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Screening of the Enterocin-Encoding Genes and Antimicrobial Activity in *Enterococcus* Species

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In the current study, a total of 135 enterococci strains from different sources were screened for the presence of the enterocin-encoding genes *entA*, *entP*, *entB*, *entL50A*, and *entL50B*. The enterocin genes were present at different frequencies, with *entA* occurring the most frequently, followed by *entP* and *entB*; *entL50A* and *L50B* were not detected. The occurrence of single enterocin genes was higher than the occurrence of multiple enterocin gene combinations. The 80 isolates that harbor at least one enterocin-encoding gene (denoted "Gene$^+$ strains") were screened for antimicrobial activity. A total of 82.5% of the Gene$^+$ strains inhibited at least one of the indicator strains, and the isolates harboring multiple enterocin-encoding genes inhibited a larger number of indicator strains than isolates harboring a single gene. The indicator strains that exhibited growth inhibition included *Listeria innocua* strain CLIP 12612 (ATCC BAA-680), *Listeria monocytogenes* strain CDC 4555, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* strain UK-1 (ATCC 68169), and *Escherichia coli* BAC 49LT ETEC. Inhibition due to either bacteriophage lysis or cytolysin activity was excluded. The growth inhibition of antilisterial Gene$^+$ strains was further tested under different culture conditions. Among the culture media formulations, the MRS agar medium supplemented with 2% (w/v) yeast extract was the best solidified medium for enterocin production. Our findings extend the current knowledge of enterocin-producing enterococci, which may have potential applications as biopreservatives in the food industry due to their capability of controlling food spoilage pathogens.

Keywords: Enterocin-encoding genes, antimicrobial activity, *Enterococcus*

Introduction

Enterococci are lactic acid bacteria (LAB) and have applicability in the food and pharmaceutical industry because of their GRAS (generally recognized as safe) status [23, 58].

Enterococci isolated from different sources, including vegetables [19], animal intestine [56] (including human intestine [63]) and fermented foods such as cheese [2, 54, 59], sausages [55], and other meat products [7, 38], have potential applicability as food preservatives and for biotechnological purposes. *Enterococcus* spp. are commonly present in food owing to their ability to tolerate and survive under adverse environmental conditions [33, 36, 51] and their ability to inhibit pathogenic microorganisms. This latter feature is due to their capacity to produce antimicrobial compounds, such as diacetyl, organic acids, hydrogen peroxide, and enterocins [18]. In general, enterocins can be found naturally in foods involving bacterial growth (*e.g.*, in natural fermentations or even in contaminated and spoiled foods) [8].

Enterocins are ribosomally synthesized antimicrobial peptides that exhibit antagonism against the same bacteria species or across genera in the context of competitive

exclusion [15, 32]. The bactericidal mechanisms of enterocins include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and/or inhibition of peptidoglycan synthesis [31].

Different enterocins have demonstrated activity against several foodborne pathogens, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*, *Mycobacterium* spp., *Escherichia coli*, and *Myxococcus* spp. [5, 13, 31, 39]. The widespread enterocins described in *Enterococcus* spp. food isolates are enterocins A, B, P, L50A, and L50B [23].

These *Enterococcus* spp. enterocins are antimicrobial peptides produced in situ by probiotic strains that exhibit specific molecular properties, including low toxicity and either broad-spectrum or narrow-spectrum activity. Owing to these properties, the antimicrobial peptides became a target of industrial interest as food biopreservatives or as alternatives to antibiotics employed against microbial pathogens [3, 16, 26, 53]. Some examples of the former application include enterocins produced by *E. faecium* RZS C5 and *E. faecium* DPC 1146 in cheddar cheese [28], enterocin produced by *E. faecalis* CECT7121 in crafted goat cheese [59], and enterocin on fresh-cut lettuce [7]. In addition, enterocin AS-48 has been used in sausages [4, 5], fruit juices [35], cooked ham [3], and other dairy products [47]. However, studies concerning the use of enterocins in food are scarce compared with other bacteriocins. Currently, the use of antimicrobial peptides as food preservatives has mainly been focused on bacteriocins produced by other members of the LAB group, predominantly *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., and *Pediococcus* sp. [47].

Considering that specific enterocins will likely have unique properties and antimicrobial potential, the isolation of new bacteriocins will be beneficial. This study was conducted to search for enterocin-producing enterococci for the potential application toward food preservation and ultimately to enhance human health.

Materials and Methods

Bacterial Strains and Growth Conditions

A collection of 135 enterococcal strains isolated from several sources, collected over the period of 2008-2010 (clinical strains) and 2011-2012 (food strains) [29, 30], was employed in the present study. The indicator strains were obtained from the American Type Culture Collection (ATCC) and Centers for Disease Control and Prevention (CDC), and included *Listeria innocua* strain designation CLIP 12612 (ATCC BAA-680), *Listeria monocytogenes* CDC 4555, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538, *Salmonella enteritidis* ATCC 13076, and *Salmonella typhimurium* strain UK-1 (ATCC 68169; exhibits the highest invasion and virulence attributes among the most frequently studied strains). *Escherichia coli* strain BAC 49LT ETEC (heat-labile enterotoxin-producing) was kindly donated by Dr. Renata Katsuko T. Kobayashi (Microbiology Department, State University of Londrina). Brain Heart Infusion medium (BHI) was used for microbial cultivation. Stock cultures were stored at –20°C in BHI broth supplemented with 20% (v/v) glycerol. Fresh cultures were prepared by inoculation of 20 µl of the frozen stock into 3 ml of BHI broth incubated for 18 to 24 h at 37°C.

PCR Genotyping for Enterocin-Encoding Genes

The most common enterocin-encoding genes are *entA*, *entB*, *entP*, *entL50A*, and *entL50B* and were amplified by polymerase chain reaction (PCR) (Table 1). All of the reactions were processed in a thermal cycler (Swift MaxPro Thermal Cycler, Esco Technologies Inc.) using total DNA according to the boiling method [44]. PCR was performed in a final volume of 20 µl containing 2 µl of total DNA, Taq buffer (10×), 2.5 mM $MgCl_2$, 0.17 mM dNTPs, 1 ρmol of each primer (forward and reverse), and 1 U of Taq DNA polymerase (Invitrogen). The negative controls contained all of the reagents except the DNA. The amplicons were separated by

Table 1. Specific primers for the PCR amplification of enterocin-encoding genes and the *cyl*A gene.

Gene	Sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)	References
entA	F: GGT ACC ACT CAT AGT GGA AA	138	55	Özdemir *et al*. [50]
	R: CCC TGG AAT TGC TCC ACC TAA			
entB	F: CAA AAT GTA AAA GAA TTA AGT ACG	201	56	De Vuyst *et al*. [21]
	R: AGA GTA TAC ATT TGC TAA CCC			
entP	F: GCT ACG CGT TCA TAT GGT AAT	87	55	Özdemir *et al*. [50]
	R: TCC TGC AAT ATT CTC TTT AGC			
entL50A/B	F: ATGGGAGCAATCGCAAAATTA	274	58	Özdemir *et al*. [50]
	R: TAGCCATTTTTCAATTTGATC			
cylA	F: ACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTT	688	54	Creti *et al*. [17]

electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized by UV light exposure using a L-PIX ST (LOCCUS) computerized photographic system.

Screening for Enterocin Activity

Screening for enterocin activity was performed according to a previously described method [37] with modifications. Isolates that harbored at least one enterocin-encoding gene (denoted Gene$^+$ strains) were screened for their capability to produce bacteriocins against nine different indicator microorganisms. The *Enterococcus* strains were streaked on plates containing BHI agar (Himedia) and were incubated at 37°C for 24 h. The plates were inverted to receive 1 ml of chloroform in the plate covers and remained closed for 20 min. The residual chloroform was evaporated by opening the plates. Through the pour plate method, each indicator strain (10^8 cells/ml) was inoculated into soft BHI agar (0.8%) and was poured into the *Enterococcus* plates forming an overlay. The plates were then incubated at 37°C for 24 h. Isolates were considered as bacteriocin producers if inhibition zones were evident around the colonies; the inhibition zones were measured in millimeters. For statistical comparisons, Fisher's exact test was used to analyze the association between the antimicrobial activity against at least one indicator strain (*ent*$^+$) and the presence of a single enterocin gene or enterocin genes in multiple combinations, with significance denoted at $p < 0.05$.

Detection of Bacteriophage Activity and Genotyping for the Cytolysin Activator-Encoding Gene (*cylA*)

Bacteriophage activity and the genotype of the *cylA* gene (putative cytolysin activity) were examined to exclude their potential role in influencing the inhibition assay. For bacteriophage activity, a portion of the inhibition zone was cut and added to 3 ml of BHI broth, and the material was then macerated with sterile tips. The suspension was held at room temperature, and a 100 μl aliquot of this suspension was mixed with 100 μl of the indicator strain culture followed by growth at 37°C for 18–24 h in BHI broth. The culture was then added to 4 ml of BHI soft agar and was poured in BHI plates forming an overlay, followed by incubation under the same conditions. The formation of plaque zones would indicate bacteriophage activity. The genotype for gene *cylA* was evaluated [17] (Table 1).

Enterocin Production under Different Growth Conditions

All of the isolates that exhibited antimicrobial activity against *L. innocua* were tested for enterocin production under different culture conditions. The media comprised BHI agar medium, MRS (deMan, Rogosa, and Sharpe) agar medium (Himedia), and BHI and MRS with supplementations: BHI 2% (w/v) glucose, BHI 4.5% (w/v) lactose, MRS 2% (w/v) yeast extract, MRS 2% (w/v) glucose, and MRS 1.5% (w/v) lactose. The growth inhibition zones around the colonies were measured in millimeters. A non-parametric test, Friedman's test, was used for the comparisons, with $p < 0.05$ representing significance.

Results

Enterocin-Encoding Genes

Among the 135 *Enterococcus* spp. isolates evaluated in this study, the presence of one or more enterocin-encoding genes was verified in 80 isolates (59.26%). Isolates harboring at least one enterocin-encoding gene were referred to as Gene$^+$ strains. Among the Gene$^+$ strains, 21 (26.25%) belonged to human colonizing samples, and 59 (73.75%) belonged to food samples. Regarding the species prevalence, *E. faecium* occurred predominantly among Gene$^+$ strains (90%) followed by *E. faecalis* (7.5%) and *Enterococcus* spp. (2.5%).

The genes *entA*, *entB*, and *entP*, which encode enterocins A, B, and P, respectively, were detected among the isolates. In contrast, the enterocin-encoding genes *entL50A* and *entL50B* were absent. The profile of enterocin-encoding genes, according to isolate source and corresponding species, is shown in Table 2. The enterocin A-encoding gene occurred at the highest frequency among the Gene$^+$ strains (69 isolates; 86.25%) and was present as a single enterocin gene in 36 isolates (45%). The *entP* gene was the second most frequent gene, was present in 42 isolates (52.5%), and was present as a single enterocin in 10 isolates (12.5%). None of the Gene$^+$ strains harboring the *entP* gene belonged to the human-colonizing samples. The *entB*-encoding gene was the least frequently detected enterocin gene among the examined isolates and only occurred together with another *ent* gene.

Table 2. Percentage of enterocin-encoding genes among *Enterococcus* Gene$^+$ strains.

Genes	Number and percentage of *Enterococcus* isolates				
	Species			Source	
	E. faecium	*E. faecalis*	*Enterococcus* spp.	Food	Human
entA (single)	34 (42.5%)	2 (2.5%)	-	17 (21.25%)	19 (23.75%)
entP (single)	9 (11.25%)	1 (1.25%)	-	10 (12.5%)	-
entA/B	2 (2.5%)	-	-	-	2 (2.5%)
entA/P	22 (27.5%)	1 (1.25%)	1 (1.25%)	24 (30%)	-
entA/B/P	4 (5%)	3 (3.75%)	1 (1.25%)	8 (10%)	-

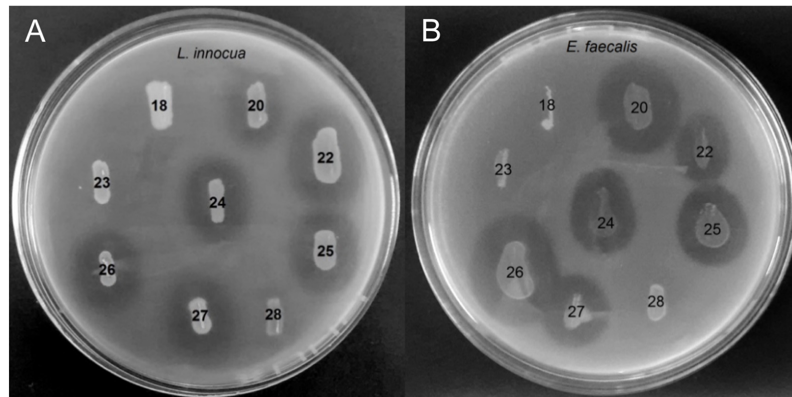

Fig. 1. Effect of antimicrobial activity of cell culture by distinct *Enterococcus* Gene$^+$ strains (18, 20, 22, 23, 24, 25, 26, 27, and 28 strains) determined by the pour plate method using BHI agar (see Materials and Methods section).
The zone inhibition are shown for (**A**) *Listeria innocua* CLIP 12612 and (**B**) *Enterococcus faecalis* ATCC 29212 indicator microorganisms.

Among these 10 isolates, two were from clinical colonization samples and the remaining were from food samples.

As shown in Table 2, the gene combination of *entA* and *entP* was the most predominant and occurred in 24 strains (30%), which was followed by *entA* and *entB* (2.5%). The combination of the three genes *entA*, *entB* and *entP* occurred in eight isolates (10%). With regard to clinical colonization samples, only two isolates harbored the *entA* and *entB* gene combination.

Screening for Enterocin Production

Our data revealed that 66 out of 80 *Enterococcus* Gene$^+$ strains (82.5%) exhibited antimicrobial activity against at least one of the tested indicator strains, as illustrated in Fig. 1. The *Enterococcus* Gene$^+$ strains that presented antimicrobial activity were denoted as *ent*$^+$ strains. The antimicrobial activity results of the *ent*$^+$ strains against different indicator strains are shown in Table 3. Most of the indicator strains exhibited growth inhibition, including *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538, *L. innocua* CLIP 12612, *L. monocytogenes* CDC 4555, *E. faecalis* ATCC 29212, and *S. enteritidis*; the remaining two gram-negative strains were not inhibited. In addition, various combinations of growth inhibition of these seven indicator strains were observed among the *ent*$^+$ strains with antimicrobial activities. The closely related *E. faecalis* species was the indicator microorganism that was inhibited by the largest number of *ent*$^+$ strains, followed by the potential pathogenic strains, including *L. innocua*, *S. aureus*, *L. monocytogenes*, and *S. enteritidis*.

With regard to the source of the *Enterococcus* strains, 17 of the 21 strains representing human-colonizing samples (80.95%) showed antimicrobial activity against at least one indicator strain. A total of 49 of the 59 strains representing food samples (83.05%) were identified as *ent*$^+$. The proportion of *ent*$^+$ strains between the two sources was equivalent, and

Table 3. Antimicrobial activity of *Enterococcus* Gene$^+$ strains against indicator strains.

Indicator strain	Antimicrobial activity[a]	No. of enterococci strains with inhibitory activity
Listeria innocua CLIP 12612	+	24 (30%)
Listeria monocytogenes CDC 4555	+	4 (5%)
Enterococcus faecalis ATCC 29212	+	44 (55%)
Staphylococcus aureus ATCC 25923	+	19 (23.8%)
Staphylococcus aureus ATCC 29213	+	18 (22.5%)
Staphylococcus aureus ATCC 6538	+	17 (21.2%)
Salmonella enteritidis ATCC 13076	+	3 (3.8%)
Salmonella typhimurium UK-1	-	0
Escherichia coli BAC 49LT ETEC	-	0

[a](+) presence of growth inhibition; (-) absence of growth inhibition.

no correlation was found between the strain origin and inhibitory activity, although there were less clinical colonization isolates than food isolates in our sample set. Among the *Enterococcus* strains that exhibited antimicrobial activity against *L. innocua*, there was a significantly higher frequency (p = 0.02) of strains harboring combined genes than strains harboring single genes.

The bacteriophage activity assay revealed the absence of plaque zones, indicating that none of the observed inhibition zones were due to phage-mediated lysis. None of the Gene$^+$ strains encoded the *cylA* gene, suggesting that none of the inhibition zones were due to cytolysin activity, which is a β-hemolytic toxin with bactericidal activity against gram-positive bacteria. In addition, none of the Gene$^+$ strains were able to produce organic acids (data not shown).

Enterocin Production under Different Culture Media Conditions

To evaluate a suitable medium for enterocin production, we employed *L. innocua* CLIP 12612 as an indicator microorganism. Among the 24 *Enterococcus* strains that showed inhibitory activity against *L. innocua*, 11 strains (20, 22, 24, 25, 26, 27, 52, 62, 69, 71, and 70) exhibited inhibition zones. For these isolates, the inhibition zone ranged from 0.8 to 2.5 mm, and the other strains exhibited zones of 0.5 mm. Therefore, these 11 strains were used to evaluate a suitable medium for enterocin production.

Differences in *L. innocua* inhibition occurred as a function of the different tested growth media (Friedman's test, p = 0.002). The MRS agar supplemented with yeast extract was the most appropriate solidified medium for enterocin production, which was followed by BHI non-supplemented, BHI glucose, MRS lactose, MRS glucose, BHI lactose, and MRS non-supplemented. Significant differences were observed between MRS yeast extract and BHI lactose, and MRS glucose and MRS without supplementations ($p < 0.05$). The remaining tested media formulations did not affect the inhibition activity against *L. innocua*. Therefore, these can be considered suitable conditions for cultivation for enterocin production in solidified medium. Inhibition activity differences among the *Enterococcus* strains (Friedman's test, p = 0.0001) were also observed. The highest enterocin producers were strains 70 and 71.

The strains with the single *entA* gene and the *entA/P* gene combination exhibited activity against almost all of the tested indicator strains, including the foodborne pathogens (Tables 2 and 3). Coincidently, these strains produced the highest bacteriocin titers against *S. aureus*, *E. faecalis*, and *L. innocua* and the lowest titers against gram-negative bacteria and *L. monocytogenes*. However, this finding could be attributed to the inherent sensitivity of each indicator microorganism towards the bacteriocins.

Discussion

The use of bacteriocins for the biopreservation of foods can be considered an additional tool to enhance microbiological safety and reduce the risk of spoilage microorganisms. Enterococci are commonly used in the food industry as biopreservatives, due to their ability to produce antimicrobial compounds, including enterocins [24].

In general, this study showed that a higher frequency of enterocin-encoding genes occurs as a single gene than as genes in multiple combinations. For the food isolates, a similar proportion of isolates harboring multiple and single genes was found. In contrast, the clinical colonization isolates displayed a higher frequency of single genes than genes in multiple combinations.

The majority of *Enterococcus* isolates that harbored at least one enterocin-encoding gene exhibited enterocin activity (*ent*$^+$ strains). The frequency of *ent*$^+$ strains was higher among *E. faecium* isolates (89.4%) than among *E. faecalis* isolates (7.6%) and *Enterococcus* spp. isolates (3%). These data are in agreement with other studies [21, 51, 60]; however, others have found a higher proportion of bacteriocin production among *E. faecalis* isolates than *E. faecium* isolates obtained from several sources [20]. The enterocin produced by *ent*$^+$ strains exhibited activity against almost all of the tested indicator strains, including foodborne pathogens, whereas it has previously been reported that most of bacteriocins produced by species of *Enterococcus* are only active against genetically related species [1].

Consumer appeal for quality products without chemical additives has stimulated industrial interest for new microbial strains with biopreservation capabilities. Examination of the genes encoding the most common enterocins (A, B, P, L50 A, and L50B) among enterococci isolates has been evaluated in several studies [21, 53, 54, 57]. In the present study, the *entA*-encoding gene occurred at high frequency among the tested *Enterococcus* strains and was present as a single enterocin gene in 45% of the strains, which is similar to the rate found in previous studies [21, 50]. As described in our study, the occurrence of a single enterocin-encoding gene is common among *Enterococcus* strains and has been described by Strompfová *et al*. [60]. However, when considering only isolates from food sources, the presence

of two or more genes was prevalent, which has also been demonstrated by other researchers [2, 21, 50, 53, 54]. A previous analysis of the presence of enterocin-encoding genes among clinical isolates revealed a high frequency of isolates harboring multiple genes [40], which is different than what was observed in the present study. On evolutionary basis, the ability to synthesize one or more bacteriocins has been a highly advantageous characteristic for survival and proliferation of the microorganism.

A combination of the genes *entA* and *entP* was frequently found among our strain collection, which is in accordance with the literature [21, 60]. Furthermore, *entB* was found associated with *entA*, which is in accordance with previous studies [21, 50, 52]. According to these authors, the association between *entB* and *entA* may be due to the absence of transport or accessory protein genes in some *Enterococcus* strains. Thus, it is possible that both enterocins may act synergistically when expressed in the same culture [9, 27]. The genes *entL50A* and *entL50B* were absent among the *Enterococcus* strains tested in this study, which was similarly reported by Sabia *et al*. [55].

The majority of Gene$^+$ strains inhibited at least one of the indicator strains, and the isolates that harbored multiple enterocin-encoding genes inhibited the growth of a larger number of indicator strains. The presence of these genes does not indicate that all enterocins were expressed at the same time, as gene expression depends on several factors, including environmental conditions and genetic mechanisms [9, 54].

In the present study, gram-positive bacteria, including *E. faecalis, L. innocua, S. aureus*, and *L. monocytogenes*, were more sensitive to enterocin activity than gram-negative bacteria. The closest related indicator strain, *E. faecalis*, was inhibited by a large number of Gene$^+$ strains, which was followed by *L. innocua*. Most bacteriocins have a narrow spectrum; hence, it is common that their activity is specific against closely related genera or species. Another possibility to explain the greater inhibition of *E. faecalis* and *L. innocua* is the fact that Class IIa bacteriocins, such as enterocins A and P, bind to specific receptors belonging to the mannose phosphotransferase system (Man-PTS) on target cells. Class IIa bacteriocins specifically target a subgroup of Man-PTSs (group I); the genera *Listeria* and *Enterococcus* exclusively contain this protein subgroup [41, 42].

In considering the Gene$^+$ strains, the inhibitory activity against *L. innocua* was dependent on single enterocin genes or enterocin genes occurring in combinations. The presence of multiple enterocin genes does not assure that all of the genes are expressed at the same time, but if enterocins were present in the same supernatant, their antagonistic activity may be higher owing to synergism. Several studies demonstrated a more efficient inhibitory activity when multiple bacteriocins were produced at the same time [9, 38, 56].

Our data revealed that growth under low amounts of glucose, as well as the presence of lactose as an additional carbon source and the level of nitrogen sources (yeast extract), has a positive influence on enterocin activity. The increase or decrease of enterocin production in response to the presence of simple carbon sources (glucose) or disaccharides (lactose) indicates that enterocin biosynthesis is strain dependent and/or dependent on the carbohydrate concentration. In accordance with the present results, Ogunbanwo *et al*. [49] obtained improved bacteriocin production by *Lactobacillus brevis* OG1 using MRS broth supplemented with low concentrations of glucose (1%) and yeast extract (2% and 3%). Foulquié Moreno *et al*. [28] demonstrated that the use of lactose as a carbon source resulted in a greater production of enterocin by *E. faecium* RZS C5.

In conclusion, modifications of the culture medium can positively influence the amount and activity of enterocins. These conditions should therefore be considered for the optimization of enterocin production.

In summary, following genotyping and antimicrobial activity screening, we identified a large number of isolates with industrial potential for applications as biopreservatives and protective agents against foodborne pathogens. However, more detailed isolate characterization and enterocin production optimization studies are required before these isolates can be used as biopreservatives in food products.

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