Ciência e Agrotecnologia
SCIENCE AND AGROTECHNOLOGY

Ciência e Agrotecnologia, 46:e008022, 2022
http://dx.doi.org/10.1590/1413-7054202246008022

Food Science and Technology
eISSN 1981-1829

Soybean flour as a substrate to obtain *Enterococcus durans* bacteriocins

Farinha de soja como substrato para obtenção de bacteriocina de *Enterococcus durans*

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Received in June 20, 2022, and approved in September 1, 2022

ABSTRACT

Natural preservatives, such as enterocins, have been the focus of several studies for use in the food industry. However, the commercial media used to obtain enterocins are still expensive, presenting a disadvantage for large-scale production. In this study was developed four formulations of culture media containing soybean flour for obtaining *Enterococcus durans* enterocins. The antilisterial activity of *E. durans* MF5 enterocins obtained in soybean and MRS media (control) was characterized, with *Listeria monocytogenes* CLIP2032 and *L. innocua* CLIP12612 as the bacterial strains. The growth of *E. durans* (CFU/mL) was significantly affected by the incubation time in the soybean and MRS media ($p < 0.05$), but the composition of the media did not affect the cell development of the enterocin-producing microorganism. When evaluating the genes encoding enterocin synthesis, positive results were obtained for enterocin A, B, P, and X. When treated with proteolytic enzymes (α-chymotrypsin, protease, and proteinase-K), enterocin was inactivated, confirming its protein character. The antilisterial activity of the enterocins obtained in soybean media was up to 6,400 AU mL^{-1}. Enterocins from soybean media M1 and M2 showed antibacterial activity at a concentration of 1 mg/mL after 6 h incubation. Thus, we show that culture media formulated with soybean flour are promising substrates for enterocin production that would allow the protocol to be expanded on a large scale.

Index terms: Agro-industrial waste; enterocins; antimicrobial peptides; food conservation.

RESUMO

Conservantes naturais, como exemplo as enterocinas, têm sido foco de diversos estudos para uso na indústria de alimentos. No entanto, os meios comerciais utilizados para obtenção de enterocinas ainda são onerosos, sendo a grande desvantagem para produção em larga escala. Neste estudo foram desenvolvidas quatro formulações de meios de cultura contendo farinha de soja para obtenção de enterocina por *Enterococcus durans* MF5. A atividade antilisterial da enterocina MF5 obtida em meio soja e MRS (controle) foi testada contra *Listeria monocytogenes* CLIP2032 e *L. innocua* CLIP12612. O crescimento de *E. durans* (UFC/mL) foi significativamente afetado pelo tempo de incubação nos meios soja e MRS (p<0,05), porém a composição do meio não afetou o desenvolvimento celular do microrganismo produtor de enterocina. Ao avaliar os genes que codificam a síntese de enterocina, foram obtidos resultados positivos para enterocina A, B, P e X. Quando tratada com enzimas proteolíticas (α-quimotripsina, protease e proteinase-K), a enterocina foi inativada, confirmando seu caráter proteico. A atividade antilisterial das enterocinas obtidas em meio de soja foi de até 6.400 AU mL-1. Enterocina produzida nos meios de soja M1 e M2 apresentaram atividade antibacteriana na concentração de 1 mg/mL após 6 h de incubação. Assim, mostramos que meios de cultura formulados com farinha de soja são substratos promissores para a produção de enterocinas que permitiriam a expansão do protocolo em larga escala.

Termos para indexação: Resíduo agro-industrial; enterocina; peptide antimicrobiano; conservação de alimentos.

INTRODUCTION

Consumers are increasingly demanding food safety alternatives to reduce the use of synthetic additives in the food industry. This situation is the reason for pressure on researchers to study alternative additives to obtain safe and healthy foods (Abbasiliasi et al., 2017; Montiel et al., 2019; Vandera et al., 2020).

Benzoates, sorbates, propionates, and nitrites are antimicrobials commonly used in food. However, many researchers have raised suspicions about synthetic preservatives, mainly related to health (Gokoglu, 2019). Therefore, replacing these synthetic preservatives with natural preservatives is safer for humans and the environment. Natural preservatives can be obtained from

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different sources, such as plants, bacteria, fungi, animals, and algae (Gyawali; Ibrahim, 2014).

Among natural preservatives, antimicrobial peptides of bacterial origin have been highlighted. Enterocins are obtained from the cultivation of several species of the genus *Enterococcus* and are characterized as antimicrobial peptides synthesized in ribosomes and released into the extracellular environment. Furthermore, due to their antagonistic activity, enterocins act against several Gram-positive and Gram-negative species, especially against *Listeria* spp (Furlaneto-Maia et al., 2020).

Enterocins exhibit heat stability and are sensitive to proteolytic enzymes, being degraded in the intestinal tract; as a result, enterocins can be applied in the food industry and are favorable for consumption (Ögundare et al., 2021). Therefore, making the use of enterocins feasible is of great interest to the food industry (Abbasiliasi et al., 2017; Zou; Liu, 2018).

Unfortunately, the commercial means used to obtain enterocins are still expensive, presenting a disadvantage for large-scale production. Given this, it is necessary to develop alternative culture media while ensuring optimal yield and a low production cost (Yang et al., 2018). Natural resources and biotechnological applications have been widely studied, especially the application of agro-industrial waste in the food industry. Soybean waste, for example, has a protein content of approximately 40% and is a promising component for use in culture media as a source of nutrients for bacterial cultivation; the protein quality of soybean waste is similar to that of whole grain (Quinaud et al., 2020).

Although the commercial media used for obtaining bacteriocins are efficient, the high cost and low yield of large-scale production preclude their use. Thus, this study aimed to develop an alternative culture medium using soybean flour for obtaining *Enterococcus durans* enterocins.

MATERIAL AND METHODS

Biological material

The enterocin-producing isolate used was *E. durans* MF5 (Tosoni et al., 2019). The indicator bacteria used were *L. monocytogenes* CLIP2032 and *L. innocua* CLIP12612. All isolates were stored at -20 °C in *brain heart infusion* (BHI) medium (supplemented with 20% (v/v) glycerol).

Media and chemicals

Reactivation of *E. durans,* antimicrobial test and indicator bacteria was performed in de Man, Rogosa & Sharpe (MRS, Sigma) broth and BHI broth, respectively, followed by incubation at 37 °C for 24 h. All the media and chemicals were purchased from Acumedia, Gibco and Sigma–Aldrich (Germany).

PCR genotyping of enterocin genes

The total genomic DNA of the MF5 isolate was obtained by the boiling method (Furlaneto-Maia et al., 2020) and used as a template for the presence of genes encoding known enterocins selected by the amplification procedure (PCR). Genes for enterocins were analyzed using the primer sequences described in Table 1.

Table 1: Primer used for the detection of enterocin structural genes.

Target gene	Sequence (5´- 3´)	Hybridization temperature (°C)	Amplicon size (bp)	Reference
ent A	ggtaccactcatagtggaaa ccctggaattgctccacctaa	55	138	Özdemir et al. (2011)
ent B	caaaatgtaaaagaattaagtacg agagtatacatttgctaaccc	56	201	De Vuyst, Moreno and Revets (2003)
ent P	gctacgcgttcatatggtaat tcctgcaatattctctttagc	55	87	Özdemir et al. (2011)
*ent*L50A/B	atgggagcaatcgcaaaatta tagccatttttcaatttgatc	55	274	Özdemir et al. (2011)
Enterocina 1071 A/B	ggggagagtcggtttttag atcatatgcgggttgtagcc	50	243	Martin et al. (2006)
Enterocin 31	cctacgtattacggaaatggt gccatgttgtacccaaccatt	50	122	Du Toit et al. (2000)
Enterocin AS48	atattgttaaattaccaa gaggagtatcatggttaaaga	50	185	Du Toit et al. (2000)
Enterocin X	cctcttaatcattaaccatac gtttctgtaaaagagatgaaac	50	500	Edalatian et al. (2012)

The PCRs were performed in a thermocycler (ESCO/Swift-Max-Pro) with an initial stage of 95 °C for 5 min; 30 cycles of 94 °C for 30 s, oligonucleotide annealing temperature (Table 1) for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The PCR products (10 µL) were separated by electrophoresis in 2% agarose gels (Sigma–Aldrich, Germany) buffered with 1 x TAE (Merck, Germany) containing 1 µg/mL ethidium bromide (Sigma–Aldrich, Germany) and visualized by ultraviolet light.

Development of culture medium containing soybean flour and enterocins collection

Four formulations of culture media were prepared (soybean medium M1, M2, M3, and M4) containing different concentrations of soybean flour and important components for bacterial growth (Table 2). All formulations and the MRS reference medium had their pH adjusted to 6.5 and were autoclaved at 121 °C for 15 min.

Table 2: Formulations of the culture medium containing soybean flour.

Composition (g/L)	M1	M2	M3	M4
Soybean meal	10	16	10	15
Dextrose	20	20	-	-
Tween 80	1	1	1	1
Potassium phosphate	2	2	2	2
Sodium chloride	-	-	5	5
Tryptone	-	-	15	15

The MF5 isolate was cultured in each of the formulations at a cell density of $1x10^6$ CFU/mL and incubated at 37 °C under agitation at 120 rpm. The control treatment was the inoculum in MRS commercial medium, which is the most strongly indicated medium for the production of enterocin. At 0, 6, and 18 h, aliquots were taken to quantify the number of total bacterial cells per colony-forming unit (CFU).

To obtain enterocin, the culture after 24 h of incubation was centrifuged at 12,298 x g for 15 min. Then, the pH of the supernatant was adjusted to 6.5 (1 M NaOH), treated with catalase (1 mg/mL) and sterilized by filtration (0.22 µm Millipore® membrane). The bacteriocin was semi-purified as described previously by Rocha et al. (2019). Bacteriocina was precipitated with ammonium sulphate (40% saturation), the following centrifugation at 12,298 × *g* for 15 min at 4 °C. The resulting floating pellicle was dissolved in 1 mL of 5 mmol/L potassium phosphate buffer (pH 6.5) and 15 volumes of a mixture of chloroform/methanol (2:1) was added and vortexed thoroughly. The sample was centrifuged at 12,298 × *g* for 15 min and the aqueous metanol layer was removed from the top of the sample. The pellet was was concentrated by lyophilization (L101 Liotop, São Paulo, Brazil).

The protein content was estimated by the Bradford method at each step of the purification process (Rocha et al., 2019).

Antibacterial activity and arbitrary units of enterocin produced in soybean media

To evaluate the antagonistic action of semi-purified enterocin obtained in the different formulations of soybean medium, an agar well diffusion assay (AWDA) were used (Harris et al., 1989). For this, 30 µL of the semi-purified enterocin were deposited in 5 mm wells on 0,8% BHI agar containing L. *innocua* CLIP12612 (1.0 x 10^6 cells/mL). Finally, the plates were incubated at 37 °C for 24 h, and the inhibition halo ≥ 2.0 was considered to be a positive result.

The inhibitory activity of enterocins against *L. innocua* CLIP12612 was quantified and expressed as arbitrary units (AU) per milliliter. For this experiment, semi-purified enterocin at

1:1 (v/v) dilutions were deposited onto well on 0,8% BHI agar containing *L. innocua* CLIP12612 (1.0 x 10^6 cells/mL). AU was defined as the highest dilution that showed an inhibition halo, multiplied by 100 (Mayr-Harting; Hedges; Berkeley, 1972).

The inhibitory activity of semi-purified MF5 enterocins (1 mg/mL) was evaluated at different incubation times (0, 6, and 18 h) with *L. monocytogenes* CLIP2032 and *L. innocua* CLIP12612. The test was performed on microtiter plates, and the optical density (OD_{540nm}) indicating cell growth was measured in a spectrophotometer (Bio Tek, USA).

Scanning electron microscopy (SEM)

The cell injury caused by semi-purified MF5 enterocin was observed with scanning electron microscopy (SEM) following the protocol established by Rocha et al. (2019). For this purpose, *L. monocytogenes* CLIP2032 and *L. innocua* CLIP12612 were grown in the presence of MF5 from soybean media. Subsequently, the cells were recovered by centrifugation, washed twice with PBS, and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 18 h at 4 °C. The samples were carefully washed with 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M sodium. Then, the samples were

dehydrated in an ethanol gradient (50% to 100% ethanol), dried in CO_2 (BALTEC DCP 030 Critical Point Dryer), and coated with gold (BALTEC SDC 050 Sputter Coater). Images were captured under an electron microscope (Quanta 200, FEI Company, Netherlands).

Stability of enterocin concerning temperature and proteolytic enzymes

The thermostability of semi-purified MF5 enterocin was verified at different temperatures. Volumes of MF5 were heated to 80 °C/10 min and 100 °C/20 min (Ammor et al., 2006). For the proteolytic enzyme sensitivity test, a volume of MF5 enterocin was treated with 1 mg/mL (Sigma) α-chymotrypsin, protease, and proteinase-K and incubated at 37 °C for 60 min (Garriga et al., 1993). The residual antimicrobial activity of each treatment was evaluated according to the protocol described by De Vuyst; Leroy (2007) against the more resistant indicator *L. innocua* CLIP12612.

Statistical analysis

The data were analyzed using GraphPad Prism 7.0® software. The results of the control and posttreatment, performed in triplicate, were analyzed using the analysis of variance (ANOVA)-Tukey test. Data with $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The *E. durans* MF5 isolate was selected for this study because it has been previously demonstrated to exhibit antibacterial activity (data not shown). The extracted genomic DNA was amplified by PCR with oligonucleotides targeting eight known enterocin genes, and positive results were obtained only for enterocin A, B, P, and X, according to the amplification of the DNA fragments, with 138, 201, 87, and 500 bp, respectively. These genes encode class II enterocins, which show potential antilisterial action and have disulfide bridges that guarantee thermostability, corroborating the results obtained in this study (Du et al., 2017; Merzoug et al., 2019; Schueler et al., 2021).

Thus, semi-purified enterocins was obtained in different formulations of culture media containing soybean flour (soybean media) called M1, M2, M3, and M4. The process of production and semi-purification of enterocins, it may contain residues of other proteins present in the medium. However, previous treatment with proteolytic enzymes and thermostability indicates bacteriocin. The growth of *E. durans* MF5 measured as CFU/mL was significantly affected by the incubation time in soybean medium and MRS ($p < 0.05$, Figure 1). Notably, the composition of the medium did not affect the cellular development of the enterocins-producing microorganisms. In addition the production of bacteriocins depends on the growth conditions including medium composition, pH, temperature, water activity, and others (Pato et al., 2022a). Fact, to cell growth, peptide-rich substrates may act as inducers or precursors of bacteriocin biosynthesis (Parlindungan; Dekiwadia; Jones, 2021; Vázquez et al., 2005; Yang et al., 2018).

In addition to the protein content, the presence of surfactants such as Tween 80 is an important factor in increased bacteriocin production due to the effect of the surfactant on the permeability of the cell membrane and acceleration of peptide diffusion (Garsa et al., 2014). The formulations prepared in this study contained Tween in their composition, thus allowing the exteriorization of enterocin.

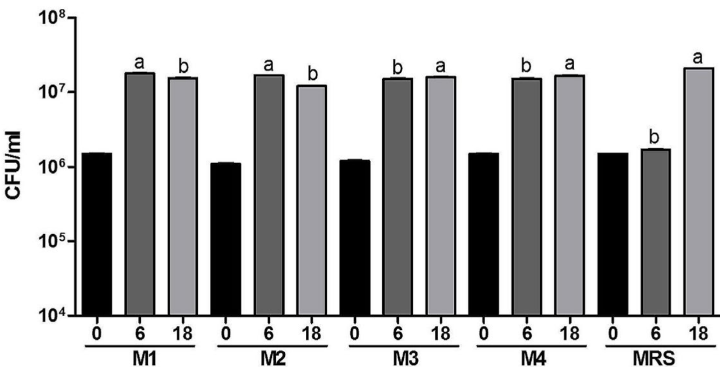

Figure 1: Total bacterial count of *E. durans* MF5 (CFU/mL) in the formulated culture media (M1, M2, M3, and M4) and MRS media, evaluated at 0, 6, and 18 h. Different lowercase letters indicate significant differences, evaluated by Tukey's test ($p < 0.05$).

The enterocins obtained in the different soybean media formulations (M1, M2, M3, and M4) showed antagonistic activity against *L. monocytogenes* and *L. innocua*, as observed in the diffusion well assay (Figure 2A). The inhibition halo formed by enterocins produced in MRS showed a significant difference for *L. innocua* but did not differ for *L. monocytogenes.* Furthermore, regardless of the formulation, enterocins production and antagonistic activity were observed in the isolates tested, thus showing the viability of using a soybean medium for enterocins production.

MRS medium is the most widely used medium for maximizing bacteriocin production; however, it is an expensive substrate (Juárez-Tomás et al., 2002; Moradi; Molaei; Guimarães, 2021; Zhao et al., 2021). Our results showed that bacterial development and enterocins production in soybean media did not differ from those obtained in MRS; notably, soybean medium is a less expensive and easily accessible substrate for large-scale production.

The AU/mL value of enterocins differed according to the target bacterial isolate. However, the enterocins produced in the M1, M2, and M3 media showed a relatively AU (6,400 AU mL^{-1}) for both microorganisms tested, which is a good indicator of antimicrobial activity (Figure 2B). There was no change in enterocins production and action in the formulations containing tryptone.

After the inhibition and AU tests, enterocins produced in the M1 and M2 media were selected for subsequent inhibitory activity and SEM analyses. Total protein dosage ranged from 73 µg/mL in MRS and 92.6 to 96 µg/mL in M1 and M2 media respectively. We emphasize that both soybean media contained 10% or 16% soy flour, dextrose, Tween 80, and potassium phosphate. Several authors report values of 5,000 AU mL^{-1} as satisfactory for antagonistic action. In this study, enterocins produced in soybean media and MRS stood out for having high AU values (6,400 AU mL^{-1}) (Al-Seraih et al., 2017; Du et al., 2017; Furlaneto-Maia et al., 2020; Ghrairi et al., 2008).

When treated with proteolytic enzymes (α-chymotrypsin, protease, and proteinase-K), enterocin MF5 was completely inactivated, confirming its protein character. Similar results were obtained by Pato et al. (2022b), which determined the loss of bacteriocin antimicrobial activity after enzymatic treatment. After heat treatment at 80 °C for 10 min and 100 °C for 20 min, the enterocins produced in MRS and the soybean media showed no change in antagonistic activity with *Listeria* spp., confirming its thermostability.

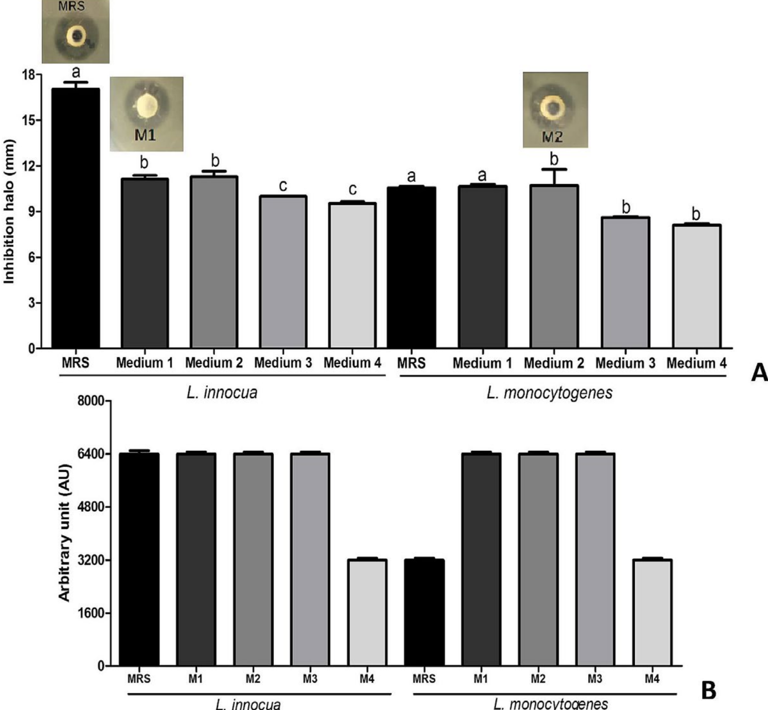

Figure 2: Antilisterial activity of enterocins obtained in different formulations of culture media containing soybean flour (M1, M2, M3, and M4), evaluated by inhibition halo (A) and arbitrary units (AU) (B). Different, lowercase letters indicate significant differences, evaluated by Tukey's test ($p < 0.05$).

The enterocins obtained in the M1 and M2 soybean medium at 6,400 AU mL^{-1} showed antibacterial activity after 6 h of incubation, which was maintained for up to 18 h (Figure 3). Compared to the control (without enterocin), up to 30% inhibition of *L. monocytogenes* CLIP2032 growth and 49% inhibition of *L. innocua* CLIP12612 growth were obtained when samples were treated with enterocins obtained in M1 and M2 media (Figure 3 B). These values did not differ from those for enterocins produced in MRS, thus emphasizing the viability of using other culture components for enterocin production. Similar inhibition was demonstrated with *Escherichia coli* STEC (Tosoni et al., 2019).

Figure 4 confirms the antilisterial action of enterocins obtained in M1 (Figures 4 B and 4 E) and M2 (Figures 4 C and 4 F) media. It can be observed that cells post-treated with enterocins present membrane rupture due to extravasation of the intracellular content when compared to untreated cells (4 A and 4 D), which maintained typical rod shapes with unchanged surfaces.

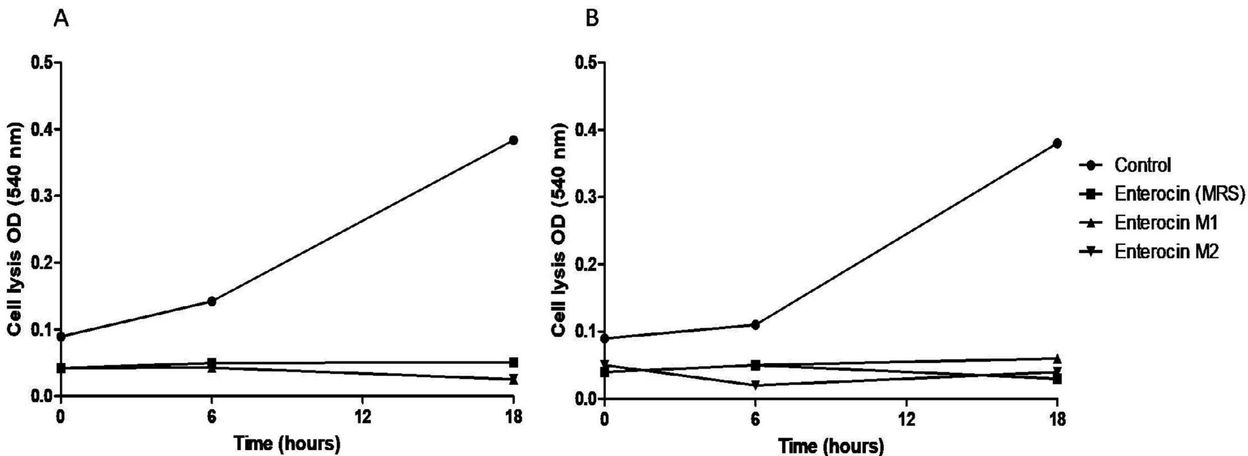

Figure 3: Inhibitory activity of enterocins obtained in soybean media M1 and M2 against *L. monocytogenes* (A) and *L. innocua* (B) determined by measuring the optical density (OD_{540nm}) at different incubation times (0, 6, 12, and 18 h). The control corresponds to the bacterial strains in the culture medium without treatment with enterocins.

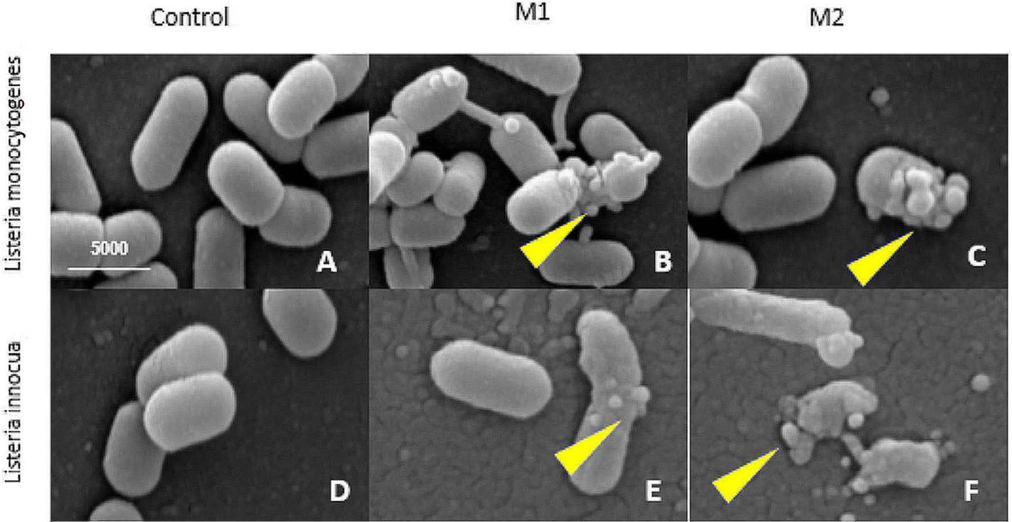

Figure 4: Scanning electron microscopy (SEM) images of *L. monocytogenes* and *L. innocua* pre- and post-translation with enterocins obtained in the M1 and M2 soybean media at 50,000x magnification. Yellow arrows indicate cell damage.

According to the ultrastructural analysis of *L. monocytogenes* CLIP2032 and *L. innocua* CLIP12612 cells (Figure 4), membrane damage occurred, as indicated by cell shrinkage or rupture; such damage was observed with enterocins produced in both M1 and M2 soybean medium. Class II enterocins can induce the formation of pores in the cell membrane, binding to the mannose-phosphotransferase system and influencing cell viability (Meade; Slattery; Garvey, 2020; Shastry; Arunrenganathan; Rai 2020). Several reports suggest that cell disruption caused by bacteriocin is a mode of action on foodborne pathogens (Klayraung; Okonogi; 2009; Kouakou et al., 2010).

Comparing the enterocins obtained in the two culture media (M1 and M2) indicates that the soybean medium provided enterocins; *i.e.*, the amount of soybean flour influenced bacterial growth and aided in antimicrobial activity. Thus, the enterocins obtained in the M2 culture medium showed greater antagonistic activity against *L. innocua* CLIP12612 strains than those obtained in the M1 culture medium, since M1 and M2 differed only in the concentration of soybean flour used (10 g and 16 g, respectively).

Considering the antimicrobial action and considerable yield of enterocins, soybean media M1 and M2 have the potential to obtain antimicrobial peptides for food preservation, since enterocins should be applied based on their activity and mode of action under conditions that reproduce those used in food products (Zou; Liu, 2018).

Enterocins can be added as food preservatives from the incorporation of the starter culture of the producing bacterium and the crude extract of bacteriocin itself or through the incorporation of purified bacteriocin (Abbasiliasi et al., 2017).

CONCLUSIONS

In the current study, was demonstrated that the culture medium M1 and M2 containing soybean meal is a good source for the production of bacteriocins, been pioneer. As soybean meal is a by-product of the food-industry, this substrate is easily accessible and low cost. Enterocins produced in the M1 and M2 culture medium did not differ in antimicrobial action when compared to the enterocins produced in MRS, therefore, the culture medium are potent producers of enterocins. Additionally, can be used effectively as bio preservative for food products.

AUTHOR CONTRIBUTIONS

Conceptual Idea: Maia, L.F.; Methodology design: Maia, L.F., Bússolo, T.B.; Data collection: Bússolo, T.B., Souza, C.M., Souza, N.A.A.; Data analysis and interpretation: Maia, L.F., Bússolo, T.B.; and Writing and editing: Maia, L.F., Bússolo, T.B., Souza, C.M., Souza, N.A.A., Bona, E. and Furlaneto, M.C.

ACKNOWLEDGMENT

This work was supported by UTFPR-Londrina Campus and State University of Londrina-Paraná. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001. The authors thank the "Laboratório Multiusuário da UTFPR Campus Londrina (LabMulti-LD)¨ for the analyses.

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