CAPÍTULO 2

PURIFICATION OF MULTIFUNCTIONAL SUBSTANCES ACTIVE AGAINST *Shigella sonnei*

Data de aceite: 28/09/2022

Jaqueline S. Moreira
Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Jamil S. Oliveira
Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Marcelo P. Bemquerer
EMBRAPA Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Brasília, DF, Brazil

Ricardo A. Machado-de-Ávila
Unidade de Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil

Daniel M. Santos
Fundação Ezequiel Dias, Belo Horizonte, MG, Brazil

Desielle C. Matos
Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Bruna T. Maria
Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Paula P. Magalhães
Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Luiz M. Farias
Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

ABSTRACT: *Shigella* is the etiological agent of shigellosis. Antimicrobial peptides and proteins are biologically active substances produced by prokaryotes and eukaryotes that may present antagonistic activity against a wide range of microorganism. In this study, the intracellular extract of a *Shigella sonnei* isolate was precipitated with 75% ammonium sulfate and purified by sequential chromatography steps using ion exchange, molecular exclusion, and reversed-phase columns. Analysis by mass spectrometry identified three substances with molecular masses of 7.2, 9.2 and 10.7 kDa, active against another *Shigella sonnei* isolate. The amino acid sequences of the active substances were evaluated with the aid of BLAST - P software. The antagonistic substances were identified, respectively, as 50S ribosomal protein L29 of *Escherichia coli*, DNA-binding protein HU-beta and ribosome hibernation promoting factor both of *Shigella sonnei*. Data demonstrated that S*higella sonnei* synthesizes three antimicrobial substances that present other classical functions, active against another isolate of the same species.

KEYWORDS: *Shigella sonnei*, shigellosis, multifunctional substances, antimicrobial peptides and proteins.

INTRODUCTION

Shigella is a genus of Gram-negative enteric pathogens including four species, *Shigella sonnei, S. flexneri, S. dysenteriae*, and *S. boydii*, that are heterogeneously worldwide distributed (Puzari et al. 2018; Sheikh et al. 2019). All of them are agents of shigellosis, a disease that affects about 165 million people around the world with approximately one million deaths per year, mostly children from underdeveloped regions (González-Torralba et al. 2018; McCrickard et al. 2018; Aslam and Okafor, 2019). When infected, the individual may present from watery diarrhea to a severe inflammatory disease, with abscesses and ulcerations in the mucous intestinal membrane. The infection is also characterized by abdominal pain, anorexia, vomiting, fever, and mucous bloody feces (Niyogi 2005; Phalipon and Sansonetti, 2007; Marteyn et al. 2012; Carayol and Nhieu, 2013; Nygren et al. 2013; The et al. 2016; Kotloff et al. 2018; Aslam and Okafor, 2019). In some cases, the patient may develop sequelae after infection healing, such as arthritis, sepsis, seizures, and haemolytic uremic syndrome (Duggan and Mostowy, 2018). The development of resistance to antibiotics by *Shigella* is an emerging problem and according the World Health Organization (WHO), *Shigella* is among the twelve pathogens that require urgent intervention, such as discovery and development of new antibiotics targeting the organism (WHO report, 2017; Duggan and Mostowy, 2018; Micoli et al., 2022).

Antimicrobial proteins and peptides may be synthesized by several organisms including prokaryotes and both upper and lower eukaryotes (Wilson et al. 1999; Nuding et al. 2007; Meyer-Hoffert et al. 2008; Hegedus and Marx, 2013; Saggese et al. 2022). Examples of such compounds synthesized by ribosomes active against bacteria, fungi, and viruses include defensins produced by vertebrates, arthropods, and plants (Bulet et al. 1999; Ganz, 2003; Wilson et al. 2016); cecropin (Ouyang et al. 2015) and drosomycin (Bulet et al. 1999 ; Lee et al. 2002a) synthesized by insects; magainin II and ranatuerin 2Mb produced by amphibians (Rollins-Smith, 2009); bacteriocins expressed by Gram-negative (Rebuffat 2011; Micenková et al. 2016) and Gram-positive bacteria (Rea et al. 2011; Ha, 2016); histone-like proteins produced by fishes (Park et al. 1996; Noga et al. 2001; Bergsson et al. 2005), toads (Cho et al. 2009), and bacteria (Carvalho et al. 2010); ribosomal proteins synthesized by mice (Meyer-Hoffer et al. 2008), fishes (Bergsson et al. 2005), and bacteria (Putsep et al. 1999; Lee et al. 2002a,b; Carvalho et al. 2010, 2018).

Antimicrobial peptides (AMPs) are generally substances made up of as many as 100 amino acids with cationic or anionic charges, although anionic peptides are less commonly reported (Bahar and Ren, 2013; Leite et al. 2019). The attraction between the antimicrobial peptide and the target bacterium culminates in formation of electrostatic bonds between

either the anionic or the cationic portions of the peptide with the bacterium (Brogden, 2005). Thus, anionic AMPs are generally rich in glutamine and aspartic acid and some of the use metal ions, as zinc, to create cationic salt bridges with negatively charged substances of microbial membranes, which allows its penetration by the membrane and acting against intracellular components, such as ribosomes (Jeżowska-Bojczuk and Stokowa-Sołtys, 2018). Already the cationic AMPs are attracted to the anionic structures of the bacterial wall, such as anionic phospholipids and phosphate groups on lipopolysaccharide (LPS) of Gram-negative bacteria and the teichoic acids on the surface of Gram-positive bacteria (Brogden, 2005; Jenssen et al. 2006). After this contact, it translocate the bacterial cell wall and may act against the bacterial target, which may be the cytoplasmic membrane and intracellular components (Brogden, 2005; Jenssen et al. 2006; Bahar and Ren, 2013) In this study, we purified three antibacterial substances produced by *S. sonnei* that express antagonism against an isolate of the same species.

MATERIAL AND METHODS

Bacterial strains

Two strains of *S. sonnei*, named as SS9 and SS12 were included in the study as producer of antagonist substance(s) and indicator of antagonism expression, respectively. They were obtained from fecal specimens of children presenting acute diarrhea who searched for assistance at Hospital Infantil João Paulo II, Belo Horizonte, Brazil (Sousa et al. 2010). Bacterial isolates have been kept in Brucella Broth (BBL, Sparks, MD, USA) added with 10% glycerol (v/v) at -80 °C. Before use, they were cultivated for three consecutive days in Tryptic Soy Agar (TSA; Difco, Sparks, MD, USA) at 37 °C, for 24 h, in aerobiosis (Sousa et al. 2010).

Isoantagonist activity assay

Antimicrobial activity of SS9 against SS12 was confirmed by the double-layer diffusion test (Farias et al. 1994; Sousa et al. 2010). Ten microliters of SS9 culture was spotted onto the surface of TSA. After drying of the drop the plate was incubated at 37 °C, for 24 h, in aerobiosis. The viable cells were eliminated with chloroform vapor for 30 min followed by keeping the plate partly open, in sterile environment, for evaporation of residual chloroform. The plate was covered with 3.5 mL of semisolid TSA (TSB + 0.7% agar) added with 10 μL of a 24h culture of the SS12 and incubated as described above. The positive result for isoantagonistic action was determined by observation of an inhibition halo around the spot of SS9.

Intracellular protein extraction

Colonies of *S. sonnei* SS9 obtained from the third day of cultivation were inoculated

in 60 mL of Tryptic Soy Broth (TSB; Difco) and incubated at 37 ºC, for 24 h, under aerobic conditions. After this period, the culture was added to 1,740 mL of TSB and incubated at 37 °C, in aerobiosis. After 12 h, the culture was centrifuged at 16,200 *g*, for 30 min, at 4 °C and the sediment was suspended in 20 mmol.L^{-1} Tris-HCl buffer, pH 8.0, centrifuged and resuspended in the same buffer. The suspension was sonicated at 50 W for 12 cycles of 1 min with 30 s intervals, in an ice bath. After that, the material was centrifuged and the supernatant was kept in an ice bath and undergone protein precipitation by means of ammonium sulfate addition (concentrations 0-30 and 30-75% p/v; Vetec, Duque de Caxias, RJ, Brazil). After centrifugation at 29,830 *g*, for 30 min, at 4° C sediments were dissolved in the same buffer, generating the intracellular extracts named as C-30 and C-75. C-75 was dialyzed against the same buffer at 4°C overnight, aliquoted, and kept at -80 °C (Moreira, 2011, Sousa et al. 2013).

Evaluation of antagonism expression, titration and determination of protein concentration

Antagonism expression was evaluated by the double-layer diffusion test. A 10 µL aliquot of C-75 was dripped onto the surface of TSA. After exposure to chloroform vapor for 30 min and evaporation of residual chloroform, 3.5 mL of semisolid TSA added with 10 μL of a 24 h culture of the indicator strain was poured over TSA layer. After incubation at the same conditions described before, the presence of inhibition zones was considered indicative of antagonistic activity. Tris-HCl buffer and ultrapure water were employed as controls (Booth et al. 1977; Sousa et al. 2013). The assay was used to determine the antagonistic activity of the samples obtained from the purification steps.

C-75 and the pools of the fractions of chromatography on ion exchange and molecular exclusion columns were titrated through serial dilutions in ultrapure water and tested for antagonistic activity. Results were expressed as arbitrary units per milliliter (AU.mL^{-1}) which is defined as the highest dilution that results in a clear zone of inhibition of the indicator strain (Batdorj et al. 2006).

Protein concentration of C-75 and the pools of the fractions of chromatography on ion exchange and molecular exclusion columns were determined according to the methodology described by Bradford (1976) using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as standard.

Purification of antibacterial peptides

C-75 was submitted to sequential chromatography steps as specified. The first purification step was performed in a Mono-Q™ ion exchange column, Tricorn 5/50 GL™ (GE Healthcare, Uppsala, Sweden) coupled to a FPLC system (Pharmacia, GMI, Ramsey, MN). The column was previously equilibrated in 20 mmol L^{-1} Tris-HCl buffer, pH 8.0, at a flow rate of 60 mL.h^{-1}. For elution, the same buffer with addition of 1 M of NaCl was used in a linear gradient from 0-100% in 60 min with detection at 280 nm. Fractions were

collected, lyophilized, re-suspended in ultrapure water, and assessed for antagonist activity. Selected fractions were combined into a pool that was submitted to a Superose 12HR 10/30 molecular exclusion column (Pharmacia Biotech, Piscataway, NJ, USA) coupled to a FPLC system. The mobile phase was Tris-HCl buffer, pH 8.0, at a rate of 30 $mL.h^{-1}$. Fractions were monitored at 280 nm. The fractions were lyophilized, solubilized, and tested concerning their antagonism expression. Selected pools of fractions were separately chromatographed in a C8-Supelco reversed-phase column (4,6 mm x 25 cm, 5 μm) coupled to an HPLC (Shimadzu Prominence, Kyoto, Japan) system. The column had been previously eluted with H_2O:ACN:TFA (20:80:0.1, by volume) and equilibrated with H_2O:TFA (100:0.1, by volume). The fractions were eluted with at a flow rate of 1.0 $mL.min^{-1}$, with the following solvent conditions: H_2O:TFA (100:0.1) for 10 min, followed by a linear gradient to H_2O:ACN:TFA (20:80:0.1) during 30 minutes and an isocratic condition at H_2O:ACN:TFA (20:80:0.1) for 40-45 min. The fractions were monitored at ƛ 220 and ƛ 280 nm. They were lyophilized, solubilized, and tested concerning their antagonism expression. Selected fraction and pool were separately injected into a C18-Sephasil™ Peptide reversed-phase column (4,6 mm x 25 cm x 5 μm) coupled to HPLC, following the same protocol described for C8 column. Active fractions analyzed by mass spectrometry (Moreira, 2011).

Mass spectrometry

Fractions and purified proteins were lyophilized, and solubilized in 0.1 % TFA solution. Then, 1 μL of protein solution was mixed to 3 μL of matrix solution (α-cyano-4-hydroxycinnamic acid, α-CHCA,), which was prepared at 50 mmol/L in in H_2O:ACN:TFA (1:1:0.3, v:v:v). This mixture spotted onto the MALDI acquisition plate (MTP Anchor Chip 384 x 600, Bruker Daltonics, Billerica, MA, USA) that was kept at room temperature. For determining the protein molecular mass, an Autoflex® III MALDI-TOF/TOF spectrometer (Bruker Daltonics) was operated in the positive linear mode. For external linear calibration, either Peptide Calibration Standard II, or Protein Calibration Standard I (Bruker Daltonics) were employed. Mass analysis ranged from m/z 500 to m/z 4000 for peptides and from m/z 4,000 to m/z 20,000 for proteins, using the respective voltage parameters determined by Bruker Daltonics. Partial peptide sequences employed for protein identification were obtained by in-source dissociation (ISD-MALDI) in an Ultraflex III mass spectrometer (Bruker Daltonics). As matrix, a solution of 1,5-diaminonaphthalene at 60 mmol/L was prepared in H_2O:ACN:TFA (1:1:0.3, v:v:v). This solution (1 μL) was mixed with an aqueous solution (1 μL) of the purified proteins and spotted onto the MALDI acquisition plate. The following analytical voltage parameters were employed: ion source 1, 25.00 kV; ion source 2, 22.40 kV; lens, 9.40 kV; reflector, 26.50 kV; reflector 2, 13.46 kV. The m/z values are not calibrated. One analyzed the results by using the Flex Analysis program (Bruker Daltonics).

Amino acid sequencing of the antagonist substances

The sequence of amino acid residues of the purified antibacterial substances was

determined by fragmentation Lift and ISD on a mass spectrometer. Sequences obtained were analyzed by comparison with known protein sequences using the BLAST-P software.

RESULTS AND DISCUSSION

S. sonnei SS9 is able to produce proteic antagonist substances active against the same species. Following lysis by sonication and protein precipitation, it was possible to recover 23 mL of C-75. The extract was active against SS12, with an antagonistic activity titer 3.60 x 10^{18} AU/mL and a protein concentration of 113 mg/mL. The high titer obtained is important considering that loss of material should be expected as a result of chromatographic processes employed for purification.

C75 was subjected to purification by chromatographic sequential steps. The pools of the selected active fractions obtained from ion exchange and molecular exclusion chromatography were analyzed by titration of the antagonistic action, volume and protein dosage (Tab.1). The same parameters were not analyzed for the samples obtained from reversed-phase chromatography steps due to the reduced amount of material obtained. Forty active fractions were recovered from the ion exchange column. Among them, fractions 1 to 7 that presented positive or neutral liquid charge were pooled and submitted to molecular exclusion chromatography. Among the 25 fractions generated, 14 were active against SS12. Two pools including fractions 2 to 6 and 7 to 9 were subsequently submitted to reserved-phase chromatography. Active fractions named B15 (P1) obtained from pool 2 to 6, C1 to C3 (P2) and C4 to C10 (P3) from pool 7 a 9 were *re-chromatographed in* Sephasil™ Peptide reversed-phase column. The active fractions 35 (F1), 34 (F2) and 34 (F3) generated by this last chromatographic step, originated from P1, P2 and P3, respectively, were eluted in 67%, 64% and 64% acetonitrile, suggesting that they are hydrophobic molecules (Figs. 1, 2, 3).

Mass spectrometry analysis of sample F1 showed a purified active substance with molecular mass of 9,215 Da (Fig. 4). The following partial fifty-two amino acid residues sequence was obtained: **MNKSQLIDKIAAGADISKAAAGRALDAIIASVTESLKEGDDVALVGFGTFAV**. The sequence demonstrated 100% identity and query coverage with DNA-binding protein HU beta from *S. sonnei*, which is composed of 90 amino acid residues (Tab. 2). One must point out that the neither glutamine may be distinguished from lysine nor leucine may be distinguished from its isomer isoleucine in the MALDI mass spectrometry method employed.

The purified active sample F2 corresponded to a substance with a molecular mass of 10,742 Da (Fig. 5). The partial sequencing of the substance identified nineteen amino acid residues correspondent to the sequence **LREFVTAKFAKLEQYFDRI,** which presented 100% identity and query coverage with ribosome hibernation promoting factor from *S. sonnei*, a protein that is constituted of 95 amino acid residues (Tab. 2).

The sample F3 presented a purified substance with molecular mass of 7,271 Da. (Fig. 6). Sequence analysis of seventeen amino acids residues, **SVEELNTELNLNLRKEF**,

identified the substance as 50S ribosomal protein L29 of *Escherichia coli*, which is composed of 63 amino acid residues. The analysis demonstrated 83% identity e 100% query coverage with the reported sequence (Tab.2).

Antimicrobial peptides present from 12 to 100 residues of amino acids which are usually cationic and amphiphilic. Regardless of their mechanism of action, the antibacterial action depends on the contact with the cell membrane promoted by electrostatic and hydrophobic interactions. Therefore, the first contact between the antimicrobial peptide and the target bacteria is given by the electrostatic interactions between the cationic peptide and the negatively charged component found in the bacterial envelope, as the phosphate of lipopolysaccharide and anionic phospholipids of Gram-negative bacteria or the lipoteichoic acid of Gram-positive bacteria. After their first contact, the hydrophobic interactions occur, by the insertion of the antibacterial peptide into the outer membrane of Gram-negative bacteria, which provides a perturbation that promotes the entry of the peptide into the periplasmic space. In the periplasmic space the peptide may integrate with the negatively charged lipids present in the monolayer of the cytoplasmic membrane, or they may cross the membrane and interact with intracellular compounds (Brogden 2005; Jenssen et al; 2006, Bahar and Ren, 2013; Gupta et al. 2014; Seo et al. 2017; Jeżowska-Bojczuk and Stokowa-Sołtys, 2018).

Carvalho et al. (2010) purified a DNA-binding HV histone from *Lactobacillus. sakei* subsp. Sakei 23K supernatant that showed antagonistic action against *Listeria monocytogenes* and *Enterococcus faecalis*. The authors suggest that the antagonistic action of the substance may be due to the interaction of the molecule with DNA structure and replication. Similar results are known in the literature for a histone fragment, denominated as Buforin II, isolated from the gastric tissue of the Asian toad (*Bufo bufo garagrizans*). The molecule is able of crossing the cell membrane of the target bacterium and of strongly interacting with nucleic acids causing bacterial death (Park et al. 2000). Other peptides with antibacterial action derived from histones were also isolated, such as oncorhyncin II, oncorhyncin III, HLP1, and H2B from *Oncorhynchus mykiss* (Rainbow trout); hipposin-I from *Hippoglossus hippoglossus* (Atlantic halibut); and SAMP H1 from *Salmo salar* (Atlantic salmon) (Valero et al., 2013).

Another interesting result was obtained by Mryer-Hoffert et al. (2008). The authors isolated a ribosomal protein (L29) from mouse's lung and small intestine that showed antagonist activity against intestinal and pulmonary microorganisms *L. monocytogenes, Streptococcus pyogenes, E. coli*, and different lineages of *Pseudomonas aeruginosa*. Similar data were obtained by Pidutti et al (2018), that isolated from the extracellular medium of *Lactobacillus salivarius* SGL03 the L27 and L30 ribosomal proteins belonging to 50S subunit that present antagonistc action against *S. pyogenes*, *Streptococcus uberis* and *Enterococcus faecium*. Furthermore, these two substances presented bactericidal action against *S. pyogenes*. Four ribosomal proteins and a translation initiation factor IF-1 peptide

of *L. sakei* were also active against *Listeria* spp., *E. faecalis* and *L. sakei* isolates (Carvalho et al. 2018). In addition, hemoglobin derived peptides Hbβ P1, P2, and P3 obtained from *Ictalurus punctatus* (Channel catfish) showed antibacterial and antiparasitc properties (Ullal et al. 2008). Other hemoglobin derived peptide, denominated as SHβAP, presented antagonist activity against Gram-positive bacteria, such as *Bacillus subtilis, Staphylococcus aureus,* and *Streptococcus iniae* as well as Gram-negative bacteria, such as *E. coli*, *P. aeruginosa, Salmonella enterica, S. sonnei*, and *Vibrio parahaemolyticus* (Seo et al. 2014). Astacin-1, a hemocyanin derived peptide originating from *Pacifastacus leniusculus* (Crayfish) also shows antibacterial action (Lee et al. 2003).

In conclusion, *S. sonnei* SS9 isolate is able to produce three proteic multifunctional compounds that express antimicrobial activity against other *S. sonnei* isolates. These substances may be candidates for new drugs against shigellosis. Other studies to evaluate their mechanism of action, stability, activity against indigenous microbiota and eukaryotic cells, among others, should be performed for verifying the biotechnological potential of these molecules.

ACKNOWLEDGEMENTS

This study was supported by grants from the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), proc. n° CBB-APQ-02068-15, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Conselho de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Pró-reitoria de Pesquisa da Universidade Federal de Minas Gerais (PRPq/UFMG), Brazil. To the Laboratório de Proteômica belonging to the Centro de Laboratórios Multiusuários (CELAM) for making the equipment available for viewing the results.

REFERENCES

Aslam A, Okafor CN. *Shigella* (Shigellosis) [Updated 2019 Feb 8]. In: StatPearls [Internet]. Treasure Island (FL): Stat Pearls Publishing; 2019 Jan-.Available from: https://www.ncbi.nlm.nih.gov/books/NBK482337/ Accessed 20 april 2019.

Bahar AA, Ren D. (2013) Antimicrobial peptides. Pharm 6:1543-1575.

Batdorj B, Dalgalarrondo M, Choiset Y, Pedroche J, Métro F, Prevost H, Chobert JM, Haertlé T. (2006) Purification and 168 characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. J Appl Microbiol 101:837-848.

Bergsson G, Agerberth B, Jörnvall H, Gudmundsson GH. (2005) Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). Febs J 272:4960-4969.

Booth SJ, Johnson JL, Wilkins TD. (1977) Bacteriocin production by strains of *Bacteroides* isolated from human feces and the role of these strains in the bacterial ecology of the colon. Antimicrob Agents Chemother 11:718-724.

Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Brogden KA. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat 3:238-250.

Bulet P, Hetru C, Dimarcq JL, Hoffmann D. (1999) Antimicrobial peptides in insects; structure and function. Development Comparat Immunol 23:329-344.

Carayol N, Nhieu GTV. (2013) The inside story of *Shigella* invasion of intestinal epithelial cells. Cold Spring Harb Perspect Med 3:1-13.

Carvalho KG, Bambirra FHS, Kruger MF, Barbosa MS, Oliveira JS, Santos AMC, Nicoli JR, Bemquerer MP, Miranda A, Salvucci EJ, Sesma FJM, Franco BDGM. (2010) Antimicrobial compounds produced by *Lactobacillus sakei* subsp. *sakei* 2a, a bacteriocinogenic strain isolated from a Brazilian meat product. J Ind Microbiol Biotechnol 37:381–390.

Carvalho KG, Bambirra FHS, Nicoli JR, Oliveira JS, Santos AM, Bemquerer MP, Miranda A, Franco BD. (2018) Characterization of multiple antilisterial peptides produced by sakacin P-producing *Lactobacillus sakei subsp. sakei* 2a. Arch Microbiol 200:635-644.

Cho JH, Sung BH, Kim SC. (2009) Buforins: histone H2A-derived antimicrobial peptides from toad stomach. Biochim Biophys acta (*bba)*-Biomem 1788:1564-1569.

Duggan GM, Mostowy S. (2018) Use of zebrafish to study *Shigella* infection. Dis Model Mech11:1-11.

Farias LM, Totola AH, Miranda CMS, Carvalho MAR, Damasceno CAV, Tavares CAP, Cisalpino EO, Vieira EC. (1994) Extraction, partial purification and characterization of a bacteriocin (fragilicin) produced by a strain of *Bacteroides fragilis* isolated from *Callithrix penicillata*. Res Microbiol 145:9-16.

Ganz T. (2003) Defensins: antimicrobial peptides of innate immunity. Nat Ver Immunol 3:710-720.

González-torralba A, García-esteban C, Alós JI. (2018) Enteropatógenos y antibióticos. Enferm Infecc Microbiol Clin 36:47-54.

Gupta A, Gupta R, Kurwardkar S. (2014) Liposome-encapsulated antimicrobial peptides: Potential infectious diseases therapy. In: Soni S, Salhotra A, Suar M (eds) Handbook of research on diverse applications of nanotechnology in biomedicine, chemistry, and engineering, 1st edn. IGI Global, Pensilvânia, pp 301-332.

Ha EM. (2016) *Escherichia coli*-derived uracil increases the antibacterial activity and growth rate *of Lactobacillus plantarum*. J Microbiol Biotechnol 26:975-987.

Hegedüs N, Marx F. (2013) Antifungal proteins: more than antimicrobials? Fungal Biol Rev 26:132-145.

Jenssen H, Hamill P, Hancock REW. (2006) Peptide antimicrobial agents. Clil Microbiol Ver 19:491–511.

Jeżowska-Bojczuk M, Stokowa-Sołtys K. (2018) Peptides having antimicrobial activity and their complexes with transition metal ions. Eur J Med Chem 143:997-1009.

Kotloff KL, Riddle MS, Platts-Mills JA, Pavlinac P, Zaidi AKM. (2018) Shigellosis. Lancet. 391:801-812.

Lee DG, Park Y, Kim HN, Kim HK, Kim PI, Choi BH, Hahm KS. (2002a). Antifungal mechanism of an antimicrobial peptide, HP (2–20), derived from N-terminus of *Helicobacter pylori* ribosomal protein L1 against *Candida albicans.* Biochem Biophys Res Commun 291:1006-1013.

Lee DG, Kim HN, Park Y, Kim HK, Choi BH, Choi CH, Hahm KS. (2002b) Design of novel analogue peptides with potent antibiotic activity based on the antimicrobial peptide, HP (2–20), derived from N-terminus of *Helicobacter pylori* ribosomal protein L1. Biochem Biophys Acta 1598**:**185-194.

Lee SY, Lee BL, Soderhall K. (2003) Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*. J Biol Chem 278:7927-7933.

Leite ML, Sampaio KB, Costa FF, Franco OL, Dias SC, Cunha NB. (2019) Molecular farming of antimicrobial peptides: available platforms and strategies for improving protein biosynthesis using modified virus vectors. An Acad Bras Cienc 91:1-23.

Marteyn BS, Gazi AD, Sansonetti, PJ. (2012) *Shigella*: A model of virulence regulation in vivo. Gut Microbes 3:104-120.

Mccrickard LS, Crim SM, Kim S, Bowen A. (2018) Disparities in severe shigellosis among adults - Foodborne diseases active surveillance network, 2002-2014. BMC Public Health 18:1-8.

Meyer-Hoffert U, Hornef M, Henriques-Normark B, Normark S, Andersson M, Pütsep K. (2008) Identification of heparin/heparan sulfate interacting protein as a major broad-spectrum antimicrobial protein in lung and small intestine. Faseb J 22:2427-2434.

Micenková L, Bosák J, Štaudová B, Kohoutová D, Čejková D, Woznicová VMV, Ševčíková A, Bureš J, Šmajs D. (2016) Microcin determinants are associated with B2 phylogroup of human fecal *Escherichia coli* isolates. Microbiol Open. 5:490-498.

MICOLI F, NAKAKANA U N, BERLANDA S F.(2022) Towards a Four-Component GMMA-Based Vaccine against *Shigella*. Vaccines. 10: 1-14.

Moreira JS (2011) Extração, purificação e caracterização de substância antagonista produzida por *Shigella sonnei*. Monografia, Universidade Federal de Minas Gerais.

Niyogi SK. (2005) Shigellosis. J Microbiol 43:133-143.

Noga EJ, Fan Z, Silphaduang U. (2001) Histone-like proteins from fish are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum.* Parasitol 123:57-65.

Nuding S, Fellermann K, Wehkamp J, Stange EF. (2007) Reduced mucosal antimicrobial activity in Crohn's disease of the colon. Gut 56:1240-1247.

Nygren BL, Schilling KA, Blanton EM, Silk BJ, Cole DJ, Mintz ED. (2013) Foodborne outbreaks of shigellosis in the USA, 1998–2008. Epidemiol Infect 141:233-241.

Ouyang L, Xu X, Freed S, Gao Y, Yu J, Wang S, Ju W, Zhang Y, Jin F. (2015) Cecropins from *Plutella xylostella* and their interaction with *Metarhizium anisopliae*. PloS one10:1-16.

Park CB, Kim MS, Kim SC. (1996) A novel antimicrobial peptide from *Bufo bufo gargarizans*. Biochem Biophys Res Commun 218:408-413.

Park CB, Yi KS, Matsuzaki K, Kim MS, Kim SC. (2000) Structure activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. Proc Nat Acad Sci USA 97:3245–3250.

Phalipon A, Sansonetti PJ. (2007) *Shigella*'s ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival? Immunol Cell Biol 85:119-129.

Pidutti P, Federici F, Brandi J, Manna L, Rizzi E, Marini U, Cecconi D. (2018) Purification and characterization of ribosomal proteins L27 and L30 having antimicrobial activity produced by the *Lactobacillus salivarius* SGL03. J Appl Microbiol. 124:398-407.

Pütsep K, Brändén CI, Boman HG, Normark S. (1999) Antibacterial peptide from *H. pylori*. Nat 398: 671-672.

Puzari M, Sharma M, Chetia P. (2018) Emergence of antibiotic resistant *Shigella* species: A matter of concern. J Infect Public Health. 11:451-454.

Rea MC, Ross RP, Cotter PD, Hill C (2011) Classification of bacteriocins from Gram-positive bacteria. In: Drider D, Rebuffat S (eds) Prokaryotic antimicrobial peptides: from genes to applications, 1st edn. Springer, New York, pp. 29-53.

Rebuffat S (2011) Bacteriocin from Gram-negative bacteria: A classification? In: Drider D, Rebuffat S (eds) Prokaryotic antimicrobial peptides: from genes to applications, 1st edn. Springer, New York, pp. 55-71.

Rollins-Smith LA. (2009) The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochim Biophys acta (bba)-Biomem 1788:1593-1599.

SAGGESE A, LUCA Y, BACCIGALUPI L, RICCA E. (2022) An antimicrobial peptide specifcally active against *Listeria monocytogenes* is secreted by *Bacillus pumilus* SF214. BMC Microbiol. 22:1-11.

Seo JK, Lee MJ, Jung HG, Go HJ, Kim YJ, Park NG. (2014) Antimicrobial function of SHβAP, a novel hemoglobin β chain-related antimicrobial peptide, isolated from the liver of skipjack tuna, *Katsuwonus pelamis*. Fish & Shellfish Immunol 37:173–183.

Seo J, Kim D, Oh R, Park K, Lee I, Cho S, Lee K, Nam B. (2017) Antimicrobial effect of the 60S ribosomal protein L29 (cgRPL29), purified from the gill of pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol 67:675-683.

Sheikh AF, Moosavian M, Abdi M, Heidary M, Shahi F, Jomehzadeh N, Seyed MS, Saki M, Khoshnood S. (2019) Prevalence and antimicrobial resistance of Shigella species isolated from diarrheal patients in Ahvaz, southwest Iran. Infect Drug Resist 12: 249–253.

Sousa MAB, Mendes EN, Apolônio ACM, Farias LM, Magalhães PP. (2010) Bacteriocin production by *Shigella sonnei* isolated from faeces of children with acute diarrhea. APMIS 118:125-135.

Sousa MAB, Farias LDM, Oliveira PLD, Moreira JS, Apolonio ACM, Oliveira JS, Santoro MM, Mendes EN, Magalhaes PP. (2013) Antagonistic activity expressed by *Shigella sonnei*: identification of a putative new bacteriocin. Mem Inst Oswaldo Cruz 108:724-729.

The HC, Thanh DP, Holt KE, Thomson NR, Baker S. (2016) The genomic signatures of *Shigella* evolution, adaptation and geographical spread. Nat Rev Microbiol 14: 235-250.

Ullal AJ, Litaker RW, Noga EJ. (2008) Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). Dev Comp Immunol 32:1301-1312.

Valero Y, Chaves-Pozo E, Meseguer J, Esteban MA, Cuesta A (2013) Biologial role of fish antimicrobial peptides. In: Seong MD, Hak YI (eds) Antimicrobial peptides: properties, functions and role in immune response, 1st edn. Nova Science Publishers, New York, pp.31-60.

Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez-Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC. (1999) Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defense. Sci 286:113-117.

Wilson SS, Wiens ME, Holly MK, Smith JG. (2016) Defensins at the mucosal surface: latest insights into defensin-virus interactions. J Virol 90:5216-5218.

World Health Organization (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. http://www.who.int/medicines/publications/WHO-PPL Short_Summary_25Feb-ET_NM_WHO.pdf. Accessed 3 february 2019.

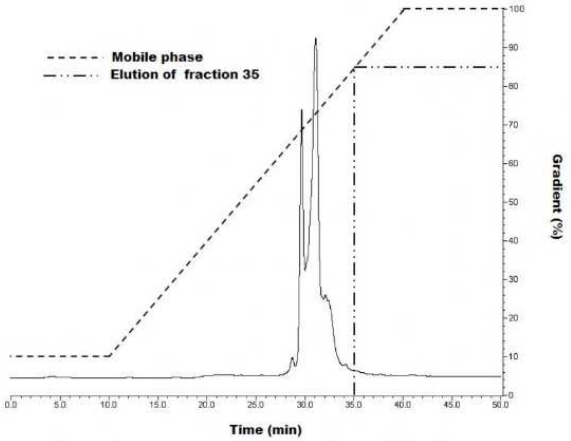

Fig.1 C18-Sephasil™ Peptide reversed-phase column chromatography. The inverse order for obtaining the sample is as follows: fraction B15 (P1) of C8-Supelco reversed-phase chromatography; a pool of fractions 2 to 6 of molecular exclusion chromatography, which pool of fractions 1 to 7 of ion exchange chromatography; intracellular extract of *S. sonnei* SS9 precipitated at 75% ammonium sulfate. The fractions were eluted with at a flow rate of 1.0 mL/min, with the following solvent conditions: H_2O:TFA (100:0.1) for 10 min, followed by a linear gradient to H_2O:ACN:TFA (20:80:0.1) during 30 minutes and an isocratic condition at H_2O:ACN:TFA (20:80:0.1) for 40-45 min. The purified fraction 35 is highlighted.

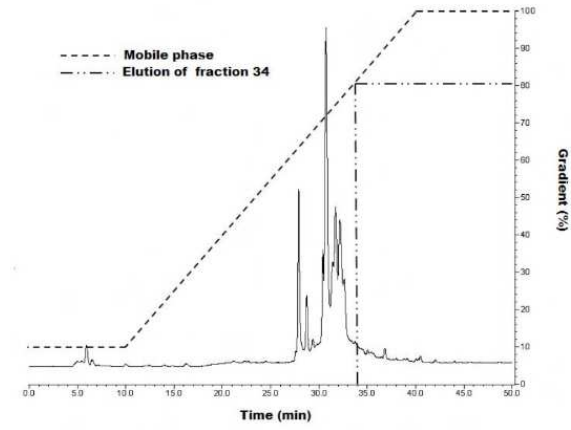

Fig.2 C18-Sephasil™ Peptide reversed-phase column chromatography. The inverse order for obtaining the sample is as follows: pool fractions C1 to C3 (P2) of C8-Supelco reversed-phase chromatography; pool of fractions 7 to 9 of molecular exclusion chromatography; pool of fractions 1 to 7 of ion exchange chromatography; intracellular extract of *S. sonnei* SS9 precipitated at 75% ammonium sulfate. The fractions were eluted with at a flow rate of 1.0 mL/min, with the following solvent conditions: H_2O:TFA (100:0.1) for 10 min, followed by a linear gradient to H_2O:ACN:TFA (20:80:0.1) during 30 minutes and an isocratic condition at H_2O:ACN:TFA (20:80:0.1) for 40-45 min. The purified fraction 34 is highlighted.

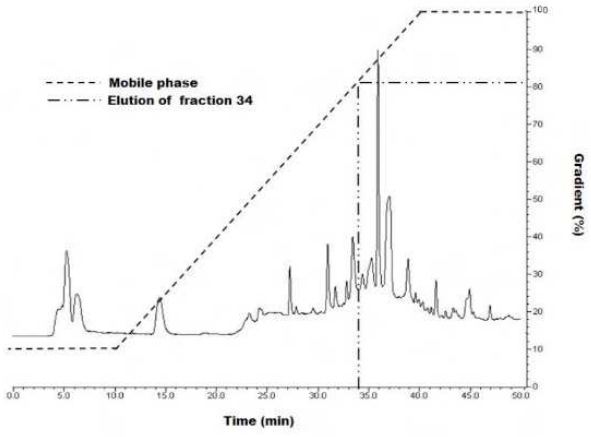

Fig. 3 C18-Sephasil™ Peptide reversed-phase column chromatography. The inverse order for obtaining the sample is as follows: pool fractions C4 to C10 (P3) of C8-Supelco reversed-phase chromatography; pool of fractions 7 to 9 of molecular exclusion chromatography; pool of fractions 1 to 7 of ion exchange chromatography; intracellular extract of *S. sonnei* SS9 precipitated at 75% ammonium sulfate]. C18-Sephasil™ The fractions were eluted with at a flow rate of 1.0 mL/min, with the following solvent conditions: H_2O:TFA (100:0.1) for 10 min, followed by a linear gradient to H_2O:ACN:TFA (20:80:0.1) during 30 minutes and an isocratic condition at H_2O:ACN:TFA (20:80:0.1) for 40-45 min. The purified fraction 34 is highlighted.

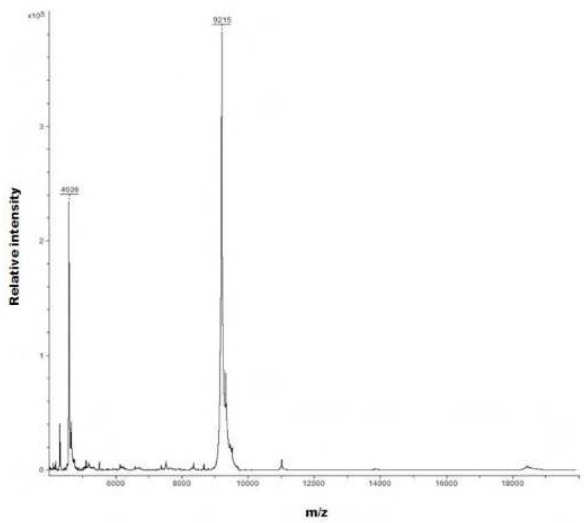

Fig. 4 MALDI mass spectrum of F1 sample, obtained in a Bruker Autoflex® equipment operated in the linear positive mode (Bruker Daltonics, Bilerica, MA). The inverse order for obtaining the sample is as follows: fraction 35 of C18-Sephasil™ Peptide reversed-phase chromatography; fraction B15 of C-8 Supelco reversed-phase chromatography; pool of fractions 2 to 6 of molecular exclusion chromatography; pool of fractions 1 to 7 of ion exchange chromatography.

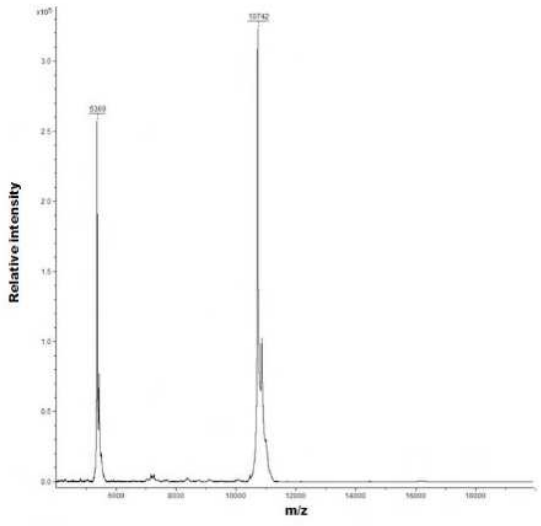

Fig. 5 MALDI mass spectrum of F2 sample, obtained in a Bruker Autoflex® equipment operated in the linear positive mode (Bruker Daltonics, Bilerica, MA). The inverse order for obtaining the sample is as follows: fraction 34 of C18-Sephasil™ Peptide reversed-phase chromatography; pool fractions C1 to C3 of C-8 Supelco reversed-phase chromatography; pool of fractions 7 to 9 of molecular exclusion chromatography; pool of fractions 1 to 7of ion exchange chromatography.

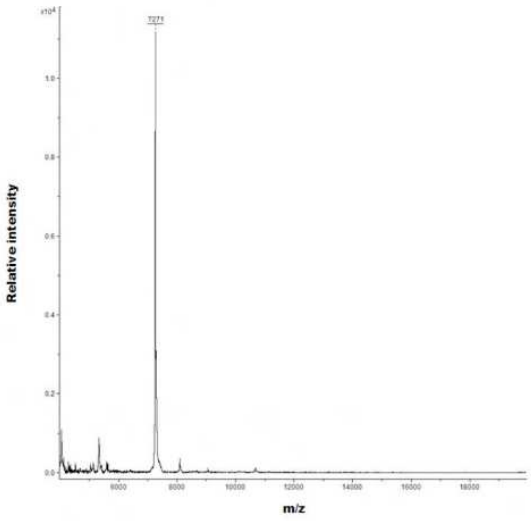

Fig. 6 MALDI mass spectrum of F3 sample, obtained in a Bruker Autoflex® equipment operated in the linear positive mode (Bruker Daltonics, Bilerica, MA). The inverse order for obtaining the sample is as follows: fraction 34 of C18-Sephasil™ Peptide reversed-phase chromatography; pool fractions C4 to C10 of C-8 Supelco reversed-phase chromatography; pool of fractions 7 to 9 of molecular exclusion chromatography; pool of fractions 1 to 7 of ion exchange chromatography.

Sample	Volume (ml)	Titration of the antagonistic action (AU/ml)	Total activity (AU)[a]	Pr otein (mg/ml)	Total protein (mg)	Specific activity (AU/mg)
C-75	23	3.6×10^{18}	8.3×10^{19}	113	2599	3.2×10^{16}
Pool of fractions 1 to 7 of Mono-Q™ ion exchange chromatography	8	1.4×10^{16}	1.1×10^{17}	24	192	5.7×10^{14}
Pool of fractions 2 to 6 of Superose 12 molecular exclusion chromatography	3	2.5×10^{4}	7.5×10^{4}	17	51	1.5×10^{3}
Pool of fractions 7 to 9 of Superose 12 molecular exclusion chromatography	1.8	800	1.4×10^{3}	0.9	1.6	8.7×10^{2}

[a]: arbitrary unit

Tab. 1 Volume, titration of the antagonistic action, total activity, protein, total protein, specific activity of the C-75 extract and the partially purified fractions produced by *Shigella sonnei* SS9

	Mass spectrometry							
Sample	**Experimental molecular mass (Da)**	**Amino acid sequence identified**	**Identification of the molecule**	**Amino acids**	**Identity**	**Query coverage**	**Accession [a]**	**Predicted isoelectric point [b]**
F1	9,215	MNKSQL IDKIAAGA DISKAAA GRALDAII ASVTESLK EGDDVAL VGFGTFAV	DNA-binding protein HU-beta (*Shigella sonnei*)	90	100%	100%	WP_ 052978778	8.9
F2	10,742	LREFVTA KFAKLE QYFDRI	Ribosome hibernation promoting factor (*Shigella sonnei*)	95	100%	100%	WP_ 052983145.1	6.3
F3	7,271	SVEEL NTELNL NLRKEF	50S ribosomal protein L29 (*Escherichia coli*)	63	83%	100%	WP_ 023146509.1	9.5

[a]: The purified substances were identified by comparison with the deposited sequences on the Basic Local Alignment Search Tool (BLAST- P) program. (https://blast.ncbi.nlm.nih.gov)

b: The isoelectric point was determined using protein isoelectric point calculator website (http://isoelectric.org/calculate.php)

Tab. 2 Identification of molecular mass, partial amino acid sequence and predicted isoelectric point of the purified antagonist substances F1, F2 and F3 synthesized by *Shigella sonnei* with isoantagonist action