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Cloning and heterologous expression of recombinant microcin S in *Escherichia coli*

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ABSTRACT

New antibacterial agents against antibiotic-resistant pathogens have clearly attracted global attention. Microcins, as peptide antibiotics, offer novel antimicrobial strategies for therapeutic uses. This study aimed to assess heterologous expression of recombinant microcin S (MccS) from *Escherichia coli* G3/10 in *E. coli* BL21 Star™ (DE3). The physicochemical properties of the MccS protein were analyzed using bioinformatics methods. The desired sequence of the MccS gene was optimized and synthesized in the pMAL-p5X expression vector, positioned downstream and in frame with the maltose-binding protein (MBP) coding sequence. Verification of recombinant protein expression in *E. coli* BL21 Star™ (DE3) was carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Based on the results, the expression of MBP fusion MccS protein with a molecular weight of ~52.742 KDa was achieved in *E. coli* BL21 Star™ (DE3). Furthermore, the expressed protein exhibited 60% solubility, which could be partially attributed to its fusion with MBP. In the current study, the production of recombinant MccS was reported for the first time. The designed expression system proved to be efficient in producing the desired protein. Therefore, in future studies, it will be important to investigate the antibacterial and probiotic activity of MccS. Additionally, it may also act as an efficient biological preservative.

Keywords: Cloning; Heterologous expression; Microcin S; Recombinant protein

INTRODUCTION

The *Enterobacteriaceae* family is a heterogeneous group of gram-negative microorganisms that can be found in human microbiome as commensals and pathogens. In this family (mostly *Escherichia coli*), two classes of bacteriocin are recognized: colicins, which are larger molecules (26–75 kDa), and microcins, which are typically smaller (1–9 kDa) [1]. Microcins (Mcc) are hydrophobic and highly stable peptides, overproduced under stress conditions such as starving, with nanomolar-level antibacterial activity against closely related bacteria [2].

Microcin S (MccS), which is currently the largest microcin known (11.67 kDa), was discovered through analysis of the mechanism behind the successful effect of a probiotic commonly used in functional gastrointestinal disorders. It was identified as a product of *E. coli*

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G3/10, a component of the probiotic medicine Symbioflor 2. The microcin S gene cluster on megaplasmid pSYM1 consists of the four clustered genes *mcsS, mcsI, mcsA,* and *mcsB* and has a genetic organization similar to other class IIa microcins [3, 4]. Due to the strong antimicrobial efficacy of microcins and their selective targeting of specific bacteria, they are promising candidates for use in food preservation or as substitutes to conventional antibiotics [2]. Among them, MccS inhibits the adherence of virulent *E. coli* pathotypes, including enterohemorrhagic (EHEC) and enteropathogenic (EPEC) strains to intestinal epithelial cells [4].

Regarding the microcin S, similar to other bacteriocins, low production yields by native strains is due to the high-energy synthesis process in the cell. Isolation of these natural products from wild-type strains is also a costly and time-consuming process, commonly resulting in low-scale amounts of the products in most cases. To overcome these limitations, recombinant approaches are of particular importance to enhance the product yields [5]. Thus, optimization strategies are used for the production of these microcins by modifying growth factors and peptide expression in highly efficient or peptide-resistant heterologous hosts [6]. As *E.coli* BL21 (DE3) and its derived strains lack the Lon and OmpT protease, these strains are generally used for the expression of microcins [7, 8]. Consequently, the insertion of the MccS coding sequence into this host could significantly develop the production process and provide a suitable platform for applied research.

A well-established strategy to enhance solubility and facilitate affinity purification of recombinant proteins is using amino acid tags at their N- or C-terminus. Among the various tags utilized for this purpose, the *E. coli* maltose-binding protein (MBP) has been extensively employed due to its ability to improve both expression levels and the solubility of target proteins while simultaneously facilitating purification. In this study, the pMAL-p5x plasmid was utilized as an expression vector, enabling the translational fusion of MBP to the N-terminus of the target protein [9, 10].

Despite reports on the antibacterial activity of several microcins, such as MccJ25, MccE492, and MccV [11-13], there is limited information on the biological activity of microcin S, and limited experimental evidence has been provided on the expression of this protein. This knowledge gap highlights the need to investigate the expression of this microcin. The aim of the present study was the expression of MccS recombinant protein in *E. coli* strain BL21 Star™ (DE3) using pMAL-p5x vector, and to confirm its expression using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. While prior studies have primarily characterized the inhibitory effects of native microcin S on pathogenic bacterial adhesion [4], the present study establishes a recombinant expression system for this protein. By transitioning from native isolation to recombinant production, this approach facilitates high-yield synthesis and standardized purification. This development provides a scalable platform for subsequent functional investigations into its antibacterial properties and its potential application as a high-efficiency, natural bio-preservative in the food industry.

MATERIALS AND METHODS

Bacterial strain and expression plasmid: pMAL-p5X plasmid was chosen as expression vector and *E.coli* BL21 Star™ (DE3) was used as the expression host.

Plasmid construction: The amino acid sequence of MccS was extracted (Accession number. AFH37358.1) from National Center for Biotechnology Information (NCBI). A 6×His tag sequence was subsequently added to the C‑terminal end of the target protein. Following this, the amino acid (AA) sequence was converted into the corresponding nucleotide (DNA) sequence using the Benchling online platform (https://www.benchling.com). To ensure efficient expression, the obtained sequence was codon‑optimized using the Codon Optimization Tool available at Integrated DNA Technologies (https://eu.idtdna.com/CodonOpt). The codon adaptation index (CAI) was subsequently calculated using the Rare Codon Analysis Tool

provided by GenScript (https://www.genscript.com/tools/rare-codon-analysis). The resulting sequence was designed to be cloned into the pMAL‑p5x plasmid, positioned directly downstream and in-frame with the *E. coli mal*E sequence (MBP coding sequence), for subsequent expression. Finally, the synthesis and cloning of this sequence into the pMAL-p5X plasmid were performed by GenScript (USA).

Physiochemical characterization and solubility prediction: The MccS sequence modified by the addition of a C-terminal 6×His tag was subjected to *in silico* analysis. The physicochemical properties of this construct, includeing molecular weight, theoretical IP, net charge, instability index, aliphatic index, and grand average hydropathy (GRAVY) were obtained by the Expasy's ProtParam server (https://web.expasy.org/protparam). The solubility of this protein was predicted using the Protein-sol online server (https://protein-sol.manchester.ac.uk). The resulting solubility index was then compared to the Population Average Solubility (PopAvrSol) index for *E. coli* soluble proteins, which is 0.45. A predicted value greater than 0.45 indicates higher solubility relative to the average *E. coli* soluble protein, while a value below 0.45 suggests lower solubility [14].

Secondary structure prediction: Secondary structures prediction of the MccS protein was performed by the SOPMA (https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html) that indicates the amino acids arrangements as α-helices, β-sheets, turns and coils structures.

Three-dimensional structure prediction: The three-dimensional structure of MccS was predicted using the Protein Homology/analogY Recognition Engine, version 2.2 (Phyre2).

Evaluation of MccS expression: Bacterial culture and expression induction. An overnight culture of *E.coli* BL21 Star™ (DE3), transformed with the recombinant plasmid, was prepared on Luria Bertani (LB) agar (HiMedia HiMedia, India) supplemented with 100 µg/mL ampicillin. A single colony was inoculated into LB broth supplemented with ampicillin; cultures were incubated at 37°C with shaking at 200 rpm. Once cell density reached to OD = 0.6-0.8 at 600 nm, 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) was introduced for induction of protein expression. Samples (2 mL of each) were collected from the bacterial culture at 2, 3, 4, and 5 hours post-IPTG induction for subsequent protein expression analysis.

SDS-PAGE and Western blot analysis: The harvested bacterial cultures were transferred to microtubes and subjected to centrifugation to obtain the bacterial pellets. The resulting pellets were suspended in SDS-PAGE sample buffer and subsequently boiled for 15 minutes. The whole-cell lysates were examined by performing 12% polyacrylamide gel electrophoresis. Subsequently, the gels were subjected to staining with Coomassie Brilliant Blue R-250 (Merck, Germany). To carry out the western blotting, the separated proteins by SDS-PAGE were electrotransferred onto a PVDF membrane utilizing a Trans-Blot semidry apparatus (Bio-Rad, USA). The membrane was blocked with 1× Tris-Buffered Saline with Tween-20 (TBST) containing 10% bovine serum albumin (BSA) overnight at room temperature while rotating. To verify the expression of the His-tagged recombinant fusion protein, the membrane was incubated with a 1:1000 dilution of monoclonal anti-polyhistidine–HRP conjugate antibody (Sigma, USA). The colour development was done using H_2O_2/DAB substrate/chromogen (Sigma, USA).

RESULTS

The pMAL-p5X vector was successfully engineered to express MccS, positioning the coding sequence downstream and in-frame with the *E. coli malE* sequence (Fig. 1). The resulting fusion protein comprised 492 amino acids with an expected molecular weight of

52.742 kDa and a predicted isoelectric point (pI) of 6.110; the detailed amino acid sequence is presented in figure 2. Regarding the physiochemical properties of the His-tagged MccS peptide (126 amino acids), it exhibited a molecular weight of 12.50 kDa, a pI of 9.49, and a positive net charge of +5 at pH 7. The negative GRAVY value of –0.47 indicated that MccS is hydrophilic, while the predicted instability index of 37.65, falling below the threshold of 40, suggested that the protein is likely stable. Analysis of the hydropathy profile via the ProtScale tool (Kyte–Doolittle algorithm) revealed alternating hydrophobic and hydrophilic regions (Fig. 3). The solubility of the recombinant MccS protein was predicted using the SOLpro server. The results yielded a solubility probability score of 0.64 for MccS, which is notably higher than that of the control protein, PopAvrSol (0.45). This score indicates a high probability of protein solubility under physiological conditions, suggesting that MccS is likely to remain in a soluble state when expressed in *E. coli*.

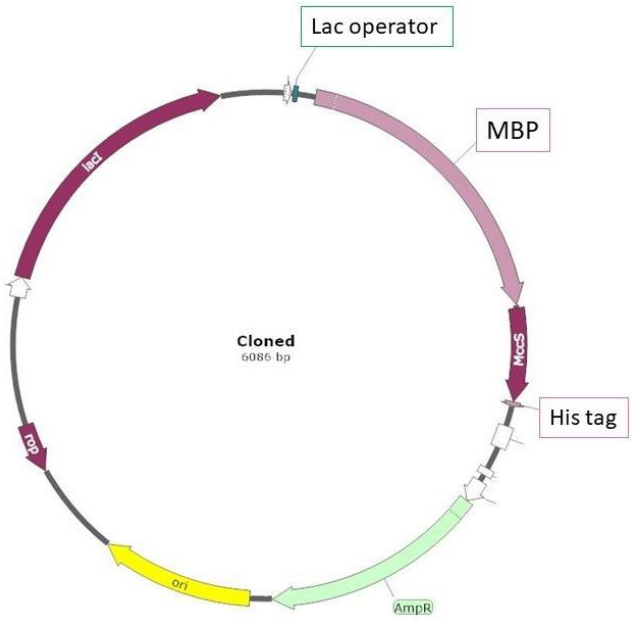

Figure 1: The map of pMAL-p5x vector containing MccS coding sequence. The MccS coding sequence positioned directly downstream and in-frame with the *E. coli mal*E sequence (MBP coding sequence).

Figure 2: The designed protein sequence: Protein Length: 492 aa; MBP: 4-366; MccS: 367-486 (underlined) ; 6xHis tag: 487-492; MW: 52.742Kda; Predicted pI:6.110.

Structural analysis of MccS using the SOPMA server indicated that the peptide is predominantly composed of random coils (66.67%), followed by extended β-strands (17.46%) and α-helices (15.87%). The sequence-based distribution map showed that these α-helical and β-sheet regions are interspersed throughout the sequence, with the probability graph confirming higher confidence levels for coil and sheet formation (Fig. 4). Furthermore, the tertiary structure of MccS was modeled using the Protein Homology/analogY Recognition Engine (Phyre2.2), as illustrated in figure 4. Finally, Western blot analysis confirmed the successful expression of the

recombinant protein in *E. coli* BL21 Star™ (DE3) induced with 1 mM IPTG at 37 °C. The recombinant protein exhibited an apparent molecular weight of approximately 52.742 kDa, consistent with the predicted size, with the highest expression observed 3 h post-induction (Fig. 5).

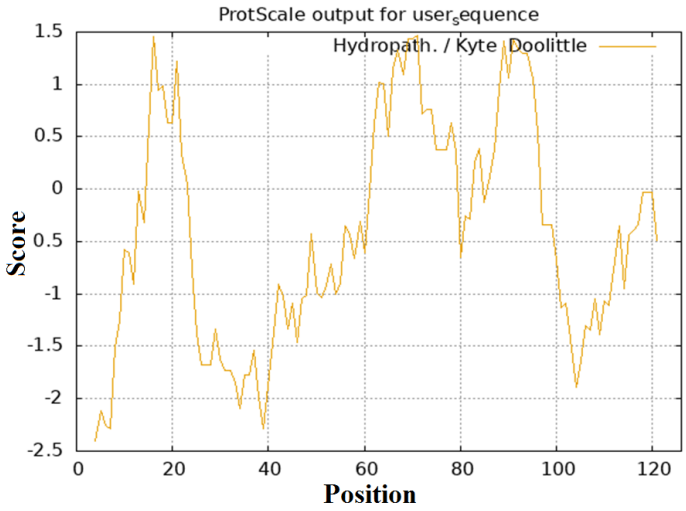

Figure 3: The hydropathy profile of Mcc S calculated using the Kyte-Doolittle algorithm. Positive values (peaks) indicate hydrophobic amino acids, and negative values (valleys), while negative values (valleys) represent hydrophilic amino acids.

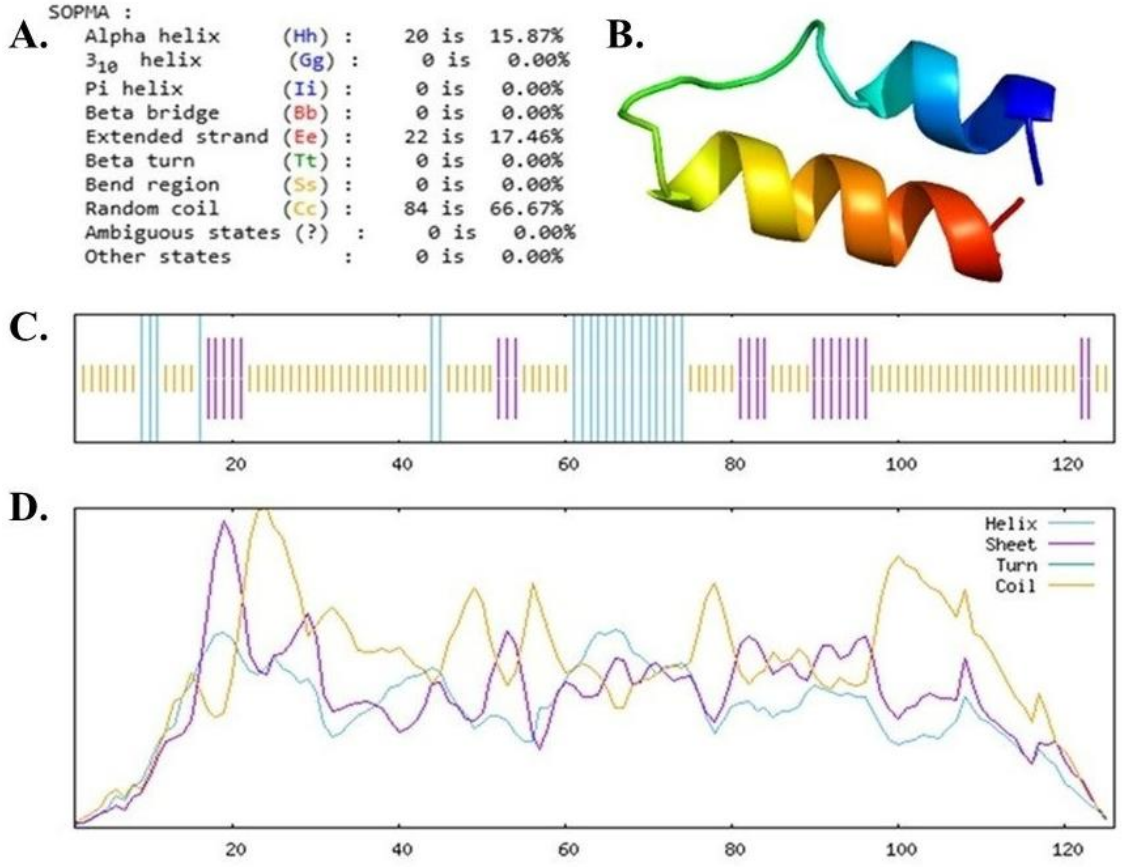

Figure 4: Predicted secondary and tertiary structure of microcin S. (A) Summary of predicted secondary structure composition (SOPMA Analysis), (B) The three-dimensional model predicted using Phyre2 server, (C) Sequence-based distribution of secondary structure elements (The colours are the same as D), (D) The probability graph for secondary structure formation, illustrating the local confidence scores for helix, sheet, turn, and coil.

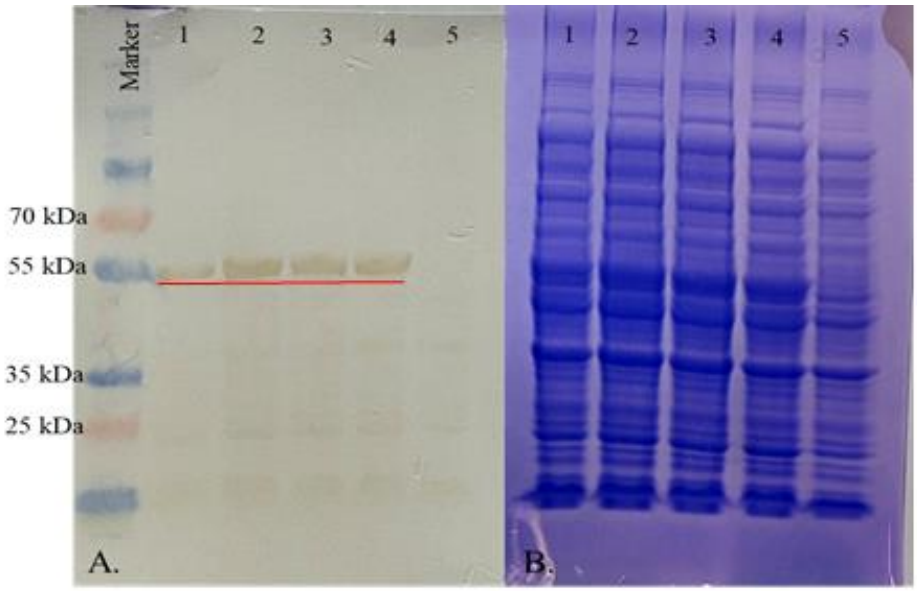

Figure 5: Western blot (A) and SDS-PAGE (B) of recombinant Mcc S. Lane 1: 2h post IPTG induction, Lane 2: 3h post IPTG induction, Lane 4: 4h post IPTG induction, Lane 5: without IPTG. The red line indicates the position of the target protein.

DISCUSSION

The need for new antibacterial agents against antibiotic-resistant pathogens has clearly attracted global attention. Microcins, peptide antibiotics secreted by members of the family *Enterobacteriaceae*, offer novel antimicrobial strategies for preventive and therapeutic uses [15]. Among them, MccS was first produced by *E.coli* G3/10, its role in inhibiting the attachment of EPEC to intestinal epithelial cells was shown [4].

In general, the isolation and purification of microcins from native hosts is time-consuming and difficult. Although the MccS -encoding gene is expressed in *E. coli* G3/10 strain, its expression under the natural conditions of the strain has limitations. Therefore, in this study, for the first time, the direct heterologous expression of recombinant MccS in *E. coli* strain BL21 Star™ (DE3) was investigated. The reason for choosing *E. coli* BL21 Star™ (DE3) strain was that this strain is deficient in Lon and OmpT proteases, and the lack of these proteases reduces the degradation of heterologous proteins expressed in the cells, making this strain an appropriate strain [16].

E. coli BL21 (DE3) and its derivatives have been widely used as expression hosts for bacteriocin production [17, 18]. However, the aggregation of recombinant proteins in their cytoplasm as inclusion bodies following overexpression is a frequently encountered challenge, which adversely affects protein yield. In this study, the pMAL-p5X plasmid was chosen as the expression vector, which has the advantage of adding an MBP tag to the N-terminus of the target protein. The incorporation of an N-terminal MBP in fusion proteins confers a significant advantage in solubility, in addition to facilitating more robust expression [19-21].

Also in this work, the physicochemical properties of this microcin were characterized for the first time. The results showed that this recombinant MccS is a 126-amino-acid hydrophilic peptide with good solubility and stability, and it has an isoelectric point of 9.49 and a net positive charge.

According to the results of the present study, the *mcsS* gene was successfully expressed in the pMAL-p5X vector after induction with IPTG at a final concentration of 1 mM for 3 h at 37 °C in *E. coli* BL21 Star™ (DE3) strain, and the expression of this microcin was demonstrated using SDS-PAGE and confirmed by Western blotting. Based on the findings of the current

study, the utilization of *E. coli* BL21 Star™ (DE3) strain as an expression host in combination with the pMAL-p5X plasmid represents a suitable system for the heterologous expression of microcin. The bioinformatics analysis showed that MccS is a stable and soluble protein. The MccS fused to MBP had 60% solubility which may be attributed not only to the properties of MccS but also to the addition of MBP.

This innovative approach in recombinant production of MccS, can pave the way for future research related to the probiotic properties and the antibacterial activity of this microcin, as well as its use as an efficient biological preservative.

In order to identify the gene responsible for the production of MccS, Zschüttig et al. (2012) cloned genes of the MccS operon (*mcsS*, *mcsA*, *mcsB*, and *mcsI*) and showed that the *mcsS* gene plays a role in the bactericidal activity of MccS, and thus indirectly demonstrated the expression of this microcin based on biological activity [4]. In comparison with the previous study, the present study demonstrated the direct expression of MccS in a heterologous system using SDS-PAGE and Western blotting.

Le et al. (2005) compared the Mcc S gene cluster of *Klebsiella pneumoniae* and *K. quasipneumoniae* strains with *E. coli* G3/10. Microcin amino acid sequence analysis identified two types, S-a and S-b, which had 73.4% and 52.8% identity with MccS of *E. coli* G3/10, respectively. Genomic analysis revealed that the MccS region has the conserved sequence [22]. Although the study by Le et al. mainly focused on the genetic and structural aspects of MccS, our research investigated the heterologous expression of this microcin.

Previous studies on MccS have yielded seemingly contradictory results. Zschüttig et al. (2012) observed that the presence of the MccS gene significantly inhibited EPEC adherence and growth, and also screened for *mcsS* gene in *Enterobacteriaceae* family, including *Salmonella*, *Shigella*, and *E. coli*, and revealed that *mcsS* gene was not detected in any of the strains [4], While Le et al. (2025) showed limited antibacterial activity and also reported the prevalence of the MccS - encoding gene in the isolates studied to be 5% [22]. These differences may be due to variations in MccS -producing strains, as well as differences in the isolates studied for the presence of the *mcsS* gene and the methods for evaluation of antibacterial activity. Therefore, in the present study, the successful expression of this recombinant microcin provides suitable conditions for the mass production of the desired peptide, purification, and also the accurate assessment of the biological activity of the microcin.

The expression of some microcins, such as microcin B-like, was investigated in *E. coli* BL21 Star™ (DE3), and it was found that the heterologous host successfully produced this microcin. Microcin B targets the DNA gyrase and inhibits the growth of several species of the genus *Pseudomonas* and also members of *Enterobacteriaceae* [8]. The present work concentrated on expression of MccS, investigation of antimicrobial and probiotic properties, were beyond the scope of this study. However, many studies, have shown that microcins can inhibit the growth of pathogenic and multidrug-resistant strains. For example, Yu et al. (2022) demonstrated that MccJ25 has potent antibacterial and anti-inflammatory activities [23]. Also, in another study conducted by Sablé et al. (2003), the antimicrobial activity of MccL of *E. coli* LR05 was investigated against *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis, and the results showed strong antibacterial effect against these pathogens [24]. These characteristics indicate that microcins are a feasible option for controlling pathogenic microorganisms. Thus, although the biological activity of MccS was not analyzed in this study, cloning and recombinant expression of this microcin could be a prelude to progress on the antimicrobial activity research. Despite the successful expression of MccS in the present study, one of its limitations is the lack of investigation of the biological activity of the expressed microcin; therefore, evaluation of *in vitro* and *in vivo* antibacterial activity of this peptide is recommended.

Overall, the results of this research suggest that choosing *E.coli* BL21 Star™ (DE3) as a host for recombinant MccS production is suitable. Future research could focus on the purification and assessment of the antimicrobial activity of this peptide and the engineering of probiotic strains.

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Authors' Contribution: VD: investigation, visualization, writing – original draft. **SSS:** conceptualization, supervision, funding acquisition. AD: conceptualization, supervision, methodology, writing – review & editing, funding acquisition. AMB: methodology, visualization, writing – review & editing. VE: methodology (bioinformatics design), visualization, writing – review & editing. FH: conceptualization, methodology. All authors read and approved final version of this manuscript.

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