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Partial characterization of a bacteriocin-like inhibitory substance produced by *Streptococcus equinus* C6I9, a bovine rumen isolate

Caracterizaçao parcial de uma substancia inibitória tipo bacteriocina produzida por *Streptococcus equinus* C6I9, um isolado do rumen bovino

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ABSTRACT

The present study aimed to evaluate the production and the factors that influence the antimicrobial activity of a bacteriocin-like inhibitory substance (BLIS) produced by *Streptococcus equinus* C6I9, a bovine rumen isolate. The antagonist activity was evaluated by the spot-on-lawn assay. Antimicrobial activity of the crude extracts obtained using NaCl acidic solution (pH 2, 100 mM) was evaluated along the bacterial growth and in growth medium containing different carbon and nitrogen sources, by agar diffusion method. Crude extract was evaluated regarding temperature stability, storage conditions, and pH sensitivity. *S. equinus* C619 inhibited both Gram-positive and Gram-negative strains. The crude extract was sensitive to proteinase K, and showed great stability to different temperatures and pH (2 to 12). The presence of meat extract (1.5 g/L) and sucrose (8 g/L) resulted in higher production of BLIS by *S. equinus* C6I9. The BLIS molecular mass was determined as approximately 3.5 kDa. The results obtained in this study evidenced the production of a broad spectrum activity, thermo- and pH stable BLIS by the ruminal isolate *S. equinus* C6I9, important features for its biotechnological application.

Keywords: Rumen; *Listeria monocytogenes*; Antimicrobial activity**.**

RESUMO

O presente trabalho objetivou avaliar a produção e os fatores que influenciam a atividade antimicrobiana de uma substância inibidora semelhante à bacteriocina (BLIS) produzida por *Streptococcus equinus* C6I9, isolado do rumen bovino. A atividade antagonista foi avaliada pelo método de sobrecamada. A atividade antimicrobiana dos extratos brutos obtidos com solução ácida de NaCl (pH 2, 100 mM) foi avaliada ao longo do crescimento bacteriano e em meio de cultura contendo diferentes fontes de carbono e nitrogênio, pelo método de difusão em ágar. O extrato bruto foi avaliado quanto à estabilidade de temperatura, condições de armazenamento e sensibilidade ao pH. *S. equinus* C619 inibiu tanto linhagens Gram-positivas como Gram-negativas. O extrato bruto foi sensível à proteinase K e apresentou grande estabilidade a diferentes temperaturas e pH (2 a 12). A presença de extrato de carne (1,5 g/L) e sacarose (8 g/L) resultou em maior produção de BLIS por *S. equinus* C6I9. A massa molecular da BLIS foi determinada como sendo aproximadamente 3,5 kDa. Os resultados obtidos neste estudo evidenciaram a produção de uma BLIS de amplo espectro de atividade, termoestável e estável ao pH pelo isolado ruminal *S. equinus* C6I9, características importantes para aplicação biotecnológica.

Palavras-chave: Rumen; *Listeria monocytogenes*; Atividade antimicrobiana.

INTRODUCTION

The rumen is a complex ecosystem, in which a large number of microbial species are found, including bacteria (10^{10} - 10^{11} cells/mL), protozoa (10^{4} - 10^{6} /mL), anaerobic fungi (10^{3} - 10^{5} zoospore/mL) and bacteriophages (10^{8} - 10^{9} /mL) (HUNGATE, 1975; KAMRA, 2005). Considered an open, continuous and favorable system to the development of the microbial population, the rumen acts as a fermentation chamber, being an anaerobic environment, with a temperature of 39-42°C and a pH between 6.0 and 7.0 (SOLTIS et al., 2023).

Naturally present ruminal bacteria are commonly capable of synthesizing antimicrobial compounds, such as bacteriocins (MANTOVANI et al., 2001; WHITFORD et al., 2001; de OLIVEIRA et al., 2022). Bacteriocins are produced by Gram-negative and Gram-positive bacteria, as well as by some strains of the Archaea domain, and they have bactericidal or bacteriostatic activity against the target cell (DARBANDI et al., 2022). The bacteriocins comprise

a heterogeneous group of ribosomally synthesized peptides, which differ in size, structure, specificity and mechanism of action (ARBULU et al., 2015; DARBANDI et al., 2022).

Bacteriocins produced by ruminal bacteria have shown a spectrum of activity similar to nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, and used as a food preservative. Additionally, bacteriocins synthesized by ruminal bacteria show great stability in the rumen environment, since they evolved in this highly competitive ecosystem (MANTOVANI and RUSSELL, 2002), and may also be an alternative for the manipulation of rumen fermentation, replacing the use of ionophores (MANTOVANI AND RUSSELL, 2002; KISIDAYOVÁ et al., 2009; GARSA et al., 2019).

Considering that bacteriocin production is a common feature among ruminal *Streptococcus* stains (de SOUSA et al., 2021) and antimicrobial peptides have been considered promising biomolecules with varied applications in human medicine, veterinary, agriculture and biotechnology (NEGASH AND TSEHAI, 2020), this study aimed to evaluate and characterize the antimicrobial substance produced by the ruminal isolate *Streptococcus equinus* C6I9.

MATERIAL AND METHODS

Microorganisms and growth conditions

S. equinus C6I9, previously isolated from bovine rumen and identified by 16S rRNA gene sequencing (MF045075 GenBank access number) (SABINO *et al.*, 2019), was maintained in -80 ºC, in glycerol-containing Brain Heart Infusion (BHI with 10 % glycerol final concentration), at the culture collection of the Rumen Microbiology Laboratory, Embrapa Gado de Leite, Juiz de Fora, MG, Brasil.

Before each experiment, the bacterium was cultured twice in anaerobic BHI broth, at 39 °C, overnight. The culture medium was prepared under a CO_2 atmosphere, free of oxygen, distributed in tubes sealed with a rubber stopper and aluminum seal. The solid media were distributed in Petri dishes, in anaerobic chamber (atmosphere: 85% N_2, 10% H_2 e 5% CO_2).

The indicator strains used at this study were: *Bacillus cereus* ATCC 14579, *Bacillus cereus* ATCC 33018, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Lactobacillus acidophilus* ATCC 4356, *Staphylococcus aureus* ATCC 25923, *Lactococcus lactis* DPC 3147, *Listeria monocytogenes* ATCC 5779, *Streptococcus salivarius* 20P3, and the ruminal isolates *Streptococcus uberis*, *Streptococcus macedonicus, Streptococcus equinus*, *Klebsiella sp*., *Pseudomonas fluorescens*, and *Pseudomonas sp*. The indicator strains were grown in BHI media, in aerobiosis at 37ºC or in anaerobiosis at 39°C, depending on the bacterial strain.

Evaluation of the antimicrobial activity of *S. equinus* C6I9

The antibacterial activity was evaluated by the spot-on-law assay (BOOTH et al., 1977). *S. equinus* C6I9 was spotted onto BHI agar and incubated in anaerobiosis, at 39 ºC, for 18 h. Molten semi-solid BHI (0.75% agar) inoculated with the indicator bacteria (10^5 CFU mL^{-1}) was poured over agar plates already containing *S. equinus* C6I9 colonies. Plates were incubated in aerobiosis at 37ºC or in anaerobiosis at 39°C (depending on the bacterial strain), and the presence of inhibition zones around the colonies was verified after 18 h of incubation (> 6 mm diameter).

Exclusion of interfering factors

To evaluate the presence of bacteriophage, a piece of the agar (3 mm) was aseptically removed from the inhibition zone observed in the spot-on-lawn method, macerated in saline (NaCl 0.9%, pH 7.0), and centrifuged (12,000g, 20 min). Then, 200 µL of the supernatant were transferred to 3 mL BHI medium previously inoculated with the indicator microorganism. After 10 min incubation at room temperature, 200 µL was added to 3.5 mL of semi-solid BHI, which was poured over a prior layer of solid BHI (TURNER AND JORDAN, 1981). After 24 h incubation at 39 ºC, the plates were inspected for the presence of lytic zones, indicative of bacteriophage action.

To exclude the possibility of the production of acidic compounds being responsible for the observed antagonism, after the spot-on-lawn test, the pH inside and outside the growth inhibition zones was determined using pH test strips (Advantec Narrow Range pH Test papers, from 5.4 to 7.0 pH).

Sensitivity of the antimicrobial compounds to proteinase K

In order to determine the protein nature of the antagonistic substances detected on the spot-on-lawn method, sensitivity to proteinase K was evaluated. *S. equinus* C6I9 was inoculated on BHI agar plates and incubated in anaerobiosis, at 39 °C, in duplicates. After 18 h of growth, in one of the plates, proteinase K (5 mg mL^{-1}) was added (5 µL) next to each colony, and the plates were incubated, at 37 °C, for 3 h, in aerobiosis, to allow the enzymatic activity. Thereafter, a semi-solid medium containing *L. monocytogenes* ATCC 5779 as indicator organism was poured over the plates. The proteolytic activity on the antagonistic substances was observed by alterations of the inhibition zones of the indicator growth (i.e., evidence of growth of the target organism where the proteinase K has been added), as compared to the control plates (absence of proteinase K).

Preparation and determination of the antagonistic activity of *S. equinus* C6I9 extracts

Bacterial extracts were obtained as described by Mantovani et al. (2002), with modifications. Stationary phase cultures of *S. equinus* C6I9 were heat treated (70 °C, 30 minutes)

and centrifuged (12,000 rpm, 15 minutes). Cell-free supernatant (2 mL) was separated and the pellet obtained was suspended in NaCl acidic solution (100 mM, pH 2). Part of the sample was stirred for 2 hours at room temperature and the other part was autoclaved (121 °C, 15 minutes). Subsequently, the samples were centrifuged (12,000 rpm, 15 minutes), the supernatant was lyophilized and the pellet was kept under refrigeration for later use. After suspension in acidic NaCl solution (5 mM, pH 2), the lyophilized material from the supernatant was named "crude extract".

The antagonist activity of the crude extracts and pellets was evaluated by agar diffusion method (HOOVER, 1997). Stationary phase cultures of the indicator bacteria were mixed with solid BHI medium and plated. After solidification of the medium, 5 mm wells were drilled and the tested samples (15 μL) were applied into each well (extract obtained in autoclave, extract obtained at room temperature, supernatants, pellet after extraction in autoclave and pellet after extraction at room temperature). The plates were incubated overnight at 4°C and then incubated at 37°C, for 18 h. *L. monocytogenes* ATCC 5779 was used as indicator microorganisms. Antimicrobial activity was determined by the reciprocal of the highest dilution where it was possible to visualize inhibition halos (≥ 9 mm in diameter), being expressed in arbitrary units per milliliter (UA/mL) (LEWUS AND MONTVILLE, 1991).

Growth and antimicrobial activity of *S. equinus C6I9*

The antagonistic activity was monitored along the growth curve of *S. equinus* C6I9, to determine the growth phase in which the maximum production of the antimicrobial compound would be obtained.

The growth curve was performed in BHI broth, under anaerobic conditions, at 39°C. Bacterial growth was monitored over 24 hours, and aliquots were collected at intervals of 30 or 60 minutes to measure the optical density (600 nm) in a spectrophotometer. After defining the logarithmic and stationary phases, the extracts were obtained and the antimicrobial activity was evaluated by the agar diffusion method, using *L. monocytogenes* ATCC 5779 as indicator microorganism.

Influence of temperature, pH and proteolytic enzymes on the biological activity of crude extracts

Thermal stability of the crude extracts was evaluated as described by Ribeiro-Ribas et al. (2009), with modifications. Aliquots of the crude extracts were diluted into NaCl solution (5 mM, pH 6.5) and stored at different temperatures (-80°C and 4°C for 60 minutes and 45°C, 70°C and room temperature for 30 and 60 minutes), autoclaved (121°C, 15 minutes) or stored for 21 days at -80°C and 4°C.

The pH stability was evaluated using Britton-Robinson buffer (BRITTON AND ROBINSON, 1931) (ortho-phosphoric acid (0.04 M), acetic acid (0.04 M) and boric acid (0.04 M)). Solutions with different pH's (2.0; 4.0; 8.0; 10.0 and 12.0) were prepared and filtered (0.22 µm). The crude extracts were diluted (1:2) in the different buffer solutions and kept at room temperature for 2 hours.

The effect of proteinase K on the crude extracts was also evaluated. Aliquots of the crude extracts were diluted in proteinase K stock solution (20 mg/mL) (1:2) and incubated for 15, 30 or 60 minutes at 37°C.

After all the treatments, the antagonist activity of the samples was evaluated by the agar diffusion method, using *L. monocytogenes* ATCC 5779 as indicator bacterium, and expressed in AU/mL.

Influence of carbon and nitrogen sources on bacterial growth and production of antimicrobial compounds

The effect of different carbon and nitrogen sources on the growth and production of antimicrobial compounds by *S. equinus* C6I9 was evaluated as described by Carvalho (2009). Basal medium was used, without nitrogen or carbon source (amount per liter: 292 mg K_2HPO_4, 292 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg MgSO4.7 H_2O, 64 mg $CaCl_2.H_2O$, 500 mg cysteine, 4 g Na_2CO_3). Meat extract, yeast extract or trypticase were used as nitrogen sources (1.5 g/L); glucose, sucrose or lactose were used as carbon sources (4 and or 8 g/L). When the nitrogen source was varied, glucose (16 g/L) was added to the basal medium as carbon source; when the carbon source was varied, trypticase (1 g/L) was added as nitrogen source.

S. equinus C6I9 was grown at 39ºC, in anaerobiosis, for 16 h, and samples were collected to determine the optical density (600 nm). Crude extracts were obtained after treatment with NaCl acidic solution, at room temperature, for 2 h. The antagonist activity of the crude extracts and pellets was evaluated by agar diffusion method, using *L. monocytogenes* ATCC 5779 as indicator bacterium.

Molecular mass determination

The molecular mass of the protein compounds on crude extract obtained from *S. equinus* C6I9 was estimated by polyacrylamide gel electrophoresis (SDS-Tricine-PAGE, 16.5% acrylamide) (SHÄGGER and VON JAGOW, 1987).

The crude extract was mixed with the dye (1:4), heated (90 °C, 3 minutes) and applied to the gel. The compounds were separated at 120 V and 50 mA, for 4.5 h (Bio-Rad's mini-PROTEAN II electrophoresis apparatus). The gel was washed for 1 min in Milli Q water and fixed using three solutions: fixative 1 (orthophosphoric acid solution (2%) and ethanol (30%)) (3 times, 30 minutes each); fixative 2 (orthophosphoric acid (2%)) (3 times, 10 minutes each);

fixative 3 (orthophosphoric acid (2%), ethanol (18%) and ammonium sulfate (12%)) (once, 30 min). Coomassie blue G-250 solution (2%, 500 µL) was added to the gel immersed in fixative 3; the gel was kept under agitation, overnight, until the bands were visualized. Low molecular weight standard (SIGMA-ALDRICH) was used (bands of 31.0; 20.4; 19.7; 16.9; 14.4; 6.1; 3.5 kDa).

Biological activity of the compounds on SDS-Tricine-PAGE was evaluated by the biorevelation test. A layer of semi-solid BHI medium (20 mL), containing *L. monocytogenes* ATCC 5779 (10^7 CFU/mL), was placed over the polyacrylamide gel in a Petri dish, and incubated at 37°C for up to 36 h (BHUNIA et al., 1987).

RESULTS

The antagonistic activity of the ruminal isolate *S. equinus* C6I9 is summarized in Table 1. The antibacterial substance inhibited the growth of all indicator bacteria evaluated (n = 14). *Streptococcus equinus, Streptococcus macedonicus* (both isolated from ruminal fluid) and *Listeria monocytogenes* ATCC 5779 were the most sensitive bacteria, as the largest inhibition zones were observed on spot-on-lawn assays when these bacteria were used as target strains.

Table 1 - Inhibitory spectrum of the antagonistic substance produced by *S. equinus* C6I9, using the spot-on-lawn method.

Target strain	Antagonistic activity
Bacillus cereus ATCC 14579	+
Bacillus cereus ATCC 33018	+
Enterococcus faecalis ATCC 29212	+
Escherichia coli ATCC 25922	+
Lactobacillus acidophilus ATCC 4356	+
Staphylococcus aureus ATCC 25923	+
Lactococcus lactis DPC 3147	+
Listeria monocytogenes ATCC 5779	++
Streptococcus salivarius 20P3	+
Streptococcus uberis	+
Streptococcus equinus	++
Streptococcus macedonicus	++
Klebsiella sp	+
Pseudomonas fluorescens	+

"+" zone of inhibition >10mm; "++" zone of inhibition >16mm; "-" absence of inhibition

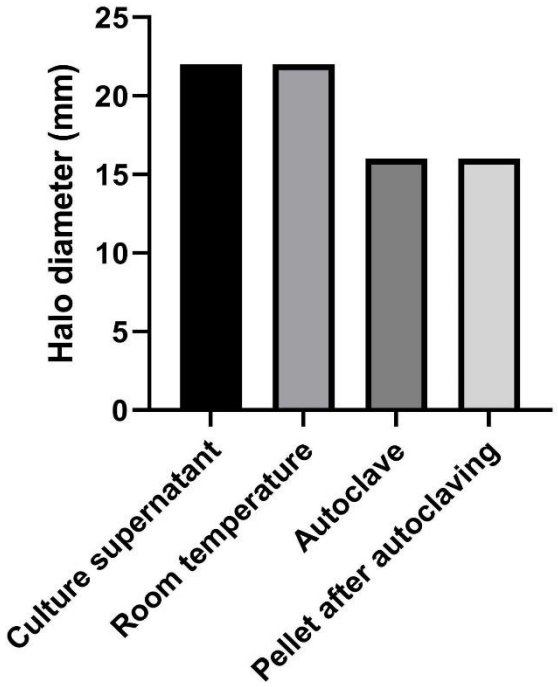
Source: survey data (2023)

Bacteriophage activity was excluded for all strains, as no plaque lysis was detected. Antimicrobial activity due to acid production was also excluded, since an average pH of 6.2 and 6.0 was observed inside the halos and in solid BHI medium, respectively.

Since the interference of bacteriophage and acid production on the antagonistic activity were excluded, the antimicrobial compound produced by *S. equinus* C6I9 was evaluated regarding its peptidic character. The antagonistic compound was sensitive to proteinase K, as a loss of ability to inhibit the growth of the indicator microorganism was observed, being evidenced by the deformation of the inhibition zones. Based on these results, the antimicrobial compound produced by *S. equinus* C6I9 was determined as a bacteriocin-like inhibitory substance (BLIS) (data not shown).

Regarding the antagonistic activity of *S. equinus* C6I9 extracts against *L. monocytogenes* ATCC5779, halos of 22 mm were observed with the *S. equinus* C6I9 culture supernatant and with the extract obtained at room temperature; halos of 16 mm were obtained with the extract and pellet after autoclave extraction (Figure 1).

Figure 1 - Antimicrobial activity of *S. equinus* C6I9 stationary phase culture supernatant, extract obtained at room temperatures, extract obtained in autoclave and pellet after autoclaving. Antimicrobial activity was evaluated by agar diffusion method, using *L. monocytogenes* ATCC 5779 as indicator microorganism.



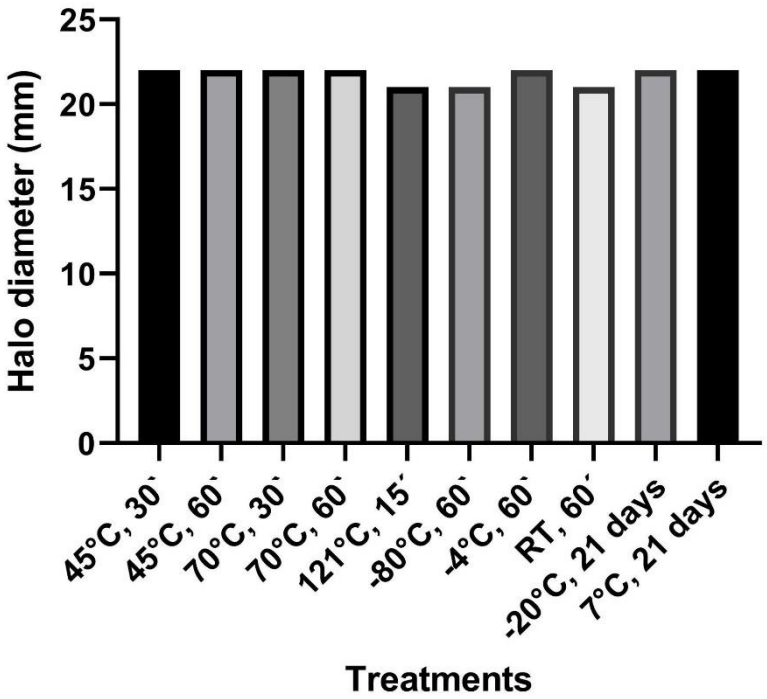
Source: survey data (2023)

The production of BLIS by *S. equinus* C6I9 was observed in crude extracts obtained with NaCl acidic solution at room temperature during the logarithmic and stationary phases (activity of 533 AU/mL and 1066 AU/mL, respectively, against *L. monocytogenes* ATCC 5779).

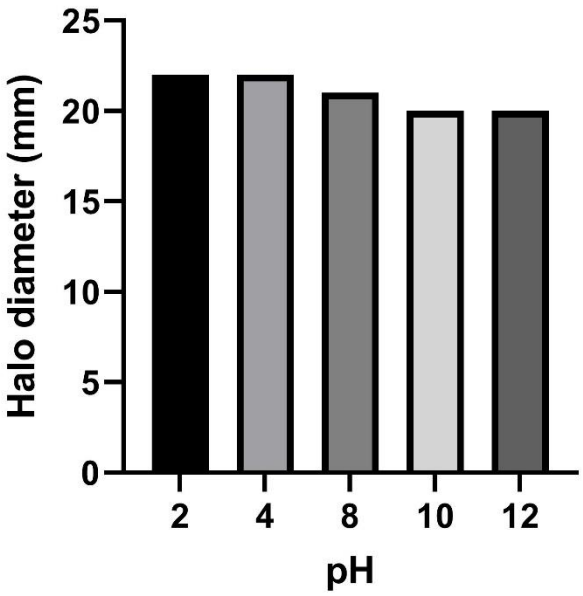
The crude extract of *S. equinus* C6I9 obtained with acidic NaCl solution showed stability at all temperatures evaluated (-80 to 121 °C), and zones of inhibition with an average diameter of 22 mm were observed against *L. monocytogenes* ATCC 5779 (Figure 2A), being the activity of 1066 AU/mL (antimicrobial activity until 1:16 dilution). The crude extract remained stable even after storage for up to 21 days at low temperatures, maintaining the activity when stored at -20°C (halos of 22 mm diameter) and at 7°C (halos of 22 mm diameter). The crude extract obtained with NaCl acidic solution at room temperature also remained stable at all evaluated pHs (2 to 12), maintaining the activity against *L. monocytogenes* ATCC 5779 (halos varying from 20 to 22 mm diameter) (Figure 2B).

Figure 2 - Antimicrobial activity of the crude extract of *S. equinus* C6I9 obtained with acidic NaCl solution. (A) Effect of temperature and storage. (B) Effect of pH. The antimicrobial activity was tested by the agar diffusion method, using *L. monocytogenes* ATCC 5779 as an indicator microorganism.

A



B



Source: survey data (2023)

Proteinase K, as noted earlier in the spot-on-lawn test, also inactivated the crude extract obtained from *S. equinus* C619, since a loss of antimicrobial activity against *L. monocytogenes* ATCC 5779 after treatment for 15, 30 or 60 minutes was observed.

. The growth of *S. equinus* C6I9 as well as the antimicrobial activity of the BLIS were detected in all nitrogen sources tested (Table 2). However, the highest activity of the crude extract was observed when meat extract was added to the growth medium (Table 2). Regarding the carbon source, the antimicrobial activity of the crude extract was only detected in the presence of glucose (4 and 8 g/L) and sucrose (8 g/L). The highest activity was observed in the presence of sucrose (Table 3).

Table 2 - Influence of nitrogen sources on antimicrobial activity of BLIS produced by *S. equinus* C19. The extracts were obtained with NaCl acidic solution at room temperature. The antimicrobial activity was tested by agar diffusion method, using *L. monocytogenes* ATCC 5779 as indicator microorganism.

Nitrogen Source	Inhibition zone (mm)	Activity (AU/mL)
Yeast extract	15	266
Meat extract	24	1,066
Trypticase	14	266

Source: survey data (2023)

Table 3 - Influence of carbon source on antimicrobial activity of BLIS produced by *S. equinus* C19. The extracts were obtained with NaCl acidic solution at room temperature. The antimicrobial activity was tested by the agar diffusion method, using *L. monocytogenes* ATCC 5779 as indicator microorganism.

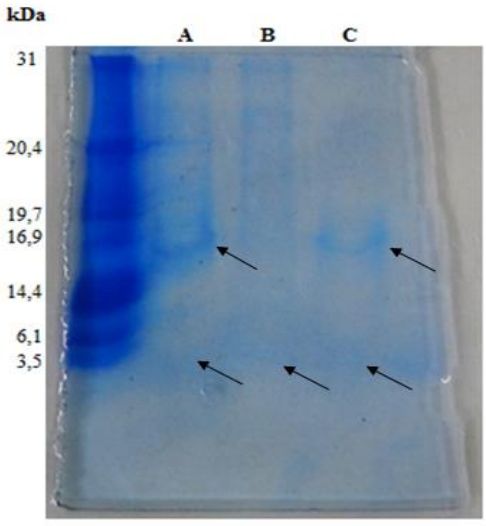
Carbon Source	Concentration (g/L)	Inhibition zone (mm)	Activity (AU/mL)
Glucose	4	7	133
	8	11	266
Sucrose	4	-	-
	8	21	1066
Lactose	4	-	-
	8	-	-

(-) absence of antimicrobial activity

Source: survey data (2023)

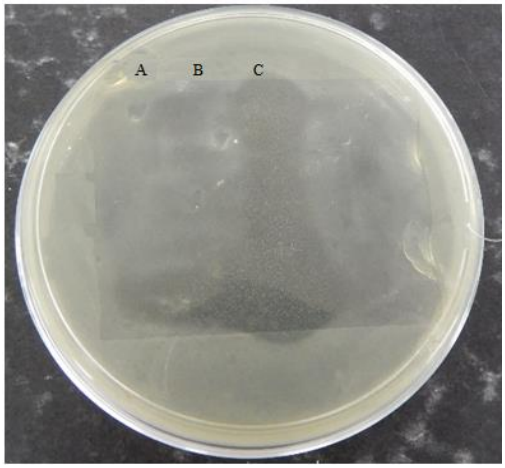
The crude extract, pellet and supernatant obtained from *S. equinus* C6I9 cultures were submitted to separation by polyacrylamide gel electrophoresis (SDS-Tricine-PAGE). Both in the crude extract and in the supernatant, it was possible to visualize two bands: one more intense, with a molecular mass between 14.4 and 16.9 kDa, and a more diffuse band, with an approximate molecular mass of 3.5 kDa (Figure 3). The biorevelation assay evidenced the presence of antagonist activity against *L. monocytogenes* ATCC 5779 at the bottom of the gel, where the protein molecules with a molecular mass of 3.5 kDa were located (Figure 4).

Figure 3 - Polyacrylamide gel electrophoresis (16.5%) of the crude extract (A), pellet (B) and supernatant (C), obtained from *S. equinus* C6I9 culture. The gel was stained with Coomassie blue G-250. The arrows show the bands present on the gel. M - Low molecular weight standard.



Source: survey data (2023)

Figure 4 - Biorevelation assay. The polyacrylamide gel containing samples of the crude extract (A), pellet (B) and supernatant (C) of *S. equinus* C6I9 was placed on a Petri dish and BHI medium containing the indicator microorganism *L. monocytogenes* ATCC 5779 was poured over the gel; plates were incubated at 37°C for up to 24 h.



Source: survey data (2023)

DISCUSSION

In complex ecosystems, with high microbial diversity, as the ruminal environment, the production of bacteriocins can be considered a competitive advantage for the producer cells (MANTOVANI et al. 2001; WHITFORD et al. 2001). Bacteria belonging to the genus *Streptococcus*, *Enterococcus*, *Ruminococcus,* and *Butyrivibrio* have been characterized as the main bacteriocin producers among ruminal strains (WANG et al. 2012; CHAKCHOUK-MTIBAA et al. 2014).

A wide variety of bacteriocins, belonging to all four main classes (lantibiotics, non-lantibiotics, Class III, and the unusual cyclic peptides), have been identified and characterized from nearly every *Streptococcus* species, isolated from different environments (GARCIA-GUTIERREZ *et al.*, 2020; WATANABE *et al.*, 2021; CHRISTOPHERS *et al.*, 2023). Recently, Sugrue et al. (2023) isolated two strains of *Streptococcus equinus* that produce a nisin variant, named nisin E. Specifically for *Streptococcus equinus* C6I9, used in our study, post-translational modification genes of the lantibiotic class of bacteriocins (*lan*B, *lan*C, *lan*M) were not detected, which may indicate that other lantibiotic post-translational modification genes are present or that the produced bioactive compound(s) belong(s) to other classes of peptides (SABINO *et al.*, 2019).

Usually, bacteriocins and BLIS produced by Gram-positive bacteria have low activity against Gram-negative bacteria, since the presence of the outer membrane in the last group limits the activity of the antimicrobial compounds (ORTOLANI et al. 2010). The BLIS produced by *S. equinus* C6I9 showed a broad spectrum of activity, since it was capable of inhibiting the growth

of phylogenetically related bacteria (such as *S. equinus* isolated from bovine rumen), but also inhibited Gram-negative strains. Activity against Gram-negative strains is not a common feature among bacteriocins produced by LAB. Other characterized bacteriocins that showed activity against Gram-negative bacteria is Nisin Z produced by *Lactococcus lactis* (MULDERS et al., 1991), and Nisin S produced by a strain of *Ligilactobacillus salivarius* (SEVILLANO et al., 2023).

Among the bacteria that were sensitive to the BLIS produced by *S. equinus* C6I9 we can highlight *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus species*, *Klebsiella* sp., *Pseudomonas* sp., and *Bacillus cereus*, bacteria that can act as opportunistic pathogens in both humans and other animals, causing diseases during microbial unbalance or alterations of the immune system.

The inactivation of the BLIS produced by *S. equinus* C6I9 after proteinase K treatment confirmed the protein nature. Susceptibility to proteolytic enzymes is reported for most bacteriocins and BLIS produced by *Streptococcus* species (GEORGALAKI *et al*., 2002; RASHID *et al*, 2009; WHITFORD *et al*., 2001; XIAO *et al*., 2004).

The extraction procedure using NaCl acidic solution at room temperature was more effective for recovering BLIS from *S equinus* C6I9 producing cells, and a more pronounced anti-*Listeria* activity was observed when compared to the extraction procedure using autoclave. As the extraction procedure was efficient, it is likely that the BLIS, after being synthesized, remains adhered to the cell envelope of *S. equinus* C6I9, being the interactions with the cell envelope reduced or undone by the action of the acidic pH of the NaCl solution.

Some bacteriocins are stable at different pH, varying from 2.0 to 8.0, and the biological activity is not affected by this parameter. Moreover, thermostability is a common feature among bacteriocins, and can be explained by the low molecular weight or by the particular structure of these peptides (TODOROV, 2010). The crude extract containing BLIS obtained from *S. equinus* C6I9 showed stability at different pH values (2-12) and excellent thermal stability, both at high and low temperatures.

Many bacteria are able to synthesize BLIS or bacteriocins at the growth deceleration phase (end of the logarithmic growth phase and the beginning of the stationary phase), presumably as an anti-competitor mechanism. Other BLIS or bacteriocins are produced since the beginning of the bacterial growth, as a constitutional factor, and the amount of the antimicrobial compound produced increases with increasing number of cells in culture. *S. equinus* C6I9 produced BLIS in both log and stationary phases, but the highest biological activity of BLIS was detected in the extract obtained from the culture of *S. equinus* C6I9 in the stationary phase.

The production of antimicrobial compounds can be affected by media composition, temperature and pH, as well as by incubation conditions (GARSA et al., 2014). These factors are essential for the production of such substances, and the optimal conditions are variable among the

bacteria (GARSA et al., 2014; ZALAN et al., 2005). The determination of the best conditions for the production of the peptides are important for the maximization of the production and for biotechnological application. The addition of sugars, vitamins and nitrogen sources to the culture medium can maximize the growth and bacteriocin production by lactic acid bacteria. On the other hand, a very rich growth medium can lead to inhibition of bacteriocin production (ABBASILIASI et al., 2017).

S. equinus C6I9 grew and produced BLIS in culture medium containing yeast extract, meat extract and trypticase as nitrogen sources. Meat extract-containing medium yielded higher production of BLIS, and highest anti-*Listeria* activity in crude extract was observed. Carvalho *et al*. (2009) obtained the highest activity of bovicin HC5 and cell mass production when yeast extract and trypticase were added to the basal medium of *Streptococcus bovis* HC5. The maximum production of BLIS by *Lactobacillus paracasei* LA07 was obtained when tryptone (3 g/L of nitrogen equivalent) was added to the medium; higher concentrations of nitrogen (5, 10 and 20 g/L) decreased both bacterial growth and BLIS production (ABBASILIASI *et al*., 2011). Regarding carbon sources, the presence of sucrose (8 g/L) in the culture medium resulted in increased BLIS production by *S. equinus* C6I9. In contrast, the highest production of bovicin HC5 by *S. bovis* HC5 was obtained when glucose was added as the carbon source in the culture medium (CARVALHO *et al*., 2009).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the crude extract and supernatant obtained from cultures of *S. equinus* C6I9 revealed two bands, with molecular weights around 16 kDa and 3.5 kDa. The protein compound with biological activity was the lowest molecular weight band. Low molecular weight BLIS produced by *Streptococcus* spp. isolated from rumen have been characterized (MANTOVANI *et al*., 2001; WHITFORD *et al*., 2001). Nisin variants produced by *Streptococcus* strains (Nisin E, Nisin G, Nisin H, Nisin P and Nisin U) have molecular weight from 2.3 to 3.4 kDa (O'CONNOR et al., 2015; ALDARHAMI et al., 2020; LASAGNO et al., 2019; SUGRUE et al., 2023; LAWRENCE et al., 2022). Bacteriocins produced by other lactic acid bacteria, such as *Lactobacillus curvatus* (Arla-10), *Enterococcus faecium* (JFR-1), *Lactobacillus paracasei* subsp. *paracasei* (JFR-5) and *Streptococcus thermophilus* (TSB-8), also show molecular mass between 4.5 to 6 kDa (YANG *et al*, 2018).

CONCLUSION

The BLIS produced by *S. equinus* C619 may be a commercially valuable bacteriocin. It shows desirable features for biotechnological applications, such as heat and pH stability, and a broad spectrum of activity. BLIS production occurs throughout the entire bacterial growth, varying according to the source of carbon and nitrogen added to the culture medium.

Understanding the behavior of this bacterial strain can be useful to optimize BLIS production and extraction processes.

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