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Original article

Evaluation of the biological function of ribosomal protein S18 from cattle tick *Rhipicephalus microplus*

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ABSTRACT

Rhipicephalus (Boophilus) microplus, also known as the cattle tick, causes severe parasitism and transmits different pathogens to vertebrate hosts, leading to massive economic losses. In the present study, we performed a functional characterization of a ribosomal protein from *R. microplus* to investigate its importance in blood feeding, egg production and viability. Ribosomal protein S18 (RPS18) is part of the 40S subunit, associated with 18S rRNA, and has been previously pointed to have a secondary role in different organisms. *Rhipicephalus microplus* RPS18 (RmRPS18) gene expression levels were modulated in female salivary glands during blood feeding. Moreover, mRNA levels in this tissue were 10 times higher than those in the midgut of fully engorged female ticks. Additionally, recombinant RmRPS18 was recognized by IgG antibodies from sera of cattle naturally or experimentally infested with ticks. RNAi-mediated knockdown of the RmRPS18 gene was performed in fully engorged females, leading to a significant (29 %) decrease in egg production. Additionally, egg hatching was completely impaired, suggesting that no viable eggs were produced by the RmRPS18-silenced group. Furthermore, antimicrobial assays revealed inhibitory activities against gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus* bacteria, affecting bacterial growth. Data presented here show the important role of RmRPS18 in tick physiology and suggest that RmRPS18 can be a potential target for the development of novel strategies for tick control.

1. Introduction

Rhipicephalus microplus, also known as the cattle fever tick, is a species described in tropical and subtropical regions of the world, where its parasitism can lead to significant economic losses related to livestock production, especially by the capacity to disseminate different pathogens that cause diseases in cattle (Estrada-Peña et al., 2006; Grisi et al., 2014). In recent decades, several immunogenic molecules produced by ticks have been investigated as anti-tick vaccine targets in an attempt to develop a tick control strategy to be adopted as an alternative to chemical acaricides (Pereira et al., 2022). Among them, defense molecules, as members of the antimicrobial peptide family (AMPs), have also been described (Radulović et al., 2014). Antimicrobial molecules have been identified in different tick organs, including salivary glands, suggesting a role related to microbiota control at the feeding site (Garcia et al., 2020; Kim et al., 2020; Tirloni et al., 2014). Moreover, microplusin, a member of AMPs and secreted in *R. microplus* tissues, presented a positive regulation of gene expression in ovaries until the start of oviposition, indicating its role in the protection of the female reproductive tract and embryos (Esteves et al., 2009).

In the present study, our group identified an immunogenic peptide corresponding to the *R. microplus* ribosomal protein S18 after screening an ORFeome phage display library constructed from salivary glands transcripts of partially fed female ticks. Ribosomal protein S18 (RPS18) is one of the approximately 80 proteins that compose the 40S and 60S subunits of ribosomes from eukaryotic cells (Graifer and Karpova, 2015; Woolford and Baserga, 2013) and is considered a housekeeping, non-secreted protein in ticks. However, secondary functions unrelated to protein synthesis have been described in other organisms, either for RPS18 and other ribosomal proteins, including antimicrobial and

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anti-inflammatory activities (Hurtado-Rios et al., 2022; Zhou et al., 2015). Despite the many ribosomal proteins previously identified in saliva from different tick species (Esteves et al., 2017; Feng et al., 2019; Radulović et al., 2014; Tirloni et al., 2017), secondary functions in tick saliva and other tissues, such as the midgut and ovary, have not been evaluated thus far. Then, to investigate alternative functions in *R. microplus* ticks, *R. microplus* ribosomal protein S18 (RmRPS18) was expressed in an *Escherichia coli* heterologous expression system and tested in antimicrobial experiments. A better understanding of the secondary functions of ribosomal proteins produced by ticks might contribute to the development of new strategies for controlling ticks and tick-borne diseases.

2. Materials and methods

2.1. ORFeome phage display library

An ORFeome phage display library was constructed from salivary transcripts of partially engorged *R. microplus* female ticks, according to Becker et al. (2015) methodology and screened against anti-tick saliva IgG elicited in a Hereford cattle exposed to experimental infestations with ticks. After two biopanning rounds, eluted phages were recovered, and the salivary peptides identified using the Basic Local Alignment Search Tool (BLAST) (Supplementary material).

2.2. Ticks, cattle serum, and ethics statement

Rhipicephalus microplus ticks (Porto Alegre strain) used in the present study were obtained from the colony maintained at the Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. The Hereford cattle sera were obtained from animals used in colony maintenance or naturally exposed to ticks in the following cities from Rio Grande do Sul state: Santa Vitória do Palmar (33° 31′ 08″ S 53° 22′ 05″ W), Aratiba (27° 23′ 38″ S, 52° 18′ 0″ W) and Rio Grande (32° 2′ 6″ S, 52° 5′ 56″ W). Serum from Nelore cattle obtained 15 days after *R. microplus* larval experimental infestation (Andreotti et al., 2002) was also utilized in the present study.

The research was handled in accordance with the ethics and methodological guidance, in agreement with the International and National Directives and Norms by the Animal Experimentation Ethics Committee of Universidade Federal do Rio Grande do Sul (UFRGS) (projects 27559 and 24651).

2.3. RNA extraction and cDNA synthesis

Partially engorged *R. microplus* female ticks were manually removed from experimentally infested cattle and grouped according to their body mass, resulting in four groups ($n = 5$) with mean weights of 30±0.2, 45 ±0.7, 75±11 and 265±24 mg. Salivary glands were dissected in 0.9 % NaCl using a stereomicroscope and fine tip tweezers and transferred individually to 1.5 mL microtubes containing 200 μL of TRIzol® Reagent (Invitrogen, CA, USA). Tissues were homogenized, and total RNA was extracted according to the manufacturer's instructions. After treatment with TURBO™ DNase (Invitrogen, CA, USA) and quantification in a NanoVue Plus (GE Healthcare, NJ, USA) spectrophotometer, 1 μg of total RNA was employed for each cDNA synthesis reaction using the ImProm-II™ Reverse Transcription System (Promega, WI, USA). Total RNA samples from salivary glands, midguts and ovaries from the fully engorged females (>250 mg) up to 24 h after natural detachment ($n = 4$) were also extracted and used in cDNA synthesis by the same reverse transcription system.

2.4. Relative expression quantification

Quantitative polymerase chain reaction (qPCR) was performed to assess the relative quantification of RmRPS18 transcripts in female salivary glands during blood feeding or between fully fed female salivary glands, ovaries and midguts. Four individual biological samples obtained from different ticks were utilized in each group. Reactions containing 5 μL of Power SYBR Green PCR Master Mix Kit (Applied Biosystems, CA, USA), 1 μL of the previously produced cDNAs (diluted 5x) as a template, 50 nM of primers (Forward - 5′ TCACCATCATGTCGAACCCC and Reverse - 5′ TGGGCACGGATCTTCTTCAG), and Milli-Q water to a final volume of 10 μL. A negative control was performed without template and 1 μL of DNAse-treated RNA samples (diluted 5x) were also used as negative control, to confirm the absence of genomic DNA contamination. The reactions were conducted in 96-well plates using a StepOnePlus™ System (Applied Biosystems, CA, USA) at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative quantification data were presented according to the $2-\Delta\Delta Ct$ method (Livak and Schmittgen, 2001), using Elongation Factor 1-α (ELF1α) (XM_037421720.1) (Forward - 5′ CGTCTACAAGATTGGTGGCATT and Reverse - 5′ CTCAGTGGTCAGGTTGGCAG) as a reference gene (Nijhof et al., 2009).

2.5. Cloning and expression of recombinant rmrps18

The RmRPS18 coding DNA sequence (CDS) (XM_037425300.1) was amplified by PCR using 1 μL of partially engorged tick female cDNA as a template and 200 nM of the following primers: forward (including a NdeI restriction site) 5′ CATATGATGTCTCTCGTGATTCCTGAC and reverse (including a Bpu1102I restriction site) 5′ GCTCAGCCTAGTTCTTCTTCTTGGACA). The 20 μL reaction was prepared using Platinum™ Taq DNA Polymerase (Invitrogen, CA, USA), and DNA was amplified using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, CA, USA) under the following cycles: 94 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s, and a final step of 72 °C for 5 min. Amplified DNA was verified in a 1 % agarose gel and ligated into a pGEM®-T Easy Vector (Promega, WI, USA) then transformed into E. coli DH5α cells. Plasmids containing the RmRPS18 sequence were purified using the Fast-n-Easy Plasmid Mini-Prep Kit (Cellco Biotec, SP, Brazil). The RmRPS18 DNA insert was recovered by digestion with NdeI and Bpu1102I restriction enzymes and cloned into the same sites in the pET28a expression vector. E. coli Rosetta™(DE3) competent cells (Sigma–Aldrich, MO, USA) were used in this transformation. The plasmid construct was checked by DNA sequencing. For expression, the bacterial culture in LB broth was kept at 37 °C and 180 rpm (revolutions per minute) until the exponential phase (OD600 0.4–0.8). Recombinant protein expression was induced by adding isopropyl b-D-1-thiogalactopyranoside (0.2 mM) and shaking at 180 rpm at 18 °C for 16 h. Purification of rRmRPS18 (recombinant RmRPS18) was performed by affinity chromatography using a Ni2+ -NTA column (Qiagen, Hilden, Germany) connected to a ÄKTA system (GE Healthcare, Uppsala, Sweden). Cell lysate proteins solubilized in 50 mM Tris–HCl, 300 mM NaCl, 6 M urea, and 6 mM 2-mercaptoethanol, pH 7.5, were loaded on to resin previously equilibrated with the same buffer. Washes and elution were performed in 40 mM and 200 mM imidazole concentrations, respectively. Eluted fractions containing the recombinant protein were pooled, dialyzed in 50 mM Tris–HCl, NaCl 200 mM, pH 7.5 buffer, quantified using the Bradford (1976) method, and stored at 4 °C.

2.6. Western blotting

Western blotting was performed to confirm rRmRPS18 expression and IgG recognition by sera from animals previously infested by ticks. Recombinant RmRPS18 was applied in SDS–PAGE (12 %) and transferred to nitrocellulose membranes using a Trans-Blot® SD semidry transfer cell (Bio-Rad, CA, USA) at 15 V for 30 min. Membranes were blocked with 5 % milk powder diluted in PBS (Phosphate saline buffer)/0.1 % Tween 20 (Sigma–Aldrich, MO, USA) for 2 h at room temperature and washed three times with PBS/0.1 % Tween 20 for 5 min. After washing, the membranes were incubated individually with mouse anti-

6xHisTag antibodies (Sigma–Aldrich, MO, USA) (1:3000) or sera from Nelore or Hereford cattle (1:200) in PBS/0.1 % Tween 20 for 2 h. After three more washes, the membranes were incubated with peroxidase-conjugated anti-mouse (1:50,000) or anti-bovine (1:25,000) IgG (Sigma–Aldrich, MO, USA) in PBS/0.1 % Tween 20 for 1 h. Detection was performed in the Chemiluminescence imaging system Alliance 4.7 (Uvitec Cambridge, UK) using SuperSignal West Femto ECL substrate (Thermo Fisher Scientific, IL, USA).

2.7. RmRPS18 gene knockdown

The RNA interference (RNAi) technique was employed to perform RmRPS18 gene expression knockdown through dsRNA (double-stranded RNA) injection in *R. microplus* ticks. dsRNA was prepared using the 432 bp RmRPS18 PCR product as a template and the primers: Forward - 5′ TAATACGACTCACTATAGGGAGAATGTCTCTCGTGATTCCTGAC and Reverse – 5′ TAATACGACTCACTATAGGGAGACTAGTTCTTCTTCTTGGACA for synthesis reaction using the T7 RiboMAX Express RNAi System (Promega, WI, USA). Then, dsRNA was purified by ethanol precipitation, eluted in RNAse-free water, and analyzed by 1 % agarose gel electrophoresis. After quantification by reading absorbance at 260 nm in a spectrophotometer, the dsRNA was dried and resuspended in sterile PBS at a final concentration of 2 μg/μL. Nonrelated dsRNA was prepared using GFP (green fluorescent protein) DNA (MN114103.1) as the template with the primers Forward – 5′ TAATACGACTCACTATAGGAGTGCTTCAGCCGCTACCC and Reverse – 5′ TAATACGACTCACTATAGGGCGCTTCTCGTTGGGGTC and was injected into the ticks of the control group. Fully engorged female ticks (av weight 282±43 mg) approximately 72 h after engorgement were divided in two groups ($n = 25$ per group) and injected with 1 μL (2 μg) of RmRPS18 or GFP dsRNA at the festoon's region using insulin syringes (5 mm x 0.23 mm). Then, specimens were individually maintained in 12-well plates inside incubator under controlled conditions of temperature (28 °C) and semicontrolled conditions of humidity (>80 % relative humidity). Three days after dsRNA injection, total RNA from midgut and ovary samples ($n = 5$) were extracted and used in cDNA synthesis. The mRNA levels were quantified by qPCR according to the methodology explained above. Five individual biological samples obtained from different ticks were utilized in each group. Eggs produced by each female ($n = 14$) were weighed and counted, and the egg production index calculated according to the following formula: weight of eggs/weight of engorged female X 100 (%) (Bennet, 1974). The egg viability based on larval hatching was also assessed by the formula: number of larvae/number of eggs X 100 (%).

2.8. Bacterial growth affect

Antibacterial activity of rRmRPS18 was evaluated using gram-negative *E. coli* and gram-positive *Staphylococcus aureus*. In vitro assays were carried out as 4 individual experiments according to Wang et al. (2021) modified methodology. Briefly, *E. coli* 35218™ and *S. aureus* 23235™ (ATCC, VA, USA) were cultured in LB media at 37 °C and 180 rpm until exponential phase. Then, the cells were washed three times in 50 mM Tris–HCl, 200 mM NaCl, and 7.5 pH buffer and diluted to a final concentration of 1 × 105 cells/mL. The cell suspension (71 μL) was mixed with 79 μL of rRmRPS18 diluted in the same buffer and 30 μL of LB broth in a sterile 96-well plate. The positive control for inhibition was kanamycin at 50 μg/mL. The plate was incubated in a Microplate Shaking incubator BT919 (Benchtop Lab Systems, MO, USA) at 37 °C at 900 rpm. Bacterial growth was quantified by 600 nm absorbance measured at 2 h intervals in a VersaMax™ microplate reader (Molecular Devices, CA, USA). Recombinant mammalian cystatin A (GenBank accession number: XP_006755392.1) expressed with a N-terminal 6xHistag was utilized as a negative control. (Supplementary material). After 20 h, 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide) (3.5 mg/mL) was added to each well, and the culture was incubated for 1 h to verify cell metabolic activity.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). The statistically significant difference between two groups with parametric distribution data was determined by Student's T test, while the Mann–Whitney test was employed for nonparametric data. ANOVA and Tukey's posttest were used to determine significant differences between three or more groups with parametric distribution data, while the Kruskal–Wallis test and Dunn's posttest were used for nonparametric data.

3. Results

3.1. Rhipicephalus microplus ribosomal protein S18

A phage display ORFeome library was constructed using cDNA of salivary glands from partially engorged *R. microplus* female ticks and screened using anti-salivary proteins IgGs purified from cattle after several experimental infestations.

After DNA sequencing, a nucleotide sequence corresponding to RmRPS18 was identified and selected for further experiments. The RmRPS18 CDS (GenBank accession number: XM_037425300.1) is represented by a 462 bp nucleotide sequence that encodes a predicted intracellular protein with a molecular weight and isoelectric point (pI) of 17.8 kDa and 10.7, respectively. Moreover, the amino acid sequence matched the 40S ribosomal protein S18 domain (PTZ00134) (Supplementary material).

RPS18 from the 40S subunit is strongly conserved among eukaryotes. ClustalW protein alignment revealed identity levels over 97 % to other tick RPS18 orthologs and 85 % to humans and bovines (Fig. 1).

3.2. RmRPS18 gene expression

Transcripts corresponding to RmRPS18 were detected in *R. microplus* salivary glands from female ticks ranging from 30 to 265 mg, representing different phases of hematophagy. RmRPS18 mRNA levels were similar in partially fed ticks from the groups with mean weights of 30, 45 and 75 mg. However, there was a significant decrease of 28 % in salivary glands mRNA levels in the 265 mg group ($p < 0.05$) of engorged female ticks in comparison to the 75 mg group (Fig. 2).

The RmRPS18 gene was also differentially expressed in salivary glands in comparison to the ovary and midgut of fully engorged female ticks after detachment (>250 mg). Despite the decrease in mRNA levels in salivary glands at the end of blood feeding, the mean expression level was approximately 10 times higher than the expression level observed in the midgut ($p < 0.05$), but there was no significant difference in comparison to the expression in ovaries (Fig. 3).

3.3. rRmRPS18 expression and purification

Recombinant RmRPS18 (rRmRPS18) was expressed in an *E. coli* cell system. rRmRPS18 had a predicted molecular weight and pI of 20 kDa and 10.88, respectively, with the 6xHistag at the N-terminal region, which allowed affinity purification using Ni2+ -NTA resin. The proteins from induced cell lysates (Fig. 4A) were loaded on a Ni2+ -NTA column. Purified rRmRPS18 was shown as a major protein band in SDS–PAGE (Fig. 4B).

3.4. Western blotting

Western blotting was performed to investigate whether IgG antibodies elicited after tick infestation of two different breeds of cattle were able to recognize rRmRPS18. rRmRPS18, recognized by the anti-6xHisTag IgG (Fig. 5A), was also evaluated against different serum samples. Serum from Hereford cattle experimentally infested with *R. microplus* recognized rRmRPS18. Moreover, cattle naturally exposed

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Rhipicephalus microplus      MSLVIPDKFQHILRVLNTNIDGRRKVMFALTAIKGVGRRFSNMVCKKADVDLNKRAGELT  60
Ixodes scapularis            MSLVIPDKFQHILRVLNTNIDGRRKVMFALTAIKGVGRRFSNMVCKKADVDLNKRAGELT  60
Amblyomma americanum         MSLVIPDKFQHILRVLNTNIDGRRKVMFALTAIKGVGRRFSNIVCKKADVDPNKRAGELS  60
Bos taurus                   MSLVIPEKFQHILRVLNTNIDGRRKIAFAITAIKGVGRRYAHVVLRKADIDLTKRAGELT  60
Homo sapiens                 MSLVIPEKFQHILRVLNTNIDGQRKIAFAITAIKGVGRRYAHVVLRKADTDLTKRAGELT  60
                             ******:***************:**: **:*********::::* :*** * .******:

Rhipicephalus microplus      DEEVEKLITIMSNPRQYKIPDWFLNRQKDIKDGKYSQVTSNALENKLREDLERLKKIRAH  120
Ixodes scapularis            EEEVEKLITIMSNPRQYKIPDWFLNRQKDIKDGKFSQVTSNALENKLREDLERLKKIRAH  120
Amblyomma americanum         EEEVEKLITIMSNPRQYKIPDWFLNRQKDIKDGKYSQVTSNALENKLREDLERLKKIRAH  120
Bos taurus                   EDEVERVITIMQNPRQYKIPDWFLNRQKDVKDGKYSQVLANGLDNKLREDLERLKKIRAH  120
Homo sapiens                 DDEVERVITIMQNPRQYKIPDWFLNRQKDVKDGKYSQVLANGLDNKLREDLERLKKIRAH  120
                             ::***::****.*****************:****:*** :*.*:****************

Rhipicephalus microplus      RGLRHFWGLRVRGQHTKTTGRRGRTVGVSKKKN    153
Ixodes scapularis            RGLRHFWGLRVRGQHTKTTGRRGRTVGVSKKKN    153
Amblyomma americanum         RGLRHFWGLRVRGQHTKTTGRRGRTVGVSKKKN    153
Bos taurus                   RGLRHFWGLRVRGQHTKTTGRRGRTVGVSKKK-    152
Homo sapiens                 RGLRHFWGLRVRGQHTKTTGRRGRTVGVSKKK-    152
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Fig. 1. ClustalW multiple alignment of amino acid sequences of RPS18 proteins of *Rhipicephalus microplus* (XP_037281197.1), *Ixodes scapularis* (XP_029848945.1) and *Amblyomma americanum* (JAG90328.1) ticks and *Homo sapiens* (EAW79789.1) and *Bos taurus* (NP_001028786.1) vertebrates. * (asterisk) indicates positions that have a single, fully conserved residue,: (colon) indicates conservation between groups of strongly similar properties and . (period) indicates conservation between groups of weakly similar properties.

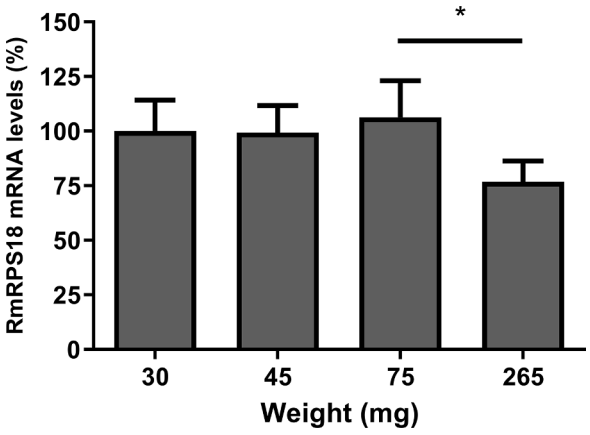

Fig. 2. Relative quantification by quantitative PCR of mRNA levels corresponding to the RmRPS18 gene in the salivary glands of *Rhipicephalus microplus* females with different body weights. Mean mRNA level in 30 mg female group was set as 100 %. Mean ± SD; $n = 4$ biological replicates per group. ANOVA-Tukey's test – $^*p < 0.05$.

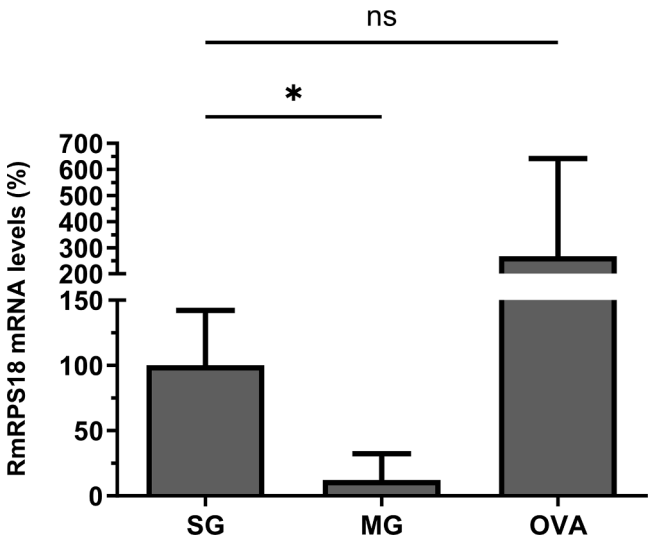

Fig. 3. Relative quantification by quantitative PCR of mRNA levels corresponding to the RmRPS18 gene in salivary glands (SG), midgut (MG) and ovary (OVA) from engorged *Rhipicephalus microplus* females. Mean mRNA level in salivary glands was set as 100 %. Mean ± SD; $n = 4$ biological replicates per group. Kruskal–Wallis-Dunn's test – $^*P < 0,05$; ns – nonsignificant.

to tick infestations in different areas from Rio Grande do Sul state also had antibodies that recognized the recombinant protein at variable levels, as well as serum from tick-resistant Nelore cattle that was experimentally infested (Fig. 5B).

3.5. RmRPS18 knockdown

Transcripts corresponding to RmRPS18 were detected in different tick organs from engorged female ticks, including the midgut and ovary. Therefore, to investigate a possible role of this protein in embryogenesis, gene knockdown was performed by RNA interference (RNAi), and reproductive parameters evaluated. Seventy-two hours after dsRNA injection, RmRPS18 mRNA levels showed a 54 % reduction in the ovary compared to the control group ($p < 0.05$) (Fig. 6A). However, the transcript level decrease in the midgut was not significant in dsRNA-injected female ticks (Fig. 6B).

The reproductive parameters evaluated revealed that RmRPS18 gene knockdown affected egg production. The results showed that silenced female ticks produced 112 mg of eggs on average, a 29 % reduction in comparison to the 156 mg observed in the control group (Fig. 7A). Moreover, the egg production index was also affected, with ticks in the RmRPS18 knockdown group able to convert only 41 % of their body mass into eggs, while the conversion index in the control group was 52 % (Fig. 7B).

Egg viability was strongly affected by RmRPS18 gene knockdown. The larval hatching rate in the control group was 31.5 % with 78.5 % of the female ticks from these groups to produce viable eggs. However, the eggs produced by RmRPS18 knockdown female ticks did not result in any larvae (Fig. 8A). Moreover, these eggs did not exhibit development of embryos 18 days after the start of oviposition; embryo development was present in most eggs from control group (Fig. 8B).

3.6. Bacterial growth affect of rRmRPS18

To investigate the potential antimicrobial activity of rRmRPS18, different concentrations of the purified protein were added to gram-negative *E. coli* or gram-positive *S. aureus* cultures in liquid media.

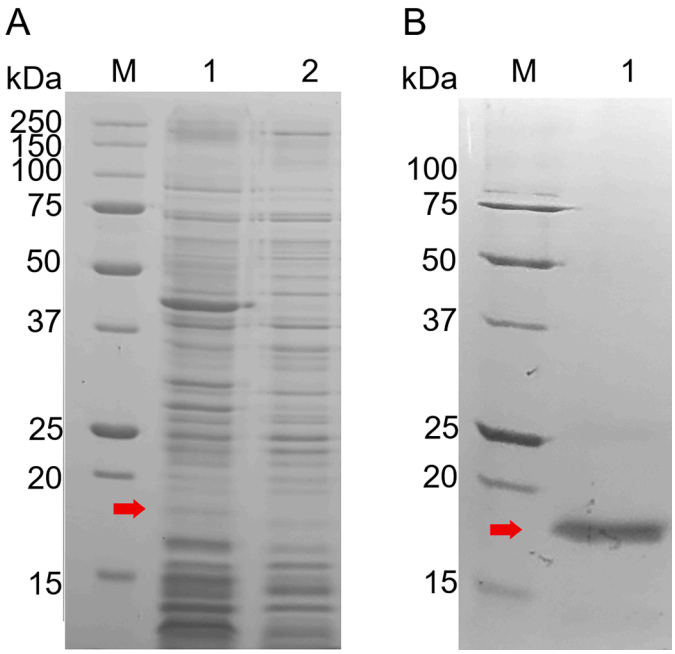

Fig. 4. SDS–PAGE (12 %) of rRmRPS18 expression in E. coli cells: M – Molecular weight markers; 1 – Lysate proteins from noninduced cells; 2 – Lysate proteins from IPTG-induced cells (A). 1 - Purified rRmRPS18 after affinity Ni2+-NTA chromatography (B). The arrow indicates the purified protein.

During culture incubation, the recombinant protein was able to slow bacterial growth in a dose-dependent manner. After 20 h incubation, the E. coli growth, as measured by absorbance, was significantly lower in wells containing 2 μM rRmRPS18. The bacterial growth affect was observed for *S. aureus* after the fourth hour of incubation, while the effect was seen from the second hour of growth for *E. coli*. A reduction of 24 % and 20 % in growth was observed for *E. coli* and *S. aureus*, respectively, after 20 h incubation when compared to the negative control (Fig. 9). Although rRmRPS18 was able affect the growth of *E. coli* and *S. aureus* cultures, no interruption of bacterial cell proliferation was observed.

4. Discussion

In the present work, some functions of RPS18 from *R. microplus* were functionally characterized which suggested secondary roles in tick feeding and reproductive parameters. RPS18 is a component of the 40S ribosomal subunit in eukaryotic cells and was previously described as a structural molecule since it binds specifically to 18S rRNA, fixing its structure and contributing to the assembly of the 40S subunit (Malygin and Karpova, 2009). However, despite its documented role in ribosomal structure, there was no other information about any secondary function in tick physiology until now.

Quantitative PCR data revealed that RmRPS18 gene expression was modulated in salivary glands during blood feeding, with a significant decrease in the transcript levels at the end of the feeding process when tick detachment occurred. This finding suggests that ribosomal protein-encoding gene expression might be related to tick–host interactions, since other transcripts corresponding to proteins present in saliva and important to success in blood acquisition were also regulated during the feeding process (Tirloni et al., 2020). Giachetto et al. (2020) identified transcripts corresponding to P2 and L19 ribosomal proteins upregulated in salivary glands of *R. microplus* ticks partially fed in susceptible and resistant cattle, respectively. Interestingly, P2 was also found in the *I. scapularis* saliva proteome from ticks fed on rabbits (Tirloni et al., 2017), indicating that other aspects, such as host species and breed, might influence ribosomal protein-encoding gene expression in salivary glands and the presence of these proteins in saliva. Moreover, qPCR results also showed that the mRNA levels of ribosomal proteins were different between tick tissues, since transcripts in the salivary glands of engorged females were higher than those observed in the midgut, suggesting that the increased expression in this organ could be related to a secondary function at the feeding site. rRmRPS18 was recognized by IgG antibodies present in the serum of cattle that had previous contact with tick saliva in different regions were *R. microplus* and/or other tick species are present. Moreover, these antibodies were produced either by the Hereford or Nelore cattle breeds. Despite not evaluating sera samples

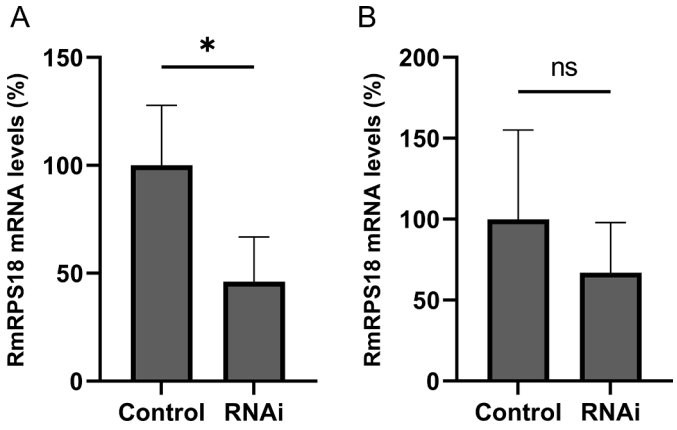

Fig. 6. Relative quantification by quantitative PCR of mRNA levels corresponding to the RmRPS18 gene in the ovary (A) and midgut (B) of fully fed *R. microplus* females 72 h after dsRNA treatment. Mean mRNA level in control group was set as 100 %. Mean ± SD; $n = 5$ biological replicates per group. Student's T test *$p < 0.05$; ns = not significant.

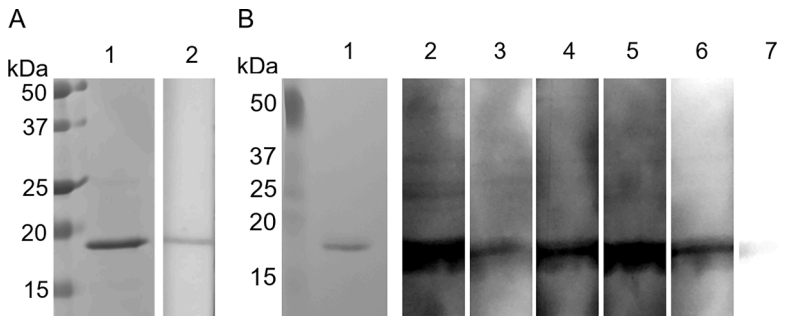

Fig. 5. SDS–PAGE (12 %) of rRmRPS18 – 1; and western blotting with mouse anti-6xHisTag IgG – 2 (A) or with different bovine sera (B): 1 – SDS–PAGE (12 %); 2 - Serum of Hereford cattle experimentally infested by *R. microplus*; Sera of Hereford cattle naturally exposed to ticks in the different regions of Rio Grande Sul state of 3 – Santa Vitória do Palmar; Rio Grande and 5 - Aratiba; 6 - Serum of Nelore cattle experimentally infested by *R. microplus*; 7 – Negative control without primary antibodies.

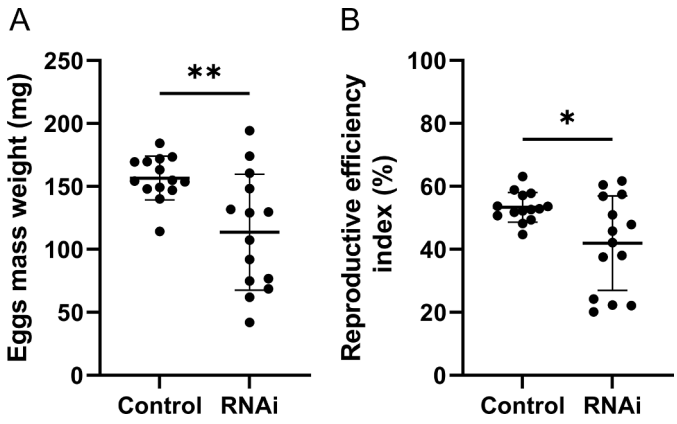

Fig. 7. Egg mass weight (A) and egg production index (B) of fully fed *R. microplus* females after RmRPS18 RNAi treatment. Mean ± SD; $n = 14$ ticks per group. Mann–Whitney test; $^{**}p < 0.01$; $^{*}p < 0.05$. Egg production index = egg mass weight/female weight x 100 (%).

from naïve cattle raised in a controlled environment, some samples presented a minor binding signal, such as the sera of cattle from Santa Vitória do Palmar region, which is a legally recognized *R. microplus*-free area. However, outbreaks have been previously reported in this region (Schild et al., 2008), mainly caused by the introduction of animals from endemic areas, as well as the presence of other tick species, probably associated with wild animals (Evans et al., 2000). Therefore, the lower signal observed in western blotting for the serum of cattle from the *R. microplus*-free area could also be caused by a cross-reaction with anti-saliva antibodies elicited by different tick species or even by other ectoparasites. Although the antibodies recognized rRmRPS18, further studies must be performed for better comprehension of the antigenicity and immunogenicity of RPS18 antigens from different tick species or parasites, since this protein is highly conserved among eukaryotes. Nevertheless, saliva from partially fed *I. scapularis* females also elicited antibodies against RPS18 in rabbits (Lewis et al., 2015), supporting its immunogenicity in this specific host.

RmRPS18 gene expression knockdown was observed in female ovaries after RNAi treatment, affecting *R. microplus* egg production. In addition to the decrease in egg mass weight, embryo development and larval hatching were severely affected, with a complete loss of egg viability. Considering the antimicrobial inhibitory activity of RmRPS18 against bacteria growth, these data might indicate an important role in embryo protection. Similarly, RNAi-mediated knockdown of S27 ribosomal protein-encoding gene expression led to comparable results in reproductive parameters of Hemaphysalis longicornis females, strongly affecting egg production (Rahman et al., 2020). However, although this ribosomal protein has been identified as an antiviral agent in shrimp (Diao et al., 2019), a secondary role of this molecule has not been investigated in ticks thus far. Furthermore, Esteves et al. (2009) showed that microplusin, an antimicrobial protein produced by *R. microplus*, is present in tick eggs and that the transcript levels gradually increased

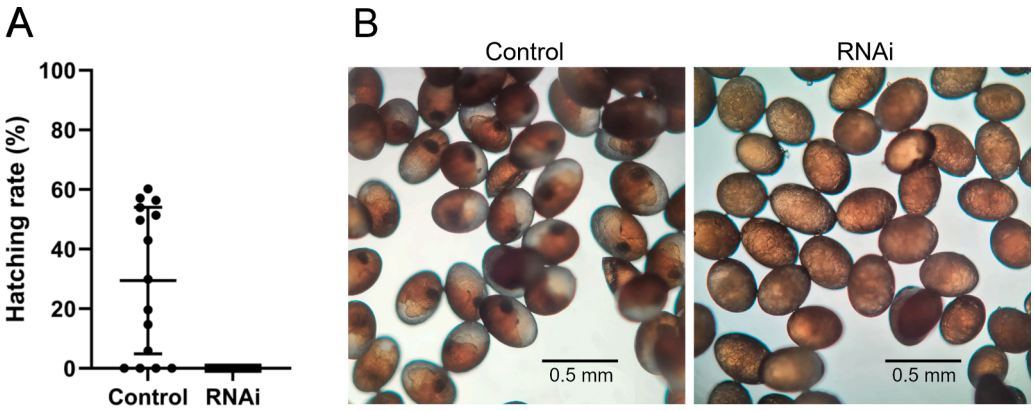

Fig. 8. Larvae hatching and embryogenesis analysis. Larvae hatching rate (%) - Mean ± SD. $n = 15$ ticks per group (A) and eggs produced by *R. microplus* females in the control or RmRPS18 RNAi treatment groups, 18 days after the start of oviposition (B).

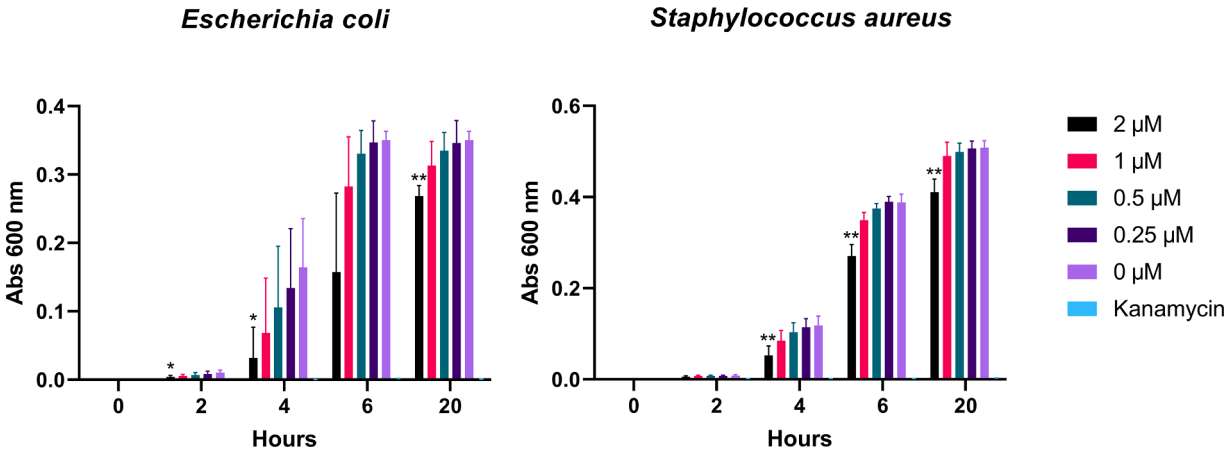

Fig. 9. Effect of rRmRPS18 against gram-negative *E. coli* and gram-positive *S. aureus* cells cultured in liquid media. Mean ± SD; Data from 4 individual experiments. Absorbance (OD600) values obtained with different RmRPS18 concentrations in each time point were individually compared to control (0 µM) by Student's T test $^{*}p < 0.05$; $^{**}p < 0.01$.

until larval hatching, indicating the importance of protection against microbial pathogens to embryo viability. The vitellin-degrading cysteine endopeptidase (VTDCE) from *R. microplus* was also found in eggs and, in addition to vitellin hydrolysis, presented antimicrobial activity against gram-positive bacteria (Oldiges et al., 2012; Seixas et al., 2003). Although RmRPS18 gene silencing in the midgut and ovary severely impacted egg viability, the presence of the protein and its possible location in tick eggs remain to be elucidated. Xavier et al. (2019) performed a proteome of *R. microplus* Gené's organs of fully fed females, and different ribosomal proteins previously described as antimicrobial molecules were identified, including RPS18. This organ is very important since it produces the wax layer that coats the eggs, protecting them against desiccation and pathogens (Arrieta et al., 2006; Booth, 1992; Zimmer et al., 2013). Yu et al. (2012) observed proteins (nonidentified) of approximately 20 kDa in the wax aqueous extract from *Amblyomma hebraeum* eggs. Therefore, if RmRPS18 is present in the wax of *R. microplus* eggs, it could contribute to the antimicrobial protection against pathogens in the environment.

RmRPS18 affects growth of gram-negative *E. coli* and gram-positive *S. aureus* bacterial strains. Other studies of RPS18 from different chordate animals have shown antimicrobial activity. Wang et al. (2021) showed that RPS18 of zebrafish has a protective effect in embryos against bacterial infections. The activity of RmRPS18, by slowing bacteria growth, might indicate a role in embryo protection. The zebrafish ribosomal protein binds to peptidoglycan, lipopolysaccharides and lipoteichoic acid from the bacterial membrane, resulting in the depolarization of cell membranes and causing cell death. Due to their conserved amino acid sequence, similar results were found for the RPS18 protein from the amphioxus *Branchiostoma japonicum* (Chen et al., 2021). RPS18 and other ribosomal proteins contain a high number of basic residues, usually clustered (Chan et al., 1991), resulting in molecules with pI > 10, which might favor interaction with negatively charged molecules of the bacterial membrane (Chen et al., 2021; Wiesner and Vilcinskas, 2010). Thus, targeting immunogenic and basic ribosomal proteins in tick saliva might be a good strategy for tick control.

The mechanism behind the secretion of ribosomal proteins in tick saliva has not been elucidated, but proteomic analysis of exosome-like vesicles found in *Hemaphysalis longicornis* saliva revealed the presence of several nonsecreted housekeeping proteins, including RPS18 (Nawaz et al., 2020). Secretion of these proteins in saliva could be controlled by the tick to play different roles at the feeding site. These recent findings show the need to study intracellular molecules present in tick saliva from different points of view since they could influence different aspects of tick–host interactions and be potential targets for the development of anti-tick strategies.

5. Conclusion

In the present study, a functional characterization of the ribosomal protein S18 from *Rhipicephalus microplus* tick in blood feeding and reproductive parameters was performed, revealing a possible role as in tick feeding sites and in the reproductive system. Moreover, the data presented suggest that the RmRPS18 protein could be a potential target employed in the development of new strategies to control tick infestations.

CRediT authorship contribution statement

Gabriel C.A. Costa: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fernando A.A. Silva:** Writing – review & editing, Methodology. **Ricardo J.S. Torquato:** Methodology. **Itabajara Silva Vaz:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Luís F. Parizi:** Writing – review & editing, Methodology. **Aparecida S. Tanaka:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2024.102333.

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