA, USA)

–Absence of signal

+Weak (less than 100 units)

++Medium (between 100 and 300 units)

+++High expression (more than 300 units)

Table 3 Purification of bacteriocin produced by *E. hirae* ST57ACC from a 40-mL cell-free supernatant (CFS) by gel-filtration (GF), solid-phase extraction (SPE), and RP-HPLC

Step	Volume (mL)	Protein concentration (mg/mL)	Bacteriocinogenic activity (AU/mL)*	Total activity (AU)	Total protein (mg)	Specific activity (AU/mg)*	Purification (fold)*	Yield (%)*
CFS	40	7.82	6400	256,000	312.8	818.41	1	100
GF	20	1.23	1600	32,000	24.6	1300.81	1.58	12.5
SPE	1	0.26	3200	3200	0.26	12,307.69	15.03	1.25
RP-HPLC	1	0.04	100	10	0.004	2500	3.05	3.9

*Bacteriocinogenic activity (AU/mL): (reciprocal of the highest dilution $\times$ 1000) / volume of bacteriocin; specific activity (AU/mg): (total activity of subsequent step / total protein of the same step); purification fold: (specific activity of subsequent step / specific activity of CFS), yield: (total activity of subsequent step / total activity of crude preparation) $\times$ 100

including most bacteriocins from different classes, where the leader peptide is processed by the peptidase domain of the N-terminal region in an ATP-dependent manner for bacteriocin translocation [29, 30]. It has been recognized that the same ABC transporter system can be simultaneously involved in secretion of bacteriocins and quorum sensing signaling molecules, according to environmental conditions in Gram-positive bacteria [31]. Bifunctional transporters are also involved in the transport of other peptide products across bacterial species and this ability of intercommunication can confer fitness advantages to LAB during bacteriocin production and secretion in vivo, contributing to competitive colonization [27, 32]. Besides, different transport systems may result in most efficient bacteriocin production and in LAB. The effects of combination of SunT transporter and double glycine leader sequence were studied for mesentericin Y105 production, revealing that association of different systems resulted in increased production levels compared to the general secretion and replacement of original double glycine type by the sec-dependent signal result in considerable bacteriocin production [33, 34]. A complex expression and exteriorization system was also reported for hiracin JM79, produced by *E. hirae* DCH5, described also as sec-dependent [12, 35]. The obtained data indicated the potential role of ABC-transporter system in the expression and transport of bacteriocin produced by *E. hirae* ST57ACC (Table 2), leading to deeper studies on these mechanisms and regulation. Efforts have been made to increase and improve the bacteriocin production on a large scale and the understanding of varied transport systems and mechanisms involved in its regulation, may help to develop different strategies for efficient industrial bacteriocin production by *E. hirae* ST57ACC.

Bacteriocin produced by *E. hirae* ST57ACC was successfully purified in a three-step protocol in this study (Table 3) and previous results about bacteriocin encoding genes have shown that *E. hirae* ST57ACC does not harbor enterocin A, enterocin P, enterocin B neither enterocin L50B genes, and the bacteriocin produced by this strain might be a novel bacteriocin [14]. Purified bacteriocins can be a strategy to improve safety and quality in food products as well as it can facilitate their use in human and veterinary medical industries.

Bacteriocins produced by *Enterococcus* spp. have been purified previously using a range of different chromatographic methods [20]. For example, a final recovery of 1.6% of enterocin DD14 produced by *E. faecalis* 14, using cation-exchange and size-exclusion chromatographic methods was achieved, along with successful purification of enterocin AS-48 from *E. faecalis* subsp. *liquefaciens* A-48-32, with a final recovery of 74.95% [24]. The purification process has always been considered the main bottleneck for industrial applications of bacteriocins, as bacteriocin yields tend to be low, most likely due to the high number of steps involved in the purification [36, 37]. The recovery and purification of bacteriocins produced by LAB are usually achieved using salt precipitation from culture supernatants, as well as a combination of GF, ion exchange chromatography, hydrophobic interaction chromatography, and RP-HPLC [37]. These methods produce satisfactory results on a small scale, but are usually of low yield, very expensive, difficult to handle, time-consuming, and unsuitable when tested on a large scale [24]. The yields can be variable depending on the adopted method, and it has been previously shown that RP-HPLC contributes the most to the loss of bacteriocin activity [38].

Here, despite low purification yields, sufficient amounts of bacteriocin were obtained to evaluate its cytotoxicity against a human colon adenocarcinoma cell line, HT-29. After treatment with both the CFS and a semi-purified fraction of bacteriocin, HT-29 cells were found to remain viable, indicating no cytotoxic effect. Most bacteriocins produced by *Enterococcus* spp. have been shown to present low cytotoxicity against eukaryotic cells [20, 39]. However, it has been reported that mild toxicity can occur when higher concentrations of bacteriocins are used [40, 41]. Different toxicities can be observed depending on differences in the membrane composition, metabolic activity of target cells, exposure time, and the cytotoxicity assay employed [40, 41]. Despite cytotoxic effects being considered a positive characteristic for treatment of carcinogenic cells, cytotoxicity itself is not desirable for the use of compounds either as bio-preservatives in food or as pharmaceutical preparations. Therefore, for these reasons the safety of each bacteriocin needs to be performed to clarify their potential use in these industries.

Conclusions

In this study, *E. hirae* ST57ACC was found to express different ABC transporters, including a bacteriocin transporter, which may help to identify the bacteriocin produced by this strain. No disadvantages were observed when the strain was grown in a bioreactor, suggesting that cultivation of *E. hirae* ST57ACC in industrial fermenters may be a strategy for implementing large-scale production of this bacteriocin. Furthermore, with no cytotoxic effects observed on a human cell line, this bacteriocin may be considered safe for use as a potential bio-preservative tool in the future.

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