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IMUNIDADE HUMORAL DE *Rhodnius prolixus*: IMPACTO SOBRE A MICROBIOTA E DESENVOLVIMENTO DE *Trypanosoma cruzi e Trypanosoma rangeli.*

CECILIA STAHL VIEIRA

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Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitária

Orientador: Prof. Dra. Patrícia de Azambuja Penna

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Rio de Janeiro, 25 de junho de 2015.

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Imunidade Humoral de *Rhodnius prolixus*: impacto sobre a microbiota e desenvolvimento de *Trypanosoma cruzi* e *Trypanosoma rangeli*.

RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Cecilia Stahl Vieira

Rhodnius prolixus é um dos principais insetos vetores de *Trypanosoma cruzi* e de *Trypanosoma rangeli* na América Latina. A produção de peptídeos antimicrobianos (AMPs) no trato digestivo ou corpo gorduroso do inseto é vital para evitar a proliferação de microrganismos patogênicos além de manter a homeostasia da microbiota nativa. O presente trabalho focou na modulação da imunidade humoral do intestino médio de *R. prolixus* desafiados oralmente com a bactéria Gram-positiva *Staphylococcus aureus* e Gram-negativa *Escherichia coli*, além de seus tripanosomatídeos naturais *T. rangeli* e *T. cruzi*, considerando a influência do desenvolvimento dos parasitas sobre a microbiota intestinal. Em condições normais, a região anterior do intestino médio houve maior abundância de transcritos de genes de lisozimas (*lis*) e defensinas (*def*), enquanto na posterior, do gene da prolixicina (*prol*). Insetos alimentados com bactérias Gram-negativas apresentaram maior quantidade de transcritos de *defC* e *prol*, enquanto a ingestão de bactérias Gram-positivas induziu a expressão de *defA* e *defB* no intestino médio. A infecção por *T. rangeli* cepa Macias diminuiu a atividade fenoloxidásica, os níveis de expressão de *lisozimas* e *prolixicina*, ao mesmo tempo em que induziu aumento de atividade antibacteriana e dos níveis de *defensina C* no tubo digestivo do inseto, também modificando a composição de bactérias nativas. Além disso, foi verificado que as diferentes cepas de *T. cruzi* Dm 28c e Y modulam a resposta imune e a microbiota no intestino médio de *R. prolixus* de forma variável. *T. cruzi* Dm 28c induziu um aumento na expressão de genes de defensina C e uma diminuição da expressão de genes de prolixicina, reduzindo drasticamente a população bacteriana cultivável. Em contraste, *T. cruzi* Y não foi capaz de induzir a expressão de AMPs no intestino médio nem de reduzir consideravelmente a microbiota neste mesmo órgão. Nossos resultados sugerem que *R. prolixus* em resposta a ingestão de microrganismos, modula diferencialmente a expressão de AMPs pelas células epiteliais do intestino que acarreta a redução da microbiota e um favorecimento do desenvolvimento de *T. rangeli e T. cruzi,* dependendo do genótipo do parasita.

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Humoral immunity of *Rhodnius prolixus*: impact on microbiota and *Trypanosoma cruzi* and *Trypanosoma rangeli* development.

ABSTRACT

PHD THESIS IN BIOLOGIA PARASITÁRIA

Cecilia Stahl Vieira

Rhodnius prolixus is a major vector of *Trypanosoma rangeli* and *Trypanosoma cruzi*, in Latin America. The production of antimicrobial peptides (AMPs) in the midgut of the insect is vital to control possible infection, and to maintain the microbiota already present in the digestive tract. This work focuses on the modulation of the humoral immune responses of the midgut of *R. prolixus* orally challenged with Gram positive and Gram negative bacteria as well with *T. rangeli* Macias strain, *T. cruzi* Dm 28c and Y strain, considering the influence of the parasites on the intestinal microbiota. Our results showed that the anterior midgut contents of control insects contain a higher inducible antibacterial activity and AMPs transcript abundance than those of the posterior midgut. Insects orally fed with Gram-negative bacteria presented higher amount of *defC* and *prol* transcripts, while the ingestion of Gram-positive induced *defA* and *defB* expression in the midgut. *T. rangeli* Macias strain successfully colonized *R. prolixus* midgut through a decreasing in PO activities, *prolixicin* and *lysozyme* levels, while at the same time induced an increase in antibacterial activity and upregulated *defC* levels in the insect anterior midgut. *T. rangeli* infection also diminishes the amount of cultivable gut bacteria as well modified the composition of indigenous microorganisms. Furthermore, different *T. cruzi* strains present distinct profiles of immune system and microbiota modulation in *R. prolixus* midgut, where *T. cruzi* Dm 28c was able to induce an increase in defensin C genes and a depression in prolixicin genes, while drastically reduce the cultivable bacteria population. In the other hand *T. cruzi* Y was not competent to induce AMPs expression in the gut or considerably reduce the microbiota in the anterior midgut. Our findings suggest that *R. prolixus* modulates AMP gene expression upon ingestion of bacteria and tripanosomatids with patterns that are distinct and dependent upon the species of the invading pathogen. Besides, the trypanosome ability to induce immune peptides in epithelial cells seems to favor its development in the insect digestive tract by decreasing intestinal microbiota, depending on the parasite genotype.

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ÍNDICE DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

µL	Microlitro
Água Milli-Q	Água deionizada purificada no sistema Milli-Q
AMPs	Peptídeos antimicrobianos
BHI	Meio de cultura de infusão de cérebro e coração
cDNA	DNA complementar
CFU	Unidade formadora de colônia
defA	Defensina A
defB	Defensina B
defC	Defensina C
DNA	Ácido desoxirribonucléico
IMD	Imunodeficiência (via de sinalização)
KDa	Quilodalton
kDNA	DNA do cinetoplasto
lisA	Lisozima A
lisB	Lisozima B
LPG	Lipofofoglicano
LPS	Lipopolissacarídeos
M	Molar
Mg	Miligrama
mL	Mililitro
mm	Milímetro
mRNA	RNA mensageiro
NF-kB	Fator de transcrição nuclear k
nm	Nanômetro
NO	Óxido nítrico
NOS	Óxido nítrico sintase
^{o}C	Graus Celsius
PAMPs	Padrões moleculares associados a patógenos
PAP	Enzima ativadora da profenoloxidase
PBS	Tampão salino-fosfato
PCR	Reação em cadeia da polimerase
PGN	Peptidoglicano

PGRP	Proteína reconhecedora de peptidoglicanos
PO	Fenoloxidase
PPO	Profenoloxidase
Prol	Prolixicina
PRP	Proteínas de reconhecimento padrão
qPCR	PCR quantitativo em tempo real
RNA	Ácido ribonucleico
rRNA	RNA ribossômico
RT	Transcrição reversa
TSB	Meio de cultura caldo triptona de soja

1 INTRODUÇÃO

1.1 Rhodnius prolixus

Rhodnius prolixus é um inseto classificado na ordem Hemíptera, família Reduviidae, subfamília *Triatominae*, cujo desenvolvimento ocorre por hemimetabolia, ou seja, de insetos que apresentam metamorfose incompleta. Seu ciclo de vida é constituído por ovo, cinco estádios de ninfa e adultos. Os insetos adultos apresentam asas desenvolvidas e dimorfismo sexual (Lent 1948; Lent & Wygodzinsky 1979; Schofield & Galvão, 2009).

A subfamília Triatominae é composta por insetos de hábito alimentar hematófago. Os processos fisiológicos da muda, que culminam com a ecdise dos estádios imaturos, ocorrem após o repasto sanguíneo do inseto (Wigglesworth, 1972). *R. prolixus* pode ingerir uma quantidade de sangue e atingir até dez vezes seu peso inicial em uma única alimentação ninfal (Romoser, 1996). Sabe-se que a distensão abdominal associada à alimentação sanguínea são fatores essenciais para a produção do hormônio protoracicotrópico (HPTT), através das células neurossecretoras cerebrais do inseto, o qual liberado na hemolinfa estimula a glândula protorácica a sintetizar ecdisona, o hormônio responsável pela muda (Wigglesworth, 1933,1972; Garcia et al., 1975, Azambuja et al., 1997).

Além de *R. prolixus, Panstrongylus megistus* e *Triatoma infestans* também são considerados vetores de *Trypanosoma cruzi*, agente etiológico da doença de Chagas (Rey 2001; Guhl, 2007; Vallejo et al., 2009). Estas espécies são antropofílicas, geralmente se dispersam de forma passiva além de apresentarem alta susceptibilidade à infecção por diferentes cepas de *T. cruzi* (Vallejo et al., 2009; Coura, 2015). A doença de Chagas é considerada endêmica em 21 países, abrangendo a América do Sul, Central e parte da América do Norte. Aproximadamente 90 milhões de pessoas estão possivelmente expostas ao *T. cruzi* e, estimativas recentes, indicam que sete milhões de pessoas estejam infectadas com este parasita. Uma vez que a principal forma de transmissão da doença nas Américas é a vetorial, o controle de populações do inseto vetor é forma mais eficaz de prevenir a incidência da doença de Chagas no continente (Durvasula et al., 1997; Taracena et al., 2015; WHO, 2015).

Desde os clássicos trabalhos do Dr. Vincent Wigglesworth, iniciados na década de 1930, *R. prolixus* é considerado um importante modelo experimental para estudos fisiológicos de insetos. A grande vantagem reside no fato desta espécie aceitar

alimentação em aparato artificial, possibilitando a alteração de dietas com adição de diferentes drogas, microrganismos, ou mesmo o acréscimo de formas de cultura de *T. cruzi*, facilitando assim a infecção pelo parasita (Garcia et al., 1975; 1984), bem como as análises das respostas fisiológicas ou imunes dos insetos.

Devido à grande relevância do *R. prolixus* e ao avanço de técnicas de sequenciamento, seu genoma foi elucidado, num esforço conjunto de diversos grupos de pesquisa (Huebner, 2007). Mais recentemente, Ribeiro e colaboradores em 2014 publicaram um trabalho sobre o transcriptoma do trato digestivo desta espécie, com sequenciamentos de cDNAs e análises das expressões de genes, especialmente os envolvidos na digestão e no sistema imune dessa espécie.

1.1.1 Trato digestivo de *R. prolixus*

Em insetos hematófagos, especialmente aqueles vetores de parasitas causadores de doenças em humanos tais como a leishmaniose, malária e doença de Chagas, o tubo digestivo se configura como um primeiro órgão, e, portanto chave, para o desenvolvimento dos protozoários (Azambuja et al., 2005; Weiss & Aksoy, 2011), sendo as células epiteliais intestinais do inseto um dos principais sítios de interação com o parasita com e microbiota (Boulanger et al., 2004; Tzou et al., 2000).

O trato digestivo de triatomíneos está envolvido tanto no desenvolvimento de tripanosomatídeos, como também na modulação da resposta imune do inseto (Coura, 2006, Schaub, 2008, 2009; Garcia et al., 2010). *T.cruzi,* uma vez ingerido e presente no intestino médio do inseto, é capaz de se aderir às células epiteliais e membranas perimicrovilares, ou, como no caso do *Trypanosoma rangeli,* atravessá-lo e invadir a hemolinfa do inseto vetor (Azambuja et al., 2005).

De uma forma geral, o tubo digestivo dos insetos hematófagos tem semelhanças em sua anatomia e função. Em Triatominae, divide-se em três porções distintas: intestino anterior, intestino médio e intestino posterior. O intestino anterior é composto pela faringe e esôfago, já o intestino posterior é composto pela ampola retal e o reto (Ramirez-Perez 1969; Romoser, 1996; Coura, 2006; Garcia et al., 2010). Diferentemente dos dípteros vetores, os triatomíneos exibem uma segmentação adicional no intestino médio, onde a primeira porção, o intestino médio anterior, também chamada de “estômago”, se caracteriza como um saco dilatado, e o intestino médio posterior, também chamado de “intestino” propriamente dito, tem aparência

tubular e delgada (Ramirez-Perez 1969; Romoser, 1996; Coura, 2006; Garcia et al., 2010). O intestino anterior e o intestino posterior são de origem ectodérmica, cujas células são recobertas por uma cutícula quitinosa, que dificulta a absorção de nutrientes. Já o intestino médio tem origem endodérmica, cujas células são revestidas por um epitélio colunar rico em microvilosidades, as quais permitem aumentar a superfície de contato para a absorção de micro e macromoléculas digeridas (Ramirez-Perez, 1969; Coura, 2006).

Em triatomíneos, o intestino médio anterior é o compartimento onde o sangue é concentrado e estocado por algumas semanas durante o processo digestivo (Wigglesworth, 1943; Ramirez-Perez, 1969; Wigglesworth, 1972; Terra, 1990, Romoser, 1996). Nesse compartimento o pH é neutro-básico e onde se concentra a microbiota natural do inseto (Terra, 1990, Azambuja et al., 2004). O intestino médio posterior, apresentando pH de caráter ácido, é o compartimento onde ocorre a digestão de proteínas derivadas do sangue alimentar, principalmente devido as ações de enzimas do tipo catepsina (Terra, 1990, Waniek et al., 2012). Os túbulos de Malpighi, separando a porção do intestino médio e posterior, desempenham a importante função de reabsorção e de excreção de água e sais minerais provenientes da alimentação. O intestino posterior é o compartimento onde ficam contidos fezes e urina para intensa eliminação do conteúdo, principalmente durante o ato de alimentação no hospedeiro vertebrado (Wigglesworth, 1972).

1.1.2 Microbiota intestinal de *R. prolixus*

Os animais abrigam em seus intestinos, uma vasta comunidade constituída por diferentes microrganismos, que compõem sua microbiota natural. Como não poderia ser diferente, o trato digestivo dos insetos também é composto por uma miríade de microrganismos, muitos deles essenciais à manutenção da homeostase do hospedeiro (Dillon & Dillon, 2004; Dillon et al., 2010; Lindh & Lehane, 2011).

A dieta sanguínea dos triatomíneos é especialmente rica em proteínas e aminoácidos essenciais, entretanto, é deficiente em alguns nutrientes vitais para o inseto, como os carboidratos, lipídeos e a vitaminas do complexo B (Ribeiro, 1996; Romoser, 1996). Nesse contexto, a presença de microrganismos simbiontes no tubo digestivo de insetos hematófagos é essencial para suprir suas deficiências nutricionais. Sabe-se que algumas bactérias constituintes da microbiota intestinal suplementam a dieta dos triatomíneos sintetizando vitamina B e adicionalmente

facilitando a digestão das proteínas do sangue (Ribeiro, 1996; Dillon & Dillon, 2004; Yassin, 2005; Azambuja et al., 2005).

Utilizando meios de cultura, alguns microrganismos foram isolados do trato digestivo de algumas espécies de triatomíneos, inclusive *R. prolixus,* tais como diferentes espécies de fungos (Moraes et al., 2004). Dentre as espécies de bactérias mais frequentes foram destacadas as Gram-positivas, *Rhodococcus rhodnii* e *Enterococcus faecalis,* e as Gram-negativas, *Serratia marcenscens e Enterobacter cloacae* (Duncan, 1926; Dias, 1934; Wigglesworth, 1936; Figueiro et al., 1995; Azambuja et al., 2004). Na tentativa de demonstrar a importância do *R. rhodnii* no ciclo de vida do inseto, alguns autores eliminaram ou suprimiram esta espécie por esterilização dos ovos ou tratamento por antibiótico dos insetos. De fato, os insetos aposimbióticos apresentaram alterações morfofisiológicas, como por exemplo, a redução do sistema traqueal, como tiveram baixas taxas de muda, impedindo assim o completo desenvolvimento para o estágio adulto (Brecher & Wigglesworth, 1944; Ben-Yakir, 1987; Eichler & Schaub, 1998).

Posteriormente, a partir do uso de técnicas independentes de cultivo, foi demonstrado que, ao contrário da complexidade microbiana observada no trato digestivo de algumas espécies de insetos, tais como os mosquitos dos gêneros *Anopheles* e *Aedes* (Lindh et al., 2005; Gusmão et al., 2010; Kuechler et al., 2011; Gendrin & Christophides, 2013), em triatomíneos, a microbiota é relativamente simples e composta por apenas algumas gêneros de bactérias como *Arsenophonus*, *Serratia, Wolbachia* e *Candidatus Rohrkolberia*. Além disso, foi demonstrado que a composição de bactérias está associada ao gênero de triatomíneo (Da Motta et al., 2012). No caso de *Rhodnius* spp, *Serratia, Candidatus e Rohrkolberia* foram os gêneros bacterianos mais comumente encontrados, embora *Wolbachia* também tenha sido identificado em insetos provenientes da Amazônia (Da Motta et al., 2012). Cabe destacar que *S. marcescens,* tem sido frequentemente encontrada albergando intestino de *Rhodnius* spp e *Triatoma* spp mantidos em laboratórios, bem como coletados no campo (Azambuja et al., 2004, Da Motta et al., 2012, Gumiel et al., 2015). *S. marcescens* por seu potencial hemolítico, bem como tripanolítico, tem importante participação no processo digestivo e no desenvolvimento de tripanosomatídeos (Azambuja et al., 2004). Uma vez que trabalhos têm demonstrado que a presença de algumas espécies de bactérias pode interferir na capacidade de desenvolvimento de parasitas em seus hospedeiros invertebrados (Azambuja et al., 1983; Eichler & Schaub, 2002; Garcia et al., 2007; Garcia et al., 2010), o estudo da microbiota de

insetos vetores principalmente no contexto da interação com seus parasitas naturais, tem se intensificado bastante nos últimos anos.

1.2 Trypanosoma cruzi

O trabalho de Carlos Chagas, em 1909, narrou a descoberta de uma nova doença humana, a tripanosomíase americana, que tempos depois ficou conhecida como a doença de Chagas. Nesse trabalho, o autor não só descreve o agente etiológico da doença, o protozoário flagelado *Trypanosoma cruzi*, como também descreve as diferentes formas evolutivas desse parasita nos hospedeiros vertebrados e no inseto vetor, os triatomíneos, popularmente chamados de barbeiros.

T. cruzi é um protozoário heteroxênico, ou seja, se desenvolve ciclicamente entre hospedeiros invertebrados (insetos triatomíneos) e vertebrados mamíferos, inclusive o homem. Durante seu ciclo de vida, o parasita adota diferentes tipos morfológicos que variam em função e metabolismo no organismo hospedeiro (Hoare, 1972; Lent & Wygodzinsky, 1979; Garcia & Azambuja, 1991). De uma maneira geral, as formas multiplicativas são extracelulares nos hospedeiro invertebrados (epimastigotas) e intracelulares nos hospedeiros vertebrados (amastigotas) (de Souza, 2000; Undarneta-Morales, 2014). No inseto, as epimastigotas se diferenciam em tripomastigotas metacíclicas no intestino posterior, as quais são infectantes para os hospedeiros vertebrados (Garcia & Azambuja, 1991; Garcia et al. 1999; Kollien & Schaub, 2000; de Souza, 2000). Em mamíferos, as formas infectantes, tripomastigotas sanguíneas, penetram nas células, primeiramente nos macrófagos, transformam-se em amastigotas (primeiramente macrófagos), sofrem multiplicação e, finalmente são liberadas na circulação como tripomastigotas sanguíneos (Brener, 1973; Zeledon, 1987; Garcia & Azambuja, 1991; de Souza, 2000; Coura, 2006). A figura 1 ilustra o ciclo de vida do parasita nos seus hospedeiros vertebrados e invertebrados.

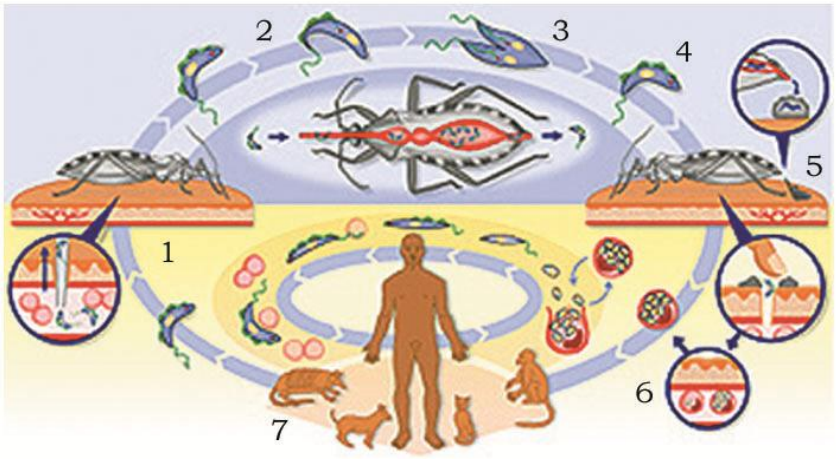

Figura 1 - Ciclo do *Trypanosoma cruzi*

1 – Inseto pica um hospedeiro vertebrado infectado, ingerindo tripomastigotas sanguíneas de *T. cruzi*. 2 - tripomastigotas atingem o intestino médio do inseto, onde se transformam em epimastigotas. 3 - epimastigotas sofrem intensa multiplicação no intestino médio. 4 - epimastigotas alcançam o reto do inseto onde, partes das formas se diferenciam para tripomastigotas metacíclicos. 5 - Triatomíneo infectado com *T. cruzi* se alimenta em um hospedeiro vertebrado eliminando tripomastigotas metacíclicas pelas fezes. O hospedeiro ao coçar o local da picada facilitando a penetração dos parasitas por esta via. 6 – As formas infectantes, metacíclicas, do parasita invadem principalmente macrófagos situados ao redor do local da picada do barbeiro. Uma vez interiorizados nas células, evoluem para amastigotas, onde se multiplicam e se transformam em tripomastigotas sanguíneos, finalmente rompendo macrófagos, 7 – os parasitas liberados alcançam a circulação sanguínea, podendo infectar outros tipos celulares do hospedeiro vertebrado. Adaptado de WHO/TDR.

T. cruzi é um protozoário posicionado taxonomicamente na família Trypanosomatidae, ordem Kinetoplastida, gênero *Trypanosoma* (Chagas, 1909; Coura, 2006). Na ordem kinetoplastida encontram-se protozoários que exibem uma estrutura mitocondrial exclusiva, o cinetoplasto, onde se concentra o DNA mitocondrial ou k-DNA (Vickerman, 1985). Um extenso conjunto de populações heterogêneas constitui a espécie *T. cruzi*. O parasita circula entre os mais variados hospedeiros vertebrados e invertebrados, e, nesse sentido, justifica-se o alto grau de variações morfológicas e fisiológicas observadas, bem como em relação à sua virulência aos vários hospedeiros (Miles et al., 1978; 1980; 2009). Tal fato pode estar associado aos distintos fenótipos da doença de Chagas observados nas diferentes regiões geográficas (Miles et al., 1981).

Nas últimas décadas, o emprego de metodologias moleculares avançadas, como o uso da PCR (reação em cadeia da polimerase), permitiu uma análise mais

profunda da diversidade genética observada nas mais diferentes cepas de *T. cruzi*. Inicialmente, partindo da amplificação de regiões intergênicas, o mini-éxon e do RNA ribossomal do parasita, foi proposto que as cepas fossem agrupadas em duas principais linhagens filogenéticas (Souto et al., 1996; Fernandes et al., 1998). Atualmente, após diversas revisões sobre o novo consenso para nomenclatura intraespecífica, *T. cruzi* é subdividido em TcI, TcII, TcIII, TcIV, TcV e TcVI (Zingales et al., 2009).

1.2.1 Interação *T. cruzi* x inseto

Os triatomíneos ao se alimentarem, inserem seus pares mandibulares e maxilares, localizados internamente na probóscide, diretamente nos capilares sanguíneos de seus hospedeiros vertebrados. Juntamente com o sangue alimentar de um hospedeiro infectado, tripomastigotas de *T. cruzi* podem ser ingeridos. No intestino médio anterior do inseto, os tripomastigotas sanguíneos desenvolvem-se em epimastigotas onde iniciam a sua multiplicação. Alguns trabalhos sugerem que neste compartimento do trato digestivo, as condições ambientais não sejam as mais favoráveis para o desenvolvimento do *T.cruzi*, não só devido ao pH ácido, como também pela competição com a microbiota bacteriana, entre outros fatores citotóxicos ao parasita (Garcia & Azambuja, 1991; Mello et al., 1996; Ratcliffe et al., 1996; Kollien & Schaub, 2000). Sabe-se que os parasitas, ao se estabelecerem na porção delgada do intestino médio ou no intestino posterior, se desenvolvem melhor e podem sobreviver por longos períodos de jejum, como foi observado em *R. prolixus* e *Triatoma infestans* (Kollien & Schaub, 2000, Cortez et al., 2012).

Epimastigotas, no intestino médio, sofrem intensa multiplicação por divisão binária especialmente na porção posterior, onde ocorre a digestão e absorção de nutrientes. Algumas destas formas evolutivas, uma vez atingindo o intestino posterior, se diferenciam em tripomastigotas metacíclicas que são posteriormente eliminadas junto às fezes do inseto (Dias, 1934; Ramirez-Perez, 1969; Brener, 1973; Garcia & Azambuja, 1991, Coura, 2006; Garcia et al., 2007). O ciclo de vida de *T. cruzi* no tubo digestivo do inseto pode ser observado na figura 2.

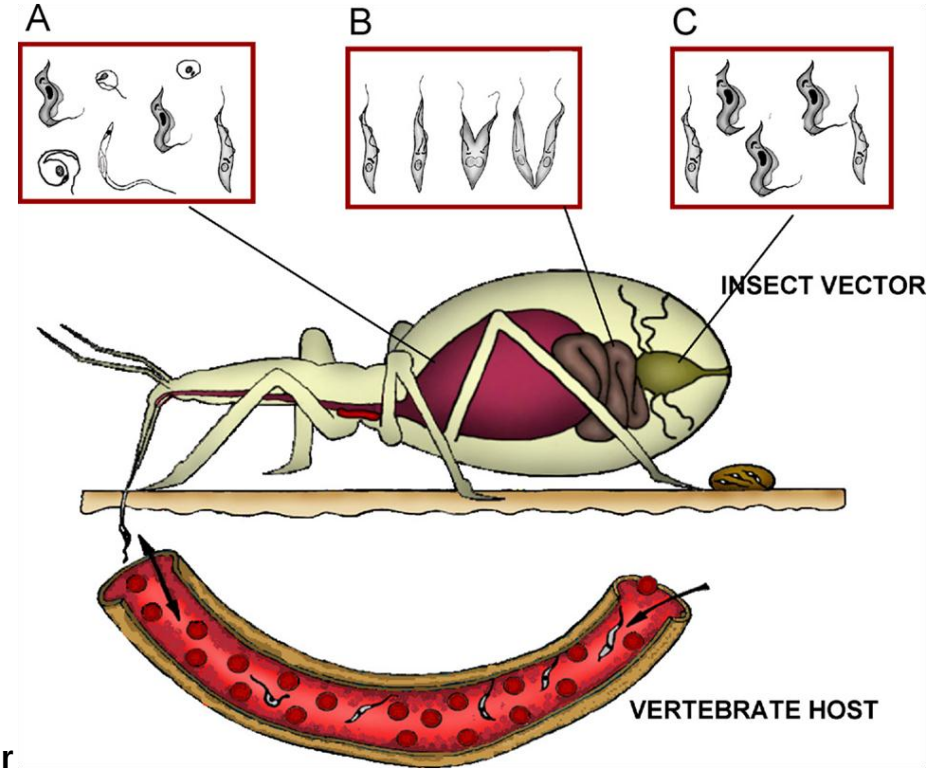

Figura 2 - Ciclo de *Trypanosoma cruzi* no inseto vetor
A- Desenvolvimento de tripomastigotas sanguíneas em epimastigotas no intestino médio anterior (estômago). B- Epimastigotas se multiplicam por divisão binária no intestino médio posterior. C- Epimastigotas migram para o intestino posterior e lá se diferenciam em tripomastigotas metacíclicas. Retirado de Garcia et al., 2007.

Em contraste com a maioria dos parasitas transmitidos por artrópodes, *T. cruzi* não é inoculado no hospedeiro pela picada do inseto, uma vez que o desenvolvimento do parasita está confinado exclusivamente ao tubo digestivo do inseto, não atravessa o epitélio intestinal e, por conseguinte não interage diretamente com a hemocele nem com as glândulas salivares do inseto (Kollien & Schaub, 2000; Buarque et al., 2013).

Nesse contexto, a importância do tubo digestivo de triatomíneos é realçada, uma vez que as interações do *T. cruzi* com triatomíneos incidirão nesse órgão. Em contato com as células do epitélio intestinal, moléculas do parasita poderão interagir com receptores presentes na superfície luminal. O reconhecimento do parasita, como organismo estranho, desencadeará uma série de respostas imunológicas no inseto as quais poderão limitar ou até bloquear o desenvolvimento do *T. cruzi* (Ursic-Bedoya & Lowenberguer, 2007; Waniek et al., 2011, Castro et al., 2012). Contudo, a literatura mostra que o estabelecimento de *T. cruzi* no hospedeiro invertebrado varia de acordo com a cepa do parasita e a espécie de triatomíneo, sugerindo que as diferentes cepas de *T. cruzi* possam ser capazes de modular as respostas imunes do inseto de uma

maneira espécie-específica (Garcia & Azambuja, 1991; Castro et al., 2012; Araújo et al., 2014). A interação deste tripanosomatídeo com o triatomíneo vetor envolve diversos fatores celulares e moleculares que modulam esta relação (Garcia & Azambuja, 1991). Tem sido evidenciado que o sucesso da infecção por *T. cruzi,* pelo menos da cepa Dm 28c, está associada a adesão do parasita com a membrana perimicrovilar, que recobre as células epiteliais do intestino médio de *R. prolixus*, etapa que parece ser crucial para o desenvolvimento do parasita (Gonzalez et al., 2006, Alves et al., 2007; Albuquerque-Cunha et al., 2009).

A capacidade de *T.cruzi* de modular as respostas imunes do inseto vetor parece ser um fator decisivo para o desenvolvimento de diferentes cepas do parasita em algumas espécies de triatomíneos.

1.3 Trypanosoma rangeli

T. rangeli é um protozoário hemoflagelado, pertencente à família Trypanosomatidae, ordem Kinetoplastida, gênero *Trypanosoma*, classificado no subgênero *Tejeraia* (Añez, 1982), que é transmitido pela picada de insetos triatomíneos (Vickerman, 1976; Grisard et al., 1999; Guhl & Vallejo, 2003). Assim como *T. cruzi*, são protozoários heteroxenos, que alternam seu ciclo de vida no inseto vetor como também em hospedeiros vertebrados (Hoare, 1972; Steindel et al., 1991; Coura et al., 1996). De maneira similar a *T. cruzi*, *T. rangeli* também infecta uma variedade de mamíferos, inclusive o homem, tendo sido identificado em diferentes hospedeiros invertebrados e vertebrados não humanos, em várias partes do Brasil (D'Alessandro 1976; Steindel et al., 1991; Coura et al., 1996). Nos hospedeiros vertebrados, a infecção por *T. rangeli* pode durar de alguns meses até vários anos, sendo a patogenicidade deste parasita ao homem tema de controvérsias (Herbig-Sandreuter, 1957; Ramirez et al., 1998; Guhl & Vallejo, 2003; Stoco et al., 2014).

A importância epidemiológica de *T. rangeli* está relacionada ao fato deste parasita exibir características biológicas e distribuição geográfica muito semelhante à de *T. cruzi*, o que acarreta, em alguns casos, infecções mistas com as duas espécies *de Trypanosoma* no mesmo hospedeiro. Neste sentido, *T. rangeli* é bastante estudado no contexto da epidemiologia da doença de Chagas (Vallejo et al., 1988; Guhl & Vallejo 2003; Stoco et al., 2014). Além disso, a resposta imune induzida pela infecção em mamíferos estimula a produção de anticorpos contra *T. rangeli*, mas que também

evidenciam reação sorológica cruzada com *T. cruzi*, complicando, assim o diagnóstico da doença de Chagas (Grögl & Kuhn, 1984; Guhl et al., 1987; Hudson et al., 1988; de Moraes et al., 2008).

Em decorrência desses fatos, recentemente, o genoma de *T. rangeli* foi elucidado com o intuito de se conhecer melhor características moleculares desse tripanosomatídeo. Através da análise genômica comparativa, foi possível observar novas características genéticas marcantes que diferem *T. rangeli* de *T. cruzi*, fornecendo assim, novas ferramentas para auxiliarem no diagnóstico desses dois tripanosomatídeos (Stoco et al., 2014).

1.3.1 Interação *T. rangeli* x inseto

No que diz respeito a seu desenvolvimento no inseto vetor, *T. rangeli* apresenta uma característica não observada em *T. cruzi*: a capacidade de atravessar o epitélio do intestino médio, colonizar a hemolinfa e posteriormente invadir as glândulas salivares do hospedeiro invertebrado **(**Grewal, 1957; Tobie, 1970; D'Alessandro, 1976; Garcia et al., 2004). Sabe-se que determinadas cepas do parasita se desenvolvem bem no intestino do inseto e ainda são aptas a invadirem e se multiplicarem na hemocele, enquanto outras, não sobrevivem às adversidades do trato digestivo do hospedeiro invertebrado (Machado et al., 2001), dependendo assim de uma superação às barreiras fisiológicas e imunológicas encontradas nesses compartimentos (Garcia et al., 2009; 2012).

O ciclo de *T. rangeli* se inicia quando o inseto, ao se alimentar em hospedeiro vertebrado previamente infectado, ingere tripomastigotas sanguíneas. Os parasitas atingem o intestino médio do inseto, onde se diferenciam em epimastigotas, que se multiplicam e aderem ao epitélio intestinal pelo flagelo (Tobie, 1970; Hecker et al., 1990; Oliveira & Souza, 2001). Alguns parasitas aderidos são capazes de invadir a hemocele por penetração em regiões onde as células epiteliais do intestino possuem uma densidade menor de organelas. Contudo, também tem sido demonstrada uma desorganização ultraestrutural nas células invadidas, em decorrência da movimentação de vários parasitas por entre as células (Watkins, 1971; Hecker et al., 1990; Oliveira & Souza, 2001; Gomes et al., 2002). A baixa taxa de penetração de parasitas na cavidade geral do inseto tem sido atribuída as limitações na sensibilidade de detecção do parasita pelo uso de técnicas de quantificação clássica por microscopia óptica (Hecker et al., 1990). Este fato também pode estar relacionado ao

fenômeno de invasão de *T. rangeli* em hemócitos, o que o torna de difícil detecção (Tobie, 1970). Uma vez na hemolinfa, os epimastigotas, dentro dos hemócitos, ou livremente, se multiplicam para posteriormente migrarem e penetrarem as glândulas salivares do inseto vetor, sítio onde o parasita se desenvolve em tripomastigotas metacíclicas, as quais são infectantes para os hospedeiros vertebrados (Garcia et al., 2004; Vallejo et al., 2009).

É notório, portanto, que a colonização de *R. prolixus* por *T. rangeli* se configura de forma mais invasiva aos tecidos que a observada por *T. cruzi*. Como consequência, *T. rangeli* precisa desenvolver mecanismos de escape às respostas imunes humorais e celulares (Gomes et al., 2003; Garcia et al., 2004). Neste sentido, foi demonstrado que o parasita é capaz de suprimir algumas reações imunológicas do hospedeiro invertebrado, como a ativação da cascata da profenoloxidase, fagocitose e microagregação dos hemócitos, garantindo sua sobrevivência e multiplicação no inseto (Gomes et al., 2003; Garcia et al., 2004; Figueiredo et al., 2008; Garcia et al., 2009). Curiosamente, verificou-se a incapacidade de *T. cruzi* em colonizar a hemolinfa de *R. prolixus*, ao contrário do observado com *T. rangeli*, a partir da inoculação desses parasitas diretamente na hemolinfa do triatomíneo (Mello et al, 1995). Sabe-se que diferentes cepas de *T. rangeli* são capazes de sobreviver e se multiplicar na cavidade geral do inseto, sugerindo assim que não só o inseto responde imunologicamente de forma diferenciada frente a estas duas infecções, como também o *T. rangeli* apresenta uma estratégia distinta de desenvolvimento no hospedeiro invertebrado (Tobie, 1968; Mello et al, 1995, Gomes et al., 2003; Vallejo et al., 2009).

No que diz respeito à susceptibilidade de infecção por *T. rangeli* nas diferentes espécies de triatomíneos, até o momento, apenas o gênero *Rhodnius* foi encontrado infectado naturalmente por *T. rangeli* nas glândulas salivares (D'Alessandro & Mandel, 1969; Guhl & Vallejo, 2003).

1.4 Sistema imune de insetos

A classe Insecta é um grupo altamente diverso, com mais de 1 milhão de espécies já descritas, representando aproximadamente 75% dos animais no planeta. Além de serem maioria em número de espécimes na Terra, os insetos são capazes de colonizar quase todos os nichos, como florestas, restingas, inclusive ambientes como águas poluídas, fezes, excrementos e carcaça de animais em decomposição (Bulet & Stocklin, 2005; Klowden 2007; Ratcliffe et al., 2011). Portanto, estão

constantemente expostos a diferentes microrganismos, muitos deles potencialmente patogênicos. Um dos fatores que possibilitam o grande sucesso evolutivo dos insetos tem sido atribuído a sua rápida e eficiente resposta imune contra microrganismos invasores (Cociancich et al., 1994; Ferrandon et al., 2007; Klowden, 2007).

A primeira barreira protetora dos insetos está relacionada ao seu rígido exoesqueleto. A cutícula, constituída majoritariamente por quitina, é a principal barreira física que dificulta a invasão de microrganismos na hemocele (Vilmos & Kurucz, 1998; Armstrong et al., 1996). Contudo, algumas bactérias ou protozoários, ao serem ingeridos ou ultrapassando a barreira cuticular, podem ser reconhecidos por receptores celulares presentes no epitélio intestinal ou nas células da cavidade geral dos insetos (hemócitos), as quais reconhecerão o invasor como partícula estranha no organismo do hospedeiro invertebrado, desencadeando uma série de respostas imunológicas (Vilmos & Kurucz, 1998; Hoffman et al., 1999; Nurnberger et al., 2004; Buchmann 2014).

Todos os animais são passíveis de se infectarem por diferentes classes de agentes patogênicos. Tanto mamíferos como insetos evoluíram num complexo sistema de produção de células do sistema circulatório e fatores humorais, relacionados ao sistema imune, que o tornam aptos a combaterem infecções no organismo hospedeiro. É evidente que o sistema imune varia entre os grupos do reino animal, porém, todos tem em comum a imunidade inata, que atua de forma rápida e geralmente eficiente no combate a organismos invasores (Muller et al., 2008). O sistema imune de insetos é apenas inato, ou seja, não possui elementos da resposta imune adaptativa tal qual ocorre nos vertebrados, caracterizado pela produção de células de memória e anticorpos. De maneira geral, a imunidade inata em insetos é bastante competente, constituída de respostas humorais, como ativação do sistema profenoloxidase, e cascata proteolítica associada, produção de peptídeos antimicrobianos (AMPs) ou moléculas que atuam sobre os diferentes patógenos; e respostas celulares onde há participação ativa dos hemócitos (células que circulam na hemolinfa) na eliminação dos microrganismos, como por exemplo, a fagocitose (Hoffmann 1995; Vilmos & Kurucz, 1998; Hoffmann et al., 1999). Vale ressaltar que as respostas humorais e celulares, comumente, atuam de forma integrada, no sentido de conter a infecção de forma mais rápida e eficaz (Azambuja et al 1991; 1999; Ferrandon et al., 2007).

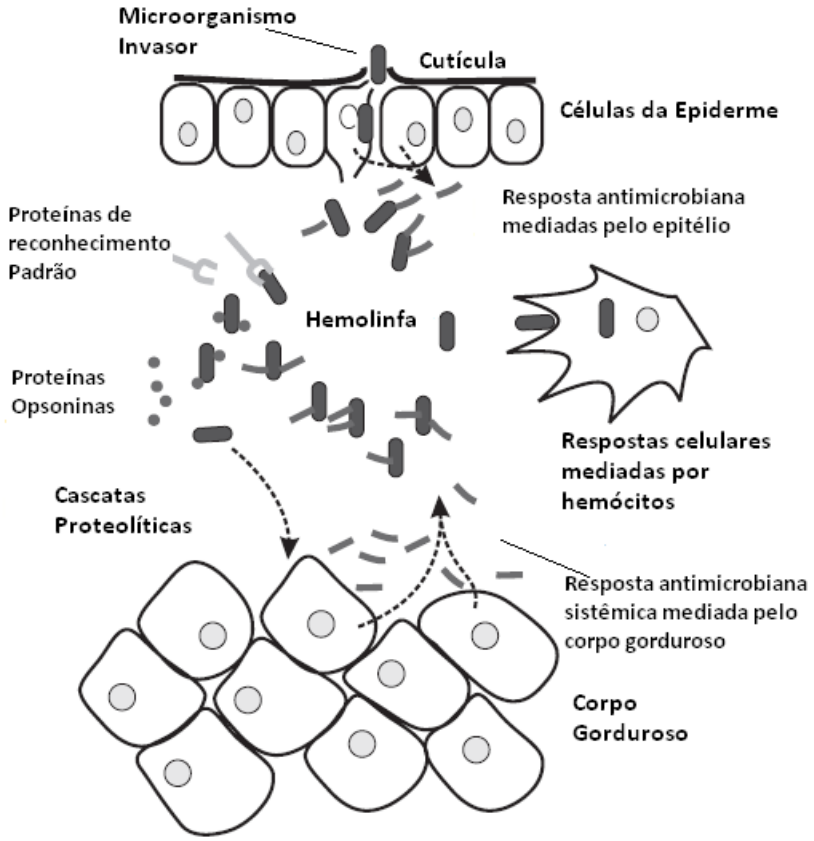

Figura 3 - Esquema de possíveis respostas imunológicas de insetos disparadas por microrganismos invasores. Adaptado de Klowdem, M.J. Physiological systems in Insects, 2007.

1.4.1 Imunidade celular

1.4.1.1 Hemócitos

O principal mediador da resposta celular em invertebrados são os hemócitos, tipos celulares morfologicamente distintos e presentes na hemolinfa, a qual preenche a cavidade geral dos insetos, e corresponde ao sangue dos vertebrados (Lackie, 1988). Os Hemócitos são responsáveis pelas três respostas imunes celulares conhecidas em insetos: fagocitose, nodulação (microagregação de hemócitos) e encapsulação celular (Ratcliffe 1985; Hoffman 1995; Vilmos & Kurucz, 1998). A presença de microrganismos invasores na hemolinfa, quando detectada por receptores específicos na membrana dos hemócitos, induz uma rápida multiplicação dessas células, ao mesmo tempo em que as mobiliza para combater ativamente a infecção (Beaulaton 1979; Lemaitre & Hoffmann, 2007).

A classificação dos hemócitos é baseada em sua morfologia e função, porém, diferentes critérios têm sido utilizados nos estudos, gerando muita controvérsia (Götz

& Boman, 1985; Lackie, 1988). Não obstante, a literatura mostra que alguns tipos de hemócitos são comuns em vários grupos de insetos, desempenhando a mesma função (Ribeiro & Brehelin, 2006). Os tipos mais frequentemente encontrados em insetos são: prohemócitos, esferulócitos, oenócitos, granulócitos e plasmatócitos (Lavine & Strand, 2002). Em *R. prolixus*, o estudo dessas células teve início com os trabalhos de Wigglesworth (1933; 1955), que posteriormente foi refinado a partir da utilização da técnica de microscopia óptica de contraste de fase (Jones, 1965), bem como por microscopia eletrônica de transmissão (Oliveira e Souza, 2003). Um estudo comparativo da morfologia dos hemócitos encontrados em diferentes triatomíneos foi realizado por Azambuja e colaboradores (1991). Neste trabalho, os autores descreveram a presença de sete tipos celulares nesses insetos: prohemócitos, plasmatócitos, cistócitos, oenocitóides, adipohemócitos, células granulares e gigantes.

1.4.1.2 Fagocitose

No que diz respeito às respostas celulares, a fagocitose, de certo, é o mecanismo mais bem conservado entre os seres vivos. Trata-se de uma forma de endocitose, na qual partículas estranhas ao organismo são reconhecidas e sequestradas para dentro das células em grandes vesículas (Lavine & Strand, 2002; Figueiredo et al., 2006). Essas vesículas são fusionadas a lisossomos, transformando-se em fagolisossomos, onde ocorre a digestão do material exógeno. Os restos não digeridos são expelidos da célula fagocítica através da exocitose (Götz & Boman, 1985). Sabe-se que os plasmatócitos e oenocitóides são os principais tipos celulares envolvidos no processo de fagocitose em insetos (Lavine & Strand, 2002).

1.4.1.3 Nodulação

A nodulação também é um processo altamente conservado, entre os invertebrados, que envolve a agregação dos hemócitos, onde um ou mais tipos celulares podem ser recrutados, no sentido de agregar e reter microrganismos ou materiais bióticos e abióticos estranhos (Ratcliffe & Gagen, 1977; Satyavathi et al., 2014). Os nódulos, além de conterem os patógenos de forma mais eficiente, também podem fagocitar e sintetizar ao redor a melanina, acelerando o processo de eliminação da infecção (Brookman et al., 1989).

1.4.1.4 Encapsulação

A encapsulação celular é um mecanismo imune no qual são formadas camadas sobrepostas de hemócitos ao redor de organismos estranhos e grandes demais para serem fagocitados pelas células, ou ainda, aprisionados pelos nódulos, como por exemplo, nos casos de nematoides, ovos ou larvas de parasitas invasores (Ratcliffe, 1982; Ratcliffe e Gotz, 1990). A encapsulação também pode estar associada ao processo de melanização, decorrente da ativação do sistema profenoloxidase, levando a morte do microrganismo por asfixia (Gillespie et al., 1997; Satyavathi et al., 2014). Ademais, dentro da cápsula, o patógeno pode ser morto pela produção de radicais livres, como reativos de nitrogênio e oxigênio, a partir dos hemócitos envolvidos na encapsulação (Nappi et al., 1995; Satyavathi et al., 2014).

1.4.2 Imunidade humoral

O sistema imune humoral consiste na síntese específica de moléculas, peptídeos ou enzimas que possam atuar de forma tóxica sobre o microrganismo invasor (Dunn, 1990; Klowden, 2007). O estudo dessas moléculas nos insetos teve início, principalmente com o trabalho de Hultmark e colaboradores, na década de 80, onde foi demonstrado que pupas da mariposa *Hyalophora cecropia*, após uma injeção com bactérias na hemolinfa, sintetizaram peptídeos com atividade antibacteriana (Hultmark et al., 1982). Esse peptídeo foi chamado de cecropina, o primeiro peptídeo antimicrobiano (AMP) detectado em insetos. Posteriormente outros AMPs foram identificados em insetos, tais como a diptericina e a defensina, ambas isoladas da mosca *Phormia terranovae* (Dimarcq et al., 1988; Lambert et al., 1989). Hoje, se conhece mais de duzentos tipos de AMPs, inúmeros deles descritos em diferentes espécies de insetos (Brey & Hultmark, 1997). Outros fatores antimicrobianos também fazem parte do arsenal humoral dos insetos, como a produção de moléculas citotóxicas, por exemplo, as denominadas espécies reativas de oxigênio e nitrogênio (Rivero, 2006; Whitten et al., 2007) e a ativação da cascata de profenoloxidase, que induz a produção de melanina, cujos produtos intermediários são tóxicos aos microrganismos (Soderhall & Cerenius,1998; Cerenius et al., 2008).

1.4.2.1 Cascata da profenoloxidase

O estudo e dissecção dos diferentes órgãos e tecidos dos insetos levou a observação do fenômeno de escurecimento gradual da hemolinfa quando a mesma é exposta ao ar. Ainda no século XIX, suspeitou-se que esse fenômeno poderia estar relacionado à atividade de uma enzima oxidativa, uma fenoloxidase (Biedermann & Moritz, 1898). Os primeiros trabalhos que descreveram as características bioquímicas da enzima responsável pelo escurecimento da hemolinfa foram publicados apenas meio século depois (Sussman, 1949; Wyatt, 1961). Atualmente, sabe-se que esse processo se deve à produção de melanina a partir da ativação do sistema profenoloxidase (PPO), em fenoloxidases ativas (PO), sendo que hoje, em insetos, estão descritos dois tipos dessa enzima (Söderhäll & Cerenius, 1998; González-Santoyo & Aguilar, 2012). Um tipo está relacionado ao processo de esclerotização e escurecimento da cutícula, são as enzimas tipo lacases; o outro tipo possui atividade de tirosinases, com capacidade de hidroxilar tirosinas e também de oxidar difenóis em quinonas (Dittmer et al., 2004; Arakane et al., 2005; Gorman et al., 2007). Esse último tipo é a enzima envolvida na resposta imune de insetos. As quinonas produzidas pela ativação da PPO ainda sofrem uma série de reações enzimáticas e não enzimáticas adicionais que resultam na polimerização e síntese da melanina (Kanost & Gorman, 2008).

Como já foi dito, as profenoloxidase (PPOs) estão presentes na hemolinfa de insetos sob a forma de zimogênios, ou seja, pró-enzimas inativas. As PPOs são predominantemente produzidas por determinados tipos de hemócitos (Cerenius & Soderhall, 2004), e até o momento não se tem registro de nenhum outro tipo celular que sintetize essa enzima em invertebrados. Em mariposas e mosquitos os hemócitos responsáveis pela síntese das PPOs são os oenócitos (Jiang et al., 1998; Hillyer & Christensen, 2002; Castillo et al., 2006; Shrestha & Kim, 2008), enquanto em *Drosophila* são as células denominadas cristal que as sintetizam (Rizki et al., 1985; Williams, 2007). Adicionalmente, acredita-se que a disponibilidade das PPOs na hemolinfa seja derivada da lise desses hemócitos, uma vez que essas enzimas não possuem uma sequência de peptídeo sinal que indique uma localização específica para sua secreção (Ashida & Brey, 1997; Kanost & Gorman, 2008). A lise dos hemócitos ocorre principalmente após exposição a microrganismos (Hofmann, 1970; Brehélin et al., 1989), mas também pode ocorrer de forma espontânea, a fim de manter níveis basais da enzima PO na hemolinfa (Ashida & Brey, 1997; Kanost & Gorman,

2008). Cabe ressaltar que, recentemente, alguns trabalhos demonstraram a presença de atividade fenoloxidásica no tubo digestivo de insetos, inclusive em *R. prolixus* (Genta et al., 2010; Castro et al., 2012). Entretanto, até o momento, é desconhecida a origem das POs nesse tecido, estando em aberto se são transportadas da hemolinfa ou secretadas por um tipo celular ainda não descrito na literatura.

As PPOs são polipeptídeos com peso molecular aproximado de 80 KDa, contendo dois átomos de cobre por molécula. Já a enzima ativa, a PO, possui peso molecular de aproximadamente 60 KDa (Ashida & Brey, 1997). No que diz respeito à capacidade de usar sítios reativos contendo átomos de cobre para catalisar reações que requerem oxigênio molecular como substrato, essas enzimas são similares às tirosinases de mamíferos (Kanost & Gorman, 2008). Após a clivagem da PPO em PO, seja por injúria tecidual ou por infecção, por um mecanismo de ativação e regulação que será descrito mais detalhadamente a frente, ocorre a hidroxilação de tirosinas com formação de diidroxifenilalanina (DOPA), a qual é oxidada em dopaquinona. Esta, por sua vez, sofre uma reação não enzimática espontânea e se converte em dopacromo (Nappi & Vass, 1993; Nappi & Christensen, 2005). O dopacromo sofre ainda uma descarboxilação, gerando o 5-6 diidroxiindol, que é então oxidado formando as indolquinonas. As indolquinonas são finalmente polimerizadas em melanina (no caso a eumelanina, o tipo mais comum de melanina) (Napolitano et al., 2000; Nappi & Christensen, 2005). Existe ainda uma via alternativa para produção de diidroxiindol, através da ação da enzima DOPA descarboxilase, que desloca CO_2 do DOPA, gerando, portanto a dopamina (Nappi & Christensen, 2005). A formação de melanina está esquematizada na figura 4.

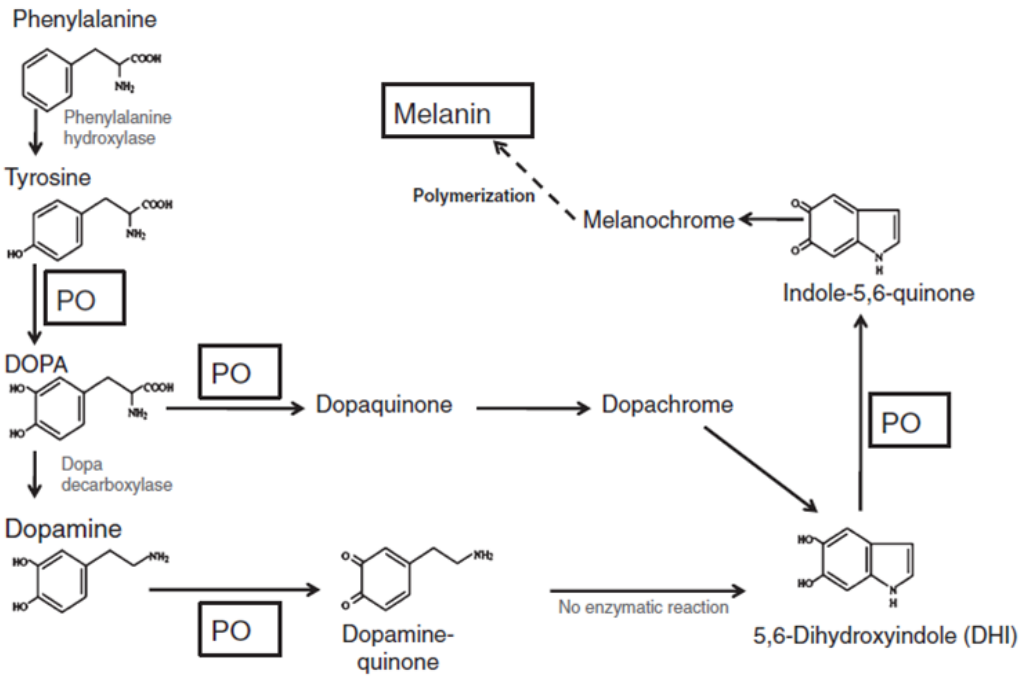

Figura 4 - Síntese de melanina em insetos através da ação da fenoloxidade. Retirado de González-Santoyo & Aguilar, 2012.

A ativação do sistema PPO pode ser disparada em resposta a injúrias teciduais, ou a partir do reconhecimento de agentes invasores ou de seus componentes celulares, como peptidoglicanos de parede celular de bactérias, β-1-3 glucanas de parede celular de fungos entre outros microrganismos ou parasitoides (Cerenius & Soderhall, 2004; Nappi & Christensen, 2005). Agentes ativadores da PPO disparam uma alteração na conformação da enzima, tornando o sítio ativo acessível ao substrato, ou ainda criando um rearranjo específico no sítio ativo para catalisar reações de oxidação (Kanost & Gorman, 2008; González-Santoyo & Aguilar, 2012). A primeira protease ativadora da PPO (PAP) foi purificada da cutícula de *Manduca sexta* (Aso et al., 1985). Posteriormente, foram purificadas da hemolinfa de *M. sexta*, serino proteases que ativam diretamente a PPO e demonstradas as similaridades a outra proteína induzida por infecção bacteriana em *Drosophila* (Jiang et al., 1998). Neste mesmo trabalho foi observada a necessidade de cofatores proteicos para ativação da PPO. Dessa forma, nota-se que a PAP também se encontra em forma de zimogênio (pró-PAP) e precisa ser ativada por outra proteína como parte da cascata proteolítica formada por serino proteases.

Experimentalmente a PPO pode ser ativada por serino proteases isoladas de insetos ou pela adição de enzimas tipo tripsina ou quimotripsina, purificadas de outros

animais. Essas proteases quebram a cadeia polipeptídica na porção carboxil de aminoácidos específicos, induzindo a clivagem da ligação peptídica a tornando PO, que é a enzima ativa (Aso et al., 1985; Asada & Sezaki, 1999; Hedstrom, 2002). As tripsinas e quimotripsinas são bastante utilizadas em diversos modelos experimentais de ativação da PPO em insetos (Aso et al., 1985; Gomes et al., 2003; Genta et al., 2010)

Em suma, o início da ativação do sistema PPO depende da ativação da cascata pró-PAP em PAP, estimulada pelo reconhecimento de microrganismos invasores. Proteínas receptoras que se ligam a sítios específicos localizados na superfície de microrganismos invasores parecem estar envolvidas nessa etapa de ativação da PAP, um processo que ainda é controverso na literatura (Ashida et al., 1983; Yu et al., 2003; Kanost & Gorman, 2008).

O resultado final dessa cascata é a produção de melanina, que pode ser depositada na superfície de parasitas encapsulados, em nódulos de hemócitos ou mesmo dispersa diretamente na hemolinfa. Tanto a melanina quanto as espécies químicas reativas formadas durante a sua síntese, auxiliam na morte de patógenos e parasitas invasores (Söderhäll & Cerenius, 1998; Cerenius et al., 2008). Os produtos gerados pela ativação da cascata da PPO também são tóxicos às células do organismo hospedeiro, e, portanto, a existência de um mecanismo de regulação dessa via é fundamental. Atualmente sabe-se que as proteínas inibidoras envolvidas nessa regulação são serino proteases, membros da superfamília das serpinas (Gettins, 2002). Serpinas já foram isoladas e caracterizadas de diferentes insetos, como nas mariposas *M. sexta* e *Hyphantrea cunea* (Park et al., 2000; Jiang et al., 2003; Tong & Kanost, 2005), em *Drosophila* (De Gregório et al., 2002; Nappi & Christensen, 2005) e mais recentemente no inseto triatomíneo *Panstrongylus megistus* (Moreira et al., 2014). As serpinas podem inibir diretamente a PO ativa, como um mecanismo de regulação negativa da cascata da PPO, evitando danos às próprias células do hospedeiro (Tong & Kanost, 2005; Kanost & Gorman, 2008). O processo de reconhecimento de um organismo invasor, culminando com a síntese de melanina e seu mecanismo de regulação pode ser observado de forma simplificada na figura 5.

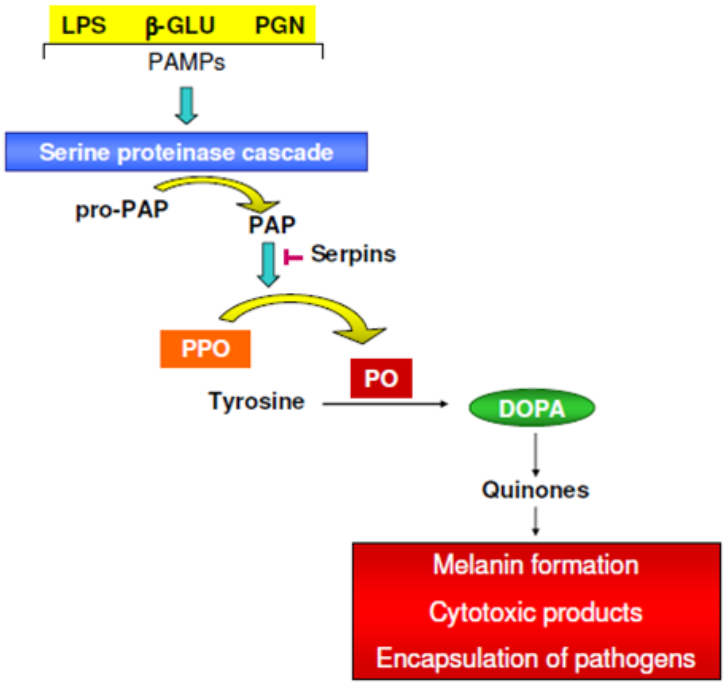

Figura 5 - Ativação da cascata da profenoloxidase após reconhecimento de padrões moleculares associados a patógenos. Retirado de Garcia et al., 2010.

1.4.2.2 - Espécies reativas de oxigênio e nitrogênio

Outro arsenal de respostas humorais em insetos está relacionado com formação de radicais livres, como as espécies reativas de nitrogênio (RNS) e espécies reativas de oxigênio (ROS), que podem ser geradas por diferentes processos metabólicos, como a ingestão e digestão de sangue em insetos hematófagos, respiração celular e ainda pela infecção por parasitas (Whitten et al., 2001; Whitten et al., 2007; Herrera-Ortiz et al., 2011). Radicais livres são moléculas que apresentam um elétron não pareado na última camada, o que define seu grande potencial oxidativo. ROS e RNS interagem com as membranas celulares dos microrganismos através da peroxidação de lipídeos, proteínas e DNA (Freeman & Crapo, 1982; Ha et al., 2005), desempenhando papel antimicrobiano que auxilia no combate a infecções e ainda na regulação da microbiota intestinal dos insetos (Fang, 1997; Rivero, 2006; Molina-Cruz et al., 2008).

ROS são radicais livres derivados do metabolismo do oxigênio, formados durante o processo de respiração celular. Dentre os ROS estão os ânions superóxidos (O_2^-), radicais hidróxi (OH) e o peróxido de hidrogênio (H_2O_2) (Thannickal & Fanburg, 2000; Bonekamp et al., 2009). Estudos tem demonstrado a participação de ROS tanto na manutenção da microbiota intestinal do inseto bem como na infecção por parasitas.

A infecção por *Plasmodium berghei* no mosquito *Anopheles albimanus* induz um aumento de peróxido de hidrogênio no tubo digestivo do inseto, que parece ser essencial para estimular a expressão de peptídeos antimicrobianos, outra resposta imune humoral importante contra a infecção por protozoários e outros patógenos (Lanz-Mendoza et al., 2002; Herrera-Ortiz et al., 2011). Em *Drosophila* foi observado que ROS é ativamente produzido no tubo digestivo em níveis basais, porém, a infecção oral com bactérias aumenta consideravelmente a produção desses reativos (Ha et al., 2005; Ha et al., 2009a; Ha et al., 2009b).

Dentre os reativos de nitrogênio, destaca-se o óxido nítrico (NO), uma molécula multifacetada que vem sendo bastante estudada no âmbito da imunidade de insetos (Rivero, 2006; Whitten et al., 2007; Castro et al., 2012; Weiss et al., 2013; Eleftherianos et al., 2014). O óxido nítrico é um radical livre gasoso, altamente lipofílico, extremamente reativo e instável (Rivero, 2006; Eleftherianos et al., 2014). É produzido pela ação da enzima óxido nítrico sintase (NOS), que oxida a L- arginina gerando L-citrulina e NO (Alderton et al., 2001; Rivero, 2006). Existem várias evidências da atividade do NO na resposta imunológica de insetos contra diferentes parasitas (Ascenzi e Gradoni, 2002; Rivero, 2006 e Carton et al., 2009). Recentemente, Whitten e colaboradores em 2007 descreveram a importância do NO na resposta imune de *R. prolixus* infectados por *T. rangeli*, *T. cruzi* e componentes da parede celular de bactérias (LPS). Já em *Anopheles* a síntese de NO está envolvida com o bloqueio da proliferação de algumas espécies de *Plasmodium* (Dimopoulos et al., 1998; Herrera-Ortiz et al., 2010; Vijay et al., 2011).

Ambos os reativos intermediários de oxigênio e nitrogênio são altamente tóxicos para os microrganismos invasores, e como também os são para as células do hospedeiro, devem ser produzidos de forma equilibrada (Sobolewski et al., 2005; Rivero, 2006).

1.4.2.3 – Lisozimas

A primeira evidência da presença de moléculas com atividade antimicrobianas em seres humanos foi feita por acaso por Alexander Fleming no início da década de 1920, quando o pesquisador evidenciou o fato de que seu próprio muco nasal tinha a capacidade de inibir o crescimento de uma determinada estirpe de bactérias em cultura. Ele percebeu que a inibição bacteriana era, em grande parte, devido à ação de uma proteína proveniente do muco, que induzia a lise das células bacterianas. Por

esse motivo denominou a proteína de lisozima. Adicionalmente, Fleming relatou essa atividade em clara de ovos de galinhas, lágrimas, saliva, expectoração, e nas secreções nasais. Num estudo subsequente, Fleming ainda detectou a lisozima em soro humano, leite, e uma ampla variedade de outros fluidos (Fleming, 1922; Kollien et al., 2003; Callewaert & Michiels, 2010).

As lisozimas de animais são agrupadas em três tipos distintos: lisozimas do tipo-c (isoladas da clara de ovos de galinha), lisozimas do tipo-g (derivadas da clara de ovo de gansos) e as lisozimas do tipo-i (derivada de invertebrados). Particularmente, os insetos possuem lisozimas do tipo-c, também encontradas em outros animais como crustáceos, peixes, aves e mamíferos (Lee & Brey, 1995; Hultmark, 1996; Callewaert & Michiels, 2010).

Uma propriedade comum a todos os tipos de lisozimas é a sua habilidade de hidrolisar ligações glicosídicas β- 1,4 entre N-acetil- glucosamina e resíduos de ácido N- acetil murâmico existentes na camada de peptidoglicanos da membrana celular de bactérias Gram-positivas (Lee & Brey, 1995; Callewaert & Michiels, 2010), o que caracteriza seu principal mecanismo de ação antimicrobiano. Dessa forma, as lisozimas podem desempenhar um papel digestivo, sobretudo em insetos que ingerem um alto número de bactérias a partir de sua fonte alimentar (Regel et al., 1998), ou ainda, podem assumir uma função imunológica, prevenindo a proliferação sistêmica de microrganismos, bem como atuando em sinergismo com outros fatores imunológicos (Boman et al., 1991; Ursic-Bedoya et al., 2005; Zdybicka-Barabas et al., 2013).

Diferentes lisozimas já foram identificadas em diversas ordens de insetos, por exemplo, em Lepidoptera, Diptera e Hemiptera, inclusive na subfamília Triatominae (Kylsten et al., 1992; Ursic-Bedoya et al., 2005; Kollien et al., 2003; Araújo et al., 2006). Em *R. prolixus*, duas lisozimas distintas foram identificadas (Lisozima A e Lisozima B) no tubo digestivo e corpo gorduroso de insetos imunizados após a injeção de uma mistura de bactérias Gram-positivas e Gram-negativas. Análises filogenéticas revelaram que a lisozima A se agrupa com lisozimas de outros triatomíneos, cuja expressão ocorre majoritariamente no intestino (Kollien et al., 2003; Araújo et al., 2006; Balczun et al., 2008). Por outro lado, a lisozima B se alinha com lisozimas identificadas em mosquitos, cuja expressão ocorre predominantemente nos hemócitos e corpo gorduroso desses insetos, sugerindo que cada isoforma da enzima possa desempenhar função digestiva ou de resposta imune no inseto (Ursic-Bedoya et al., 2008).

1.4.2.4 – Peptídeos antimicrobianos

A observação da indução de fatores antibacterianos na hemolinfa de insetos, após o desafio com microrganismos vivos ou mortos, data da década de 1970 (Bákula, 1970; Boman et al., 1972). Posteriormente, foi observado que pupas da mariposa *Hyalophora cecropia*, após injeção por bactérias na hemolinfa, secretavam fatores com atividade antibacteriana nesse mesmo tecido. Esse fator antibacteriano induzido foi isolado e caracterizado como um peptídeo que atua principalmente contra bactérias Gram-negativas, sendo, portanto, a cecropina, o primeiro peptídeo antimicrobiano (AMP) isolado de insetos (Hultmark et al., 1982). Após a descoberta da cecropina, o mesmo grupo de pesquisadores suecos identificou outra classe de AMPs de *H. cecropia*, a atacina (Hultmark et al., 1983). A partir daí, uma miríade de diferentes AMPs foram identificados em diferentes espécies de insetos, cujos nomes geralmente derivam da espécie da qual foram originalmente isolados. Contudo, AMPs identificados em uma espécie pode apresentar similaridades com outros peptídeos isolados em outras espécies. Como exemplo, foi demonstrado que o AMP isolado da mosca *Sarcophaga peregrina*, a sarcotoxina, possui similaridades com a cecropina (Okada & Natori, 1985; Dimarcq et al., 1988). As principais classes de AMPs estudadas em insetos vetores são as do tipo cecropinas, atacinas, defensinas e diptericinas, cada qual apresentando diferentes estruturas e espectro de ação (Boman et al., 1991; Hoffmann, 1999; Kollien et al., 2003; Lopez et al., 2003; Araújo et al., 2006; Ursic-Bedoya et al., 2008; Telleria et al., 2013).

As defensinas têm sido exaustivamente identificadas nos mais diversos grupos de animais. Foram inicialmente descritas em animais vertebrados, e uma das principais características da proteína é a presença de vários resíduos de cisteína, um atributo comum a todas as isoformas do peptídeo até hoje estudados (Ganz, 2003). Sua caracterização em insetos se deu a partir de estudos com larvas da mosca *P. terranovae*, que após serem injetadas com bactérias, expressaram duas proteínas com atividade contra bactérias Gram-positivas. Os autores ainda demonstraram que essas proteínas eram desprovidas de atividade de lisozima, reforçando a hipótese de se tratarem de novos peptídeos antimicrobianos de insetos. A alta homologia entre as sequencias dos peptídeos isolados da mosca com a de defensinas de coelhos e humanos, induziu os autores a nomearem esses novos peptídeos com o mesmo nome (Lambert et al., 1989). Mais tarde, foram descritas defensinas em diversos outros

insetos, como em *Drosophila*, *Anopheles*, *Lutzomyia* e triatomíneos, os quais geralmente produzem mais de uma isoforma da proteína (Dimarcq et al., 1994; Richman et al., 1996; Waniek et al., 2009; Telleria et a., 2013).

As defensinas exibem baixo peso molecular, com aproximadamente de 4KDa, compostas por uma cadeia de 33 a 46 resíduos de aminoácidos. Uma característica marcante das defensinas de insetos é a presença de seis resíduos de cisteína em sua estrutura, envolvidas na formação de três pontes dissulfídicas, além de apresentarem uma estrutura mista de α-hélices e folhas-β (Bulet et al., 1999; Lamberty et al., 2001; Bulet & Stocklin, 2005). As defensinas são especialmente eficazes contra bactérias Gram-positivas, como *Staphylococcus aureus* e *Micrococcus luteus*, embora também possam atuar sobre bactérias Gram-negativas, fungos e até alguns protozoários (Shahabuddin et al., 1998 Lamberty et al., 2001; McGwire et al., 2003).

Outras famílias de AMPs que também fazem parte da imunidade humoral de diversos insetos são as diptericinas e as atacinas. As atacinas são peptídeos que agem preferencialmente sobre bactérias Gram-negativas, aumentando a permeabilidade de suas membranas através da interação com as moléculas de LPS presentes na membrana externa. Também foi evidenciado que as atacinas penetram nas células bacterianas e interagem com o DNA, interrompendo a síntese de proteínas essenciais à montagem das membranas da bactéria (Hultmark et al., 1983; Carlsson et al., 1998). A relevância do estudo das atacinas em insetos vetores se torna evidente a partir da demonstração de seu papel no estabelecimento da infecção por *Trypanosoma brucei* na mosca tsetse, sugerindo que esses AMPs podem também desempenhar papel fundamental na infecção por tripanosomatídeos em outros insetos vetores (Hao et al., 2001; Hu & Aksoy, 2006).

Recentemente, foi identificado um novo AMP em *R. prolixus*, intitulado de prolixicina, cuja sequencia possui similaridades com atacinas e diptericinas. Esse novo AMP possui potente atividade contra bactérias Gram-negativas e, em menor grau, contra bactérias Gram-positivas (Ursic-Bedoya et al., 2011). Embora a expressão de prolixicina tenha aumentado significativamente no corpo gorduroso do inseto após inoculação por *T. cruzi* na hemolinfa, testes *in vitro* com a proteína recombinante não conseguiram comprovar ação tóxica contra o parasita (Ursic-Bedoya et al., 2011).

Além da prolixicina, três isoformas de defensinas (defensinas A, B e C) e duas lisozimas já foram identificados em *R. prolixus* (Lopes et al., 2003; Usic-Bedoya et al., 2008, 2011). Lisozimas e diferentes defensinas também já foram identificadas em

outras espécies de triatomíneos como *Triatoma brasiliensis* e *Triatoma infestans* (Kollien et al., 2003; Araújo et al., 2006; Araújo et al., 2009; Waniek et al., 2009; Waniek et al., 2011). Embora pouco se conheça sobre os mecanismos de regulação desses AMPs nos triatomíneos, há evidências de que esse grupo de insetos vetores também possua um amplo arsenal de proteínas antimicrobianas.

Os AMPs são sintetizados principalmente pelo corpo gorduroso dos insetos, células epiteliais de diferentes tecidos e pelos hemócitos. Os AMPs sintetizados pelo corpo gorduroso e hemócitos são secretados na hemolinfa, de onde podem, por meio do sistema circulatório, difundir-se rapidamente por todo corpo do inseto (Bulet & Stöcklin, 2005). Por outro lado, AMPs sintetizados pelas células epiteliais do tubo digestivo são secretados diretamente no lúmen do intestino, atuando localmente (Bulet & Stöcklin 2005, Ferrandon et al., 2007).

1.4.2.4.1 - Mecanismo de ação dos AMPs

Do arsenal de respostas humorais de insetos, os AMPs são as moléculas efetoras mais importantes e bem estudados, que atuam sobre distintos microrganismos como bactérias, fungos, vírus e protozoários (Bulet & Stöcklin 2005; Boulanger et al., 2006; McGwire & Kulkarni, 2010). São pequenos peptídeos de baixo peso molecular, com até 24 KDa, comumente compostos por menos de 100 resíduos de aminoácidos. Outra importante propriedade dos AMPs de insetos é o seu caráter anfipático e catiônico, devido a sua parte apolar (hidrofóbica) e outra onde predominam resíduos de lisina e/ou arginina (hidrofílica) (Bulet & Stöcklin, 2005; Mookherjee & Hancock, 2007; Wang & Lai, 2010).

A característica anfipática dos AMPs permite que estes interajam com a bicamada lipídica, os tornando capazes de permear e atuar na membrana plasmática (Zasloff, 2002; Brogden, 2005). É importante também destacar que a natureza catiônica dos AMPs define sua capacidade de interagir mais seletivamente com células bacterianas e não com as células eucarióticas do hospedeiro. As membranas de células procarióticas possuem grandes quantidades de fosfolipídios de carga negativa, como o fosfatidilglicerol, enquanto as membranas de células eucarióticas são compostas por fosfolipídeos zwitteriônicos, neutros, como a fosfatidilcolina (Matsuzaki, 1999; Zasloff, 2002). Dessa forma, os AMPs catiônicos tendem a interagir muito mais fortemente com as membranas celulares procarióticas, de carga negativa,

do que com as células eucarióticas de carga neutra (Shai, 2002; Zasloff, 2002), como observado na figura 6.

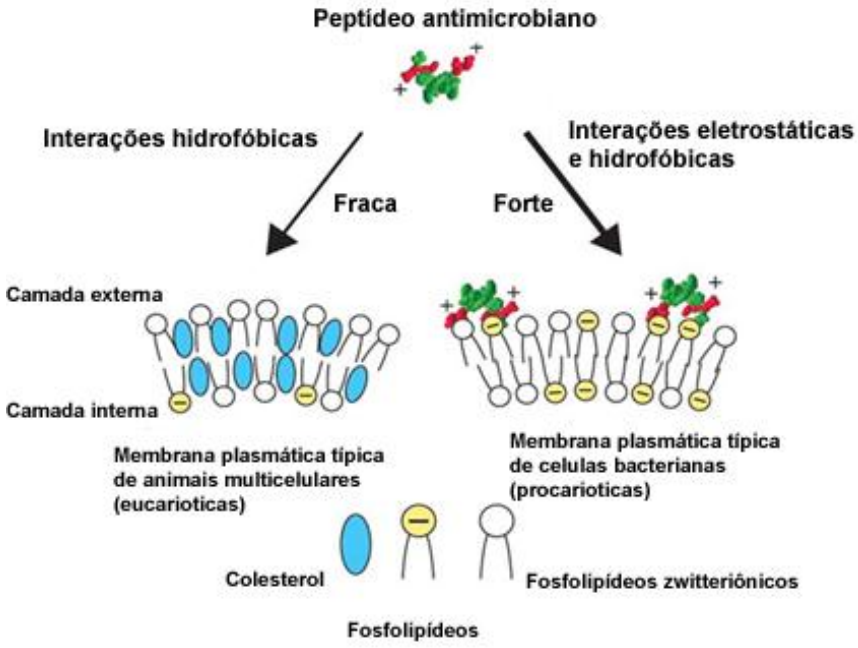

Figura 6 - Interação de peptídeos antimicrobianos com membranas de células procarióticas e eucarióticas. Retirado de Zasloff, 2002.

Diversos mecanismos de ação já foram descritos nas mais diversas famílias de AMPs. De uma forma geral, os AMPs catiônicos se ligam a seus alvos através de interações eletrostáticas entre sua carga positiva e os fosfolipídios aniônicos presentes nas membranas microbianas, bem como lipopolissacarídeos (LPS) presentes na parede celular de bactérias Gram-negativas (Epand & Vogel, 1999). Após a interação, as membranas microbianas podem ser permeabilizadas por três diferentes processos: 1 - pela formação de pequenos poros, de aproximadamente 1nm de diâmetro, chamados de poro tipo “barril”, que permite o vazamento indiscriminado de íons da célula; 2 - pela agregação de AMPs na conformação de um “tapete” sobre a membrana celular, que a penetram de forma perpendicular, e se complexam com os fosfolipídios. Esta alteração acarreta consequentemente o dobramento destes, culminando com a formação dos poros tipo “toroidais”, que facilitam o vazamento de íons e a passagem de macromoléculas; 3 - através da agregação de AMPs, formando um extenso carpete sobre a membrana, orientados de forma paralela a superfície da bicamada lipídica, resultando assim, numa completa

desestruturação da membrana (Oren & Shai, 1998; Brogden, 2005). Esses três modelos estão esquematizados na figura 7.

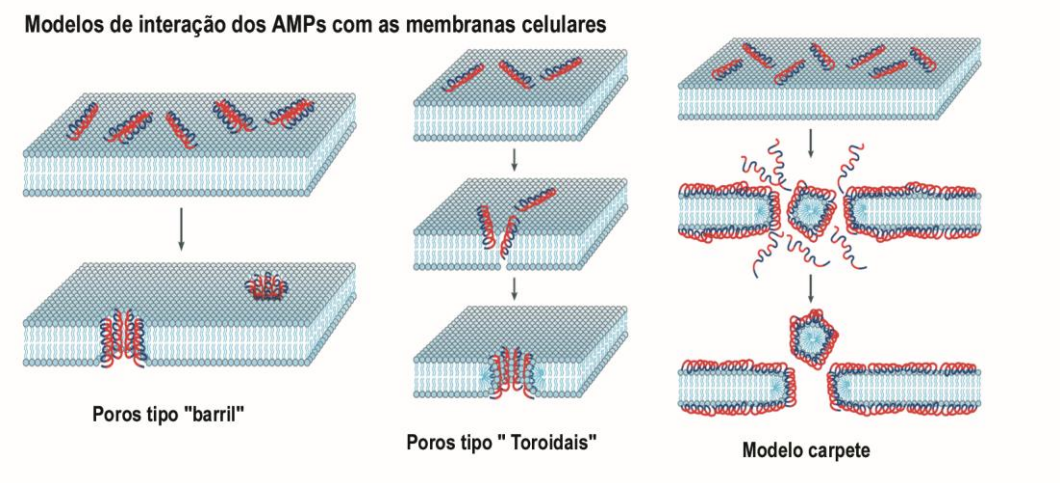

Figura 7 - Mecanismos de ação dos peptídeos antimicrobianos. Adaptado de Brogden, 2005.

Esses três modelos descritos acima se referem à AMPs formadores de poros, que matam o microrganismo através da despolarização da membrana citoplasmática e consequente lise total da célula. Deste modo, os AMPs formadores de poros são considerados líticos, com ação letal rápida, mesmo em baixas concentrações da proteína (Matsuzaki,1998; Dathe et al., 2001; Brogden, 2005). Apesar de ser um dos principais mecanismos de ação nos patógenos, os AMPs também podem atuar nos microrganismos de forma bacteriostática, sem a necessidade de desestruturação e lise total da membrana plasmática. Neste contexto, os AMPs atravessam a membrana do patógeno e se ligam a um alvo intracelular, desencadeando a interrupção de um processo metabólico, ou ainda a síntese de proteínas vitais a proliferação celular (Gallo & Huttner, 1998; Otvos, 2000; 2002).

1.4.3 – Reconhecimento e ativação do sistema imune

O inseto é capaz de reconhecer um patógeno específico a partir de um complexo sistema de transdução de sinais, que direcionam a produção de determinadas proteínas imunológicas ou a proliferação de células específicas, dependendo do microrganismo invasor (Lemaitre et al., 1997; Nurnberger et al., 2004). Ao ser desafiado por um microrganismo, células epiteliais e hemócitos do inseto secretam proteínas de reconhecimento padrão (“pattern recognition proteins - PRPs”) que poderão distinguir a classe do microrganismo invasor. O reconhecimento da

infecção pelas PRPs se dá através dos padrões moleculares associados a patógenos (“Pathogen-associated molecular pattern– PAMPs”), que são estruturas conservadas em grupos de microrganismos específicos, como peptidoglicanos presentes na parede celular de bactérias e β-1-3 glucanas presente na parede celular de fungos (Gottar et al., 2002; 2006; Leulier et al., 2003; Kaneko et al., 2004).

O atual conhecimento que se tem sobre receptores de reconhecimento padrão em insetos se deve aos inúmeros trabalhos publicados sobre imunidade de dípteros, especialmente da mosca *Drosophila melanogaster*. Em *Drosophila*, sabe-se que existem duas grandes famílias de proteínas receptoras que ativam vias de resposta imune: as proteínas ligantes de Gram-negativas (Gram-negative binding protein - GNPBs) e as proteínas reconhecedoras de peptidoglicanos (peptidoglycan recognition protein – PGRPs). Cada uma dessas famílias apresentam vários membros (Ferrandon et al, 2007; Muller et al., 2008), o que justifica como os insetos discriminam as mais variadas classes de microrganismos (Hoffmann, 2003; Leulier et al., 2003; Ferrandon et al., 2007).

Nos insetos, a síntese de AMPs é resultado da sinalização intracelular que pode ocorrer por duas diferentes vias, Toll e IMD (via da imunodeficiência), as quais poderão ser ativadas em decorrência do reconhecimento de PAMPs pelos receptores PRPs (De Gregorio et al., 2002; Gottar et al., 2002; Ferrandon et al., 2007; Muller et al., 2008). Adicionalmente, sabe-se que a partir da ativação dessas vias, fatores de transcrição NF-kB geralmente estão envolvidos na regulação da transcrição dos genes dos AMPs (Ferrandon et al., 2007; Imler, 2014).

Bactérias Gram-negativas como *Escherichia coli* e *S. marcescens* são caracterizadas por apresentarem uma camada de LPS na membrana externa de sua parede celular. Entretanto, essas moléculas não são as responsáveis por ativar nenhuma das duas principais vias de sinalização. Uma importante configuração de bactérias Gram-negativas é a presença de um resíduo de ácido mesodiaminopimélico (DAP) na camada de peptidoglicanos (PGNs), que se localiza logo abaixo da membrana externa rica em LPS. Já bactérias Gram-positivas, como *S. aureus* e *M. luteus*, não possuem a membrana externa composta por LPS, mas apenas uma grossa parede celular composta também por PGNs. Entretanto os PGNs de bactérias Gram-positivas apresentam resíduos de lisina (LYS) em sua composição. Os resíduos descritos acima caracterizam os PGNs em tipo DAP (proveniente de bactérias Gram-negativas) e tipo LYS (proveniente de bactérias Gram-positivas) (Ferrandon et al., 2007; Muller et al., 2008).

No modelo *Drosophila*, a discriminação entre os tipos de PGNs é feita pela família de receptores PGRPs e GNPBs. PGRP-LC e PGRP-LE, são receptores que se ligam a PGNs tipo DAP, reconhecem bactérias Gram-negativas e, consequentemente, ativam a via IMD (Gottar et al., 2002; Kaneko et al., 2006). Já os receptores PGRP-AS e PGRP-SD se ligam a PGNs tipo LYS proveniente de bactérias Gram-positivas, resultando no acionamento da via Toll (Michel et al., 2001; Bischoff et al., 2004). Algumas bactérias Gram-positivas e fungos também são reconhecidos por receptores GNPBs, que igualmente ativam a via Toll (Lee et al., 1996; Gottar et al., 2006).

Após a ativação extracelular dessas vias, uma cascata de reações intracelulares serão desencadeadas, culminando com a liberação de fatores nucleares de transcrição NF-kB, específicos para cada via, que então se translocam para o núcleo celular, regulando a transcrição de proteínas antimicrobianas como AMPs e lisozimas (Lemaitre & Hoffmann, 2007; Ferrandon et al., 2007; Imler, 2014).

A generalização de que bactérias Gram-positivas e fungos ativam a via Toll e que bactérias Gram-negativas ativam a via IMD, deve ser tratada com cautela, uma vez que já foi descrito que bacilos Gram-positivos ativam a via IMD (Leulier et al., 2003). Além disso, deve se considerar que os microrganismos possuem diversos padrões moleculares estruturais, que podem ativar concomitantemente as duas vias, reforçando a ideia de uma ativação cruzada de ambas as vias, como ocorre pela infecção por bacilos Gram-positivos (Lemaitre et al., 1997; Hoffmann & Reichhart 2002; Hoffmann, 2003; Leulier et al., 2003). Até o momento, pouco se sabe sobre a ativação dessas vias após infecção por protozoários como os tripanosomatídeos. Contudo, em modelos experimentais diferentes de triatomíneos, tais como na mosca tsé-tsé, é possível encontrar na literatura registros dos diferentes AMPs induzidos pela infecção por estes protozoários (Hao et al., 2001; Boulanger et al., 2002; 2004; Hu & Aksoy, 2006).

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o sistema de defesa humoral em 4º ou 5º estádio de ninfas de *Rhodnius prolixus* alimentadas com sangue não infectado ou contendo *Escherichia coli, Staphylococcus aureus, Trypanosoma rangeli ou Trypanosoma cruzi*.

2.2 Objetivos específicos

A - Detecção e quantificação da atividade antibacteriana em diferentes compartimentos do tubo digestivo do inseto e em diferentes dias após a alimentação sanguínea.

B – avaliação do perfil da expressão de genes da resposta imune humoral, especialmente dos peptídeos antimicrobianos (defensinas e prolixicina) e de lisozimas, em diferentes tecidos do inseto e em diferentes dias após alimentação sanguínea.

C – avaliação das atividades antibacterianas no tubo digestivo de insetos alimentados com sangue contendo os parasitas e bactérias.

D – avaliação do perfil de expressão dos diferentes genes relacionados à resposta imune humoral no intestino médio anterior (estômago) e posterior (intestino) de insetos alimentados com sangue contendo bactérias e *T. rangeli*, bem como no corpo gorduroso e epitélios intestinais de insetos infectados com diferentes cepas de *T. cruzi*.

E – avaliação da ativação do sistema profenoloxidase após alimentação com tripanosomatídeos.

F – Influência da infecção por tripanosomatídeos sobre a microbiota bacteriana nos diferentes compartimentos do tubo digestivo do inseto.

3 RESULTADOS

Artigos publicados:

3.1 Artigo 1: Humoral responses in *Rhodnius prolixus*: bacterial feeding induces differential patterns of antibacterial activity and enhances mRNA levels of antimicrobial peptides in the midgut. Vieira CS, Waniek PJ, Mattos DP, Castro DP, Mello CB, Ratcliffe NA, Garcia ES, Azambuja P *Parasites & Vectors* 2014, (7): 232.

3.2 Artigo 2: *Rhodnius prolixus* interaction with *Trypanosoma rangeli*: modulation of the immune system and microbiota population. Vieira CS, Mattos DP, Waniek PJ, Santangelo JM, Figueiredo MB, Gumiel M, Da Mota FF, Castro DP, Garcia ES, Azambuja P *Parasites & Vectors* 2015, (**8)**: 135.

Manuscrito em preparação:

3.3 Artigo 3: Antimicrobial peptides gene expression in *Rhodnius prolixus* infected with different *Trypanosoma cruzi* strains: impact on parasite survival and the gut bacterial microbiota community. Vieira CS, Waniek PJ, Mattos DP, Figueiredo MB, Castro DP, Garcia ES, Azambuja P. A ser submetido à revista *Plos Neglected Tropical Diseases*.

3.1 Artigo 1: Humoral responses in *Rhodnius prolixus*: bacterial feeding induces differential patterns of antibacterial activity and enhances mRNA levels of antimicrobial peptides in the midgut. **Vieira CS**, Waniek PJ, Mattos DP, Castro DP, Mello CB, Ratcliffe NA, Garcia ES, Azambuja P *Parasites & Vectors* 2014, (7): 232.

Parasites & Vectors

RESEARCH Open Access

Humoral responses in *Rhodnius prolixus*: bacterial feeding induces differential patterns of antibacterial activity and enhances mRNA levels of antimicrobial peptides in the midgut

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Abstract

Background: The triatomine, *Rhodnius prolixus,* is a major vector of *Trypanosoma cruzi*, the causative agent of Chagas disease in Latin America. It has a strictly blood-sucking habit in all life stages, ingesting large amounts of blood from vertebrate hosts from which it can acquire pathogenic microorganisms. In this context, the production of antimicrobial peptides (AMPs) in the midgut of the insect is vital to control possible infection, and to maintain the microbiota already present in the digestive tract.

Methods: In the present work, we studied the antimicrobial activity of the *Rhodnius prolixus* midgut *in vitro* against the Gram-negative and Gram-positive bacteria *Escherichia coli* and *Staphylococcus aureus*, respectively. We also analysed the abundance of mRNAs encoding for defensins, prolixicin and lysozymes in the midgut of insects orally infected by these bacteria at 1 and 7 days after feeding.

Results: Our results showed that the anterior midgut contents contain a higher inducible antibacterial activity than those of the posterior midgut. We observed that the main AMP encoding mRNAs in the anterior midgut, 7 days after a blood meal, were for lysozyme A, B, defensin C and prolixicin while in the posterior midgut lysozyme B and prolixicin transcripts predominated.

Conclusion: Our findings suggest that *R. prolixus* modulates AMP gene expression upon ingestion of bacteria with patterns that are distinct and dependent upon the species of bacteria responsible for infection.

Keywords: *Rhodnius prolixus*, Antimicrobial peptides, Bacteria, mRNA modulation

Background

Although insect immunity has been studied since the first half of the 20th century [1-3], the mechanisms involved have yet to be fully elucidated. The immune system in insects, unlike vertebrates, lacks the classical response to pathogens mediated by memory cells and immunoglobulin, but relies solely on an extremely efficient innate immune response [4]. This efficiency is probably one reason insects are the most abundant animal group, well adapted to many ecotopes [5]. Insect immunity includes the synchronized activation of cellular and humoral factors, such as the formation of microaggregates, phagocytosis and encapsulation by haemocytes, as well as the formation of reactive intermediates of oxygen and nitrogen, the prophenoloxidase system and antimicrobial peptides (AMPs) [6,7].

One of the major components of insect immunity is the synthesis of AMPs. Insect AMPs are usually cationic, amphipathic, often composed of 12–50 amino acid residues and have a broad activity spectrum [8]. The gene expression of AMPs occurs principally in the fat body, haemocytes and digestive tract epithelia, and the peptides

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are secreted into the haemolymph or midgut lumen [9,10]. AMP production is triggered by activation of different immune signalling pathways including Toll, Imd and Jak/STAT after recognition of non-self molecules known as the pathogen associated molecular patterns (PAMPs) [5,11].

Relatively few studies focus on the importance of the immune system in the midgut of insects, which is one of the most vulnerable tissues since it is always in contact with a variety of microorganisms [12]. Haematophagous insects, such as *Rhodnius prolixus,* ingest large amounts of blood from vertebrate hosts, often containing pathogenic microorganisms. The production of AMPs in the insect gut is therefore vital to protect against infection and to maintain homeostasis of the intestinal microbiota. The mutualistic microbiota of insects not only supplies essential nutrients but also aids digestion and the control of pathogenic microorganisms by modulating the immune responses [13,14]. Moreover, several studies have shown the importance of the microbiota in regulating insect genes involved in maintaining homeostasis of the gut [15-21].

R. prolixus is an important triatomine vector of *Trypanosoma cruzi*, the etiologic agent of Chagas disease in Latin America [22-24]. In the insect vector, *T. cruzi* remains exclusively inside the *R. prolixus* gut where, in order to survive, the parasite counteracts various host defence factors, including the AMPs [12]. Evidence indicates that in some insect vectors AMPs may be able to control parasite development [25-30]. Therefore, the study of AMPs present in the digestive tract of insects may have potential to provide new targets for control strategies.

Antimicrobial peptides are encountered in numerous organisms and are diverse even among closely related species [8]. In *R. prolixus,* six different AMPs have been identified, namely, defensin A, B and C, prolixicin and lysozymes A and B [31]. Each AMP has potential activity against a range of microorganisms. Lysozymes possess high activity against Gram-positive bacteria, by hydrolysing the 1,4-β-linkage between N-acetylmuramic acid and N-acetylglucosamine of the cell wall peptidoglycans [32,33]. Defensins are cysteine-rich peptides and are also known for their action against Gram-positive bacteria [27,34-36]. In contrast, prolixicin has high activity against Gram-negative *Escherichia coli* [37].

Despite the presence of these different AMPs in *R. prolixus*, the relative dynamics of their induction upon exposure to different species of bacteria is poorly understood. Thus, in the present study, using fifth instar nymphs of *R. prolixus*, the antimicrobial activities of the midgut *in vitro* against *Staphylococcus aureus* and *E. coli* have been investigated. We also analysed the relative abundance of mRNAs encoding AMPs in the midgut of insects fed with either *S. aureus* or *E. coli* at different days after an infected blood meal to test the hypothesis that each type of bacterium triggers a distinct immune response.

Methods

Ethics statement

For all experiments, *R. prolixus* were maintained in controlled environmental conditions and fed with defibrinated rabbit blood provided by the Laboratory Animals Creation Centre (Cecal). For feeding insects, an artificial apparatus was used, similar to that described previously [38] according to the Ethical Principles in Animal Experimentation approved by the Ethics Committee in Animal Experimentation (CEUA/FIOCRUZ, under the protocol number L-0061/08). The protocol was developed by CONCEA/MCT (http://www.cobea.org.br/), which is associated with the American Association for Animal Science (AAAS), the Federation of European Laboratory Animal Science Associations (FELASA), the International Council for Animal Science (ICLAS) and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Bacteria

S. aureus 9518 and *E. coli* K12 4401 were purchased from the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK. Bacteria were maintained frozen at −70°C in tryptone agar and 10% glycerol. For all experimental procedures, bacteria were grown with shaking (90 revolutions per minute) in 20 ml of tryptone soy broth (TSB) for 17 h at 30°C, and then 10 ml of fresh TSB were inoculated with 100 μl of the respective bacterial culture and incubated for a further 4 h under the same conditions. The bacteria were then washed in phosphate buffered saline - PBS (0.01 M phosphate buffer, 2.7 mM potassium chloride and 0.137 M sodium chloride, pH 7.4) and diluted in TSB to a final concentration of 1×10^4 cells/ml.

Insect treatment

Fifth-instar *R. prolixus* nymphs were obtained from a colony reared and maintained in Laboratório de Bioquímica e Fisiologia de Insetos IOC/FIOCRUZ at a relative humidity of 50–60% and at 27°C. The insects were randomly chosen and then fed with defibrinated rabbit blood through a membrane feeding apparatus [38]. Three groups of insects were fed as follows: blood only (control), blood containing *E. coli* or blood containing *S. aureus*. The final concentration of bacteria in the blood was 10^4/ml.

To compare the effects of whole normal plasma on the insect's antibacterial activity, insects were fed with blood after heat-inactivation of the plasma. The blood

was centrifuged at 1.890 × g for 10 min at 4°C, and the supernatant (plasma) was collected and incubated for 30 min at 55°C. After inactivation, the plasma was added back to the erythrocytes and fed to the insects. In the same experiment, a group of insects was fed with normal plasma in the blood (control).

Midgut sample preparations and antibacterial assays

For midgut sample preparations, starved or full engorged fifth-instar nymphs of *R. prolixus* were used at different days after feeding (DAF). The cuticle of the insects was cut laterally, to remove and separate the enlarged anterior midgut (stomach) from the narrow posterior midgut (intestine). The anterior midgut was separated into contents and wall for the antibacterial assays. Additionally, the antibacterial activity of the intestine was tested. All midgut preparations were collected in 1.5 ml reaction tubes always using pools of 3 insect midgut compartments diluted in 200 μl Milli-Q water, homogenized, centrifuged at 10,000 × g for 10 min at 4°C and finally sterilized by Millipore PVDF membrane filtration. Afterwards, the pools of 3 anterior midgut contents were diluted ten times in sterile water and stored at −20°C until use.

Antibacterial activity was assessed by turbidometric assays (TB) previously adapted by Castro *et al.,* 2012 [39,40]. For midgut TB assays, *S. aureus* or *E. coli,* grown as described above, were washed in PBS and diluted in TSB to a final concentration of 10^4 cells/ml. Subsequently, 10 μl of *E. coli* or *S. aureus* bacterial suspensions were incubated in each well of a sterile flat bottom 96-well microtiter plate (Nunc, Fisher Scientific, Leicestershire, UK) with 45 μl of sample (anterior midgut content, anterior midgut wall or posterior midgut) plus 5 μl of peptone 10%, to a final concentration of 1% peptone, at 37°C for 19 h. The optical densities were measured at 550 nm (OD_{550}) at hourly intervals using a Spectra Max 190 Plate Reader (Molecular Devices, Sunnyvale, California, USA). Control wells, run without midgut samples, contained 10 μl of bacteria in 1% peptone in Milli-Q water. The antibiotic ampicillin (80 μg/ml) was included in each experiment as a positive control.

All data points were subsequently blanked against time zero to account for the opacity of the midgut samples. The midgut samples were also incubated in the plate without bacteria to observe the change in sample colour after 19 h and the readings obtained were subtracted from the samples incubated with bacteria to ensure that the difference in readings were related to antibacterial activity. Then, the readings for the bacteria, *E. coli* or *S. aureus,* were subtracted from all sample readings to obtain the antibacterial activity value. All experiments were carried out in triplicate (9 pools of 3 insects, n = 27 insects). In addition, to find out how the sample dilutions affect antibacterial activity, different concentrations of the anterior midgut contents were tested against both bacteria. The anterior midgut contents of control insects at 7 DAF without dilution gave absorbance readings above the range of the standard curve and therefore in all TB assays samples were diluted 10 times which corresponded to 14.7 μg protein/μl of protein. The posterior midgut samples of control insects at 7 DAF used for TB assays contained 0.8 μg protein/μl of sample. All protein testing of midgut samples used a protein assay kit (BCA* Protein Assay Reagent, Pierce, USA) with bovine serum albumin (BSA) standards. Additionally, the differences in protein concentrations of each midgut preparation analysed in these assays were considered and are discussed below.

Concurrent with the TB assays, the anterior and posterior midgut samples (45 μl) were also incubated with 10 μl of *E. coli* or *S. aureus* (1×10^4 cells/ml) and 5 μl of peptone 10% at 37°C. At different times during incubation, samples were plated onto BHI-agar to compare the bacterial growth, by counting colony forming units (CFU), with the readings in the TB assays. Ampicillin (80 μg/ml) was incubated with both bacteria and plated on BHI-agar as a positive control of bacterial growth inhibition. The culture medium (TSB) used in the sample dilutions was also plated out as a control.

The thermal stability of the anterior midgut contents was analysed by heating the samples at 100°C for 60 min. The susceptibility of the anterior midgut contents to protease digestion was tested by pre-incubation with bovine pancreas trypsin (Sigma-Aldrich) at a final concentration of 2500 Uml^{-1} for 24 h at 37°C [40]. Samples were then centrifuged at 10,000 × g for 5 min and the supernatants assayed for antibacterial activity. Tests showed that trypsin had no adverse effects on bacterial growth and for this reason was not inhibited in the sample prior to TB assay.

Analysis of AMPs mRNA abundance by reverse transcription (RT) PCR

Steady state levels of mRNA encoding peptides involved in the innate immunity of *R. prolixus* were tested by reverse transcription (RT) PCR. Before dissection, insects were immersed in water at 55°C for 15 sec to release haemocytes from tissues [41]. From fifth instar nymphs (n = 10), unfed (15 days after ecdysis), 1 and 7 DAF (infective and non-infective), the anterior and posterior midgut walls were dissected and stored at −70°C. Total RNA was extracted using a NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and subsequently measured by a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Synthesis of cDNA was carried out with a First-Strand cDNA Synthesis Kit (GE Healthcare,

Buckinghamshire, UK) following the manufacturer's protocol using either 1.25 or 2.5 μg of total RNA. *R. prolixus* primers were designed from previously published defensin A, B and C, lysozyme A and B, prolixicin and β-actin (internal control, GenBank accession number ACPB02032143) encoding cDNA sequences as listed in Table 1 [31,37,42-44]. All defensins and the prolixicin encoding genes possess an intron and could therefore also be used as an internal control for contamination with genomic DNA.

PCRs were performed using Illustra Taq DNA Polymerase (GE Healthcare, Buckinghamshire, UK) at the following conditions: initial denaturation at 94°C for 5 min; cycling step at 94°C for 25 sec, 54°C for 25 sec, 72°C for 30 sec and a final elongation step at 72°C of 7 min. The amplification of prolixicin was conducted at an annealing temperature of 48°C. The number of cycles (25 and 30) was experimentally optimized with the gene encoding actin to eliminate signal saturation [45]. For verification of primer specificity, amplicons of all genes were excised from agarose gels, purified and sequenced in both directions by Plataforma Genômica – Sequenciamento de DNA/PDTIS-FIOCRUZ, Rio de Janeiro, Brazil. PCRs were carried out three times under the same conditions using technical replicates. As negative controls, PCR reactions were carried out without a template. All nucleic acid experiments were performed on a Veriti 96-Well Fast Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). Amplification products (5 μl) were separated on an ethidium bromide stained 2% agarose gel and documented with a Gel Doc™ XR + System (Bio-Rad, Hercules, CA, USA). Band intensity was measured with the ImageJ program (version 1.47q). Means and standard deviations of the different samples were calculated.

Statistical analyses

The results were analysed with GraphPad Prism 5 using two way ANOVA or one way ANOVA or unpaired T tests, depending on the data distribution and number of treatments. Data are reported as mean ± standard deviation (SD). Differences among groups were considered not statistically significant when $p > 0.05$. Probability levels are specified in the text and Figure legends.

Results

Midgut antimicrobial activity

In the present study, the antimicrobial activity of *R. prolixus* midgut was assessed against two bacterial species, *E. coli* and *S. aureus*. To determine in which midgut compartment the antibacterial activity are present, we tested separately the anterior midgut wall and contents as well as total posterior midgut using the TB assay (Figure 1). Results showed that the anterior midgut contents had a significantly higher activity than the anterior midgut wall and posterior midgut against both bacterial species (Figure 1; $p < 0.001$). A comparison between the anterior midgut contents and posterior midgut was also made using BHI agar plates incubating the samples with *E. coli* and *S. aureus*. In the anterior midgut contents, no bacteria grew after 19 h incubation in contrast to the rapid growth of the bacteria alone controls (Additional file 1; $p < 0.001$). In contrast, incubation with the posterior midgut samples resulted in numerous bacteria colony forming units (CFU) after 19 h (Additional file 1; $p < 0.001$). These results confirm those from the TB assay above.

Analysis was also undertaken to determine if any antibacterial activity recorded was related to the complement system of the rabbit blood. Comparison of the antibacterial activity of anterior and posterior midgut samples from insects fed on blood containing whole

Table 1 List of primers used in the present study

Gene/name	Sequence 5'-3'	Tm (°C)	Amplicon length
RPDEFAF	GAATACTCCACTCAACCGCAAC	62.7	
RPDEFAR	TAGTTCCTTTACATCGGCCA	58.4	295 bp
RPDEFBF	CAGTACCTAGGATATTCCACTCAAC	62.9	
RPDEFBR	TAGTTCCTTTACAATGGCCG	58.4	304 bp
RPDEFCF	CAGTACAGTCCTAATACCTAGCC	62.8	
RPDEFCR	CAGTTCCTACGCAACGGCCT	64.5	300 bp
RPLYS1F	TTCTTACTGGCTATTTTCGCC	58.7	
RPLYS1R	CGACCTCTGCAATGGTACTG	62.4	377 bp
RPLYS2F	CTAGTTTTAACACTATTGCTGCTG	59.4	
RPLYS2R	GCCCTTACATTTCTTGATCC	58.4	378 bp
RPPROLF	CTATAACGAGTGAACTATAAGACAA	50.0	
RPPROLR	GTGTTTAATGGCGGTAACAAATTAC	53.2	406 bp
RPACTF	CACGAGGCTGTATACAATTCCA	60.8	
RPACTR	GTAGCTGTTTAGAAGCATTTGCG	61.0	314 bp

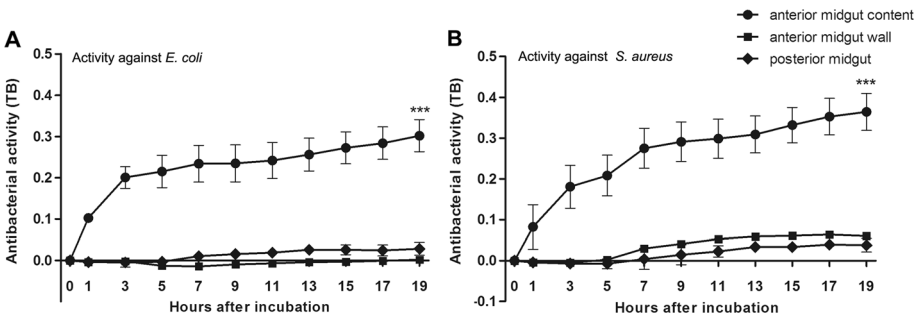

Figure 1 Antibacterial activity of the anterior and posterior midgut of *Rhodnius prolixus* 7 days after feeding. A – Activity of anterior (contents and wall) and posterior midgut samples against *E. coli*. **B** – Activity of anterior (contents and wall) and posterior midgut samples against *S. aureus*. Antibacterial activity measured by turbidometric assay (TB) (OD_{550} nm) with readings from 0 to 19 hour in plate assay. Treatments: ● bacteria incubated with anterior midgut contents; ■ bacteria incubated with anterior midgut wall; ♦ bacteria incubated with posterior midgut. Values represent the means ± SD of 9 pools using 3 insects each (n = 27) in triplicate wells. Asterisks relates to significant differences (***$p < 0.001$) obtained by a two way ANOVA.

native plasma with those fed on heat- inactivated plasma revealed no differences in activity against *E. coli* or *S. aureus* (Additional file 2).

In order to analyse the dynamics of antibacterial activity in *R. prolixus*, the anterior midgut contents were tested against *E. coli* or *S. aureus* at different days after feeding (DAF). The results showed that at 7 DAF, the activity against *E. coli* was significantly higher than 5 DAF ($p < 0.05$), as well as 1, 9 and 12 DAF ($p < 0.01$) (Figure 2A). The activity of the anterior midgut contents against *S. aureus* was also highest at 7 DAF which was significantly higher ($p < 0.05$) than all the other DAF (Figure 2B).

The antibacterial activity of the anterior midgut contents was also tested for thermal stability and susceptibility to trypsin digestion. All antibacterial activities against *E. coli* and *S. aureus* were significantly reduced after trypsin and boiling treatments compared with the untreated controls (Additional file 3A; $p < 0.01$ and $p < 0.05$, respectively). The activities against *S. aureus* were also significantly reduced with these treatments (Additional file 3B; $p < 0.001$).

Transcription of AMPs in insects

In order to categorize antibacterial activity in the digestive tract of *R. prolixus*, the gene expression profiles of AMPs in the anterior midgut and posterior midgut walls of unfed insects and insects 1 or 7DAF were studied. The relative abundance of transcripts for lysozyme A (*LysA*), lysozyme B (*LysB*), prolixicin (*Prol*), defensins A (*DefA*), B (*DefB*) and C (*DefC*) was quantified (Figure 3). In general, the AMP transcript abundance was highest at 7 DAF in both tissues, but the expression pattern over time and tissue was not the same for all AMPs analysed (Figure 3A and 3B). At 1 DAF, the abundance of transcripts of *LysB* increased approximately 15 fold in the anterior and posterior midguts, while *Prol* transcripts increased 5 fold in the posterior midgut, in comparison to unfed insects. Interesting, *DefC* abundance was significantly higher in anterior midgut samples of unfed insects ($p < 0.001$), and decreased at 1 and 7 DAF (Figure 3A). Comparing the transcripts between tissues 7 DAF, the anterior midgut showed a

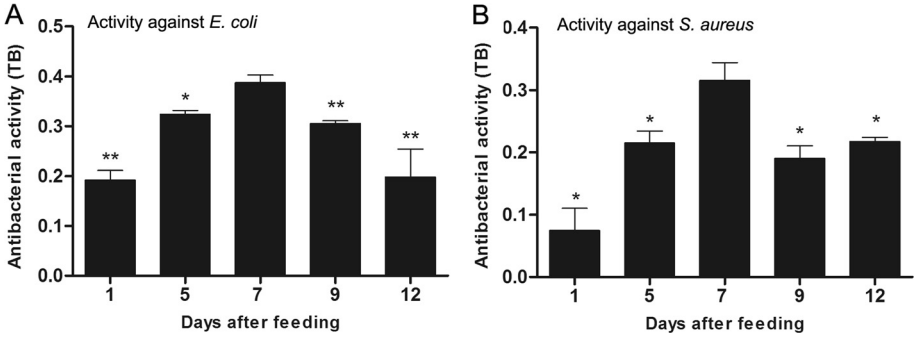

Figure 2 Antibacterial activity of the anterior midgut contents of *Rhodnius prolixus* on different days after feeding. A- Activity of anterior midgut contents against *E. coli*. **B**- Activity of anterior midgut contents against *S. aureus*. Antibacterial activity detected by turbidometric assay (TB) (OD_{550} nm) after 19 h incubation of anterior midgut content samples with different bacteria. Values represent the means ± SD of 9 pools using 3 insects each (n = 27) in triplicate wells. Asterisks relate to significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) obtained after data were compared to day seven using one way ANOVA and Mann Whitney tests.

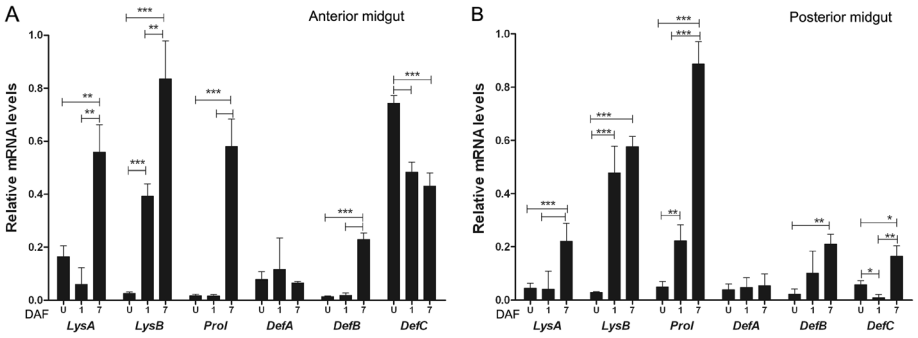

Figure 3 Relative transcript abundance of antimicrobial peptides and lysozymes encoding mRNA in *Rhodnius prolixus* midgut wall. Anterior and posterior midgut samples collected before feeding (unfed), 1 and 7 days after a blood meal. **A**- Relative mRNA levels in anterior midgut. **B**- Relative mRNA levels in posterior midgut. DAF – days after feeding. U – unfed insects. Error bars represent SD of three independent experiments. Asterisks relates to significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) obtained after data analyses using one way ANOVA and unpaired t tests.

significantly higher abundance of *LysA*, *LysB* and *DefC* than the posterior midgut (Figure 3A). Additionally, only the abundance of *Prol* transcripts was significantly higher in posterior midgut than anterior midgut (Figure 3B).

Antibacterial activity and transcription of AMPs in bacteria fed insects

R. prolixus were infected separately with Gram-positive and Gram-negative bacteria to test whether different bacteria trigger a distinct immune response, altering the

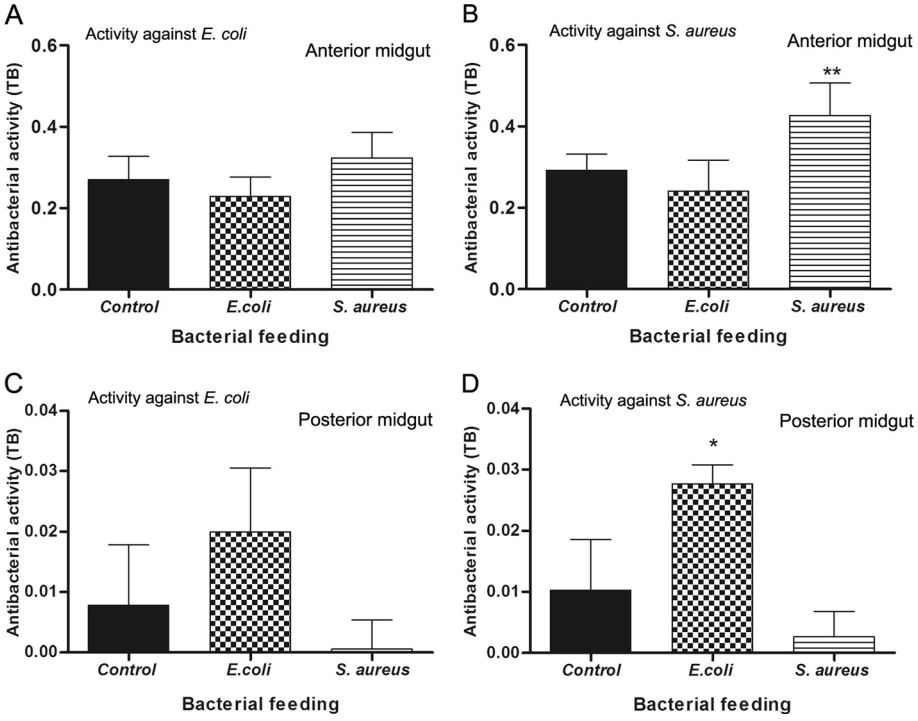

Figure 4 Antibacterial activity in *Rhodnius prolixus* midgut fed with blood containing *E. coli* or *S. aureus*. Anterior midgut contents and posterior midgut collected 7 days after feeding were tested against *E. coli* and *S. aureus*. **A-** Antibacterial activity of anterior midgut contents after feeding with *E. coli*, *S. aureus* or blood alone against *E. coli*. **B**- Antibacterial activity of anterior midgut contents after feeding with *E. coli*, *S. aureus* or blood alone against *S. aureus*. **C-** Antibacterial activity of posterior midgut after feeding with *E. coli*, *S. aureus* or blood alone against *E. coli*. **D**- Antibacterial activity of posterior midgut after feeding with *E. coli*, *S. aureus* or blood alone against *S. aureus*. Black column - antibacterial activity of control insects fed on blood alone; grid column - antibacterial activity of insects fed with blood containing *E. coli*; striped column - antibacterial activity of insects fed with blood containing *S. aureus*. Antibacterial activity measured by turbidometric assay (TB) (OD_{550} nm) after 19 h incubation of midgut samples with different bacteria. Values represent the means ± SE of three replicates. Asterisks relates to significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) in comparison to control obtained after data analyses using one way ANOVA and Mann Whitney tests.

antibacterial activity and the gene expression of AMPs. The antibacterial activities recorded were compared to control insects fed on blood without bacteria. Feeding the insects with blood containing *E. coli* failed to significantly alter the immune response of the anterior midgut contents tested against either *E. coli* or *S. aureus* (Figure 4A and 4B). In contrast insects fed with *S. aureus* recorded significantly increased antibacterial activity of the anterior midgut contents against *S. aureus* (Figure 4B; $p < 0.01$) but not *E. coli*. As with the anterior midgut contents, the oral infection with either bacterium failed to significantly change the antibacterial activities of the posterior midgut samples against *E. coli* (Figure 4C), although an increase in posterior midgut antibacterial activity was only observed afterwards when the insects were infected with *E. coli* and then tested against *S. aureus* (Figure 4D; $p < 0.05$).

In the anterior midgut at 1 DAF, oral infection with either *E. coli* or *S. aureus* increased mRNA levels of some AMPs in comparison with the control insects fed

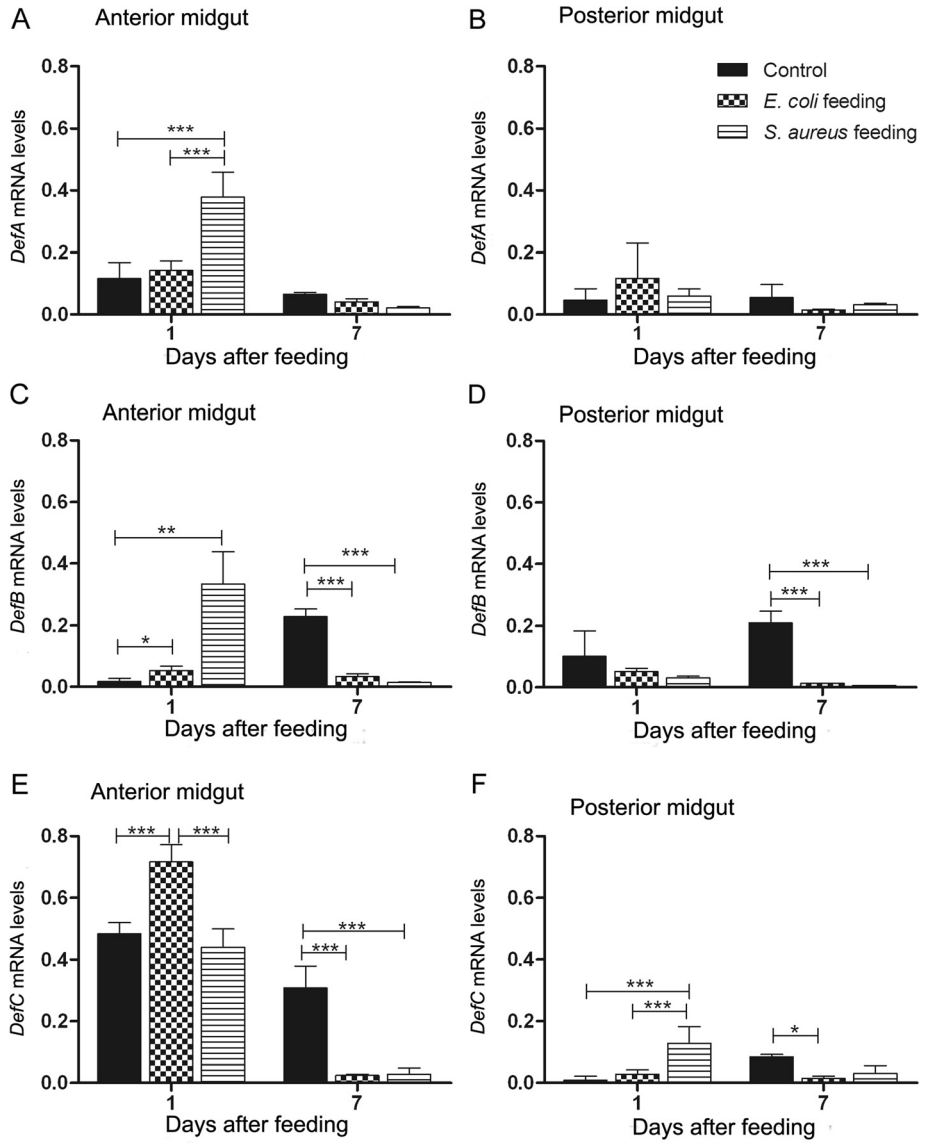

Figure 5 Relative transcript abundance of defensins encoding mRNA in *Rhodnius prolixus* after bacterial feeding. Anterior and posterior midgut samples collected 1 and 7 days after blood meal. **A, C, E**: anterior midgut relative mRNA levels. **B, D, F**: posterior midgut relative mRNA levels. **A**- *DefA* mRNA levels in anterior midgut. **B**- *DefA* mRNA levels in posterior midgut. **C**- *DefB* mRNA levels in anterior midgut. **D**- *DefB* mRNA levels in posterior midgut. **E**- *DefC* mRNA levels in anterior midgut. **F**- *DefC* mRNA levels in posterior midgut. Treatments: black column - insects fed with blood alone (control); grid column - insects fed with blood plus *E. coli*; striped column - insects fed with blood plus *S. aureus*. Error bars represent SD of three independent experiments. Asterisks relate to significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) obtained after data statistical analyses using one way ANOVA and unpaired t Test.

blood alone (Figure 5). In this tissue, *DefA* and *DefB* transcript abundance was upregulated by *S. aureus* infection (Figure 5A; $p < 0.001$ and 5C; $p < 0.01$) while *DefC* was upregulated by *E. coli* (Figure 5E; $p < 0.001$). In contrast, in the posterior midgut, at 1 or 7 DAF, bacterial feeding did not significantly increase the expression of *DefA* and *DefB* encoding genes (Figure 5B and 5D), although an increased expression of *DefC* 1 DAF occurred after *S. aureus* infection. (Figure 5F; $p < 0.001$). The transcript abundances of *DefA*, *DefB* and *DefC* were similar or even significantly lower in insects infected by either *E. coli* or S. *aureus*, in both the anterior and posterior midguts at 7 DAF when compared with control insects (Figure 5).

Concerning the *Prol* expression in both midgut tissues, only infection with *S. aureus* caused a significant increase in this AMP expression in the anterior midgut 1 DAF, when compared with control insects (Figure 6A; $p < 0.05$). In all other cases, *Prol* was significantly downregulated (Figure 6), especially at 7 DAF with bacteria (Figure 6A and 6B; $p < 0.001$).

Results with lysozyme at 1 DAF showed that *LysA* was significantly upregulated in the anterior midgut after *S. aureus* infection ($p < 0.01$) while *LysB* was significantly downregulated after *E. coli* infection (Figure 7A and 7C; $p < 0.01$). In contrast, in the posterior midgut 1 DAF with *E. coli* resulted in a significant increase in *LysA* transcript abundance compared to control (Figure 7B; $p < 0.05$). At 7 DAF, the abundance of *LysA* and *LysB* transcripts in insects infected with either bacterial species showed similar results to control insects in both tissues (Figure 7A and 7B) except for a significant decrease in *LysB,* abundance in anterior midgut tissues of *E. coli* and *S. aureus*-infected insects ($p < 0.01$) when compared with controls (Figure 7C).

Discussion

Antimicrobial peptides (AMPs) are an important part of the immune response in insects, particularly in the midgut lumen of vector species that transmit parasites during blood feeding. Furthermore, insect vectors possess gut microbiota composed of mutualistic and pathogenic bacteria [14] which are modulated by the AMPs to maintain the gut homeostasis [21]. In the present study, the results showed that oral infection with Gram-positive and Gram-negative bacteria differentially altered the antimicrobial activity and AMP expression patterns in the insect's midgut.

The AMPs detected in the gut of *R. prolixus* in the present study included transcripts for lysozyme A (*LysA*), lysozyme B (*LysB*), prolixicin (*Prol*), defensins A (*DefA*), B (*DefB*) and C (*DefC*), although probably more AMPs await discovery in *Rhodnius*. In a recent paper by Ribeiro *et al*. [46] eight defensin and five lysozyme encoding sequences were reported. From the eight reported defensin transcripts, four were identified as *DefC*, three as *DefA* and one as a truncated *def4* of *T. brasiliensis* and no *R. prolixus DefB* was identified. However, *T. brasiliensis Def4* and *R. prolixus DefA* are highly similar and are probably orthologs. Defensins are highly conserved and therefore incomplete sequences might match with the wrong sequences deposited in the GenBank. Our study analysed all full so far identified defensin genes including *DefB* which was not found by Ribeiro *et al.* [46]. In the case of lysozyme Ribeiro *et al.* [46] identified three of the five transcripts as lysozyme 1 (syn. of *R. prolixus* lysozyme A), which was included in our study. Our results also report the presence of prolixicin, another antibacterial peptide, which was not detected by Ribeiro *et al*. [46].

Both midgut compartments were analysed for antibacterial activity, since it has been shown that each midgut compartment has a highly specific environment and physiological function [47,48]. The anterior midgut of triatomines, which has a neutral-basic pH, is where the blood meal is stored and the majority of bacterial

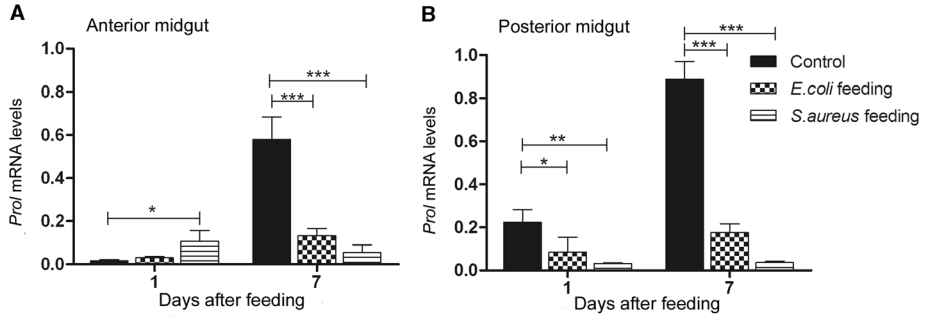

Figure 6 Relative transcript abundance of prolixicin encoding mRNA in *Rhodnius prolixus* midgut wall after bacterial feeding. Anterior and posterior midgut samples collected 1 and 7 days after feeding. **A**: anterior midgut relative mRNA levels. **B**: posterior midgut relative mRNA levels. Treatments: black column - insects fed with blood alone (control); grid column - insects fed with blood plus *E. coli*; striped column - insects fed with blood plus *S. aureus*. Error bars represent SD of three independent experiments. Asterisks relate to significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) obtained after data analyses using one way ANOVA and unpaired t tests.

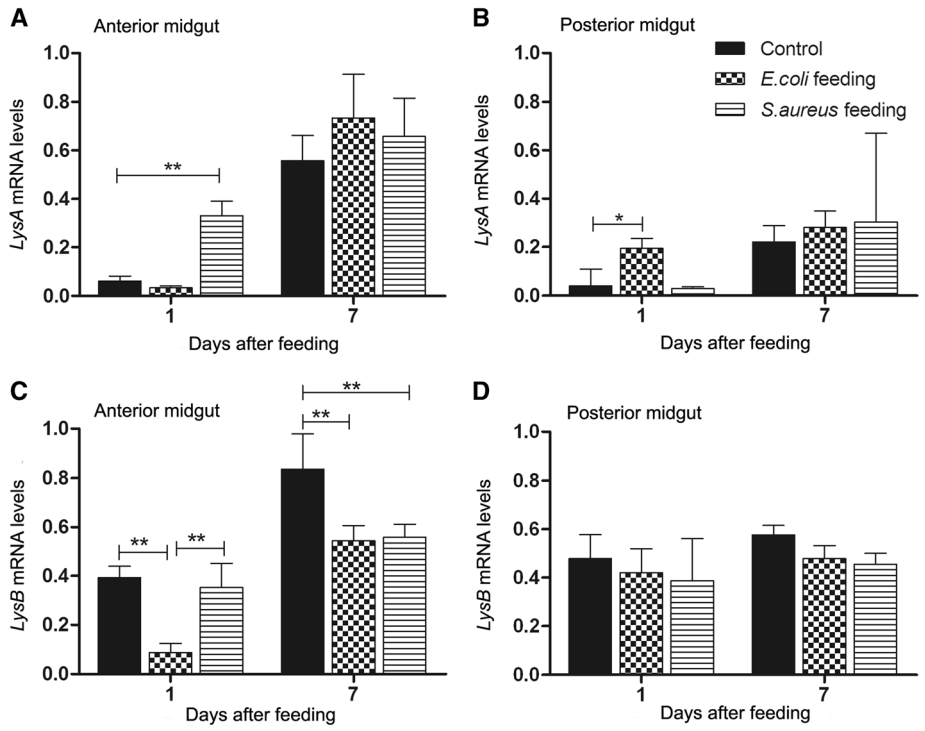

Figure 7 Relative transcript abundance of lysozymes encoding mRNA in *Rhodnius prolixus* midgut after bacterial feeding. Anterior and posterior midgut samples collected 1 and 7 days after blood meal. A, C: anterior midgut relative mRNA levels. B, D: posterior midgut relative mRNA levels. **A**- *LysA* mRNA levels in anterior midgut. **B**- *LysA* mRNA levels in posterior midgut. **C**- *LysB* mRNA levels in anterior midgut. **D**- *LysB* mRNA levels in posterior midgut. Treatments: black column - insects fed with blood; grid column - insects fed with blood plus *E. coli*; striped column - insects fed with blood plus *S. aureus*. Error bars represent SD of three independent experiments. Asterisks relate to significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) obtained after data statistical analyses using one way ANOVA and unpaired t Test.

symbionts reside. In contrast, the posterior midgut, with an acidic pH, is where protein digestion mainly occurs [45,49]. The ingested blood meal stored in the anterior midgut induces within days the transcription of AMPs and lysozymes. Concomitantly, we observed that the antibacterial activity *in vitro* was very high against both *E. coli* and *S. aureus*, reaching the highest level at 7 days after a blood meal, which may be explained by an increase of these peptides.

The results with *R. prolixus* fed with blood without microorganisms showed higher levels of mRNA encoding AMPs in the anterior than in the posterior midgut of *R. prolixus*. The anterior midgut contents also recorded a higher antibacterial activity than posterior midgut, in agreement with previous results [50]. However, results obtained by antibacterial assays on extracts from different gut regions, as in Figure 1, should be treated with caution since the anterior midgut contents are largely composed of the residual blood meal and therefore it is difficult to evaluate how much to dilute this sample to be "equivalent" to the anterior or posterior midgut walls. Thus, the protein concentration in the anterior midgut contents was 18 times higher than in the posterior midgut, and assuming that part of this protein can be related to the amount of AMPs present, then this may explain the stronger antibacterial activity detected in the anterior midgut tissues. The results showing that the most abundant AMP encoding mRNAs were present in the anterior midgut, namely, *LysA*, *LysB, DefC*, seem to confirm the elevated antimicrobial activity recorded. In addition, the inhibition of antibacterial activity observed in the anterior midgut content treated with trypsin or incubated at 100°C indicate that AMPs and lysozymes are the molecules involved [40]. Nevertheless, it is unlikely that all the antimicrobial activity recorded derived solely from these peptides since reactive oxygen (ROS) and nitrogen species (RNS) have been detected previously [21,41,50].

Regarding the results of insects fed with blood plus bacteria, this altered the pattern of antibacterial activity *in vitro* in the midgut. Feeding the insect with *S. aureus* increased the antibacterial activity against *S. aureus* in the anterior midgut and feeding the insect with *E. coli* enhanced the activity against *S. aureus* in the posterior midgut. These findings suggest that *R. prolixus* modulates antibacterial activity upon ingestion of bacteria with patterns that are distinct and dependent upon the species of bacteria present.

The results with mRNA expression showed that the *E. coli* infected insects 1 DAF expressed more *LysA* in the posterior midgut than naive insects, which may contribute to the increase in antibacterial activity against *S. aureus* observed in the posterior midgut at 7 DAF (compare Figure 4D with Figure 7B). In addition, *S. aureus* infection enhanced the anterior midgut activity against *S. aureus in vitro* and *S. aureus* infected insects showed a significantly higher *DefA*, *DefB* and *LysA* transcription abundance 1 DAF (compare Figure 4B with Figure 5A, C and Figure 7A). The significant increase in the abundance of these AMP mRNAs observed at 1 DAF may reflect an increase in antibacterial activity through to 7 DAF. This seems likely as the antibacterial activity recorded in the blood-fed controls continues to increase from 1, to 5 to 7 DAF. The enhanced *DefA*, *DefB* and *LysA* levels may explain the increase of antibacterial activity against *S. aureus* in the anterior midgut, since the respective peptides possess activity mainly against Gram-positive bacteria. Possibly other unknown *R. prolixus* antimicrobial peptides could also be responsible for the antibacterial activities observed.

In *R. prolixus,* lysozymes are involved in the digestion of polysaccharides of the symbiont *Rhodococcus rhodnii* [51]. These lysozymes may also play a role in the insect immune response [52,53]. *LysA* and *LysB* seem to have different roles in different compartments of the gut. *LysA* is expressed predominantly in the midgut and *LysB* in the fat body [31]. The rapid increase in *LysB* mRNA levels and, to a lesser extent *LysA,* in both tissues suggest a role in *R. prolixus* digestion, although a function in response to bacterial multiplication in the gut following a blood meal is also likely, as observed in other triatomines like *Triatoma infestans* and *Triatoma brasiliensis* [31,54,55]. Although phylogenetic analyses indicate that *R. prolixus LysA* groups with lysozymes that play a digestive role in other triatomine bugs [31,54,55], our results showed that *LysA* was strongly induced after *S. aureus* feeding and it was also possible to detect a slight increase of *LysA* after infection with *E. coli*, indicating also an immunological role for this lysozyme. Previous results with *Lutzomyia longipalpis* and *Galleria mellonella* have shown that there is a synergistic effect between lysozymes and other AMPs which enhances immune responses against both Gram-positive and Gram-negative bacteria [56,57]. In *R. prolixus*, synergistic effects between AMPs and lysozymes might occur as well.

Insect defensins have major activities against Gram-positive bacteria [58], but also can act against Gram-negative forms [59]. A previous study – based on structural properties – showed a high similarity between *R. prolixus DefA* and *DefB* while *DefC* differed significantly, forming two distinct groups after a phylogenetic analysis [60]. These authors suggested that the various defensins have different functions. In the present study, the analysis of transcript abundance also showed significant differences between the three *R. prolixus* defensins. In insects fed with *S. aureus* both *DefA* and *DefB* were significantly upregulated while *DefC* abundance increased only after *E. coli* infection. In unfed bugs and bugs fed solely on blood, *DefC* was the most abundant defensin transcript in the anterior midgut. The fact that in starved insects only *DefC* transcripts are abundant indicates a role of *DefC* in symbiont control whereas the upregulation of *DefA/B* after infections with unfamiliar microbes suggests a probable function of these gene products in the control of bacterial invasion. Regarding previous work, a common bacterial species found in *R. prolixus* gut was a Gram-negative bacterium, *S. marcescens* [61] and together with our findings about *DefC,* this reinforces the idea that this defensin may also play a role in the regulation of Gram-negative bacteria. As observed previously in *L. longipalpis*, high levels of defensin could be explained by microbiota control before adult emergence [62]. In *R. prolixus* fifth-instar nymphs moult to adults following a blood meal so that the high *DefC* levels could also be related to metamorphosis. Insect metamorphosis, however, is characterised not only by the need to control microbial expansion but also to mediate developmental processes and defensins have been shown to play dual roles both in immunity and development [63].

The blood meal also induces an increase of prolixicin encoding mRNA in the posterior midgut. Prolixicin, recently isolated from *R. prolixus* midgut and fat body, is a glycine-containing peptide, which is upregulated in fat body after bacterial or *Trypanosoma cruzi* haemocoel injection. The purified protein has a strong action against Gram-negative bacteria [37]. However, in the present work, prolixicin encoding gene was down-regulated in the midgut after feeding *R. prolixus* with blood containing *E. coli*. Further analyses will be necessary to clarify whether or not prolixicin is related to other microorganisms in the midgut, like e.g. different bacterial species, fungi or viruses.

In *R. prolixus*, the microbiota grows exponentially until eight days after blood feeding and thereafter decreases [50,64]. This might explain why a higher expression of peptides occurs on 7 DAF. Since AMPs may have a central role in the control of bacterial populations in the midgut. This would also explain the higher expression of the AMP encoding mRNAs and AMPs in the anterior midgut than in the posterior midgut since the anterior midgut, including the lumen, is the site of the bacterial bloom resulting from the blood meal. The anterior midgut may be a more suitable environment for lysozymes while prolixicin would control bacterial expansion in the posterior midgut.

The activation of immune responses in insects is regulated mainly by two intracellular pathways, the Toll and

the IMD pathways [65,66], that control the expression of most genes encoding the AMPs. Gram-positive bacterial infection activate the Toll pathway while Gram-negative bacteria infection induces the IMD pathway [67]. In the present work, different types of bacterial infection induced the expression of different types of AMPs in insect's midgut. Thus, AMP encoding genes induced by *S. aureus* (*DefA*, *DefB*, *Prol*) and *E. coli* (*DefC*) infections could be under different induction pathways.

Conclusion

Studies of the activation of immune responses in the gut become more relevant than those responses triggered by artificial inoculation in the body cavity of the insect, since these events occur less frequently in nature [67]. Insects and other animals live in a complex relationship with microorganisms [68] and the study of transcriptional control of AMPs can extend the understanding of how insects manage microbiota interactions and are still able to mount an efficient immune response against possible ingested pathogens.

Additional files

Additional file 1: Antibacterial activity of anterior midgut contents and posterior midgut of *Rhodnius prolixus* (7 DAF) tested against *Escherichia coli* and *Staphylococcus aureus*. The activity was measured as colony forming units (CFU/ml) after 19 hours of incubation. Values represent the means ± SD of 9 pools using 3 insects (n = 27) in triplicate wells.

Additional file 2: Antibacterial activity from *R. prolixus* anterior midgut fed on normal blood and washed erythrocytes with inactivated plasma. Antibacterial activity was measured by turbidometric assay (TB) (OD_{550} nm) with readings from hour 0 to hour 20 of incubation in plate assay. **A:** Activity against *E. coli*. **B** – Activity against *S. aureus*. Treatments: ■ bacteria incubated with content of anterior midgut from insects fed on blood; ● bacteria incubated with anterior midgut from insects fed on inactivated plasma (IP) blood; ♦ bacteria incubated with posterior midgut from insects fed on blood. ▲ bacteria incubated with posterior midgut from insects fed on erythrocytes with inactivated plasma (IP) blood. Values represent the means ± SD of 9 pools using 3 insects each (n = 27) in triplicate wells. Statistical analysis was carried out using two way ANOVA.

Additional file 3: Antibacterial activity of anterior midgut of *Rhodnius prolixus* at 7 days after blood meal. Antibacterial activity detected by turbidometric assay (TB) (OD_{550} nm) after 19 hours of incubation of anterior midgut samples with different bacteria. **A** – Antibacterial activity against *Escherichia coli*. **B** – Antibacterial activity against *Staphylococcus aureus*. Treatments: Black column - incubated with untreated anterior midgut; grid column - *bacteria* incubated with anterior midgut treated 24 hours with trypsin; striped column - *bacteria* incubated with anterior midgut heated at 100°C; Values represent the means ± SD of three replicates. Asterisks relates to significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) obtained after data statistical analyses in comparison to control using one way ANOVA and Mann Whitney test.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CSV, PJW, DPC, PA, NAR, ESG and CBM designed the study protocols and drafted the manuscript; CSV, DPM and DPC carried out the antibacterial activity experiments; CSV, PJW, DPM and DPC performed the molecular experiments. All authors analyzed the data, revised the article, approved the version to be published and are the guarantors of the paper.

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3.2 Artigo 2: *Rhodnius prolixus* interaction with *Trypanosoma rangeli*: modulation of the immune system and microbiota population. **Vieira CS**, Mattos DP, Waniek PJ, Santangelo JM, Figueiredo MB, Gumiel M, Da Mota FF, Castro DP, Garcia ES, Azambuja P *Parasites & Vectors* 2015, (8): 135.

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Parasites & Vectors

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Rhodnius prolixus interaction with *Trypanosoma rangeli*: modulation of the immune system and microbiota population

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Abstract

Background: *Trypanosoma rangeli* is a protozoan that infects a variety of mammalian hosts, including humans. Its main insect vector is *Rhodnius prolixus* and is found in several Latin American countries. The *R. prolixus* vector competence depends on the *T. rangeli* strain and the molecular interactions, as well as the insect's immune responses in the gut and haemocoel. This work focuses on the modulation of the humoral immune responses of the midgut of *R. prolixus* infected with *T. rangeli* Macias strain, considering the influence of the parasite on the intestinal microbiota.

Methods: The population density of *T. rangeli* Macias strain was analysed in different *R. prolixus* midgut compartments in long and short-term experiments. Cultivable and non-cultivable midgut bacteria were investigated by colony forming unit (CFU) assays and by 454 pyrosequencing of the 16S rRNA gene, respectively. The modulation of *R. prolixus* immune responses was studied by analysis of the antimicrobial activity *in vitro* against different bacteria using turbidimetric tests, the abundance of mRNAs encoding antimicrobial peptides (AMPs) defensin (*DefA*, *DefB*, *DefC*), prolixicin (*Prol*) and lysozymes (*LysA*, *LysB*) by RT-PCR and analysis of the phenoloxidase (PO) activity.

Results: Our results showed that *T. rangeli* successfully colonized *R. prolixus* midgut altering the microbiota population and the immune responses as follows: 1 - reduced cultivable midgut bacteria; 2 - decreased the number of sequences of the Enterococcaceae but increased those of the Burkholderiaceae family; the families Nocardiaceae, Enterobacteriaceae and Mycobacteriaceae encountered in control and infected insects remained the same; 3 - enhanced midgut antibacterial activities against *Serratia marcescens* and *Staphylococcus aureus;* 4 - down-regulated *LysB* and *Prol* mRNA levels; altered *DefB*, *DefC* and *LysA* depending on the infection (short and long-term); 5 - decreased PO activity.

Conclusion: Our findings suggest that *T. rangeli* Macias strain modulates *R. prolixus* immune system and modifies the natural microbiota composition.

Keywords: *Rhodnius prolixus*, *Trypanosoma rangeli*, Immune system, Prophenoloxidase, Antimicrobial peptide

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Background

The haemoflagellate, *Trypanosoma rangeli,* is a protozoan parasite that infects a large number of mammals, including humans, and it is vectored by triatomine insects, especially the genus *Rhodnius* [1-4]. The interaction with triatomine hosts, such as *Rhodnius prolixus,* begins with the ingestion of an infective blood meal containing *T. rangeli.* After ingestion, the parasites transform into epimastigotes, then multiplies in the insect gut, and invades the haemolymph. To perpetuate the infection they transform into the metacyclic forms in the salivary glands [1,2,5]. The life cycle of *T. rangeli* is completed with the transmission of the parasite to vertebrate hosts by the vector through its salivary gland secretions during a blood meal [6,7].

The establishment of *T. rangeli* infections in both the digestive tract and haemocoel is regulated by physiological processes of the triatomine vector [8]. The parasites survive despite the activation of innate immune reactions and complete their life cycle in the insect host [9-11]. Once inside the midgut, the parasites must interact with blood digestion products as well as midgut components including bacterial microbiota [12,13], haemolytic factors [14,15], lectins [16], the prophenoloxidase (PPO) system [17], antimicrobial peptides (AMPs) [18,19] and reactive nitrogen and oxygen species [20].

Some of these factors act as biological barriers to the infection of *T. rangeli* in the vector gut. However, the *T. rangeli* infection may lead to immunedepression of the insect host by the inhibition of phagocytosis, haemocyte microaggregation, PPO activation and eicosanoids synthesis [11,21,22]. These physiological alterations allow the parasites to overcome the immune response, reach the salivary glands and complete their life cycle.

Knowledge of the modulation of the triatomine immune system by the numerous strains of *T. rangeli* is still poorly understood. Thus, the aim of the present study was to investigate the effects of *T. rangeli* Macias strain infection on the midgut immune responses, parasite development and bacteria population of 5th instar nymphs of *R. prolixus* orally infected with parasites. In addition to the evaluation of the effects of *T. rangeli* in short-term infections, long-term infections were analysed in the 5th instar nymphs previously infected in the 4th instar stage. The present results suggest that the parasites modulate the *R. prolixus* immune responses, affecting the intestinal microbiota by inhibiting activation of prophenoloxidase, altering the abundance of antimicrobial peptide transcripts and enhancing antimicrobial activities against *Serratia marcescens*. These results provide further elucidation of the *T. rangeli-R. prolixus* interaction.

Methods

Ethics Statement

Defibrinated rabbit blood provided by the Animals Creation Center Laboratory (Cecal/Fiocruz) was provided to the insects through an artificial apparatus respecting the guidelines of the Ethics Committee on Animal Use (Ceua/Fiocruz). CEUA follows the Ethical Principles in Animal Experimentation composed by Fiocruz researchers and external consultants. The protocol number L-0061/08 was established by CONCEA/MCT [23].

Maintenance of *Trypanosoma rangeli* epimastigotes

T. rangeli Macias strain, first isolated from a human in Venezuela [24,25] and later characterized as genotype KP1+ [26], was kindly supplied by Dr. Suzete Gomes, Universidade Federal Fluminense (Rio de Janeiro, Brazil). The parasites were maintained at 28°C in brain heart infusion (BHI) media (Sigma-Aldrich, São Paulo, Brazil) supplemented with 20% heat-inactivated bovine foetal serum [27] and subcultured twice a week. This procedure keeps the parasite in the log phase growth resulting predominantly in short epimastigotes (99%). The number of parasites was quantified in a Neubauer chamber.

Bacteria preparation

Staphylococcus aureus 9518, and *Escherichia coli* K12 4401 were purchased from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK. *S. marcescens* RPH was previously isolated from *R. prolixus* [12] and maintained at Laboratório de Bioquímica e Fisiologia de Insetos. The bacteria were maintained at -70°C in tryptone agar and 10% glycerol. For all experimental procedures, bacteria were grown as previously described [28]. Briefly, bacteria were grown overnight in tryptone soy broth (TSB) at 30°C and then cultured in fresh TSB for a further 4 h under the same conditions. The bacteria were then washed in phosphate buffered saline (PBS, 0.01 M phosphate buffer, 2.7 mM potassium chloride and 0.137 M sodium chloride, pH 7.4) and resuspended in TSB to a final concentration of 1×10^4 cells/ml.

Insect oral infection: long and short-term infections

Insects were kept at 27°C and fed artificially with defibrinated rabbit blood [27]. All insects were fed on blood, after heat-inactivation of the plasma. The blood was centrifuged at 1,890 x g for 10 min at 4°C and the supernatant (plasma) was incubated for 30 min at 55°C. The plasma was added back to the erythrocytes and then received *T. rangeli* epimastigotes obtained from culture. The same procedure of plasma inactivation was undertaken for control insects.

For long-term experiments, inactivated blood containing 1×10^6 epimastigotes/mL (infected group) or blood

without parasites (control uninfected group) was given to 4th instar nymphs. After moulting to 5th instars, both insect groups received a non-infective blood meal which occurred 38 days after feeding (DAF) of the 4th instar nymphs. For short-term experiments, 5th instar nymphs were fed on blood containing 1×10^6 epimastigotes/mL or with parasite-free blood. Only fully engorged insects were used for all experiments.

Quantification of parasites in the digestive tract

Fifth instar nymphs obtained from long or short-term experiments were dissected. The anterior midgut (stomach) was collected and homogenized in 1.0 mL PBS and the posterior midgut (intestine) plus rectum was placed in 50 μL in PBS. Samples were macerated and the number of parasites was determined by counting in a Neubauer haemocytometer and expressed as parasites/mL. Parasites were quantified in three experiments with five insects each (n = 15).

Analysis of intestinal microbiota

Colony forming unit (CFU)

Anterior midgut contents obtained from 5th instar nymphs infected or uninfected with parasites (short and long-term infections) were analysed for microbiota bacterial population using CFU at 12 DAF. The midgut contents were serially tenfold diluted with PBS and 20 μL was spread on a Petri dish in BHI agar (Sigma-Aldrich) culture medium. The plates were incubated at 30°C for 24 h and the CFU counted. As a control, PBS was also plated to check the sterility of all experiments.

Metagenomic DNA extraction

Seven days after insect feeding (long-term infection), metagenomic DNA was extracted from the anterior midgut contents of four *T. rangeli* infected insects and four uninfected *R. prolixus* 5th instar nymphs by an unbiased and efficient mechanical lysis method [29]. The extraction was carried out using the commercial Fast-DNA™ Spin Kit for Soil (Qbiogene, MP Biomedicals, USA) following the manufacturer's instructions. DNA extracts were visualized on 1% agarose gels to assess their integrity and purity.

Amplification and 454 sequencing of targeted 16S rRNA gene variable region

For quantitative analysis of bacterial microbiota in long-term infected insects, ribosomal genes from metagenomic DNA samples were amplified using bar-coded primers for the 16S variable region V3-V1, cleaned up, quantified and normalized according to the HMP 3730 16S protocol version 4.2 [30], which is available on the HMP Data Analysis and Coordination Center website [31]. The PCR products obtained were then submitted to FLX-Titanium pyrosequencing in a GS Junior System (Roche).

The raw sequences were analysed using the RDP Pipeline with default parameters. Sequences with a score below the quality threshold were discarded and the sequence portions devoted to 454 sequencing were trimmed out. Sequences with more than 400 bases were then aligned using the INFERNAL aligner [32] and chimeric sequences detected (and removed) with UCHIME [33]. Taxonomical classification was assigned using the RDP classifier [34,35] with a minimum confidence level for record assignment set to 0.80.

Turbidimetric antibacterial assay

In long and short-term experiments, the antibacterial activities of the anterior midgut contents from 5th instar nymphs infected or not with *T. rangeli* were tested at 7 DAF, according to previous studies which have shown that the maximal antibacterial activity is reached at this time [17,36]. Fifth instar nymphs of *R. prolixus* were dissected to collect the anterior midgut. The midgut walls were removed and the midgut contents pooled (3 insects) in 200 μl ultrapure water, homogenized, centrifuged at 10,000 x g for 10 min at 4°C and filtered by a sterile PVDF membrane (Millipore) and stored at −20°C until use. Before assaying, the midgut content samples were diluted ten times in sterile water. Subsequently, 10 μl of *E. coli*, *S. aureus or S. marcescens* bacterial suspensions (10^4 cells/mL) were added to each well of a sterile flat bottom 96-well microtiter plate (Nunc, Fisher Scientific, Leicestershire, UK) with 45 μl of diluted midgut samples and 5 μl of peptone 10% and incubated at 37°C for 19 h. The optical densities were measured at 550 nm (OD_{550}) at hourly intervals using a Spectra Max 190 Plate Reader (Molecular Devices, Sunnyvale, USA). Control wells, run without anterior midgut samples, contained 10 μl of bacteria in a final concentration of 1% peptone in ultrapure water. Ampicillin (80 μg/ml) was included in each experiment as an antibiotic control. To exclude the opacity of the midgut samples all data points were blanked against time zero. The antibacterial activity was calculated by subtracting the bacterial growth readings (control wells) from the respective values of anterior midgut samples incubated with bacteria.

Transcript abundance of antimicrobial peptides

Transcript abundance of genes encoding antibacterial peptides in short and long-term experiments with *T. rangeli* infected and uninfected 5th instar nymphs was analysed by reverse transcription PCR (RT-PCR) as described previously [36]. In brief, the anterior and posterior midgut walls of a pool of ten insects were dissected from 5th instar nymphs 1 and 7 DAF. Total

RNA was extracted using the NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. RNA concentration was measured on a NanoDrop 2000 (Thermo Scientific, Waltham, USA). For cDNA synthesis, 1.25 or 2.5 μg of total RNA was performed using a First-Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK). Oligonucleotide primers for amplification of defensin A, B and C, lysozyme A and B, prolixicin [19,37,38] and ß-actin (endogenous control) were used. PCRs were carried out in triplicate on a Veriti 96 thermocycler (Applied Biosystems, Carlsbad, USA) using an IllustraTaq DNA-Polymerase (GE Healthcare). In negative PCR controls, ultrapure water was added instead of cDNA. PCR products were electrophoretically separated on a 2% agarose gel and stained with ethidium bromide. Gels were documented using an E-Gel® Imager (Life Technologies, Carlsbad, USA) and band intensity quantified using the ImageJ program (v. 1.47q).

Determination of phenoloxidase activity

Phenoloxidase (PO) activities were analysed in samples of the anterior midgut contents freshly prepared from 5th instar nymphs obtained from long and short-term experiments. Each midgut content was diluted in 200 μL of ultrapure water, centrifuged at 10,000 x g for 10 min and the supernatant ten times diluted. For PO analysis, five insects were used from each group. The experiments were carried out in triplicate and at 7 and 12 DAF.

PO activity was determined by measuring the dopachrome formation from DOPA using midgut samples, as described previously [39]. For assaying, 25 μL of a midgut preparation was mixed with 10 μL of cacodylate-$CaCl_2$ buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 7.4). After the addition of 25 μL of a saturated solution of DOPA (4 mg/mL), the absorbance at 490 nm was measured continually in a Spectra Max 190 Microplate Reader at 37°C for 120 min. The enzyme unit was expressed as absorbance/min × 100.

Statistical analysis

Depending on the distribution of the data and treatment number, the results obtained were analysed using 1-way ANOVA, Student's *T*-test, the Kruskal-Wallis test or the Mann–Whitney test on GraphPad Prism 5 software. Differences between groups were considered statistically significant when $p < 0.05$. The levels of probability are shown in the respective figures.

Results

Short-term infection

Quantification of parasites in the digestive tract

The infection rates of the *R. prolixus* digestive tract by *T. rangeli* were analysed on different days after feeding (DAF). Analyses of the presence of parasites in the digestive tract showed that the percentage of infected insects at 2 DAF was 100% and decreased to 26.6% and 6.7% after 7 and 12 days, respectively. The anterior midgut presented a high number of parasites, starting with 9.7×10^5/mL at 2 DAF and decreased significantly along time to 9.3×10^4/mL and 0, respectively, at 7 and 12 DAF ($p < 0.001$) (Figure 1). A similar pattern of parasite temporal distribution was observed in the posterior midgut and rectum with an infection level of 1.1×10^5/mL, 5.0×10^4/mL and 8.3×10^3/mL at 2, 7 and 12 DAF, respectively with significant difference between 2 and 12 DAF ($p < 0.05$) (Figure 1).

Analysis of intestinal microbiota (CFU)

Cultivable bacterial microbiota population in 5th instar nymphs infected with *T. rangeli* Macias strain was evaluated using CFU counts of digestive tract preparations. At 12 DAF the bacterial population in infected insects (5.7×10^7 CFU/mL) was significantly lower than the uninfected control (2.0×10^8 CFU/mL) ($p < 0.01$) (Figure 2).

Turbidimetric (TB) antibacterial assay

The antibacterial activity in 5th instar nymphs infected with *T. rangeli* was analysed *in vitro* by incubating anterior midgut content samples collected 7 DAF with different bacterial strains. Antibacterial activity of the infected insects against *S. marcescens* was significantly higher than in control insects ($p < 0.001$) (Figure 3A). The activities measured against *S. aureus* and *E. coli*

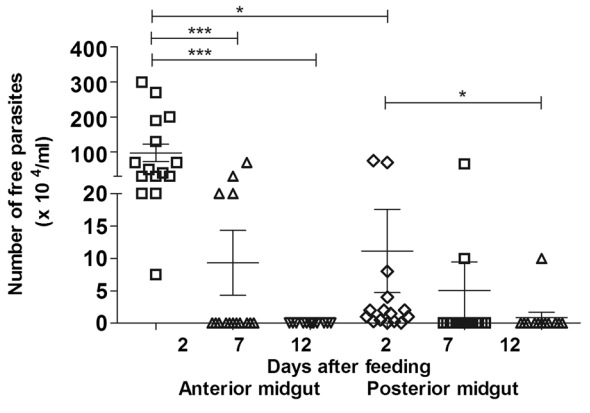

Figure 1 Short-term experiment showing *Trypanosoma rangeli* numbers in the gut of *Rhodnius prolixus*. Parasite numbers in *R. prolixus* 5th instar nymphs were analysed in anterior midgut and posterior midgut plus rectum at different days after feeding. The insects were fed on inactivated blood containing 1×10^6 epimastigotes/mL *T. rangeli* Macias strain. Bars represent mean ± SEM of three independent experiments each with five insects (n=15). Means were compared using one-way ANOVA and Kruskal-Wallis test; ***$p < 0.001$ and *$p < 0.05$.

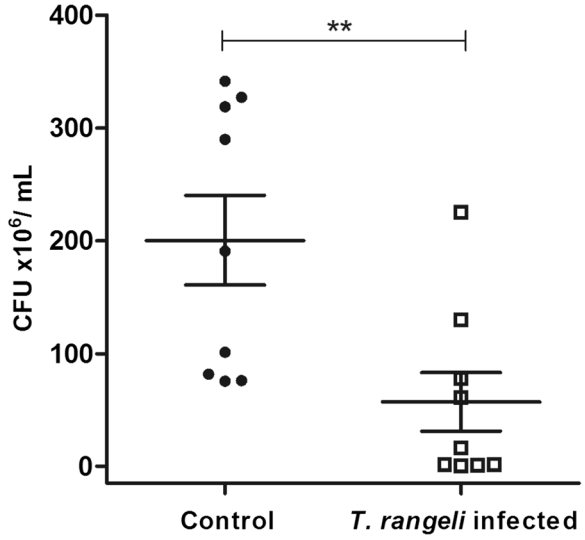

Figure 2 Short-term experiment showing bacteria population in *Rhodnius prolixus* midgut infected with *Trypanosoma rangeli*. Fifth instar nymphs were fed on inactivated blood containing 1×10^6 epimastigotes/mL of *T. rangeli*. CFU counts were made 12 days after feeding. Bars represent mean ± SEM of three independent experiments with three pools of insects (n = 9). Each point represents the CFU from one pool of three insects. Means were compared using Student *T*-test or Mann–Whitney test; **p < 0.01.

were similar in infected insects compared with the controls (Figure 3B, C).

Transcript abundance of antimicrobial peptides (AMPs)

The modification of antimicrobial activities in the anterior and posterior midguts of the 5th instar nymphs infected with *T. rangeli* was analysed by the transcript abundance profiles of AMPs 1 and 7 DAF. The relative abundance of lysozyme A (*LysA*), lysozyme B (*LysB*), prolixicin (*Prol*), defensins A (*DefA*), B (*DefB*) and C (*DefC*) was also quantified (Figure 4).

In the anterior midgut at 1 DAF, the expression of *DefB* was significantly higher ($p < 0.05$) and *LysB* was significantly lower ($p < 0.05$) in comparison between infected and control insects, respectively (Figure 4A). In contrast, at 7 DAF, three AMPs (*DefB, LysB* and *Prol*) had significantly lower levels ($p < 0.001$; $p < 0.05$; $p < 0.001$, respectively) and only one (*DefC*) had a significantly higher level ($p < 0.001$) of transcripts in infected insects when compared to control (Figure 4C). However, in the posterior midgut the differences in levels of the AMPs between the infected and control insects were less striking. Compared to control the infected insects presented higher levels ($p < 0.001$) of *DefC* transcripts at 1 DAF and lower levels ($p < 0.001$) of *Prol* at 7DAF (Figure 4B and D).

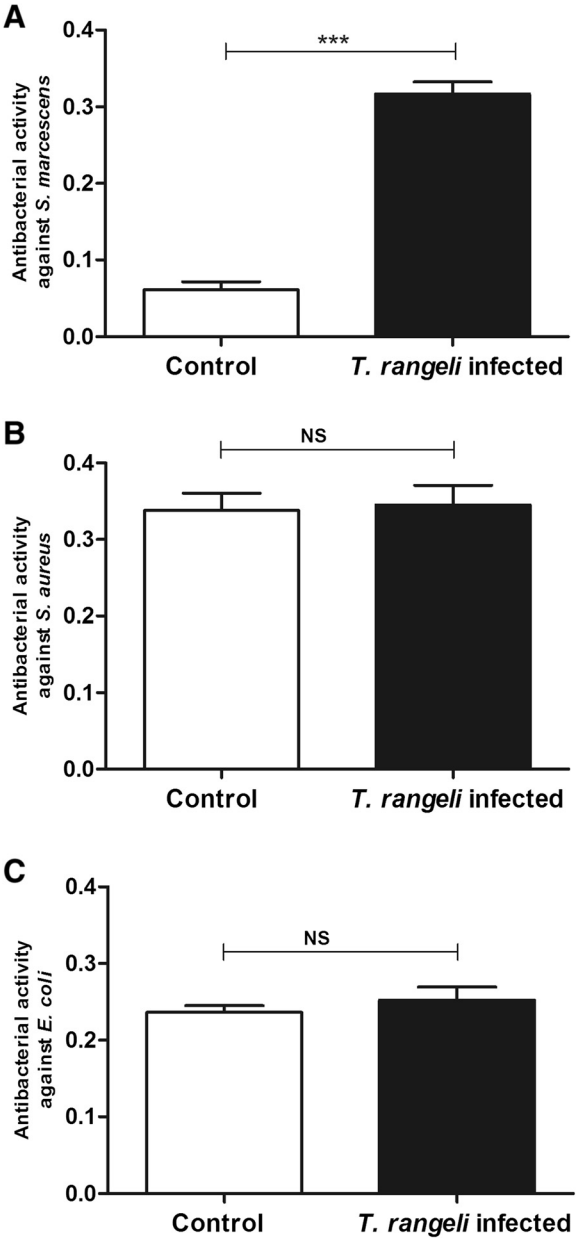

Figure 3 Short-term experiment showing antibacterial activity in the midgut of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. The antibacterial activities of anterior midgut of *R. prolixus* 5th instar nymphs 7 days after infection with *T. rangeli* were tested against **(A)** *S. marcescens* **(B)** *S. aureus* **(C)** *E. coli*. Treatments: white columns – control, uninfected group; black columns – infected group. Fifth instar nymphs were fed on inactivated blood with or without 1×10^6 epimastigotes/mL. Antibacterial activity value was measured using the turbidimetric assay (TB) (OD_{550} nm) after 19 h incubation of midgut samples with different bacteria and calculated by the difference between the optical densities of the midgut samples and bacterial control. Bars represent mean ± SEM of three independent experiments. Each experiment consisted of three pools of three insects (n = 9). Means were compared using *t*-test; ***p < 0.001 and NS = not significant.

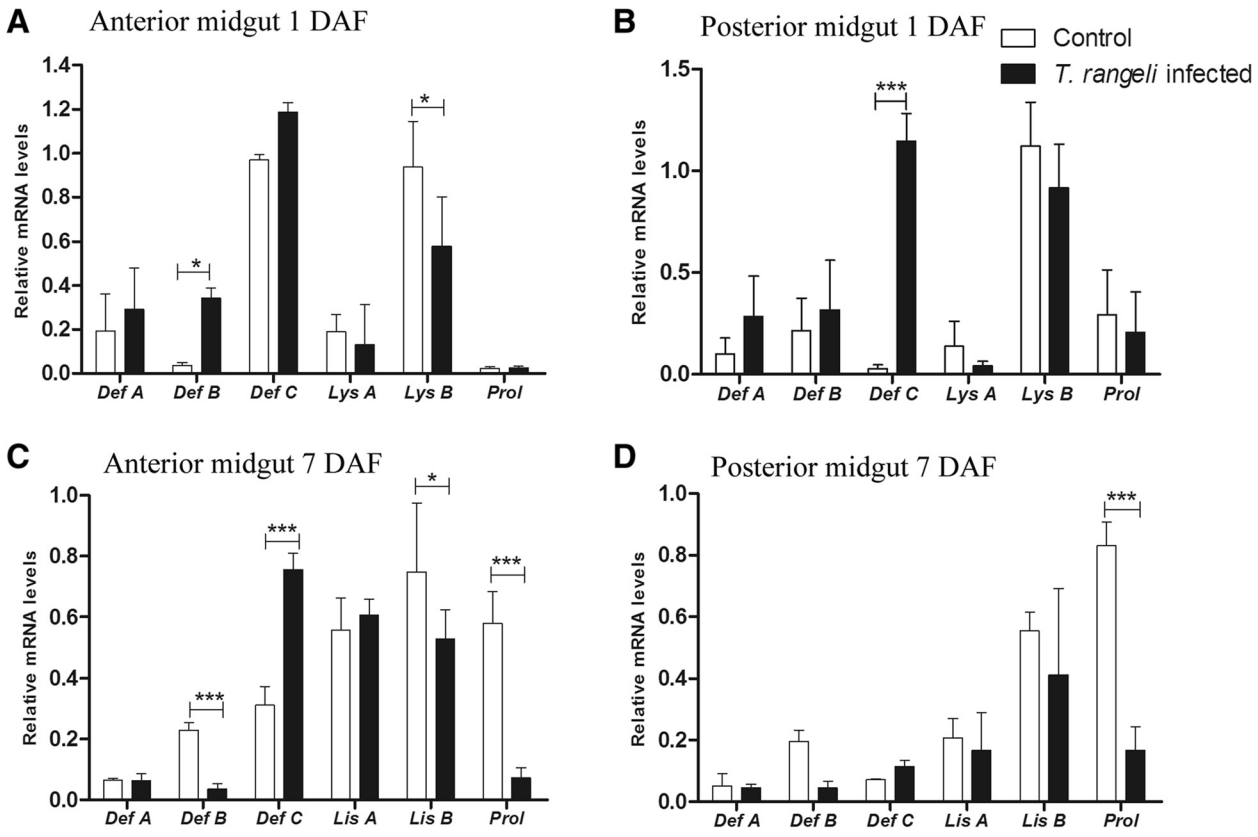

Figure 4 Short-term experiment showing relative transcript abundance of antimicrobial peptides and lysozymes in *Rhodnius prolixus* midgut. Relative AMP mRNA levels from *R. prolixus* 5th instar nymphs were analysed 1 and 7 days after feeding (DAF) **A** – anterior midgut 1 DAF; **B** – posterior midgut 1 DAF; **C** – anterior midgut 7 DAF; **D** – posterior midgut 7 DAF. Treatments: white columns – control, uninfected group; black columns – infected group. Insects were fed on inactivated blood with or without 1×10^6 epimastigotes/mL. Bars represent mean ± SEM of three independent experiments with a pool of 10 insects (n = 3). Means were compared using one-way ANOVA and Student *T*-test; ***$p < 0.001$ and *$p < 0.05$.

Determination of phenoloxidase activity

PO activities measured in the anterior midgut contents of the 5th instar nymphs at 7 DAF did not show significant differences, when comparing *T. rangeli* infected and control groups. However, at 12 DAF, the PO activity was significantly lower in infected insects than in the control ($p < 0.01$) (Figure 5). The PO activity inhibition by *T. rangeli* infection was significantly higher at 12 DAF when compared with 7 DAF ($p < 0.001$) (Figure 5).

Long term infection

Quantification of parasites in the digestive tract

The *T. rangeli* infection in the insects was also investigated in long-term experiments. Parasites were quantified in the digestive tract from the 4th instar nymphs when infection occurred and after insects moulted to 5th instar followed by a second feeding with parasite free blood. The percentages of infected insects in 4th instar nymphs at 2 and 7 days after infection were 86.7% and 93.3%, respectively. In this infected group, the number of parasites encountered in the anterior midgut was significantly higher than in the posterior midgut on both days analysed (Figure 6A). The parasite numbers reached 28.9×10^4/mL and 32.8×10^4/mL in the anterior midgut at 2 and 7 DAF, respectively, and 3.4×10^4/mL and 4.4×10^4/mL in the posterior midgut at 2 and 7 DAF, respectively (Figure 6A).

The percentages of 5th instar nymphs which showed *T. rangeli* infection in the digestive tract were 46.7% and 73.3% at 2 and 7 DAF, respectively, after an uninfected blood meal. In these 5th instar nymphs, the results were opposite to those observed in the 4th instar nymphs, in which the anterior midgut presented significantly lower numbers of parasites than the posterior midgut on both days analysed (Figure 6B). The infection level in the anterior midgut was 0 and 0.17×10^4/mL at 2 and 7 DAF respectively and in the posterior midgut and rectum was 22.4×10^4/mL and 17.7×10^4/mL at 2 and 7 DAF, respectively (Figure 6B). These results showed that *T. rangeli* successfully colonized *R. prolixus* midgut, even after moulting and a second blood meal (Figure 6).

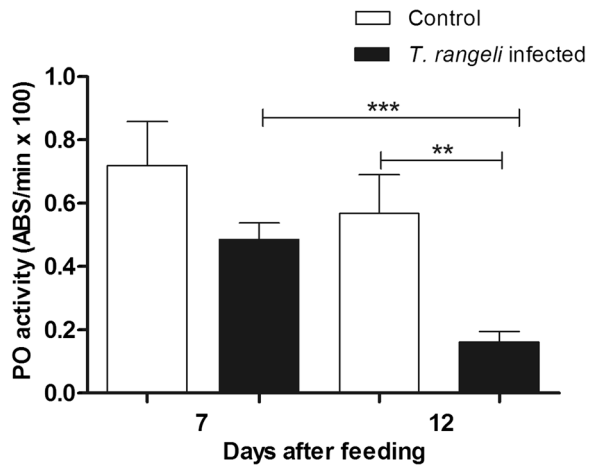

Figure 5 Short-term experiment showing phenoloxidase activity in the midgut of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. PO activities were measured in the anterior midgut of *R. prolixus* 5th instar nymphs at 7 and 12 days after infection with *T. rangeli*. The insects were fed on inactivated blood with or without 1×10^6 epimastigotes/mL. Treatments: white columns – control, uninfected group; black columns – infected group. Bars represent mean ± SEM of three independent experiments each with five insects (n = 15). Means were compared using Student *T*-test; ***p < 0.001 and **p < 0.01.

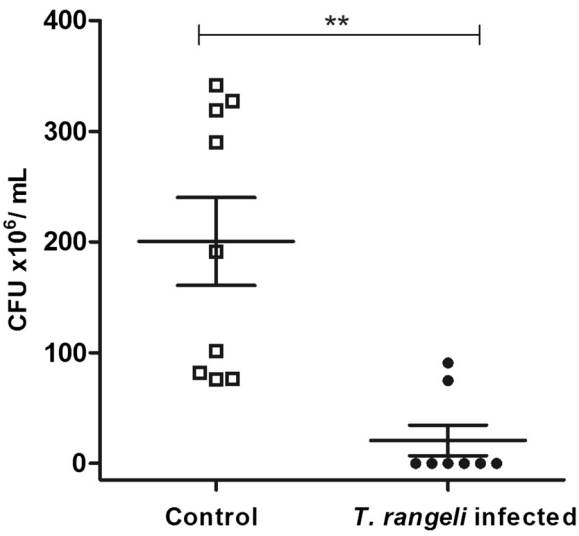

Figure 7 Long-term experiment showing bacteria population in *Rhodnius prolixus* midgut infected with *Trypanosoma rangeli*. CFU counts were made with *R. prolixus* 5th instar nymphs 12 days after feeding (DAF) on blood without parasites. Previously, 4th instar nymphs were fed on inactivated blood with or without 1×10^6 epimastigotes/mL. Bars represent mean ± SEM of three independent experiments. Each point represents the CFU from three pools of three insects (n = 9). Means were compared using Student *T*-test or Mann–Whitney test; **p < 0.01 and *p < 0.05.

Analysis of intestinal microbiota

Colony forming unit (CFU)

The cultivable bacterial microbiota population of *R. prolixus* 5th instar nymphs, infected as 4th instars with *T. rangeli* was significantly lower than control insects at 12 DAF (p < 0.01) (Figure 7).

Amplification and 454 sequencing of targeted 16S rRNA gene variable region

The bacterial microbiota in the anterior midgut was predominantly composed of Enterobacteriaceae and Enterococcaceae families, which include *Serratia* and *Enterococcus* species, respectively, as well as Nocardiaceae (Figure 8). Seven days after feeding, there was a significant decrease of Enterococcaceae in the *R. prolixus* 5th instar nymphs, infected at 4th instar with *T. rangeli* while there was significant increase of Burkholderiaceae in the infected 5th instar nymphs (Figure 8).

Turbidimetric antibacterial assay

The anterior midgut antibacterial activity of insects infected over the long-term was investigated. Comparing to the control group, infected insects presented significantly

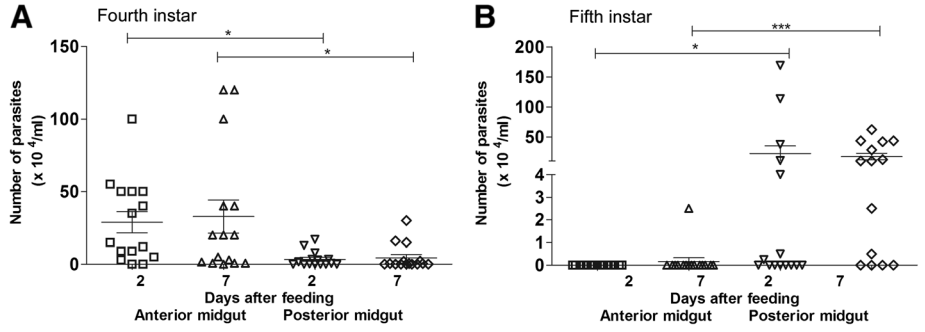

Figure 6 Long-term experiment showing *Trypanosoma rangeli* numbers in the gut of *Rhodnius prolixus*. Parasite numbers were analysed in *R. prolixus* **(A)** 4th and **(B)** 5th instar nymphs: anterior midgut and posterior midgut at 2 and 7 days after feeding. Fourth instar nymphs were fed on inactivated blood containing 1×10^6 epimastigotes/mL. After moulting, 5th instar nymphs were fed on blood without parasites. Bars represent mean ± SEM of three independent experiments each with five insects (n = 15). Means were compared using one-way ANOVA and Kruskal-Wallis test; ***p < 0.001 and *p < 0.05.

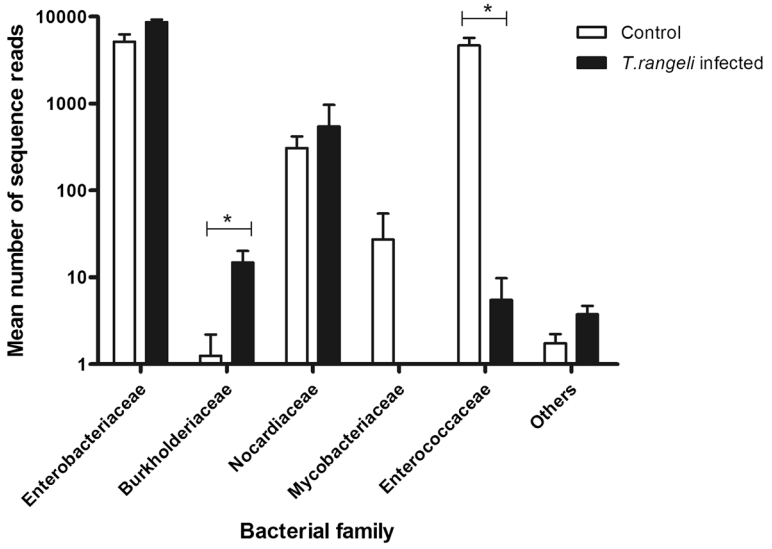

Figure 8 Bacterial composition identified by 16S ribosomal pyrosequencing in *Rhodnius prolixus* midgut infected with *Trypanosoma rangeli*. Long-term experiment showing bacterial composition at the family levels. Pyrosequencing 454 experiments of anterior midgut preparations from *R. prolixus* 5th instar nymphs 7 days after feeding (DAF) on blood without parasites. Previously, 4th instar nymphs were fed on inactivated blood with or without 1×10^6 epimastigotes/mL of *T. rangeli*. Each bar graph presents the mean number of sequence reads assigned to a given bacterial family in four insect samples. Others: represent families with only one or two sequences (Pseudomonadaceae, Comamonadaceae, Rhodobacteraceae, Phyllobacteriaceae, Bradyrhizobiaceae, Staphylococcaceae, Bacillaceae, Nitrospiraceae, Flavobacteriaceae). Means were compared using *t*-test or Mann–Whitney test; *$p < 0.05$.

higher antibacterial activity against *S. marcescens* ($p < 0.001$) and *S. aureus* ($p < 0.05$) and lower activity against *E. coli* ($p < 0.001$) (Figure 9).

Transcript abundance of antimicrobial peptides (AMPs)

The relative transcript abundance of AMPs and lysozymes encoding mRNA in the 5th instar *R. prolixus* nymphs that were infected with *T. rangeli* as 4th instar nymphs was investigated (Figure 10). The expression of *LysB* was significantly lower in both compartments of the midgut at 1 and 7 DAF in infected insects when compared to the uninfected control group. This difference in abundance of *LysB* was more significant at 1 DAF ($p < 0.001$) both in the anterior and posterior midguts of the infected insects (Figure 10A and 10B). The abundance of *LysA* transcripts was significantly lower only in the anterior midgut at 7 DAF of the infected insects ($p < 0.05$) in comparison to the control insects (Figure 10C). Compared to the control group, *DefC* transcripts in infected insects were less abundant in the anterior midgut at 1 DAF ($p < 0.001$). In the posterior midgut at 7 DAF abundance of the *DefB* transcripts was lower ($p < 0.01$) in infected insects than in the control insects (Figure 10D). Moreover, the abundance of *Prol* was significantly lower in the anterior midgut at 7 DAF ($p < 0.001$) and in the posterior midgut at 1 and 7 DAF ($p < 0.01$) of infected insects when compared to control (Figures 10 B, C and D). Only *DefC* mRNA levels in the posterior midgut were up-regulated (57-fold, $p < 0.001$) at 1 DAF in infected insects when compared to the control group (Figure 10B).

Prophenoloxidase (PPO) activity

The anterior midgut of 5th instar nymphs, previously infected with *T. rangeli* at 4th instar, were investigated. The PO activities of infected insects were significantly lower than the control insects at 7 and 12 DAF ($p < 0.001$ and $p < 0.01$, respectively) (Figure 11). Moreover, the PO activity in the control insects was lower at 12 DAF when compared to 7 DAF ($p < 0.01$) (Figure 11).

Discussion

Experiments in which *R. prolixus* were infected with *T. rangeli* H14 or Choachi strains have demonstrated that the modulation of the insect's immune responses and subsequent establishment of the infection in the digestive tract depends on the strain of the parasite [25,40-44]. It is also known that gut microbiota can be correlated to the success of the parasite infection in diverse invertebrate hosts [17,45-48]. Therefore, we infected *R. prolixus* with the *T. rangeli* Macias strain and investigated the modulation of the immune system and bacteria population of the insect's digestive tract. Our results demonstrated that the percentage of insects with intestinal parasites varied within days after infection and midgut compartments examined. In the short-term infection, *T. rangeli* predominantly colonized the anterior midgut and the number of parasites decreased over time.

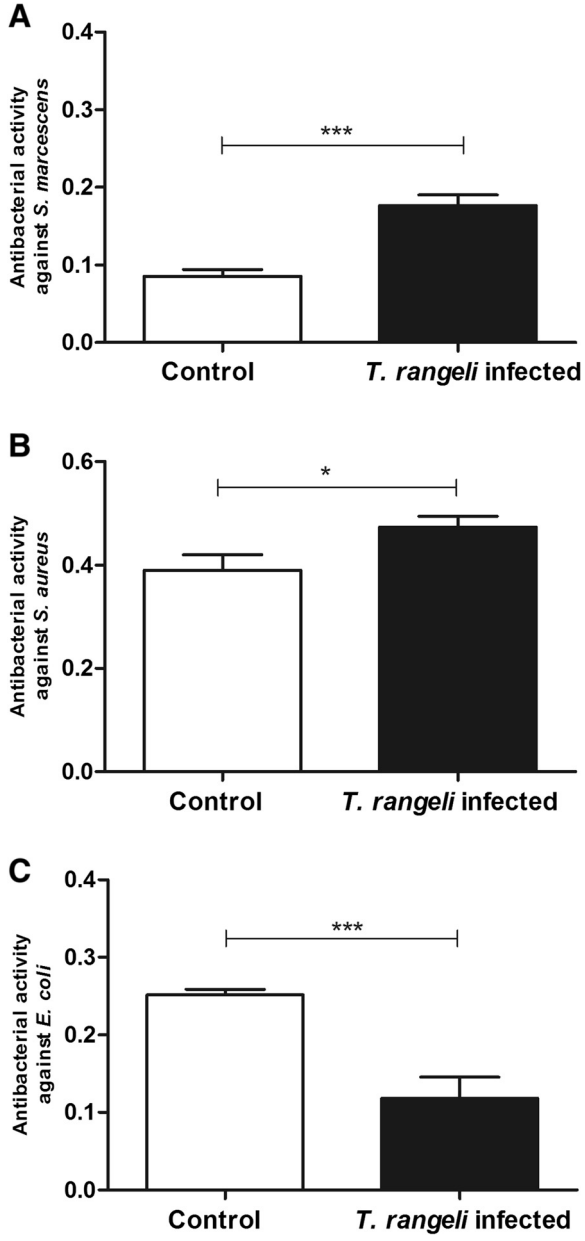

Figure 9 Long-term experiment showing antibacterial activity in the midgut of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. Antibacterial activities of anterior midgutof *R. prolixus* 5th instar nymphs 7 days after feeding (DAF) on blood without parasiteswere tested against **(A)** *S. marcescens***(B)** *S. aureus* **(C)** *E. coli*. Treatments: white columns – control, uninfected group; black columns – infected group. Previously, 4th instar nymphs were fed on inactivated blood with or without 1×10^6 epimastigotes/mL. Anterior midgut contents were collected from 5th instar nymphs 7 (DAF). Antibacterial activity was measured using the turbidimetric assay (TB) (OD_{550} nm) after 19 h incubation of midgut samples with different bacteria and calculated by the difference between the optical densities of the midgut samples and bacterial control. Bars represent mean ± SEM of three independent experiments. Each experiment consisted of three pools of three insects (n = 9). Means were compared using Student *T*-test; ***$p < 0.001$ and *$p < 0.05$.

In the long-term experiments, the parasites were found preferentially in the anterior midgut of the 4th instar nymphs; however after moulting and receiving a parasite-free blood meal, *T. rangeli* was predominantly found in the posterior midgut of the 5th instar nymphs. In terms of *T. cruzi* development in triatomines, the parasites migrate to the posterior midgut and rectum within few weeks after infection [49-52]. Depending on the nutritional conditions of the insect, the anterior midgut contains different microbiota compositions and cytotoxic components (nitrogen and oxygen reactive species, AMPs and haemolysins) that may create a hostile environment for the parasites [20,36,51,53-56].

It is known that the intestinal microbiota modulates the host immune responses and can interfere in parasite infection [12,57,58]. Moreover, the bacteria density can be regulated depending on the parasite genotype infecting the insect host as observed with *T. cruzi* infection in *R. prolixus* [17]. The present study showed lower cultivable bacterial CFU numbers in the digestive tract of *R. prolixus* infected with *T. rangeli* than in control insects (short and long-term). Additionally, bacterial microbiota analysis by pyrosequencing revealed a decrease of Enterococcaceae and Mycobacteriaceae while Burkholderiaceae increased in sequence numbers in infected insects. In other insect vectors, such as *Glossina* and *Anopheles,* bacteria from these families have also been observed and they have varied with parasite infection as well [59,60]. *Rhodococcus rhodnii* and *S. marcenscens* that have been frequently observed colonizing triatomines [12,61,62] and which belong to the families Nocardiaceae and Enterobacteriaceae, respectively, were not altered after *T. rangeli* Macias strain infection in *R. prolixus*. However, *R. rhodnii* population decreased in a study using the *T. rangeli* Choachi strain infecting *R. prolixus* [63]. Additionally, *in vitro* studies have already shown that *S. marcescens* possess cytotoxicity against some *T. cruzi* and *T. rangeli* strains [12,13,55,64,65] besides its antibiotic activity [66]. These findings indicate that bacterial communities can be modulated differently depending on the *T. rangeli* strain.

We analysed the antibacterial activity in the midgut of the *T. rangeli* infected insects and showed that this activity was related to the decrease in the bacteria population of the insect's digestive tract. The high antibacterial activity observed against *S. marcescens in vitro* can be one reason for the low number of cultivable bacteria detected in the CFU analysis. In addition, the high antimicrobial activity against *S. aureus* might reflect the decrease of *Enterococcus* in the midgut of infected samples analysed by pyrosequencing.

The production of AMPs in the insect gut has been demonstrated to be vital to maintain insect homeostasis of the intestinal microbiota which provide essential

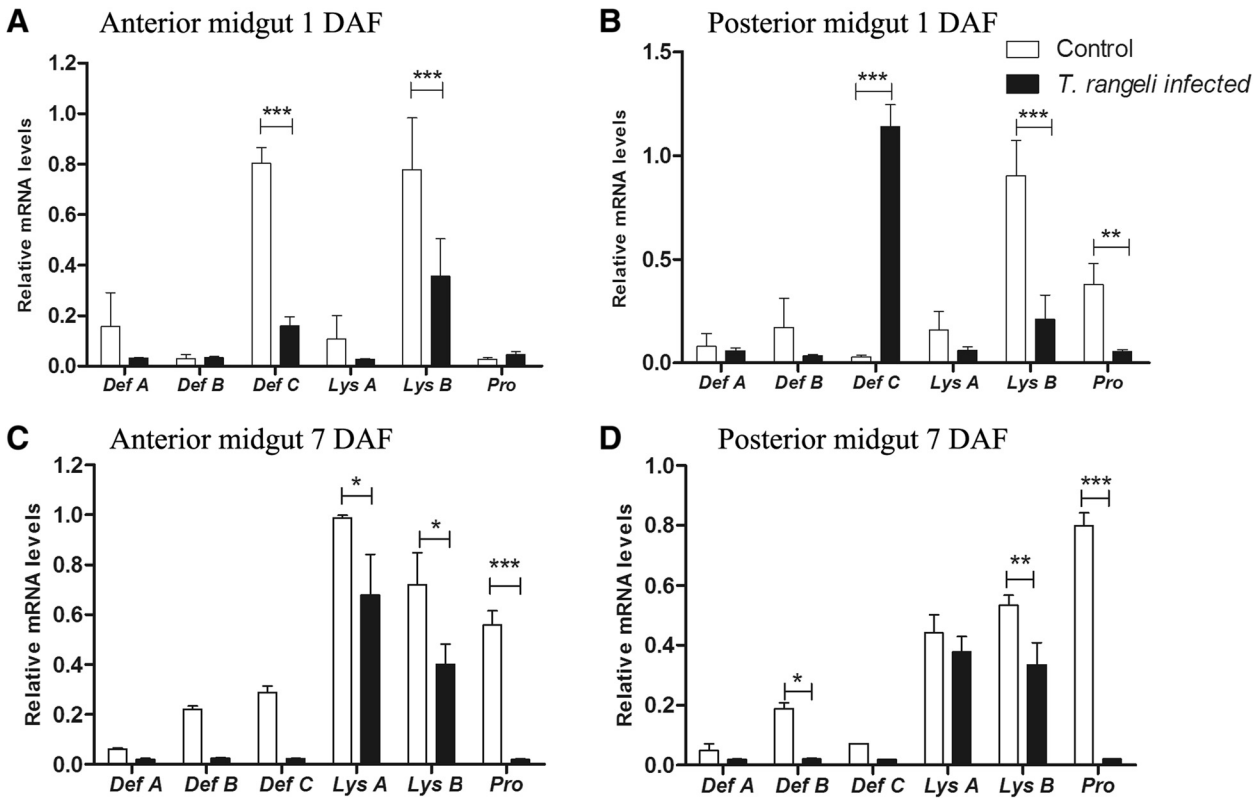

Figure 10 Long-term experiment showing relative transcript abundance of antimicrobial peptides and lysozymes in *Rhodnius prolixus* midgut. Relative AMP mRNA levels from *R. prolixus* 5th instar nymphs were analysed 1 and 7 days after feeding (DAF) on blood without parasites. **A** – anterior midgut 1 DAF; **B** – posterior midgut 1 DAF; **C** – anterior midgut 7 DAF; **D** – posterior midgut 7 DAF. Previously, 4th instar nymphs were fed on inactivated blood with or without 1×10^6 epimastigotes/mL. Treatments: white columns – control, uninfected group; black columns – infected group. Bars represent mean ± SEM of three independent experiments with a pool of 10 insects (n = 3). Means were compared using one-way ANOVA and Student *T*-test; ***p < 0.001, **p < 0.01 and *p < 0.05.

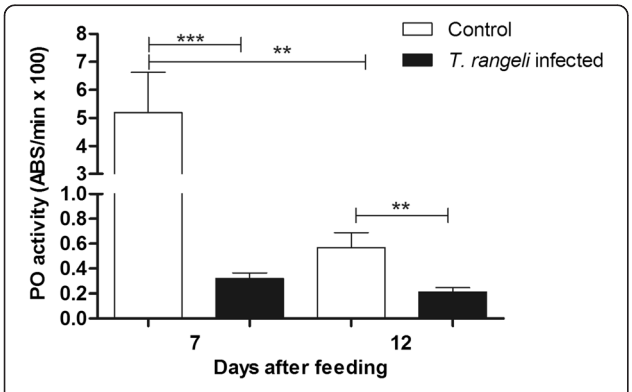

Figure 11 Long-term experiment showing phenoloxidase activity in the midgut of *Rhodnius prolixus* infected with *Trypanosoma rangeli*. PO activities were measured in the anterior midgut of *R. prolixus* 5th instar nymphs at 7 and 12 days after feeding on blood without parasites. Previously, 4th instar nymphs were fed on inactivated blood with or without 1×10^6 epimastigotes/mL. Treatments: white columns – control, uninfected group; black columns – infected group. Bars represent mean ± SEM of three independent experiments each with five insects (n = 15). Means were compared using Student *T*-test; ***p < 0.001 and **p < 0.01.

nutrients, promote digestion and control pathogenic microorganisms by modulating the immune responses [55,67-69]. In *Drosophila* the activation of signalling pathways of the immunity depends on the type of predominant microorganisms in the digestive tract [70-72].

An important immune response in the midgut lumen of insect vectors to control natural microbiota growth and pathogens is the production of AMPs [55,69]. AMPs are effectors molecules of the humoral immune system of insects that control microorganisms by disrupting cell membranes [73-75]. Analysis of the relative expression of mRNAs encoding lysozymes and AMPs in *T. rangeli* infected insects showed a different pattern in short and long-term infections. However, in general there was a suppression of most AMP genes. For example, *LysB* and *LysA* down-regulation was observed in the anterior and posterior midgut compartments. A previous work suggested that *LysA* is mainly expressed in the midgut with a digestive function while *LysB* is expressed in the fat body with an immune role [38]. Nevertheless, *S. aureus* oral infection in *R. prolixus* increased *LysA* mRNA levels in the midgut [36]. Combining these results with the suppression

of *LysA* by *T. rangeli* infection observed herein, we suggest its involvement in the immune response.

Regarding prolixicin, a previous work showed that this peptide presented antimicrobial activities against Gram-negative and Gram-positive bacteria, but no toxicity against *T. cruzi* was detected [19]. In the present work, *Prol* was down-regulated in both midgut compartments, in both the short and long-term infections with *T. rangeli*. Although cytotoxicity of prolixicin against *T. rangeli* has not been described in the literature, the present results suggest that the modulation of *Prol* expression by *T. rangeli* could be one possible mechanism that, indirectly benefits the parasite's development in *R. prolixus*.

Another group of AMPs extensively studied in insects are the defensins. These peptides are known to act mainly on Gram-positive bacteria, but also show some activity against Gram-negative bacteria [76,77] and some protozoans such as *Plasmodium* and *Trypanosoma* [78-81]. Considering the short-term infection and the parasite population dynamics in the insect's midgut, a rapid increase of *DefB* levels and its subsequent down-regulation in the anterior midgut suggests a possible role of the respective peptides in the control of microorganism density in this compartment. The role of defensins in the control of trypanosomatid infections in the vector has been suggested previously [80,82]. On the other hand, the increase of *DefC* in both midgut compartments represents an immune modulation caused by *T. rangeli* that could represent a strategy to facilitate the establishment of *T. rangeli* in the gut of *R. prolixus*. Combined, these results suggest that an increase of antimicrobial activities and a decrease of CFU numbers detected in the anterior midgut in short term infection might be a result of the increased *DefB* and *DefC* levels observed. Long-term infection resulted in a massive up-regulation of *DefC* in the posterior midgut, which can explain the decrease of bacteria population encountered and the parasite's preference to develop in this midgut compartment. These results indicate that the parasite infection can modulate the insect's immune system, which consequently can influence the microbiota population in the insect's digestive tract.

Another important biological event in the *T. rangeli* cycle, in its invertebrate host, is its ability to modulate the PPO system in the triatomine haemolymph [83-85]. The presence of *T. rangeli* also reduced the level of PPO activation *in vitro* [86] and *in vivo* in *R. prolixus* haemolymph [22,84]. The present study is the first to demonstrate that the PO activity in the *R. prolixus* midgut was also inhibited after oral infection with *T. rangeli.* The PO activity in the midgut seems to be differently regulated accordingly to the trypanosomatid species. While *T. rangeli* has the ability to inhibit the insect PPO system, *T. cruzi* infection induces an increase in this immune response [17]. Other immune modulated factors such as reactive oxygen and nitrogen species may be involved in the development of the parasite in insect's midgut [17,87-89].

Conclusion

Parasite-microbiota competition for nutrients can change the bacteria composition in the *R. prolixus* midgut and subsequently modulate the insect's immune system. A direct modulation of the immune system by the parasite can also affect the microbiota population. The strategy of certain trypanosome species for successful infection of the invertebrate host is a complex interplay and depends on a tripartite interaction between parasite, insect immune system and bacteria [46,59,60,90,91]. These interactions are an important field for research, opening up new insights into the understanding of parasite-vector relationships [92]. A better understanding of the role of bacterial species composing the gut microbiota on host immunity against pathogens can lead to the development of new strategies to control vector-borne diseases.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: CSV, PJW, DPC, ESG and PA. Carried out the biochemical experiments: CSV, DPM, MBF and DPC. Performed the molecular experiments: CSV, PJW, MG, FFM. Analysis and interpretation of data: CSV, DPM, PJW, JMS, MBF, MG, FFM, and PA. Contributed reagents/materials: ESG and PA. Wrote the manuscript: CSV, PJW, DPC and PA. All authors read and approved the final manuscript.

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3.3 Artigo 3: Antimicrobial peptides gene expression in *Rhodnius prolixus* infected with different *Trypanosoma cruzi* strains: impact on parasite survival and the gut bacterial microbiota community. **Vieira CS**, Waniek PJ, Mattos DP, Figueiredo MB, Castro DP, Garcia ES, Azambuja P. A ser submetido à revista *Plos Neglected Tropical Diseases*.

Antimicrobial peptides gene expression in *Rhodnius prolixus* infected with different *Trypanosoma cruzi* strains: impact on parasite survival and the gut bacterial microbiota community.

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Abstract:

Background: *Rhodnius prolixus* is a major vector of *Trypanosoma cruzi*, the causative agent of Chagas disease in Latin America. It has a strictly blood-sucking habit in all life stages, ingesting large amounts of blood from vertebrate hosts from which it can acquire pathogenic microorganisms. In this context, the production of antimicrobial peptides (AMPs) in the midgut of the insect is crucial to control pathogen proliferation and maintain the microbiota already present in the digestive tract. The present work focuses on the modulation of the AMPs defensins and prolixicin in midgut and fat body from *R. prolixus* orally infected with *T. cruzi* Dm 28c and Y strain, considering the influence of the parasites on the intestinal microbiota.

Methods: The presence of *T. cruzi* in the insect was confirmed through visualization of *R. prolixus* midgut samples under an optical microscope. Cultivable midgut bacteria were quantified by colony forming unit (CFU) assays. The modulation of *R. prolixus* immune responses was studied by analysis of the antimicrobial activity of different insect tissues *in vitro* against different bacteria using turbidometric tests. The relative expression of the antimicrobial peptides encoding genes *defensin* (*defA*, *defB*, *defC*) and *prolixicin* (*prol*) was quantified by RT-qPCR.

Results: Our results showed that different *T. cruzi* strains present distinct profiles of immune system and microbiota modulation in the *R. prolixus* midgut and fat body, where *T. cruzi* Dm 28c was able to induce an increase of *defC* transcripts and a reduction of *prolixicin* expression, simultaneously reducing the midgut cultivable bacteria population drastically. In the other hand *T. cruzi* Y was not able to induce AMPs expression in the gut or considerably reduce the microbiota in the anterior midgut.

Conclusion: Our findings suggest that *R. prolixus* modulates AMP gene expression upon ingestion of *T. cruzi* with patterns that are distinct and dependent upon parasite genotype. The trypanosome ability to properly induce immune peptides in midgut epithelial cells seems to favor its development in the insect digestive tract by decreasing intestinal microbiota.

Keywords: *Rhodnius prolixus*, *Trypanosoma cruzi*, immune system, antimicrobial peptides, microbiota.

INTRODUCTION

Trypanosoma cruzi is a protozoan parasite transmitted to vertebrate hosts by triatomine insects and is the causative agent of Chagas disease (Chagas 1909; Coura 2015). This disease is a public health problem and it is estimated that about 6 to 7 million people are infected with *T. cruzi* worldwide, mostly in Latin America (WHO 2015). In this context, *Rhodnius prolixus* is one of the most important *T. cruzi* vectors in Central and South America (Dias et al., 2002; Coura 2015). Therefore, the great medical relevance of this species spurred several studies on its physiology, immunology and molecular biology, especially in experimental infections with *T. cruzi* (Wigglesworth 1972; Azambuja et al., 2005; Figueiredo et al., 2006; Garcia et al., 2007; Ursic-Bedoya et al., 2011; Castro et al., 2012; Ribeiro et al., 2014; Vieira et al., 2014).

Similarly, different *T. cruzi* genotypes of a wide range of heterogeneous populations that circulates through vertebrate and invertebrate hosts were investigated. This parasite has many morphological, physiological and ecological variations, including its infectivity and pathogenicity (Miles et al, 1978;. 1980; 2009), which may explain the various forms of clinical manifestation of Chagas disease observed in different geographic regions (Miles et al., 1981). Currently, the intraspecific nomenclature of *T. cruzi* is based on grouping populations into six discrete typing units (DTUs) TcI-TcVI (Zingales et al. 2009).

These *T. cruzi* genotypes differ in the success of development inside the digestive tract of different triatomine vectors (Brener, 1973; Garcia and Azambuja 1991). Previous studies have demonstrated that *T. cruzi* Y strain, classified as TcII, is not able to colonize the *R. prolixus* gut while *T. cruzi* Dm 28c clone, classified as TcI successfully infects *R. prolixus* (Azambuja et al., 2004; Vallejo et al., 2009; Zingales

et al., 2009). Many factors from the invertebrate host can be related to the parasite development including the activation of humoral immune responses and the influence of natural gut microbiota (Garcia and Azambuja 1991; Azambuja et al., 1999, Azambuja et al., 2004).

The humoral immunity in insects is composed by a number of effector molecules, rapidly activated after microorganism invasion. One important humoral response is the production of inducible antimicrobial peptides (AMPs) (Steiner et al., 1981; Ferrandon et al., 2007). AMPs are synthesized mainly by fat body cells, released in the hemolymph, with the ability to diffuse into the entire insect body, reaching sites of infection (Lamberty et al., 2001; Bulet and Stocklin 2005). AMPs are also produced in further insect tissues, including the gut epithelial cells where the parasites interact directly, inducing a local synthesis and release of these molecules (Charroux and Royet, 2010).

Analogous to the main part of animals, insects also present a rich natural gut microbiota which is essential for diverse functions in the host, like digestion or vitamin production (Garcia et al., 2010; Charroux and Royet 2010). These observations rises up an important question: how insects could manage the AMPs synthesis after possible infections while maintaining its intestinal flora population? Regarding *R. prolixus*-*T. cruzi* interaction, previous studies have shown that the infective *T. cruzi* Dm 28c strain caused a decrease in insect cultivable microbiota in contrast to the non-infective *T. cruzi* Y strain (Castro et al., 2012). Moreover, a differential *T. cruzi* susceptibility to a lytic activity from the bacteria *Serratia marcescens*, commonly present in *R. prolixus* midgut has been observed (Azambuja et al., 2004; Castro et al., 2007a). The data obtained in the present study suggest that the success of *T. cruzi* colonization in *R. prolixus* midgut depends on the parasite DTU and its capacity to interact with the natural vector microbiota. The perspective of the present work is to understand how the spatial and temporal synthesis of antimicrobial peptides in *R. prolixus* infected with different strains of *T. cruzi* could be related with parasite survival and gut microbiota modulation.

MATERIAL AND METHODS

Rhodnius prolixus maintenance and ethics statement

R. prolixus were maintained in a colony at Laboratório de Bioquímica e Fisiologia de Insetos, Instituto Oswaldo Cruz with controlled temperature and

humidity conditions. The insects were fed with defibrina ted rabbit blood provided by the *Centro de Criação de Animais de Laboratório* (Cecal) in an artificial apparatus, similar to that described in Azambuja & Garcia 1997. The rabbit blood was obtained according to the Ethical Principles in Animal Experimentation approved by the *Comissão de Ética no Uso de Animais do Instituto Oswaldo Cruz* (CEUA/IOC) under the protocol number L-0061/08, developed by *Conselho Nacional de Experimentação Animal/Ministério de Ciência e Tecnologia* CONCEA/MCT (http://www.cobea.org.br).

Trypanosoma cruzi **culture**

T. cruzi Dm 28c clone (Contreras et al., 1988) and *T. cruzi* Y strain (Silva & Nussenzweig 1953), previously classified as TcI and TcII, respectively (Zingales et al., 2009), were maintained as epimastigote forms at 28ºC in brain heart infusion (BHI) media (Sigma-Aldrich) supplemented with 10% heat-inactivated bovine fetal serum, as described in Azambuja and Garcia, 1997. For insect infection, the parasites were used in the exponential phase. The number of parasites was quantified on a Neubauer chamber.

Bacteria cultures

Staphylococcus aureus 9518, and *Escherichia coli* K12 4401 were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK. *Serratia marcescens* RPH was previously isolated from *R. prolixus* and maintained at Laboratório de Bioquímica e Fisiologia de Insetos. All bacteria were kept at -70°C in tryptone agar and 10% glycerol.

Rhodnius prolixus **oral infection**

Fifth instar nymphs were randomly chosen and fed with defibrinated rabbit blood containing *T. cruzi* epimastigotes Dm28 clone or Y strain. The blood complement system was previously heat inactivated by centrifugation at 1,890 x g for 15 min at 4ºC, and incubation of the plasma (supernatant) for 30 min at 55ºC. Subsequently the plasma was mixed with the erythrocytes and the parasites were added to the blood at a final concentration of 1 x 10^7 epimastigotes/mL. Control insects were fed on inactivated blood without parasites. Only full-engorged fifth-instar nymphs of *R. prolixus* were used for the experiments.

Quantification of *T. cruzi* in *R. prolixus* midgut

Insect midgut were dissected and macerated individually from five insects fully engorged in three independent *T. cruzi* infections (n=15). The midgut (all digestive tract) was collected and homogenized in 1.0 mL phosphate buffered saline (PBS) and the parasites were counted using a Neubauer hemocytometer, as described by Vieira et al. (2015).

Quantification of midgut microbiota in *R. prolixus* by colony forming unit (CFU)

Three pools of either anterior or posterior midgut were dissected separately from uninfected fifth instar nymphs or insects infected with different *T. cruzi* strains. Cultivable microbiota population were quantified by counting colony forming units (CFU) at 7 days after feeding (DAF) as described by Castro et al. (2012). The midgut samples were serially tenfold diluted with sterile PBS and 20 µL was spread on a Petri dish in sterile BHI agar (Sigma-Aldrich) culture medium. The plates were incubated at 30°C for 24 h and the CFU quantified. As a control PBS was plated to check the sterility of the experiments.

Hemolymph and midgut antibacterial detection

Pools of hemolymph, anterior and posterior midgut were dissected and prepared as previously described (Vieira et al., 2014, 2015). Briefly, hemolymph samples were pooled from 10 insects 5 DAF and diluted 1:1 in ultrapure water in sterile 1.5 ml tubes containing crystals of phenylthiourea in order to avoid melanisation. For midgut sample preparations, the anterior midgut (stomach) and posterior midgut (intestine) were collected separately at 7 DAF. All midgut preparations were collected in reaction tubes (3 pools of 3 insects), diluted in 200 µl ultrapure water. All samples were homogenized, centrifuged at 10,000 x g for 10 min at 4ºC, sterilized by 0.22 µm Millipore PVDF membrane filtration and stored at -20°C until use.

The bacteria (*S. aureus, E. coli* and *S. marcescens*) were grown by shaking (90 revolutions per minute) overnight in 20 ml of tryptone soy broth (TSB) at 30ºC and then cultured in new TSB for an additional 4 h in the same conditions. The bacteria were then washed in phosphate buffered saline (PBS, 0.01 M phosphate buffer, 2.7 mM potassium chloride and 0.137 M sodium chloride, pH 7.4) and resuspended in TSB to a final concentration of 1×10^4 cells/ml.

Antibacterial activity was assessed by turbidometric assays (TB) previously modified by Castro et al. (2012). Briefly, for TB assays, 10 µl of *E. coli, S. aureus* or *S. marcescens,* grown as described above, were incubated in each well of a sterile flat bottom 96-well microtiter plate (Nunc, Fisher Scientific, Leicestershire, UK) with 45 µl of sample (hemolymph, anterior midgut content or posterior midgut) plus 5 µl of peptone 10%, to a final concentration of 1% peptone, at 37°C for 19 h. The optical densities were recorded at a wavelength of 550 nm (OD_{550}) at hourly intervals using a Spectra Max 190 Plate Reader (Molecular Devices, Sunnyvale, California, USA). Control wells, run without midgut samples, contained 10 µl of bacteria in 1% peptone in ultrapure water. All data points were subsequently blanked against time zero to account for the opacity of the samples. In the case of anterior midgut TB assays, samples were also incubated in the plate without bacteria to observe the change in sample colour after 19 h and the readings obtained were subtracted from the samples incubated with bacteria to ensure that the difference in readings were related to antibacterial activity. Then, the readings for the bacteria, *E. coli, S. aureus* or *S. marcescens,* were subtracted from all sample readings to obtain the antibacterial activity value. All experiments were carried out at least in triplicate (n=9 insects).

Quantification of antimicrobial peptides gene expression (RT-qPCR)

Three pools of each five anterior midguts, posterior midguts and fat bodies were dissected from insects at 1 and 7 DAF (*T. cruzi* infected and non-infected) as previously described (Vieira et al., 2014). Total RNA was extracted using a NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Synthesis of cDNA was carried out with a First-Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK) following the manufacturer's protocol using 2.5 µg of total RNA. cDNA was quantified by fluorescence, using a Qubit Fluorimeter (Life Technologies) with the ssDNA assay kit. The AMPs genes in tissues of *R. prolixus* infected with *T. cruzi* were quantified by the comparative Ct ($\Delta\Delta$Ct) method (Livak & Schmittgen, 2001) normalized with the *R. prolixus* reference genes α-tubulin and GAPDH. Real-time quantitative polymerase chain reactions (RT-qPCR) were conducted using an ABIPRISM 7500 Sequence Detection System (Applied Biosystems) at Health-

PDTIS/FIOCRUZ facilities (Real-Time PCR Platform RPT-09A). The present study analyzed the gene expression of three *R. prolixus* defensins (*defA*, *defB* and *defC*) and prolixicin (*prol*). The primers of each AMP genes used here and the *R. prolixus* reference genes were used as previously published or designed based on the respective sequence (Tab. 1) (Lopez et al., 2003; Ursic-Bedoya 2011; Paim et al., 2012). Each reaction was run in duplicate for each pool of insects (n=3). Each well contained 10 ng of diluted cDNA, each pair of primer (0.25 µM) and the qPCR master mix DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific, EUA), at a final volume of 20 µl. The cDNA was amplified at 95°C for 10 min followed by 40 cycles of 95°for 15 s and 60°C for 1 min. As negative controls, PCR reactions were carried out without cDNA template to verify primer-dimer formation or contamination in the reactions. A melting curve analysis was carried out to confirm that only a single product was amplified for each target.

RESULTS

Quantification of *T.cruzi* in *R. prolixus* midgut

Parasite population in *R. prolixus* 5th instar nymphs whole midgut were quantified from 2 to 7 days after feeding. It was observed that 2 DAF, the average concentration of *T. cruzi* Dm 28c (75 x 10^4 parasites/mL) was ten times higher than the amount of *T. cruzi* Y (7.5 x 10^4 parasites/mL) in the insect gut (Figure 1). In the fifth DAF it was detected a decrease in the parasite number from both *T. cruzi* strains, although the number of *T. cruzi* Dm 28c was 2 times higher than *T. cruzi* Y(Figure 1). On the seventh DAF, there was an increase in *T. cruzi* Dm 28c population in the insect gut (Figure 1). Regarding the *T. cruzi* Y strain, was not possible to detect any parasite in the *R. prolixus* gut samples analyzed in this period.

Analysis of *R. prolixus* midgut microbiota - Colony forming unit (CFU)

Cultivable bacterial microbiota population in 5^{th} instar nymphs infected with *T. cruzi* was evaluated separately in anterior and posterior midgut using CFU counts. The infection with both *T. cruzi* strains induced a significantly reduction on bacterial population in *R. prolixus* anterior midgut at 7 DAF. However, *T. cruzi* Dm 28c caused

a stronger CFU reduction (2.5 x 10^8 CFU/mL – 26-fold less) than *T. cruzi Y* (1.64 x 10^9 CFU/mL – 4-fold less) in comparison with uninfected insects (6.57 x 10^9 CFU/mL) (Figure 2). *T. cruzi* infection did not alter significantly the CFU counts in the posterior midgut, comparing with control insects.

Hemolymph and midgut antibacterial detection

In order to detect the presence of *R. prolixus* inducible humoral immune factors indirectly, the antibacterial potential of distinct tissues of 5th instar nymphs infected with *T. cruzi* was analysed *in vitro* by incubating the hemolymph, anterior and posterior midgut samples collected 5 and 7 DAF with different bacterial strains.

The antibacterial potential from *R. prolixus* hemolymph was demonstrated using TB assays. It was observed that the hemolymph of control insects already possess an antibacterial activity to significantly reduce the growth of Gram-negative and Gram-positive bacteria (Figure 3A, 3B). Hemolymph from both *T. cruzi* infected insects groups presented a significantly higher bacteria growth inhibition than hemolymph from control insects (Figure 3A, 3B).

The optical densities recorded from *R. prolixus* anterior midgut incubations with different bacteria showed that only *T. cruzi* Dm 28c infection induced a significantly increase of antibacterial activity against *S. marcescens* ($p < 0.001$) (Figure 4B). The activities measured against *E. coli* in infected insects with both *T. cruzi* strains were statistically not significant in comparison to the control insects (Figure 4A).

Antibacterial assays of *R. prolixus* posterior midgut samples demonstrated that only *T. cruzi* Dm 28c infection induced a sensible increase in antibacterial activity against *E. coli* ($p < 0.1$) (Figure 5A). No differences were recorded about the activities against *S. aureus* in infected insects with both *T. cruzi* strains in comparison with the controls insects in this same midgut compartment (Figure 5B).

Quantification of antimicrobial peptides gene expression (RT-qPCR)

The modulation in the expression of these genes of *R. prolixus* 5th instar nymphs infected with *T. cruzi* Dm 28c and *T. cruzi* Y was verified in different tissues

at 1 and 7 DAF. All obtained data were related to the gene expression in control insects, considered as the value 1.0, represented in the figures as dotted horizontal lines on the Y axis.

Concerning the *prol* gene expression, both *T. cruzi* strains significantly upregulated the transcript abundance in the hemolymph at 7 DAF in comparison to control insects (Figure 6A). However, only *T. cruzi* Dm 28c infection was able to modulate the *prol* expression in *R. prolixus* midgut, but with distinct patterns in the different compartments. In the anterior midgut *prol* transcript levels were significantly lower in infected insects than in controls at 1 DAF. At 7 DAF the *prol* transcript levels were increased in 2.5-fold in this midgut compartment of *T. cruzi* Dm 28c infected insects (Figure 6B).The opposite pattern was observed in posterior midgut. The *prol* expression was about 10-fold higher in Dm 28c infected insects at 1 DAF, while at 7 DAF the levels of *prol* transcripts were downregulated, at the same levels observed in control insects (Figure 5C). Additionally, *T. cruzi* Y infected insects presented *prol* gene expression similar to control insects in the midgut at 1 and 7 DAF (Figure 6B, C).

Regarding *defA* and *defB*, it is important to emphasize that it was not possible to detect the expression of these genes in the posterior midgut of control or infected insects. Formation of primer dimers in control or infected insects was observed in all experimental conditions tested during the present study.

Infection of *R. prolixus* with both *T. cruzi* strains induced a downregulation *defA* levels at 1 DAF in the fat body (Figure 7A). At 7 DAF a significant 2-fold increase in *defA* levels in *T. cruzi* Dm 28c infected insects was detected, while the *T. cruzi* Y infected insects presented the same gene levels of control (Figure 7A). In the anterior midgut, only the infection with *T. cruzi* Y was able to modulate the *defA* transcript levels, which were significantly lower than in control insects (Figure 7B).

The ingestion of *T. cruzi* Y induced a downregulation of *defB* levels at 1DAF in *R. prolixus* fat body and midgut (Figure 8A, B). On the other hand, *T. cruzi* Dm 28c was capable to significantly upregulate *defB* in anterior midgut, but only 1 DAF (Figure 8B). It was not possible to detect defB genes in the anterior midgut 7 DAF with both *T. cruzi* strains (Figure 8B).

Concerning *defC*, both *T. cruzi* strains, 1 DAF, strongly induced its superexpression in insect fat body. While *T. cruzi* Dm 28c increased *defC* levels in 11 times fold, *T. cruzi* Y increased *defC* in 24 times fold in comparison with non-infected insects (Figure 9A). In the anterior and posterior midgut, just *T. cruzi* Dm 28c

significantly upregulated *defC* levels 7DAF (Figure 9B, C), whereas *T. cruzi* Y downregulated these genes in posterior midgut 7DAF (Figure 9C).

DISCUSSION

T. cruzi is recognized as an extremely variable parasite, which displays diverse levels of susceptibility that depend on its genotype to triatomine bugs. TcI can successfully infect the digestive tract of *R. prolixus*, while those *T. cruzi* DTUs classified as TcII are apparently eliminated from the insect midgut or are not able to produce metacyclic trypomastigote forms, respectively (Mello et al., 1996; Azambuja et al., 2004; Araújo et al., 2014). Moreover, it is known that some bacterial species that compose the insect natural microbiota can influence the parasite development (Azambuja et al., 2005; Weiss & Aksoy, 2011; Gendrin & Christophides, 2013) as it seems to be the case for some *T. cruzi* strains inside the vector. In *R. prolixus*, Castro et al. (2012) demonstrated that *T. cruzi* Dm 28c (TcI) induces a significant reduction on the cultivable microbiota in the insect's digestive tract of *R. prolixus*, an effect that was not observed after *T. cruzi* Y strain (TcII) infection. Accordingly, these two *T. cruzi* strains present different profile of susceptibility to the lytic activity of *S. marcescens in vitro* in which *T. cruzi* Dm 28c has been more resistant than *T. cruzi* Y (Azambuja et al., 2004; Castro et al., 2007). These results suggest that the capacity of *T. cruzi* to colonize the *R. prolixus* midgut depends on their genotypic characteristics combined with their ability to modulate (directly or indirectly) the host natural microbiota.

In this context, the present study attempts to understand how TcI and TcII infection modulates the AMPs gene expression and the antibacterial activity in different insect tissues, relating the parasite survival with microbiota manipulation. The cultivable *R. prolixus* microbiota was quantified in both midgut compartments and it was confirmed that the higher numbers of bacterial population are located in the anterior midgut. The infection by both *T. cruzi* genotypes stimulated a decrease in the microbiota, however *T. cruzi* Dm 28c was capable to induce a stronger CFU reduction than *T. cruzi* Y strain. These observations corroborate previous results (Castro et al., 2012). Nevertheless, in the present study, a noticeable suppression of microbiota only occurred in the anterior portion of the midgut.

Many studies describe how the intestinal microbiota of insect vectors affects the life cycle of parasites. It has been shown that native bacteria in the midgut of *Anopheles* are able to negatively impact certain species of *Plasmodium* by direct

contact between the involved microorganisms and by the induction of the immune response mediated by commensal bacteria (Dong et al., 2009; Meister et al., 2009; Cirimotich et al., 2011). In contrast, parasitic infection can modulate the immune peptide synthesis in the host (Boulanger et al., 2004; Telleria et al., 2013; Vieira et al., 2015) which can interfere in the growth of certain bacteria species from insect microbiota, as it seems to occur in *Trypanosoma rangeli* infected *R. prolixus* (Vieira et al, 2015).

In general, it seems that the AMPs genes are suppressed and induced in a complex profile that varies between the insect tissues and depending on the *T. cruzi* infecting genotype. It is considered that the AMPs modulation observed 24 hours after parasite ingestion could correspond to a rapid response of the insect in an attempt to control the spread of the invading pathogen. Additionally, the induced fat body AMPs gene expression represent a systemic response to the infection, wherein the AMPs are secreted directly into the hemolymph, from where it can diffuse throughout the insect body (Bulet & Stocklin, 2005). In the other hand, AMPs expression on intestinal epithelial cells illustrates a local immune response activated by direct contact of the parasites with the insect tissue (Tzou et al., 2000; Bulet & Stocklin, 2005). Invasion of *R. prolixus* midgut by both *T. cruzi* genotypes triggered a rapidly enhance of transcriptional levels of *defC* genes in the insect fat body, although TcII has stimulated a much more intense expression of this gene than TcI. High levels of mature DefC in the fat body of infected insects might be directly related to the increased antibacterial activity detected in the hemolymph against both Gram-positive and Gram-negative bacteria *in vitro*.

It has been shown that the presence of bacteria or parasites in the digestive tract stimulates the systemic secretion of AMPs in the hemolymph of different insects, such as *Phlebotomus*, *Glossina* and *Drosophila*, even without invasion of these microorganisms of the hemocoel (Boulanger et al., 2001; 2002; Hao et al., 2001). The same pattern was also observed in *T. cruzi* infection in the insect vector *R. prolixus* in the present study. The activation of AMP expression on the hemolymph by parasites confined to the midgut could be due to immune signaling by molecules such as nitric oxide, (NO) representing a host anticipation strategy, in order to prevent a widespread infection (Hao et al., 2001; 2003).

Regarding *T. cruzi* Dm 28c (TcI) cycle in *R. prolixus*, it is known that until the fifth day after infection, the epimastigotes a higher abundant in the anterior midgut (stomach), and thereafter, tend to migrate to the posterior midgut, although rare

epimastigotes and intermediate forms could be detected in the insect stomach later on (Cortez et al., 2012). One day after TcI infection, we observed reduced levels of *prol* in the anterior midgut while *defB* and *prol* levels are increased in anterior and posterior midgut, respectively. Moreover, TcII infection is not capable to stimulate a general AMP upregulation, it only induced an intense *defB* suppression in the stomach of *R. prolixus*. These results show that the insect systemic response to parasite seems to be similar, though local responses to TcI and TcII infection exhibit differing profiles.

The success of *T. cruzi* infection was confirmed by parasite visualization in insect digestive tract samples under an optical microscope. Seven days after parasite ingestion it was not possible to detect *T. cruzi* Y strain in the insect gut, in contrast to Dm 28c that was present, a fact already observed in previous studies (Azambuja et al., 2004). However, it is not possible to state that the parasite was eliminated from the insect digestive tract, at least it seems that TcI developed more successful strategies that TcII in order to survive in the *R. prolixus* intestinal tract. In agreement, the return of normal *defC* levels 7 days after feeding observed in the fat body of *T. cruzi* Y infected insects may be explained by the extinction of parasites in *R. prolixus* midgut. An exacerbated activation of *defC* by TcII could play a role in parasite control and could have negative impact the host fitness, signaling the genetic machinery stop the gene expression to maintain the insect immune homeostasis. In contrast, seven days after TcI infection the levels of *defC* in *R. prolixus* fat body remains higher in comparison to control insects, but less intense as observed after 24 hours in *T. cruzi* Y strain infected insects.

Regarding the local AMPs expression in the midgut epithelium of *R. prolixus* seven days after infection, we observed that TcI stimulates a significant enhancement of *defC* and *prol* expression in the anterior midgut and *defC* in the posterior midgut, while TcII does not induce expression of any AMP studied. These observations suggest that *defC* is upregulated in response to the presence of *T. cruzi* Dm 28c once from the seventh day after infection, parasites can be detected both in the stomach and in the intestine of *R. prolixus* (Cortez et al., 2012). Interestingly, *T. rangeli* Macias strain infection also upregulated *defC* levels in the midgut compartments in which the parasite was found (Vieira et al., 2015). Similarly, the parasite *P. berghei* modulates the defensin expression in the gut and salivary gland epithelium of *Anopheles gambiae*, compartments in which the parasite can be found during its life cycle (Dimopoulos et al., 1998). *Leishmania major* infection also

induces defensin expression in *Phlebotomus duboscqi* in the hemolymph and midgut of the insect (Boulanger et al., 2004), although infection by *Leishmania mexicana* abolished *def1* expression in *Lutzomyia longipalpis* (Telleria et al., 2013). These results support the hypothesis that defensins genes are modulated by protozoan infection in the insect gut.

In *Triatoma brasiliensis* four defensin encoding genes (*def1-4*) were identified so far (Araújo et al., 2006, 2009). After the analysis of the primary defensin structure and transcript abundance of defensin encoding genes in *Triatoma brasiliensis* Waniek et al. (2009) proposed different tasks for those characterized defensins. In *R. prolixus* the situation appears to be similar. Regarding *defA* and *defB*, the absence of the expected PCR product in the posterior midgut of control or infected insects, observed the melting curve analysis, indicates that this tissue is not responsive to these genes, at least in *T. cruzi* infected insects. However, Gram-positive bacteria infection upregulates *defA* and *defB* expression in *R. prolixus* midgut (Vieira et al., 2014). In contrast, *defC* is the only AMP encoding gene which is upregulated in the fat body one day after feeding with both *T. cruzi* genotypes.

Although it was not possible to quantify the protein levels of DefC in neither *R. prolixus* midgut nor hemolymph, antibacterial activity assays in these tissues shows that after *T. cruzi* infection possibly occurs an enhancement of antibacterial molecules synthesis in different ways. The inability to stimulate over expression of AMPs in the gut corroborates the results that show that TcII infection cannot induce an increase in antibacterial activity in *R. prolixus* anterior midgut against *S. marcescens*, different that observed with TcI.

Increased expression of *defC* and *prol* in the stomach of TcI infected insect may be associated with high antibacterial activity detected in the same compartment against *S. marcescens in vitro*. These results seem to have a direct relationship with the drastic reduction of microbiota observed, which increased DefC synthesis in the anterior midgut of *R. prolixus* in response to *T. cruzi* infection Dm 28c seems to favor the parasite development to the disadvantage of insect microbiota population.

The prolixicin gene seems to be downregulated according to the local concentration of epimastigotes, as also observed in experimental infections with *T. rangeli* Macias, a strain which successfully infect *R. prolixus* midgut (Vieira et al., 2015). Prolixicin was recently identified in *R. prolixus* hemolymph and shows structural similarities with another AMP family, the attacins (Ursic-Bedoya et al., 2011). Both attacin as prolixicin presented high toxicity against the Gram-negative

bacteria *E. coli* (Hu & Aksoy et al., 2005; Ursic-Bedoya et al., 2011) suggesting that the structural similarity between these AMPs may also extend to their microbial targets. The influence of attacins on *Trypanosoma brucei* establishment in the tsetse fly has been demonstrated. It was verified that *T. brucei* infection upregulated attacin genes in the fat body and midgut of the fly. In addition, insects naturally resistant to *T. brucei* infection present high levels of attacin even in non-infected flies, suggesting that this AMP could interfere on *T. brucei* development (Hu & Aksoy, 2005; Hu & Aksoy, 2006). As prolixicin share similar characteristics with attacins, including their bacteriological targets, it is possible that prolixicin also play a toxic role against trypanosomatids. It is suggested, therefore, that suppression *Prol* genes may represent one mechanism to ensure the survival of trypanosomatids in the insect gut.

In the presente work it was possible to observe that T. cruzi Y was not able to unable to induce the AMPs synthesis in the insect stomach, that may be relate to an insufficient microbiota reduction, which consequently adhered to the *T. cruzi* Y strain inducing their lysis. The higher susceptibility of *T. cruzi* Y to lytic activity of *S. marcescens,* a common bacteria present in *R. prolixus* digestive tract, in comparison with *T. cruzi* Dm 28c was demonstrated previously (Azambuja et al., 2004; Castro et al., 2007). In this context, one of the possible factors that limit TcII development in *R. prolixus* is related to its incompetence in inducing antibacterial molecules in midgut that are capable to reduce harmful bacteria to the parasite.

Currently, efforts to control human diseases transmitted by insects are focused on vector control, specifically in the construction of genetically modified insects that inhibit or express molecules that interrupt the development of their natural parasites (Hurwitz et al, 2011; Taracena et al., 2015). The knowledge about how different genotypes of *T. cruzi* modulate the antimicrobial peptides expression in the insect, can assist in selecting genes to be manipulated in *R. prolixus*, seeking a future tool for the control of Chagas disease.

T. cruzi is a genetically variable parasite to such an extent that currently is separated into six genetic lineages (Tc-TcVI) or discret typing units (DTUs) which emerged in two hybridization events and from which TcI and TcII (parental genotypes) are most widely distributed in Brazil (Araújo et al., 2009, Zingales et al., 2009). Among others, the genetic variability of *T. cruzi* is expressed in different composition of cell surface molecules such as sugars and proteins (De Souza, 1995). It was demonstrated that *T.cruzi* populations belonging to TcI genotype presented more galactose sugar residues in its cell surface than TcII populations (Araújo et al.,

2002). Membrane proteins like the virulence factor trans-sialidase are also differently distributed in TcI and TcII genotypes (Burgos et al., 2013). Other membrane-bound proteins like mucins interact with the insect tissue and influence development and infectivity (Gonzalez et al., 2013). The results in the present study corroborate these observations, showing significantly different induction by TcI and TcII of AMP gene expression in intestine and fat body of *R. prolixus*. It is probable that the *T. cruzi* membrane structures play an important role in the recognition of the parasite, and induction of specific AMP expression and other responses. Since *R. prolixus* is predominantly found infected with TcI it is possible that this DTU is the best adapted, modulating the intestinal immune response to favor his optimum growth and development. This interaction of *T. cruzi* membrane components with tissues of the insect might be one factor of the modulation of the triatomines immune system.

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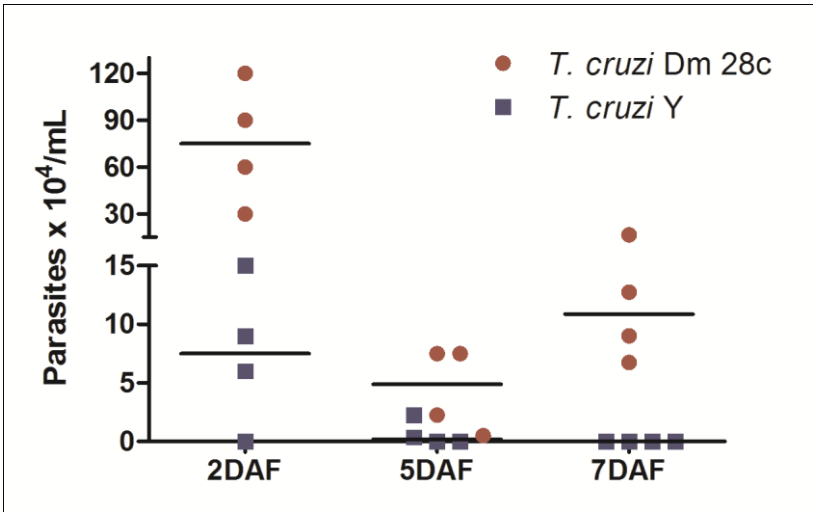

Figure 1: Parasite population in *Rhodnius prolixus* midgut. *T. cruzi* Dm 28c and *T. cruzi* Y numbers were quantified in *R. prolixus* 5th instar nymphs midgut at different days after feeding. Each point represents the number of parasites in an individual digestive tract, and bars indicate the median.

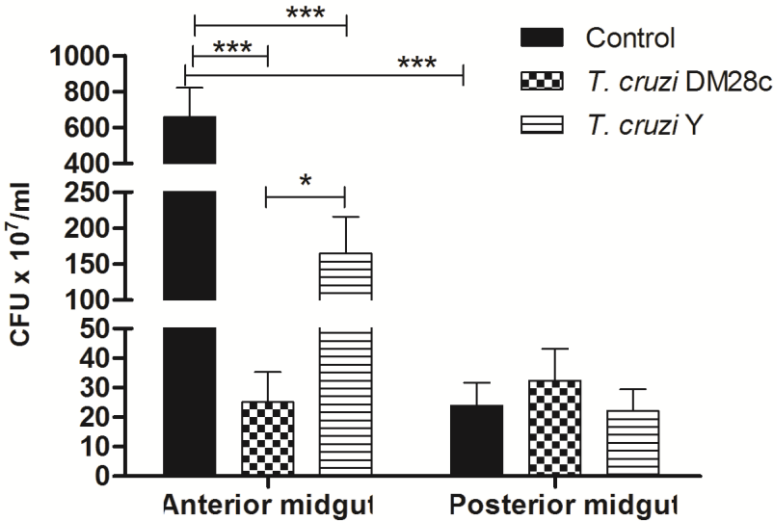

Figure 2: Bacteria population in *Rhodnius prolixus* midgut infected with two strains of *Trypanosoma cruzi.* Colony forming units (CFU) counts were made 7 days after feeding in anterior and posterior midgut. Treatments: black columns – uninfected insects; grid columns –*T. cruzi* Dm 28c infected insects; striped columns - *T. cruzi* Y infected insects. Bars represent mean ± SEM of three independent experiments with three pools of insects (n=9). Means were compared using Student T-test or Mann-Whitney test; *** $p < 0.001$, * $p < 0.05$.

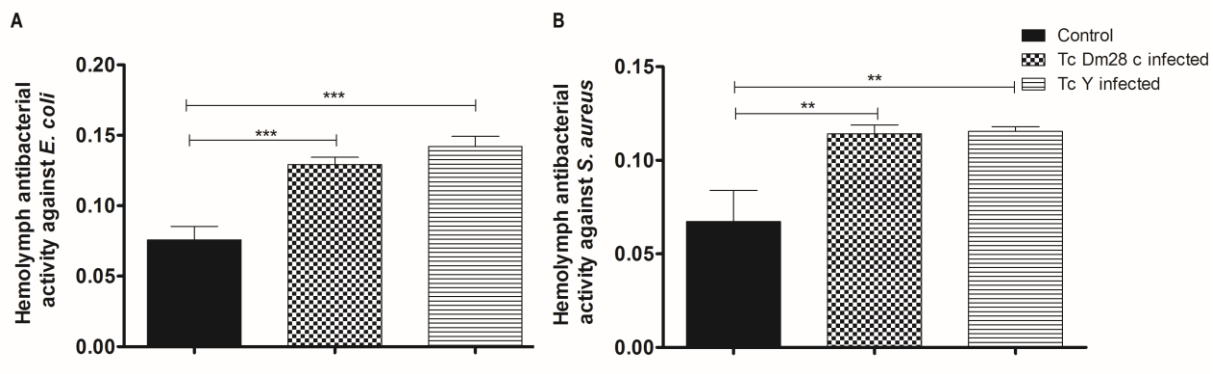

Figure 3: Antibacterial activity in hemolymph samples of *Rhodnius prolixus* infected with two strains of *Trypanosoma cruzi.* The antibacterial activities from *R. prolixus* 5th instar nymphs hemolymph 5 days after infection with different strains of *T. cruzi* were tested against (A) - *E. coli* (B) *S. aureus*. Treatments: black columns – antibacterial activity in uninfected insects hemolymph; grid columns – antibacterial activity in *T. cruzi* Dm 28c infected insects hemolymph; striped columns - antibacterial activity in *T. cruzi* Y infected insects hemolymph. Fifth instar nymphs were fed on inactivated blood with or without parasites (1 x 10^6 epimastigotes/mL). Antibacterial activity was measured through optical densities using the turbidometric assay (OD_{550} nm) after 19 h incubation of hemolymph samples with Gram-negative and Gram-positive bacteria. Each bar represents 3 experiments in triplicate wells (n=9). Means were compared using one-way ANOVA and Student T-test; *** $p < 0.001$ and ** $p < 0.01$.

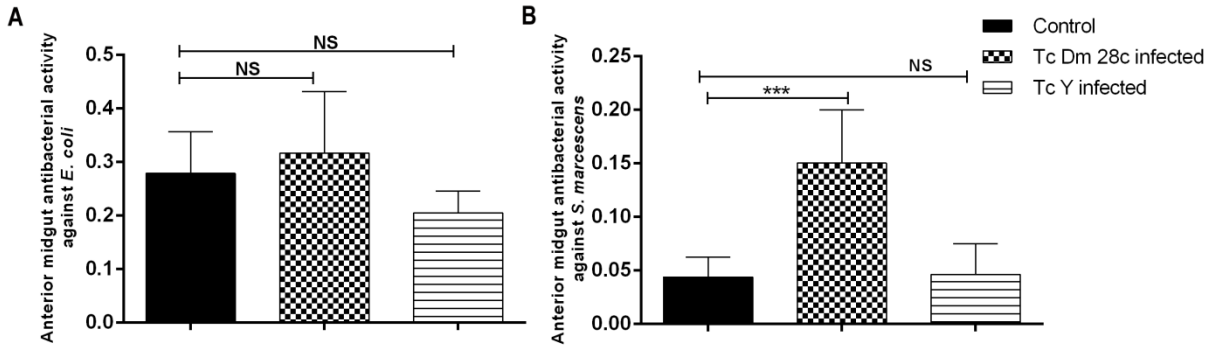

Figure 4: Antibacterial activity in the anterior midgut of *Rhodnius prolixus* infected with two strains of *Trypanosoma cruzi.* The antibacterial activities from *R. prolixus* 5th instar nymphs anterior midgut 7 days after infection with different strains of *T. cruzi* were tested against (A) - *E. coli* (B) *S. marcescens*. Treatments: black columns – antibacterial activity in anterior midgut of uninfected insects; grid columns – antibacterial activity in anterior midgut of *T. cruzi* Dm 28c infected insects; striped columns - antibacterial activity in anterior midgut of *T. cruzi* Y infected insects.). Means were compared using one-way ANOVA and Student T-test; *** $p < 0.001$. NS = not significant.

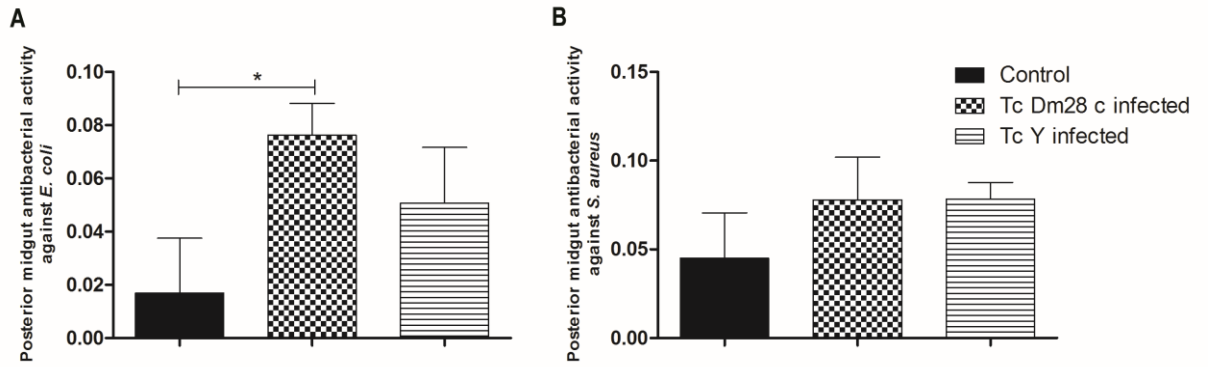

Figure 5: Antibacterial activity in the posterior midgut of *Rhodnius prolixus* infected with two strains of *Trypanosoma cruzi.* The antibacterial activities from *R. prolixus* 5th instar nymphs posterior midgut 7 days after infection with different strains of *T. cruzi* were tested against (A) - *E. coli* (B) *S. aureus*. Treatments: black columns – antibacterial activity in posterior midgut of uninfected insects; grid columns – antibacterial activity in posterior midgut of *T. cruzi* Dm 28c infected insects; striped columns - antibacterial activity in posterior midgut of *T. cruzi*

Y infected insects.). Means were compared using one-way ANOVA and Student T-test; *** p < 0.001.

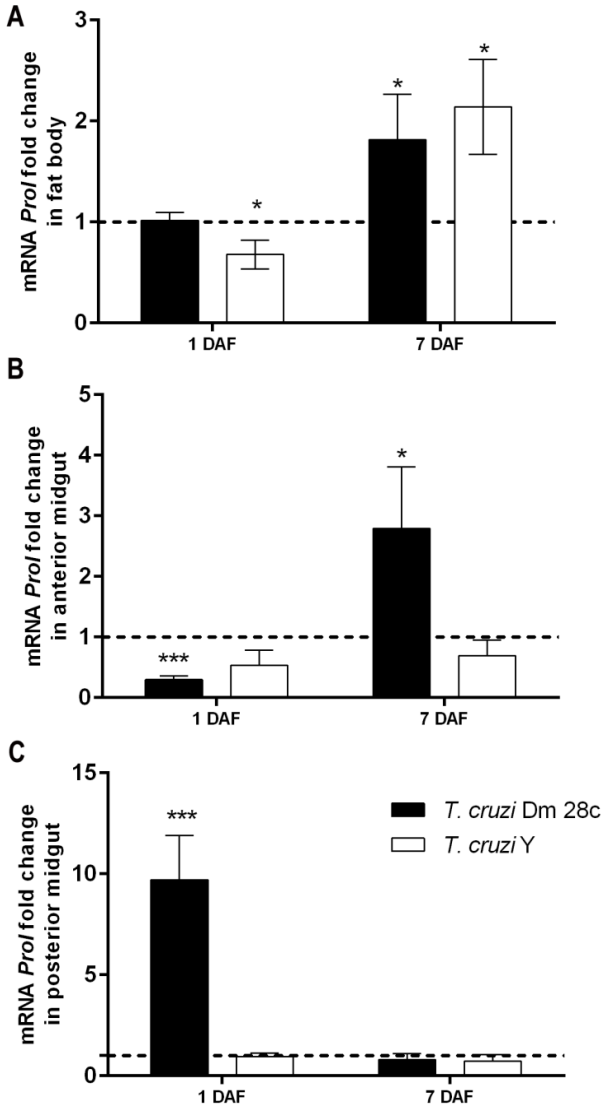

Figure 6: Spatial and temporal *prolixicin* relative gene expression in *Rhodnius prolixus* orally infected with *Trypanosoma cruzi*. Data were quantified using the gene expression of uninfected insects as the calibrator represented by the dotted horizontal line on each graphic. Data is shown as *prol* fold increase over this value. Treatments: black columns – *prol* relative expression in insects infected with *T. cruzi* Dm 28c; white columns - *prol* relative expression in insects infected with *T. cruzi* Y. Means were compared using one-way ANOVA and Student T-test; *** p < 0.001, * p < 0.1.

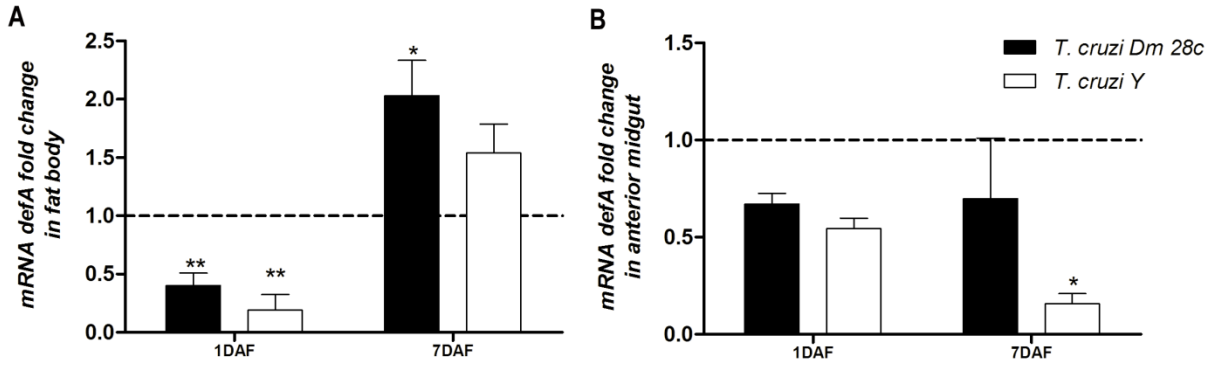

Figure 7: Spatial and temporal defensin A relative gene expression in *Rhodnius prolixus* orally infected with *Trypanosoma cruzi*. Data were quantified using the gene expression of uninfected insects as the calibrator represented by the dotted horizontal line. Data is shown as *defA* fold increase over this value. Treatments: black columns – *defA* relative expression in insects infected with *T. cruzi* Dm 28c; white columns - *defA* relative expression in insects infected with *T. cruzi* Y. Means were compared using one-way ANOVA and Student T-test; ** $p < 0.01$, * $p < 0.1$.

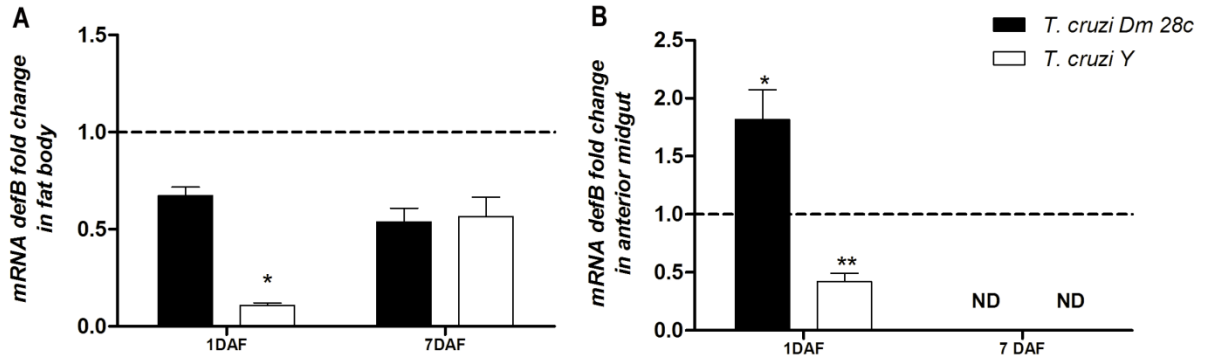

Figure 8: Spatial and temporal *defensin B* relative gene expression in *Rhodnius prolixus* orally infected with *Trypanosoma cruzi*. Data were quantified using the gene expression of uninfected insects as the calibrator represented by the dotted horizontal line. Data is shown as *defB* fold increase over this value. Treatments: black columns – *defB* relative expression in insects infected with *T. cruzi* Dm 28c; white columns - *defB* relative expression in insects infected with *T. cruzi* Y. Means were compared using one-way ANOVA and Student T-test; ** $p < 0.01$, * $p < 0.1$, ND = not determined.

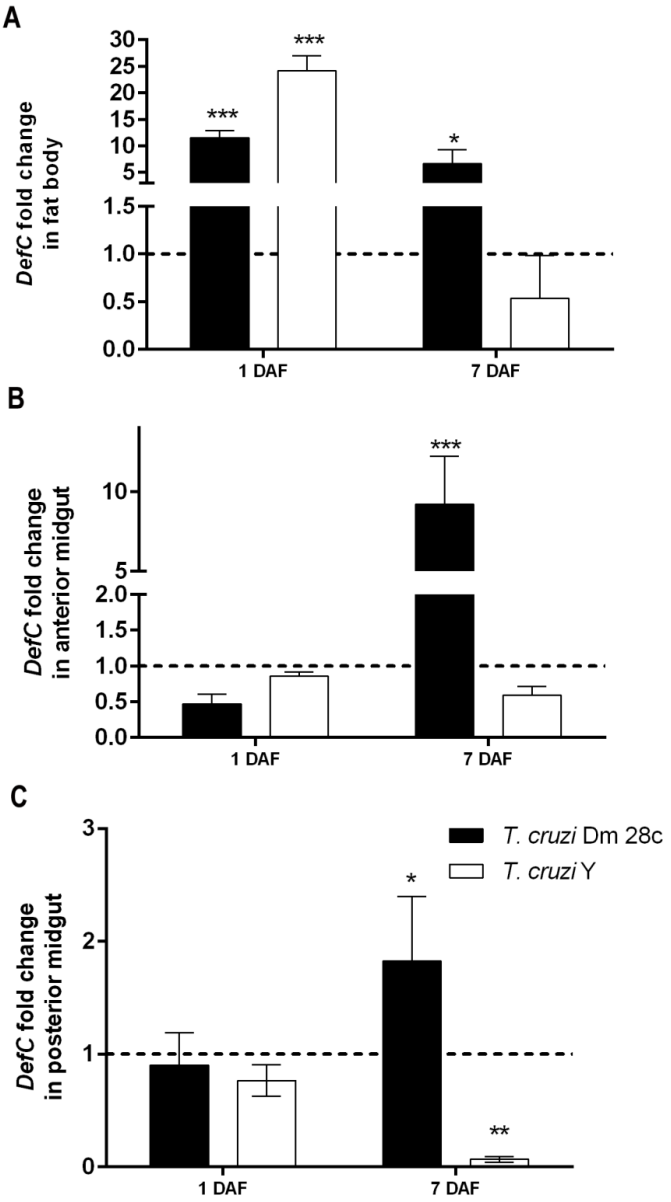

Figure 9: Spatial and temporal *defensin C* relative gene expression in *Rhodnius prolixus* orally infected with *Trypanosoma cruzi*. Data were quantified using the gene expression of uninfected insects as the calibrator represented by the dotted horizontal line. Data is shown as *defC* fold increase over this value. Treatments: black columns – *defC* relative expression in insects infected with *T. cruzi* Dm 28c; white columns - *defC* relative expression in insects infected with *T. cruzi* Y. Means were compared using one-way ANOVA and Student T-test; *** $p < 0.001$** $p < 0.01$, * $p < 0.1$.

4 DISCUSSÃO

O sucesso da relação parasita-vetor deriva de inúmeras e complexas interações moleculares entre os organismos envolvidos, que seguramente está relacionado com a história evolutiva das espécies, permitindo a adaptação entre elas (Janzen, 1980; Lymbery, 2015). O sucesso dessa interação depende tanto da tolerância do hospedeiro aos fatores de virulência do parasita, bem como da estimulação de uma resposta imune moderada, que não elimine toda a população do patógeno (Janzen, 1980; Vilcinskas, 2010). Nessa conjuntura, a microbiota intestinal dos insetos é um fator adicional a ser considerado na relação parasita-vetor, uma vez que pode influenciar a fisiologia do inseto e ainda interferir diretamente no ciclo de vida dos parasitas (Azambuja et al., 2004). Um parasita bem sucedido precisa superar as barreiras fisiológicas e imunológicas do inseto e ainda competir com a microbiota natural do hospedeiro (Eichler & Schaub, 2002; Azambuja et al., 2005; Garcia et al., 2010).

A interação entre a tríade parasita-inseto vetor-microbiota, além de complexa, é peça fundamental para o entendimento do sucesso na transmissão de determinadas cepas causadoras de doenças aos homens e animais (Azambuja et al., 2005; Weiss & Aksoy, 2011). Nesse contexto, um aspecto de extrema relevância é a ativação do sistema imune do inseto, especialmente como ele responde de forma a limitar o desenvolvimento de parasitas, enquanto ainda sustenta a microbiota nativa, essencial para manutenção de sua homeostase intestinal (Azambuja et al., 2004). A imunidade de insetos é composta por uma intrincada rede de respostas celulares e humorais, que são diferencialmente ativadas, dependendo do microrganismo invasor (Lemaitre & Hoffmann, 2007; Ferrandon et al., 2007; Garcia et al., 2010). O conhecimento detalhado do sistema imune, bem como a compreensão dos mecanismos de ativação de respostas frente às infecções por variados microrganismos é primordial para o esclarecimento da interação parasito-vetor-microbiota. A elucidação desses elementos, ainda bastante complexos, amplia as possibilidades de adoção de novas estratégias que auxiliem no controle da transmissão de microorganismos causadores de doenças por insetos vetores.

Alguns trabalhos vêm demonstrando que a produção de AMPs pode influenciar a interação de parasitas em seus insetos vetores, bem como controlar a proliferação excessiva da microbiota natural do tubo digestivo (Lowenberger et al., 1996, Lowenberger, 2001; Vizioli et al., 2001; Boulanger et al., 2006; Charroux & Royet,

2010). Nessa perspectiva, o presente trabalho teve como objetivo o estudo da resposta imune humoral de *R. prolixus*, um dos principais vetores do *T.cruzi* e *T. rangeli* na América Latina (Vallejo et al., 2009; Coura, 2015) em abordagem especial à modulação de peptídeos antimicrobianos (AMPs) no tubo digestivo de insetos infectados oralmente com bactérias Gram-positiva e Gram-negativas, bem como tripanosomatídeos. Além disso, foi verificado o efeito da infecção por tripanosomas sobre a microbiota intestinal de *R. prolixus*.

No presente estudo, analisamos a indução de fatores humorais nos diferentes compartimentos tubo digestivo do inseto e constatamos que as atividades antibacterianas das amostras do intestino médio anterior (estômago) foram superiores as observadas no intestino médio posterior (intestino) tendo sido verificadas as maiores atividades entre 7-9 dias (Vieira et al., 2014 - artigo 1). Estes resultados corroboram os encontrados por Castro et al., 2012. Analisando a abundância de AMPs no tubo digestivo de insetos em jejum e alimentados, verificamos que o repasto sanguíneo não infectivo foi capaz de induzir a transcrição de vários genes codificadores de AMPs. Adicionalmente, esses genes induzidos alcançaram seus maiores níveis no sétimo dia após a ingestão de sangue. Particularmente, o estômago apresentou maiores níveis de lisozimas e de defensinas do que os encontrados no intestino (Vieira et al., 2014 – artigo 1), confirmando a ideia de que cada compartimento do intestino médio possui distintas características fisiológicas e imunológicas (Lehane, 2005).

Sabe-se que a alimentação sanguínea é fator essencial para a ativação de diversos processos fisiológicos de *R. prolixus*, inclusive a resposta imune, seja pelos nutrientes provenientes da digestão sanguínea, tal como a hemoglobina, como pela dilatação do intestino médio anterior (Azambuja et al., 1997). Em contrapartida, o aumento da fonte nutritiva derivada da digestão do sangue, estimula a multiplicação da microbiota intestinal do inseto, especialmente a população de *Serratia marcescens* (Azambuja et al., 2004; Castro et al., 2007a).

Em contraste com o intestino, o estômago dos triatomíneos é o compartimento onde reside a maior concentração da comunidade microbiana do inseto (Azambuja et al., 2004; Waniek et al., 2011). A tendência em colonizar o estômago pelo simbionte *Rhodococcus rhodnii* foi anteriormente observada por Eichler e Schaub em 2002. Além disso, Castro e colaboradores em 2012 notaram que a densidade bacteriana cultivável do tubo digestivo de *R. prolixus* tende a crescer a partir do repasto sanguíneo do inseto, alcançando seu auge populacional por volta do oitavo dia após

a alimentação, seguida de uma queda gradual. Em vista do aumento expressivo da microbiota bacteriana verificado previamente por outros autores no intestino médio anterior de *R. prolixus* (Eichler & Schaub, 2002; Azambuja et al., 2004, Castro et al., 2012), é interessante observar a coincidência dos picos de atividade antibacteriana e dos altos níveis de expressão dos AMPs e de lisozimas no sétimo dia após a alimentação, indicando o sentido de normalizar os níveis homeostáticos da microbiota natural do inseto (Vieira et al., 2014 – artigo 1).

Da mesma forma, foi demonstrado que em *Drosophila*, a expressão gênica de AMPs é estimulada frente ao crescimento exacerbado da microbiota natural da mosca, e que esta ocorre no local específico da proliferação e não de forma sistêmica (Tzou et al., 2000; Broderick et al., 2014). Também, em *Glossina morsitans* foi comprovado que simbiontes intestinais induzem a transcrição de genes PGRP-LB, receptores envolvidos da ativação de vias imunológicas, sugerindo, portanto, a participação dos AMPs na regulação da proliferação bacteriana intestinal (Wang & Aksoy, 2012). Igualmente, foi relatado em *Anopheles gambiae* que a microbiota intestinal é controlada pela ativação da via IMD, com subsequente síntese de AMPs (Meister et al., 2009). Não obstante, outras respostas estão envolvidas na regulação da microbiota, como a ativação da cascata PPO, culminante com a melanização, previamente observado em larvas de *B. mori* (Shao et al., 2012) e a produção de ROS, demonstrada em *Drosophila* e *Anopheles* (Leulier & Royet, 2009; Kumar et al., 2010).

Em *R. prolixus*, a ingestão de *E. coli*, bactéria Gram-negativa, induziu a expressão de genes diferentes daqueles induzidos por *S. aureus*, bactéria Gram-positiva. Essas respostas foram ativadas de maneira rápida, em apenas 24 horas após infecção e especialmente na porção anterior do intestino médio (Vieira et al., 2014 – artigo 1). Esses resultados sugerem que *R. prolixus* é capaz de distinguir o tipo de bactéria invasora, expressando como resposta, diferentes perfis de moléculas imunes, conforme observado em *Drosophila* (Ferrandon et al., 2007; Imler, 2014).

De uma maneira geral, as defensinas atuam majoritariamente contra bactérias Gram-positivas embora já tenha sido relatado seu papel tóxico contra bactérias Gram-negativas (Bulet et al., 1992;1999; Lamberty et al., 2001). Desde a identificação de diferentes defensinas na mosca *P. terranovae* (Lambert et al., 1989), foi demonstrado que outros insetos também possuem mais de uma isoforma deste peptídeo. As isoformas podem ser distinguidas por pequenas discrepâncias em suas sequências gênicas, gerando diferenças marcantes nos mecanismos de ação das defensinas,

inclusive em seus microrganismos alvo (Dimarcq et al., 1994; Richman et al., 1996; Waniek et al., 2009; Telleria et a., 2013).

Em *R. prolixus,* três isoformas de defensina foram identificadas, denominadas defensina A (DefA), defensina B (DefB) e defensina C (DefC) (Lopez et al., 2003). Mais tarde, por análise filogenética das sequências gênicas destas defensinas, foi possível identificar a formação de dois grupos distintos, marcados pela alta similaridade entre *defA* e *defB*, enquanto *defC* se diferenciou significativamente (Waniek et al., 2009). Consequentemente, foi sugerido que presença de diferentes defensinas em uma mesma espécie possa estar relacionada a funções imunológicas distintas. Os resultados da presente tese evidenciaram diferenças nas expressões entre as defensinas de *R. prolixus*, corroborando com as observações feitas por Waniek e colaboradores em 2009. A abundância de transcritos de *defA* e *defB* aumentou significativamente apenas após infecção por *S. aureus,* uma bactéria Gram-positiva, enquanto que transcritos de *defC,* foram estimulados em resposta a infecção por *E. coli*, uma bactéria Gram-negativa (Vieira et al., 2014 – artigo 1).

Através de métodos cultiváveis e não cultiváveis de bactérias, tem sido demonstrado que a microbiota de triatomíneos é comumente composta por bactérias da família Enterobacteriaceae (Figueiro et al. 1995; Da Mota al., 2012). A verificação de altos níveis de *defC* em ninfas de *R. prolixus* em jejum, bem como sua indução após alimentação com *E. coli*, sugere um possível envolvimento da defensina C no controle do crescimento bacteriano de Gram-negativas (Vieira et al., 2014 – artigo 1; Vieira et al., 2015 – artigo 2). Tal sugestão deriva do fato da microbiota intestinal de *T. brasiliensis e T. pseusomaculata*, apresentar *S. marcescens*, uma bactéria também Gram-negativa, em abundancia em relação às outras bactérias nativas (Gumiel et al. 2015). De maneira complementar, análises filogenéticas entre diferentes defensinas de triatomíneos, revelaram que o gene *defC* de *R. prolixus* se agrupa com *def1* de *T. brasiliensis* (Waniek et al., 2009), indicando uma possível similaridade entre as funções dessas defensinas. Em *T. brasiliensis*, os genes que codificam a *def1* são predominantemente expressos no intestino médio anterior após alimentação sanguínea e estão provavelmente relacionados ao controle da microbiota (Araújo et al., 2006).

As lisozimas, por sua habilidade de degradação de peptidoglicanos (PGNs) da parede celular de bactérias Gram-positivas, também desempenham um papel essencial na defesa humoral dos insetos (Daffre et al., 1994; Lee & Brey, 1995; Hultmark, 1996; Araújo et al., 2006; Callewaert & Michiels, 2010). Teoricamente, a

presença de uma membrana rica em LPS, exterior a camada de PGNs, em bactérias Gram-negativas, as resguardariam da sua ação enzimática. Contudo, é interessante ressaltar que lisozimas isoladas da hemolinfa de diferentes espécies de lepidópteras também foram capazes de matar bactérias Gram-negativas (Abraham et al., 1995; Yu et al., 2002).

Por várias razões, tem sido amplamente aceito que lisozimas de insetos são agrupadas às lisozimas do tipo C, derivadas do ovo da galinha, especialmente por apresentarem alta homologia estrutural e conservação dos resíduos catalíticos (Hultmark et al., 1980; Jain et al., 2001; Yu et al., 2002; Callewaert & Michiels, 2010). Num elegante trabalho de Yu e colaboradores, foi demonstrado um aumento expressivo de lisozimas na hemolinfa de três espécies de lepidópteras após injeção de *E. coli*. Os autores mostraram que as lisozimas isoladas das mariposas lisaram bactérias Gram-positivas, e, em menor grau, também atuaram sobre bactérias Gram-negativas, diferente do que ocorre com as lisozimas do tipo C (Yu et al., 2002). Além disso, os autores notaram que anticorpos produzidos contra lisozimas de *G. mellonella*, uma das espécies estudadas, apresentaram reação cruzada com as lisozimas das outras duas espécies, mas não com as lisozimas do ovo. Com isso, sugeriram que alguma estrutura comum às três lisozimas de mariposas esteja ausente nas lisozimas do tipo C, justificando, portanto, um mecanismo de ação diferente que possa também atuar contra bactérias Gram-negativas (Yu et al., 2002).

As lisozimas além de atuarem na resposta imune de *R. prolixus* (Azambuja et al., 1991; Mello et al., 1995), estão envolvidas na digestão de polissacarídeos da bactéria simbionte *Rhodococcus rhodnii* (Ribeiro & Pereira, 1984). Com o avanço de técnicas moleculares, foi possível identificar dois genes codificantes de lisozimas induzidos após injeção de bactérias na hemocele desta mesma espécie de inseto: a lisozima A (*lisA*) e a lisozima B (*lisB*) (Ursic-Bedoya et al., 2008). Adicionalmente, foi confirmado que, em resposta a infecção por bactérias na hemolinfa, a expressão de *lisA* ocorreu predominantemente no tubo digestivo, enquanto *lisB* foi mais expressa no corpo gorduroso do inseto (Ursic-Bedoya et al., 2008).

Na presente tese, o rápido aumento dos níveis de *lisB* após alimentação sanguínea não infectante no intestino médio, sugere uma participação dessa enzima no processo digestivo de *R. prolixus*, relacionada, portanto, ao aumento de nutrientes provenientes do sangue, como já foi verificado em *T. infestans* e *T. brasiliensis* (Kollien et al., 2003; Araújo et al., 2006). Embora análises filogenéticas indiquem que a *lisA* de *R. prolixus* se agrupe com lisozimas digestivas de outros triatomíneos (Ursic-Bedoya

et al., 2008), nossos resultados mostraram uma forte indução de *lisA* após ingestão de *S. aureus* e, em menor grau, pela alimentação dos insetos com *E. coli*, sugerindo assim, uma função imunológica, além da digestiva, para essa isoforma. Este resultado contrasta com o observado em *lisB*, cuja expressão do gene não foi induzida pela ingestão de nenhuma bactéria (Vieira et al., 2014 – artigo 1).

Trabalhos prévios conduzidos em outros insetos também demonstraram ativação da expressão de lisozimas no tubo digestivo após a infecção por bactérias (Jarosz, 1993; Morishima et al., 1995; Yu et al., 2002). O aumento de atividade antibacteriana no intestino médio de ninfas de *R. prolixus* infectadas por *E. coli* e *S. aureus* pode estar relacionado diretamente ao aumento da expressão da lisozimas (Vieira et al., 2014 – artigo 1). No entanto, deve se considerar a ação sinérgica entre as lisozimas e outros AMPs, como já visto em *Lutzomyia longipalpis* e *G. mellonella* (Nimmo et al., 1997; Zdybicka-Barabas et al., 2013).

A atividade contra bactérias Gram-negativas pode ser atribuída tanto ao sinergismo com outros AMPs, como por uma ação não enzimática da lisozima, relacionada às propriedades catiônicas da proteína (Pellegrini et al., 1997; Düring et al., 1999). Análises sobre a estrutura tridimensional da lisozima M de ratos (também do tipo c) evidenciaram os sítios catalíticos responsáveis pela atividade de muramidase das lisozimas (Obita et al., 2003). Modificações genéticas da molécula no sentido de inativar os sítios catalíticos, no entanto, não provocaram a perda da atividade tóxica das lisozimas sobre bactérias Gram-positivas, como *S. aureus* e *Bacillus cereus in vitro* (Ibrahim et al., 2001). Mais tarde, outras alterações nos sítios catalíticos da lisozima, neste caso por substituições de resíduos de serina por ácido aspártico, geraram lisozimas sem atividade de muramidase, porém, ainda assim letais a bactérias Gram-positivas e Gram-negativas *in vitro* (Nash et al., 2006). Estes resultados vêm corroborando com a hipótese de que a atividade de muramidase não é essencial para ação antibacteriana das lisozimas.

Embora as expressões gênicas bem como as atividades de lisozimas de *R. prolixus* possam ser aumentadas após infecções por bactérias Gram-positivas e Gram-negativas (Azambuja e Garcia et al., 1987; Vieira et al., 2014 – artigo 1), os mecanismo de ação das lisozimas ainda é desconhecido, necessitando de estudos mais aprofundados sobre as características bioquímicas e moleculares da proteína.

Em relação ao desenvolvimento de *T. rangeli* no inseto vetor, sabe-se que o parasita deve atravessar o epitélio intestinal e invadir a hemocele do inseto como parte de seu ciclo (Guhl & Vallejo, 2003). Porém, antes disso, o parasita precisa colonizar o

trato digestivo do hospedeiro invertebrado e vencer as diversas barreiras fisiológicas e imunológicas do intestino médio (Garcia et al., 2009; 2010; 2012). Nesse contexto, o presente trabalho estudou o desenvolvimento de *T. rangeli* cepa Macias no tubo digestivo de *R. prolixus*, associado a modulação de peptídeos antibacterianos neste mesmo local, além de paralelamente, ter sido analisada as alterações nos componentes da microbiota intestinal.

Assim, realizamos infecções em curto e em longo prazo, a fim de se investigar possíveis diferenças no desenvolvimento do *T. rangeli* Macias e na modulação da resposta imune nesses dois contextos (Vieira et al., 2015 – artigo 2). Foi possível observar que *T. rangeli* Macias coloniza preferencialmente o intestino médio anterior de *R. prolixus* (Vieira et al., 2015 – artigo 2). De acordo com outros trabalhos reportados sobre o desenvolvimento de *T. cruzi* em *R. prolixus (*Kollien & Schaub, 2000; Carvalho-Moreira et al., 2003; Cortez et al., 2012; Araújo et al., 2014), a preferência por colonizar a porção posterior do intestino médio pode estar relacionada com a possível hostilidade ambiental do intestino médio anterior, onde são encontrados vários fatores adversos ao desenvolvimento do parasita (Gomes et al., 2003; Azambuja et al., 2004; 2005; Whitten et al., 2007; Waniek et al., 2009; Ursic-Bedoya et al., 2011).

Sobre a ativação da PPO em *R. prolixus*, na presente tese buscamos compreender como essa resposta é modulada dependendo do compartimento e tempo de infecção por *T. rangeli* Macias no tubo digestivo do inseto. Genta e colaboradores em 2010 analisaram a atividade fenoloxidásica em diferentes tecidos de ninfas de quinto estádio de *R. prolixus*, demonstrando uma alta atividade dessa enzima no intestino médio anterior, variável ao longo dos dias após a alimentação sanguínea (Genta et al., 2010). No que diz respeito à modulação da PPO, Gregório e Ratcliffe mostraram que a presença de *T. rangeli* H14 na hemolinfa de *R. prolixus,* não ativa o sistema PPO (Gregório & Ratcliffe, 1991a; 1991b), sugerindo que um dos fatores para o sucesso da infecção por *T. rangeli* H14 no inseto decorra da diminuíção dessa resposta. A supressão do sistema PPO foi posteriormente corroborada por Gomes e colaboradores, demonstrando que a infecção por *T. rangeli* provoca uma inibição da atividade proteolítica no corpo gorduroso, etapa que antecede a ativação da cascata de PPO na hemolinfa (Gomes et al., 2003). Por outro lado, curiosamente, foi verificado que a atividade fenoloxidásica no intestino médio aumenta significativamente em resposta a infecção oral por *T. cruzi* Dm 28c (Castro et al., 2012), o que demonstra ser a imunossupressão uma característica peculiar a infecção

por *T. rangeli* conforme já sugerido anteriormente (Gregorio & Ratcliffe, 1991a; 1991b; Gomes et al., 2003).

O sistema PPO tem sido avaliado em trabalhos que estudam a interação *Plasmodium* com *Anopheles* (Christensen et al., 2005). Sabe-se que na fase intestinal do ciclo dos parasitas da malária ocorre intensa melanização dos oocinetos no intestino médio do mosquito, importante resposta imune no sentido de controlar a multiplicação e desenvolvimento dos parasitas no inseto (Vijay et al., 2011; Fuchs et al., 2014). Neste contexto, tem sido demonstrado que o sucesso da infecção por *Plasmodium* nas diversas espécies de *Anopheles* depende também da supressão do sistema PPO (Jaramillo-Gutierrez et al., 2009; Fuchs et al., 2014). De forma complementar a essa compreensão, cepas de *Anopheles gambiae* refratárias à infecção por múltiplas espécies de *Plasmodium* apresentam um mecanismo exacerbado de melanização de oocinetos vivos, sugerindo que a ativação da cascata da PPO seja um dos principais mecanismos acionados após o reconhecimento do parasita no intestino do inseto (Collins et al., 1986).

A existência de uma flora microbiana intestinal em insetos e o reconhecimento de sua importância funcional implica na questão evolutiva dos insetos (Royet, 2011). Estes, num primeiro momento, desenvolveram mecanismos que primavam pelo controle quantitativo e qualitativo da microbiota e, posteriormente, a tolerância a certos organismos benéficos, processo pelo qual está relacionado ao sistema imune intestinal (Royet, 2011). Os mecanismos de tolerância a microbiota foram descritos em *Drosophila* e revelam o envolvimento de vários reguladores negativos da via IMD, particularmente receptores PGRP-LB e PGRP-SC, que degradam PGNs de bactérias nativas em fragmentos não imunogênicos, diminuindo a atividade de outros receptores PGRPs, que consequentemente reduzem a ativação da via IMD e a síntese de AMPs (Bischoff et al., 2006; Zaidman-Remy et al., 2006; Leulier & Royet, 2009; Paredes et al., 2011). A presença de reguladores de PGRPs já foi descrito em *Anopheles*, sugerindo a existência de um mecanismo similar de tolerância a microbiota (Waterhouse et al., 2007; Leulier & Royet, 2009), indicando se estender a outras espécies de insetos vetores, inclusive triatomíneos.

Pesquisas atuais sobre a interação de tripanosomatídeos com a microbiota intestinal de insetos tem tido atenção voltada para a bactéria Gram-negativa, *Serratia marcescens*, isolada do tubo digestivo do triatomíneo *R. prolixus* por Azambuja e colaboradores em 2004, e, principalmente, por estudos recentes que demonstram que esta espécie é frequentemente detectada em triatomíneos silvestres (DaMotta et al.,

2012; Gumiel et al., 2015). Alguns trabalhos demonstraram que *S. marcescens* possui atividade hemolítica e tripanolítica *in vitro* (Azambuja et al., 2004; 2005; Castro et al., 2007a; Castro et al., 2007b; Moraes et al., 2008), indicando uma possível interferência dessa bactéria no ciclo de vida de *T. cruzi* no inseto. Assim, é de se esperar que cepas de tripanosomatídeos bem sucedidas em colonizar o tubo digestivo do inseto, consigam manipular a microbiota a seu favor, evitando a competição por nutrientes bem como a atividade tóxica de bactérias tripanolíticas durante seu desenvolvimento.

No presente trabalho, verificamos a modulação da comunidade bacteriana cultivável no tubo digestivo de *R. prolixus* após a alimentação infectiva por *T. rangeli* Macias (Vieira et al., 2015 – artigo 2), bem como por *T. cruzi* Dm 28c, cepa que desenvolve bem seu ciclo no inseto e *T. cruzi* Y, incapaz de infectar com sucesso o tubo digestivo de R. *prolixus* (manuscrito em preparação – artigo 3), cujos efeitos serão discutidos mais a frente. No que diz respeito à infecção por *T. rangeli* Macias, foi verificada uma diminuição significativa na contagem de CFU do tubo digestivo do inseto, em comparação com insetos controle, tanto em infecções em curto como em longo prazo (Vieira et al., 2015 – artigo 2). Adicionalmente, constatamos um aumento da atividade antibacteriana contra *S. marcescens* no intestino médio de insetos infectados. Uma vez que a atividade de PO se mostrou reduzida na infecção por *T. rangeli* (Vieira et al., 2015 – artigo 2), nossos resultados indicam fortemente que a indução de AMPs poderiam estar envolvidos na regulação negativa da microbiota, como já demonstrado em outros insetos (Royet, 2011; Gendrin & Christophides, 2013).

O tempo de infecção de *T. rangeli* Macias em *R. prolixus* influenciou o perfil de expressão de genes de AMPs e lisozimas, nos diferentes compartimentos do tubo digestivo. À exemplo da modulação do gene codificante para prolixicina, constatamos que a intensidade de supressão é relativa ao tempo que *T. rangeli* se encontra colonizando o tubo digestivo do inseto. Similarmente, a expressão relativa de *lisB* também foi reprimida pela infecção por *T. rangeli* Macias (Vieira et al., 2015 – artigo 2). Sugere-se, portanto, que a supressão desses genes represente um dos possíveis mecanismos utilizados por *T. rangeli* Macias a fim de assegurar sua sobrevivência no tubo digestivo de *R. prolixus*, uma vez que AMPs e lisozimas podem ter papel tóxico a parasitas (Mello et al., 1995; Dimopoulos et al., 1998; Waniek et al., 2009).

T. rangeli também induziu uma alteração marcante nos níveis de transcritos de defensinas no epitélio intestinal de *R. prolixus*. De uma forma geral, o perfil de modulação observado sugere que *T. rangeli* se protege dos possíveis efeitos tóxicos

de um AMP (*defB*) suprimindo –o, ao mesmo tempo que manipula seu hospedeiro a superexpressar outro AMP, no caso *defC*, que possam atuar na regulação da microbiota no compartimento intestinal onde se concentram maior número de parasitas (Vieira et al., 2015 – artigo 2). A hipótese de que a defensina C favoreça o estabelecimento do parasita em detrimento da microbiota é corroborada pelas observações conduzidas em longo prazo, onde é possível observar diminuição da microbiota ao mesmo tempo em que apenas transcritos para *defC* estão superexpressados, porém agora na porção posterior do intestino médio, compartimento onde passou a residir a totalidade da população parasitária (Vieira et al., 2015 – artigo 2). Paralelamente, esses resultados confirmam hipóteses previamente levantadas, que destacam que cada isoforma da defensina pode desempenhar funções distintas no sistema imune de insetos (Waniek et al., 2009), inclusive no controle da infecção por *T. cruzi* (Waniek et al., 2011).

O avanço de técnicas de sequenciamento de DNA tem permitido estudos mais aprofundados sobre a composição da microbiota de insetos vetores, inclusive pela possibilidade de detecção de bactérias não cultiváveis (Dillon et al., 2008; Gendrin & Christophides, 2013). Com a utilização da técnica de pirosequenciamento, no presente trabalho, buscamos ampliar o conhecimento sobre a composição bacteriana de *R. prolixus* bem como analisar a alteração da mesma pela infecção dos insetos em longo prazo por *T. rangeli* Macias. Os resultados indicaram que distintas famílias bacterianas compõem a microbiota de *R. prolixus*, incluindo Enterobacteriaceae, Enterococcaceae, Nocardiaceae, Mycobacteriaceae e Burkholderiaceae (Vieira et al., 2015 – artigo 2). Observamos que a infecção por *T. rangeli* Macias altera o perfil da microbiota de *R. prolixus*, diminuindo o número de sequencias amplificadas por PCR, e referentes a família de bactérias Gram-positivas Enterococcaceae e Mycobacteriaceae. Por outro lado, foi possível notar um aumento significativo das sequencias amplificadas relativas às bactérias Gram-negativas, pertencentes à família Burkholderiaceae (Vieira et al., 2015 – artigo 2). Nossos resultados sugerem que o aumento de atividade antibacteriana contra *S. aureus*, induzido pela infecção por *T. rangeli* seja um reflexo do aumento das defensinas B e C, as quais em níveis mais altos poderiam também diminuir a concentração de bactérias Enterococcaceae e Mycobacteriaceae, favorecendo assim, a proliferação de determinadas espécies bacterianas, como por exemplo, as representantes da família Burkholderiaceae no tubo digestivo de inseto.

Os resultados do pirosequenciamento do tubo digestivo de *R. prolixus* também mostraram que as famílias Nocardiaceae, a qual inclui o simbionte *R. rhodnii* (Wigglesworth, 1936; da Motta et al., 2012), bem como Enterobacteriaceae, não foram alteradas pela infecção por *T. rangeli* Macias (Vieira et al., 2015 – artigo 2). Contudo, referente ao simbionte *R. rhodnii*, foi verificado que a infecção por *T. rangeli* cepa Choachi, acarreta diminuição significativa do número de CFU desse simbionte no intestino de *R. prolixus* (Eichler & Schaub, 2002). Esses resultados indicam que a microbiota pode ser diferentemente modulada, dependendo da cepa de *T. rangeli* que infecta o inseto. Trabalhos mostram que as famílias bacterianas identificadas em *R. prolixus* também podem compor a microbiota de outros insetos vetores, como da mosca *Glossina* e do mosquito *Anopheles*, além de também serem diferencialmente moduladas por distintas espécies de parasitas (Boissiere et al., 2012; Geiger et al., 2013; Gendrin & Christophides, 2013).

Sobre o desenvolvimento de *T. cruzi* em *R. prolixus*, a literatura mostra que as cepas do parasita classificadas como TcI, Dm 28c, Cl e C45, conseguem infectar com sucesso o tubo digestivo do inseto, enquanto as TcII aparentemente são rapidamente eliminadas do trato digestivo de seu hospedeiro, tal como ocorre com a cepa Y (Mello et al., 1996; Azambuja et al., 2004; Araújo et al., 2014). Particularmente, bactérias que compõe a microbiota natural do trato digestivo do inseto, tal como ocorre com a *S. marcescens,* podem desfavorecer o desenvolvimento de parasitas (Azambuja et al., 2005; Moraes et al., 2008; Weiss & Aksoy, 2011; Gendrin & Christophides, 2013). Relacionando o sucesso da infecção de parasitas no trato digestivo de *R. prolixus* com a modulação da microbiota, Castro e colaboradores, em 2012, mostraram que a infecção por *T. cruzi* Dm 28c (TcI) induz uma significativa redução da microbiota cultivável do tubo digestivo de *R. prolixus*, fato não observado após infecção por *T. cruzi* Y (TcII). Nesse mesmo sentido, essas duas cepas de *T. cruzi,* apresentam distintos padrões de susceptibilidade frente à atividade lítica da bactéria *S. marcescens in vitro*, onde *T. cruzi* Dm 28c se mostrou mais resistente que *T. cruzi* Y (Azambuja et al., 2004; Castro et al., 2007a). Juntos, esses resultados sugerem que a aptidão de *T. cruzi* em colonizar o tubo digestivo de *R. prolixus* depende de suas características genotípicas aliadas a sua capacidade de modular a microbiota natural do inseto vetor.

Nesse contexto, no presente trabalho buscamos compreender como a infecção por TcI ou TcII pode alterar a expressão de AMPs e atividade antibacteriana nos diferentes tecidos do inseto. Também, avaliamos a sobrevivência do parasita frente

as alterações da composição da microbiota bacteriana intestinal. Neste sentido, primeiramente, observamos que no intestino médio de *R. prolixus*, a população bacteriana encontra-se em maior número a região anterior em aumento aproximado de 100 vezes em relação a porção posterior. Neste compartimento anterior, constatamos que a infeccção por ambas as cepas de *T. cruzi* estimulou uma diminuição na microbiota, porém *T. cruzi* Dm 28c induziu uma redução de CFUs muito mais intensa do que a cepa Y de *T. cruzi*. Este resultado confirmou o observado anteriormente por Castro et al., em 2012, porém, no presente trabalho, foi possível notar que a supressão da microbiota apenas ocorre na porção anterior do intestino médio (manuscrito em preparação - artigo 3), mas não na parte posterior, digestiva do inseto.

Diversas publicações descrevem como a microbiota intestinal de insetos vetores influencia o ciclo de vida de parasitas, tanto pelo contato direto entre os microrganismos, como pela indução da resposta imune mediada pelas bactérias comensais (Dong et al., 2009; Meister et al., 2009; Cirimotich et al., 2011). Por outro lado, os parasitas também podem induzir síntese de proteínas imunes no hospedeiro (Boulanger et al., 2004; Telleria et al., 2013; Sant'Anna et al., 2014) e, consequentemente, direta ou indiretamente, interferir no crescimento de determinadas espécies da microbiota (Ursic-Bedoya & Lowenberguer, 2007; Waniek et al., 2011, Ursic-Bedoya et al., 2011; Castro et al., 2012), como parece ocorrer após a infecção de *T. rangeli* Macias em *R. prolixus* (Vieira et al., 2015 – artigo 2). Nesse contexto, recentemente foi demonstrado que a infecção prévia por *L. mexicana* em *Lu. Longipalpis* protege o inseto do efeito patogênico de *S. marcescens* (Sant'Anna et al., 2014), reforçando a hipótese de que parasitas bem sucedidos protegem seus hospedeiros, ao mesmo tempo em que criam condições favoráveis a sua colonização no inseto vetor (Dillon & Dillon, 2004).

Num panorama geral, o perfil de supressão e indução dos quatro genes de AMPs aqui analisados é bastante complexo e variável entre os tecidos do inseto, dependendo do genótipo de *T. cruzi* infectante (manuscrito em preparação - artigo 3). A expressão de genes no corpo gorduroso é considerada como uma resposta sistêmica a infecção, na qual os AMPs são secretados na hemolinfa, se difundindo por todo o corpo do inseto (Bulet & Stöcklin, 2005), enquanto a expressão nas células epiteliais do tubo digestivo representa uma resposta local ao contato direto com o parasita (Tzou et al., 2000; Bulet & Stöcklin, 2005).O reconhecimento de ambos os genótipos de *T. cruzi* no tubo digestivo do inseto rapidamente induziu o aumento da

expressão de *defC* no corpo gorduroso de *R. prolixus*, embora TcII tenha estimulado uma expressão muito mais intensa que TcI. Os altos níveis de *defC* no corpo gorduroso de insetos infectados por TcI e TcII parecem estar diretamente relacionados com o aumento na atividade antibacteriana detectada na hemolinfa (manuscrito em preparação - artigo 3). Em *Phlebotomus*, *Drosophila* e *Glossina*, foram demonstrados também que a presença de determinadas espécies de bactérias ou parasitas no tubo digestivo induzem a síntese de AMPs no corpo gorduroso, mesmo não havendo invasão dos microrganismos na cavidade geral do inseto (Boulanger et al., 2001; 2002; Hao et al., 2001). Algumas moléculas voláteis, como o óxido nítrico e reativos de oxigênio, podem estar envolvidas na sinalização ao principal órgão de síntese de AMPs na hemolinfa, o corpo gorduroso, sobre a presença de patógenos no trato digestivo, representando assim, uma estratégia de antecipação do hospedeiro, no sentido de evitar uma infecção generalizada (Hao et al., 2001; 2003).

Em relação ao ciclo de *T. cruzi* Dm 28c em *R. prolixus*, sabe-se que até o quinto dia após a infecção, os epimastigotas se concentram no intestino médio anterior e após esse período, tendem a se acumularem na porção posterior do intestino médio (Cortez et al., 2012). Os resultados da presente tese mostram que nas primeiras 24 horas de infecção pela mesma cepa, se observa um aumento na expressão de *defB* na região anterior e de *prol* no porção posterior do intestino médio. Por outro lado, a cepa Y de *T. cruzi* induziu uma drástica supressão de *defB* somente no estômago de *R. prolixus* (manuscrito em preparação - artigo 3). Esses resultados sugerem que a resposta sistêmica do inseto ao contato com o parasita é semelhante, embora as respostas nos diferentes compartimentos do trato digestivo dos insetos infectados por TcI e TcII exibam perfis discrepantes.

A positividade da infecção por *T. cruzi* foi constatada pela visualização de parasitas nas amostras do trato digestivo do inseto ao microscópio ótico. Sete dias após a ingestão dos parasitas, não foi possível detectar TcII no tubo digestivo do inseto, enquanto TcI continuava presente, fato já observado em outros trabalhos publicados por nosso grupo (Azambuja et al., 2004; Araújo et al., 2014). Embora, por contagem em câmera de Neubauer, não se possa afirmar com certeza que TcII tenha sido eliminado do inseto, ao menos parece que TcI desenvolve estratégias mais bem sucedidas que TcII no sentido de sobreviver no tubo digestivo de *R. prolixus*. Mas, o retorno dos níveis normais de *defC* observado no corpo gorduroso dos insetos alimentados com cepa Y de *T. cruzi* pode, de fato, ser resultado da ausência de parasitas no intestino de *R. prolixus*. Por outro lado, em insetos ainda positivos no

sétimo dia após infecção por *T. cruzi* Dm 28c, a expressão de *defC* no corpo gorduroso persiste maior em comparação com insetos controle, não infectados, porém um pouco menos intensa do que a observada 24 horas após infecção pela mesma cepa (manuscrito em preparação – artigo 3).

Em análises mais tardias, sete dias após a infecção, verificamos que TcI estimulou a superexpressão de *defC* e *prol* no tubo digestivo do inseto, enquanto TcII não foi capaz de induzir a expressão de AMPs (manuscrito em preparação – artigo 3). Uma vez que por volta do sétimo dia após a infecção por *T. cruzi* Dm 28c em *R. prolixus*, os parasitas são detectados tanto no estômago quanto no intestino, conforme observado por Cortez e colaboradores em 2012, é possível que a expressão de *defC* seja positivamente regulada em resposta a presença do parasita. De forma interessante, *T. rangeli* Macias também incitou a expressão de *defC* nos compartimentos do tubo digestivo onde se localizavam (Vieira et al., 2015 – artigo 2). Igualmente, Dimopoulos et al., 1998, observaram que em *A. gambiae* o protozoário *P. berghei* altera a expressão de defensinas no tubo digestivo bem como no epitélio das glândulas salivares, em ambos compartimentos que o parasita encontra durante seu ciclo evolutivo no mosquito vetor. Também, a infecção por *Leishmania major* induz a expressão de defensinas em *Phlebotomus duboscqi*, as quais foram detectadas na hemolinfa e tubo digestivo do inseto (Boulanger et al., 2004). Entretanto, a infecção por *L. mexicana* suprimiu a expressão de *def1* em *L. longipalpis (*Telleria et al., 2013), demonstrando assim que diferentes modelos de insetos e parasitas podem resultar em respostas imunológicas distintas, e que as defensinas são moduladas frente a infecção oral de insetos vetores por protozoários parasitas.

Embora não tenha sido possível quantificar a ação direta da defensina C no tubo digestivo nem na hemolinfa de *R. prolixus*, os ensaios de atividade antibacteriana nesses tecidos realçam que fatores antibacterianos foram induzidos pela infecção por *T. cruzi*. A incapacidade de induzir a expressão de AMPs no tubo digestivo, corrobora os resultados que mostram que TcII não provoca aumento na atividade antibacteriana do estômago de *R. prolixus* contra *S. marcescens*, ao contrário do observado com TcI (manuscrito em preparação – artigo 3). A elevada atividade detectada nesse mesmo compartimento contra *S. marcescens in vitro* sugere estar relacionada ao aumento da expressão de AMPs no estômago de insetos infectados por TcI, indicando por consequência, uma relação direta com a drástica redução da microbiota observada. Assim, o aumento da síntese de defensina C no intestino médio anterior de *R. prolixus*

em resposta a infecção por *T. cruzi* Dm 28c parece favorecer o desenvolvimento do parasita em detrimento de parte da população da microbiota natural do inseto.

A expressão da *prolixicina* pareceu ser negativamente regulada de acordo com a concentração local de epimastigotas de *T. cruzi*, conforme observado também nas infecções de *R. prolixus* com *T. rangeli* Macias (Vieira et al., 2015 – artigo 2). A *prolixicina*, recentemente isolada de *R. prolixus,* possui semelhanças estruturais com duas outras famílias de AMPs, as atacinas e diptericinas (Ursic-Bedoya et al., 2011). Tanto as atacinas de outros insetos, quanto a prolixicina apresentam alta toxicidade contra bactérias Gram-negativas, tal como *E.coli* (Hu & Aksoy et al., 2005; Ursic-Bedoya et al.,2011) indicando que esses AMPs atuam em alvos microbiológicos similares. Também já foi demonstrado que a infecção por *Trypanosoma brucei* induz a expressão de *atacinas* no corpo gorduroso, enquanto as suprime no tubo digestivo da mosca tsetse (Boulanger et al., 2002; Wang et al., 2008). Curiosamente, cepas da *G. morsitans* naturalmente refratárias à infecção por *T. brucei* apresentam altos níveis de *atacinas*, indicando que esse AMP seja um limitador do desenvolvimento de *T. brucei* (Boulanger et al., 2002; Hu & Aksoy, 2005; Hu & Aksoy, 2006). Assim, o fato da prolixicina possuir similaridades com atacinas, sugere de forma semelhante a este AMP, um papel tóxico também contra tripanosomatídeos. Portanto, a supressão da *prolixicina* pode representar um dos possíveis mecanismos que assegure a sobrevivência de tripanosomas no tubo digestivo do inseto.

Na presente tese observamos que *T. cruzi* Y não foi capaz de induzir a síntese de AMPs no estômago do inseto, fato que pode justificar a fraca redução da microbiota citotóxica ao parasita. A maior susceptibilidade de *T. cruzi* Y à atividade lítica da bactéria nativa do tubo digestivo de *R. prolixus*, *S. marcescens*, em comparação com *T. cruzi* Dm 28c foi demonstrada anteriormente (Azambuja et al., 2004; Castro et al., 2007a). Neste sentido, um dos fatores que limitam o desenvolvimento de TcII em *R. prolixus* poderia estar relacionado a incompetência do próprio parasita em induzir moléculas antibacterianas no tubo digestivo do inseto, sendo portanto, incapazes de reduzir as bactérias que o impactam negativamente.

Os diferentes genótipos de *T. cruzi* também exibem diferenças significativas na composição de moléculas de superfície, como açúcares e proteínas (De Souza, 1995). Araujo e colaboradores em 2002, através da incubação de lectinas conjugadas ao marcador fluorescente FITC com diferentes cepas de *T. cruzi*, mostraram que populações de parasitas TcI possuem mais resíduos do açúcar N-acetil galactosamina em sua superfície que populações TcII (Araújo et al 2002). Proteínas de membrana,

como os fatores de virulência pertencentes à família das trans-sialidades, também são diferentemente distribuídas na superfície dos genótipos TcI e TcII (Burgos et al., 2013). Outras proteínas de membrana como as mucinas, interagem com as células epiteliais do inseto e influenciam no desenvolvimento e infectividade do parasita (Gonzalez et al., 2013). Neste contexto, também foi demonstrado que moléculas de superfície como LPG de promastigotas de *Leishmania* são essenciais para o estabelecimento da infecção em flebotomíneos, bem como na indução de defensinas no tubo digestivo do inseto (McConville et al., 1992; Sacks et al., 2000; Boulanger et al., 2004). As diferenças na composição das moléculas de superfície de TcI e TcII podem justificar, em parte, os diferentes perfis de ativação de resposta imune em *R. prolixus* e modulação da microbiota por *T. cruzi* Dm 28c e *T. cruzi* Y, verificados na presente tese.

Em *R. prolixus*, os níveis de expressão das lisozimas A e B, prolixicina e defensina C indicam um envolvimento no controle da proliferação da microbiota bacteriana intestinal após o repasto sanguíneo. Enquanto as defensinas A e B parecem estar envolvidas mais diretamente na resposta contra bactérias Gram-positivas, a expressão da defensina C foi modulada frente à infecção por bactérias Gram-negativas e tripanosomatídeos. A prolixicina, por sua vez, foi regulada positivamente após a infecção por bactérias Gram-positivas, e negativamente frente à infecção por *T. cruzi* Dm28. Assim, *R. prolixus* desafiado por *T. cruzi,* além de *T. rangeli* não só resultou em modificações nos níveis dos AMPs e lisozimas, como também mostrou alterações significativas sobre a composição bacteriana da microbiota intestinal.

Atualmente, tem havido certos esforços para medidas alternativas de controle de doenças transmitidas por insetos vetores, especificamente através da introdução de bactérias geneticamente modificadas que, em ambientes naturais, uma vez colonizando o tubo digestivo dos insetos (insetos paratransgênicos), possam expressar ou inibir moléculas que interrompam o desenvolvimento de seus parasitas naturais causadores de doenças (Beard et al., 2001; Hurwitz et al., 2011; Taracena et al., 2015). A técnica de paratransgênese em *R. prolixus* foi proposta por Durvasula e colaboradores em 1997, no qual o simbionte *R. rhodnii* foi geneticamente modificado para expressar a *cecropina A*, um AMP exógeno e tóxico ao *T. cruzi*. Por outro lado, Taracena e colaboradores em 2015, demostraram que através dessa mesma técnica é possível silenciar genes do próprio inseto que possam ter um efeito na infecção por *T. cruzi*. O conhecimento sobre como a infecção por diferentes genótipos de *T. cruzi*

modulam a expressão de AMPs e a microbiota em triatomíneos, pode fundamentar à escolha dos microrganismos vetores para produção de dupla fitas dsRNA bem como dos genes alvos a serem silenciados geneticamente. A exploração de futuras ferramentas a serem utilizadas para o bloqueio do desenvolvimento do parasita em insetos, de uma maneira geral, traz novos e estimulantes desafios para a ciência.

Embora os resultados obtidos na presente tese, tenham ampliado visivelmente o conhecimento sobre a indução de diferentes tipos de lisozimas e peptídeos antimicrobianos, cujas expressões gênicas foram reguladas, positiva ou negativamente, dependendo do tipo de microrganismo empregado para infecção, futuros trabalhos que explorem a ativação da resposta imune de *R. prolixus* por outros microrganismos patogênicos, poderão melhor elucidar os possíveis padrões de resposta humoral, suas moléculas efetoras e as vias de sinalização que as regulam. Adicionalmente, através do isolamento, purificação e produção de peptídeos sintéticos será possível elucidar seus microrganismos alvo e a influência na resposta imune do inseto. Além disso, o uso combinado de outras metodologias avançadas, como por exemplo, o emprego de PCR quantitativo em tempo real e silenciamento gênico dos AMPs, poderá também esclarecer mais precisamente a dinâmica do crescimento e diminuição da comunidade bacteriana frente à infecção por diferentes microrganismos no inseto vetor *R. prolixus*.

5 CONCLUSÕES

Ninfas de quinto estádio de *R. prolixus* em jejum apresentam baixos níveis de expressão de AMPs e de lisozimas, com exceção de *defC*.

A alimentação sanguínea, não infectiva, induz a expressão de AMPs e lisozimas nos diferentes compartimentos do tubo digestivo de *R. prolixus*.

A adição de bactérias no sangue alimentar de *R. prolixus* induz um aumento da atividade antibacteriana e altera o perfil de expressão de AMPs e de lisozimas no intestino médio.

T. rangeli cepa Macias coloniza com sucesso o trato digestivo de *R. prolixus*, e modula a resposta imune no intestino médio do inseto, por inibição do sistema PPO bem como dos níveis de expressão de genes da lisozimas e prolixicina. Por outro lado, a infecção ativa a expressão de genes da defensina B e C.

A infecção por *T. rangeli* Macias de *R. prolixus* aumenta o potencial antibacteriano do intestino médio anterior contra *S. marcescens*, reduz a população bacteriana cultivável da microbiota, bem como modifica, qualitativa e quantitativamente, a microbiota bacteriana não cultivável do trato digestivo de *R. prolixus*, favorecendo a proliferação de bactérias da família Burkholderiaceae enquanto diminuiu bactérias pertencentes a família Enterococcaceae.

As atividades antibacterianas e expressões de genes de AMPs nos diferentes tecidos de *R. prolixus* são diferencialmente moduladas pela infecção oral por TcI e TcII.

A infecção por cepas Dm 28c e Y de *T. cruzi,* respectivamente, TcI e TcII, no tubo digestivo de *R. prolixus*, induz a expressão de *defC* no corpo gorduroso, sendo que TcII induziu uma expressão mais intensa que TcI.

No tubo digestivo de *R. prolixus*, a infecção por TcI suprime a expressão gênica de *prol* e induz a expressão de *defC* nos dois compartimentos do intestino médio, enquanto TcII não foi capaz de induzir a expressão de nenhum dos AMPs no mesmo órgão.

TcI induz um aumento significativo da atividade antibacteriana contra *S. marcescens*, ao mesmo tempo que reduz drasticamente a população cultivável da microbiota. TcII não é capaz de estimular aumento da atividade antibacteriana contra *S. marcescens* no intestino médio anterior, nem induzir a expressão local de AMPs.

As lisozimas são moduladas pela ingestão de bactérias Gram-positivas e Gram-negativas no tubo digestivo de *R. prolixus*.

A prolixicina, por sua vez, é regulada após a ingestão de bactérias Gram-positivas e frente à infecção por tripanosomatídeos.

As defensinas A e B são induzidas frente ao desafio por *S. aureus* no tubo digestivo de *R. prolixus*.

A defensina C está envolvida na resposta imune contra *E. coli* e tripanosomas em *R. prolixus*.

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ANEXO 1: ARTIGO PUBLICADO EM COLABORAÇÃO

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Detection and preliminary physico-chemical properties of antimicrobial components in the native excretions/secretions of three species of *Chrysomya* (Diptera, Calliphoridae) in Brazil

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ABSTRACT

Antibiotic-resistant bacteria in hospitals and communities increasingly threaten public health in Brazil and the rest of the World. There is an urgent need for additional antimicrobial drugs. Calliphorid blowfly larvae are a rich source of antimicrobial factors but the potential of Neotropical species has been neglected. This preliminary study evaluates the antimicrobial activity of the native excretions/secretions of larvae of three species of Brazilian calliphorids, *Chrysomya megacephala*, *Chrysomya albiceps* and *Chrysomya putoria*. Native excretions/secretions were collected from third instar larvae, sterile filtered and tested for antibacterial activity against *Staphylococcus aureus* 9518, *Escherichia coli* K12 4401 and *Serratia marcescens* 365. Turbidometric assays were made in micro-plates, using an ELISA reader, with readings taken up to 22 h. Bacterial suspensions at the start and end of each experiment were also serially diluted, spread on nutrient agar plates and then colony forming units counted. The physico-chemical characteristics of the native excretions/secretions were also tested by freezing/thawing, boiling, and protease digestion. The native excretions/secretions of larvae from these three *Chrysomya* species significantly inhibited bacterial growth. Therefore, Brazilian calliphorid flies could potentially provide new classes of antibiotics.

1. Introduction

Antibiotic-resistant bacteria are rapidly spreading Worldwide (WHO, 2014) with, for example, 440,000 new cases of multidrug-resistant tuberculosis emerging annually in a total of 64 countries and killing 150,000 people. At the same time, hospital-acquired infections by antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), have also been reported widely in Europe and the USA and are now recorded in Latin America and the Caribbean (Health in the Americas, 2012; Guzmán-Blanco et al., 2009; Mejía et al., 2010), and are spreading into the community in Brazil (*e.g.* Teixeira et al., 1995; Ribeiro et al., 2005; Gelatti et al., 2009, 2013; Rozenbaum et al., 2009; Carvalho et al., 2010; Rossi, 2011; Santos et al., 2010). Outbreaks of *Klebsiella pneumonia* carbapenemase-producing bacteria, resistant to most antibiotics, have occurred in Brazilian hospitals leading to a number of deaths (Marra et al., 2006). The situation in Brazil and the rest of the World is worsening so that new antibiotics are urgently required to treat these antibiotic-resistant bacteria (WHO, 2014). Unfortunately, many pharmaceutical companies withdrew from research in this area and as a result, between 1998 and 2004 only 4 new antibacterial drugs were included out of 290 listed as under development in the new drug pipeline (Shlaes et al., 2004).

One possible source of novel antibiotics is from natural products derived from insects (Ratcliffe et al., 2011, 2014). Considering the huge biodiversity of insects in Brazil and their survival in microbial infested niches for 100s of millions of years then they represent "a treasure chest of untapped resources waiting to be discovered" (Ratcliffe et al., 2014).

Likely insect candidates for investigation are calliphorid flies, such as *Lucilia sericata* that are facultative parasites normally feeding on carrion infested with massive microbial blooms that

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have to be countered by effective insect immune defences in order to survive (Ratcliffe et al., 2011, 2014). In addition, maggots are widely used for successfully treating post-operation infections, including those caused by MRSA (Thomas and Jones, 2000; Sherman, 2014). Bexfield et al. (2004, 2008) isolated and characterised a 365 Da molecule from the native excretions/secretions (NES) of *L. sericata* capable of inhibiting a range of Gram-positive and Gram-negative bacteria, including MRSA, and with potential for drug development (Bexfield et al., 2004, 2008; patent number PCT/PCT/GB2008000157, 2008). Likewise, Daeschlein et al. (2007) detected powerful *in vitro* activity of *L. sericata* secretions against a range of bacteria.

Subsequently, antimicrobial factors in *L. sericata* have received considerable attention. Thus, a *ca.* 4113 Da antimicrobial peptide (AMP), called lucifensin, has also been isolated from *L. sericata* maggots and shown to be active against clinically relevant Gram-positive bacteria including MRSA and *Streptococcus* spp. (Andersen et al., 2010; Čeřovský et al., 2010; Cerovsky et al., 2011; Čeřovský and Bem, 2014). In *Lucilia cuprina*, a similar molecule, lucifensin II, has been characterised (El Shazely et al., 2013). *L. sericata* genes have also been screened for differential expression following septic wounding and 65 novel, immune-inducible genes discovered, including 3 proline-rich AMPs (Altincicek and Vilcinskas, 2009). From the latter work, the authors predicted that the NES of *L. sericata* potentially contains additional factors, yet to be studied, and with potential for drug therapy (Altincicek and Vilcinskas, 2009). This prediction has been proven to be correct with many other antimicrobial factors now reported in *L. sericata* including an antifungal peptide, lucimycin (Pöppel et al., 2014), and 2 groups of polypeptides ranging from 130 to 700 Da and 6466 to 9025 Da in size (Kruglikova and Chernysh, 2011). Some of these peptides, including lucifensin, have also been shown to be upregulated in an infected environment (Kawabata et al., 2010; Valachova et al., 2013). Finally, other calliphorid species as well as muscoids flies have been reported to produce a variety of antimicrobial factors (reviewed in Ratcliffe et al., 2014) some of which are being developed as new antimicrobial and anti-tumour drugs (Chernysh et al., 2002; Chernysh and Kozuharova, 2013).

To date, only a few of the *ca.* 1000 species of Calliphoridae (Kutty et al., 2010) have been examined for antibacterial factors (Dimarcq et al., 1990; Chernysh et al., 2002; Huberman et al., 2007; Barnes et al., 2010) and knowledge of such factors in Neotropical calliphorids is limited to three studies (Sahalan et al., 2007; Stahl et al., 2012, Abst XL Ann Meeting Brazil Biochem Mol Biol Soc, Díaz-Roa et al., 2014). Neotropical calliphorids often occupy regions with elevated temperatures favouring rapid microbial growth so that the insect antibacterial defences would need to be even more effective than usual to ensure survival. Thus, the aim of the present study was to examine the NES of the calliphorids *Chrysomya megacephala*, *Chrysomya albiceps* and *Chrysomya putoria* present in Brazil, for novel antimicrobial factors with potential for development as therapeutic drugs. Such factors were indeed recorded and their activities are reported in the present paper together with preliminary information on their physico-chemical properties.

2. Materials and methods

2.1. Maintenance of larvae

Final instar larvae of 3 calliphorid species, namely, *C. megacephala, C. putoria* and *C. albiceps*, were used in all experiments. The larvae were derived from colonies maintained at the Laboratório de Transmissores de Leishmanioses, Setor de Entomologia Médica e Forense, IOC/FIOCRUZ RJ, Brazil, using the method described by Queiroz and Milward-de-Azevedo (1991). The larvae were fed on putrefying beef and maintained at 27 ± 1 °C with a 12:12 h light:dark regime.

2.2. Collecting larval native excretions/secretions (NES)

Final stage larvae migrate from the beef and were collected and starved for 18–24 h before washing with sterile Milli-*Q* water (M_W). They were then weighed and 100 μL M_W added per 1 g larvae in sterile 200 mL polystyrene flasks before incubation for 1 h at 37 °C. NES were then collected on ice in sterile Eppendorf tubes, centrifuged at 8000 × *g* and the supernatants filter sterilised in Millex PVDF 0.22 μm before storage at −20 °C until testing within two months of collection. NES were also collected 4 h later and treated identically to the first collected samples. The pellets were also retained for testing antimicrobial activity. Freshly collected NES samples were used in most experiments although the longevity of the activity of the NES was also tested weekly in samples stored for 1 week to 2 months at −20 °C.

2.3. Bacteria

Bacteria used in the experiments were *Escherichia coli* K12 4401 and *S. aureus* 9501 purchased from the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK, and *Serratia marcescens* 365 from the Pasteur Institute, Paris, France, kindly supplied by Dr. Cecile Wandersman. The bacteria were stored at −70 °C in liquid medium consisting of Tryptone Soya Broth (TSB) with 10% sterile glycerol. Bacteria colonies from agar slopes were grown up with oscillation for 17 h in 20 mL TSB at 30 °C, then 100 μL of this suspension were diluted in 10 mL TSB and grown for 4 h at 30 °C. The bacteria (1 mL) were then washed twice in sterile TSB at 3000 × *g* for 10 min, resuspended in 1 mL TSB, diluted, counted and adjusted to 2×10^4 mL^{-1} for all experiments.

2.4. Turbidometric (TB) assay

The TB assay was modified from Bexfield et al. (2004, 2008) and used in all experiments in order to detect antimicrobial activity of the NES. In a typical experiment carried out in a 96-well flat-bottomed ELISA plate, all the outer wells were each filled with 60 μL of sterile MW. Then three blank wells were each filled with only 60 μL 1% peptone (Primatone, Sigma P8388) while six control wells each received 50 μL 1% peptone. Another three antibiotic control wells contained 50 μL either 10 μg/mL penicillin or 80 μg/mL ampicillin antibiotics added to 1% peptone instead of the NES. The contents of each triplicate were initially prepared in an Eppendorf tube by adding 144 μL of experimental (NES or antibiotic) correspondent to 20 mg NES protein/mL, plus 16 μL 10% peptone and then pipetting 50 μL into each well. The experimental wells containing the test NES were set up in triplicate with different test bacteria or NES treatments. Finally, all the experimental and control wells received 10 μL bacteria suspension (2×10^4 bacteria/mL) to give a final volume of 60 μL. All plates were incubated at 37 °C for up to 24 h and the optical densities measured at 550 nm (OD_{550}) at hourly intervals with a Molecular Devices Spectra Max 190, Plate Reader (Sao Paulo, Brazil) using the kinetics mode.

2.5. Colony-forming (CFU) units

In order to relate the optical density readings obtained to actual bacterial numbers, 10 μL of the bacterial suspensions at the start and end of each experiment were serially diluted 6 times in 990 μL TSB, spread on nutrient agar plates, incubated overnight at 30 °C and then CFUs counted. Likewise, samples of the NES immediately after collection as well as the MW used for the assays and reagents

were tested on agar plates for assessment of sterility/levels of contamination.

2.6. Physicochemical properties

The thermal stability of the NES was investigated by heating 1 mL in a water bath at 100 °C for 60 min or by 10 cycles of freezing and thawing from –80 °C to room temperature. The susceptibility of the NES to protease digestion was tested by pre-incubation with trypsin for 24 h at 37 °C (Bexfield et al., 2004) before assaying for antimicrobial activity.

2.7. Effect of lipopolysaccharide (LPS) on NES activity

In most experiments, it was not feasible to produce sterile larvae of the species used and to retain antimicrobial activity of the NES. Therefore, the influence of bacterial LPS on the antimicrobial activity of the NES was examined. Fifty microliters of NES in triplicate wells of an ELISA plate were pre-incubated with 5 μL 20 μg/μL stock solution of *E. coli* 0111:B4 LPS (Sigma-Aldrich) for 30 min at 37 °C, *E. coli* bacteria were then added and the TB assay run as usual at 37 °C for antimicrobial activity. Controls wells were set up with the NES pre-incubated with 5 μL of MW instead of LPS before addition of bacteria.

2.8. Protein levels of NES

All protein levels determination of NES samples used a protein assay kit (BCA* Protein Assay Reagent, Pierce, Thermo Scientific, USA) with bovine serum albumin (BSA) standards.

2.9. Statistical analyses

Data are expressed as arithmetic means ± SEM. The significance of differences between sample values was assessed using two-tailed unpaired Student's *t*-tests with significance set at $p \leq 0.05$. Results were analysed by GraphPad prism 5 programme using the two-way ANOVA or Student's *t* test according to the results.

3. Results

3.1. Turbidometric (TB) assay

In the TB assays, the NES supernatants, and not the NES pellets, of *C. megacephala, C. putoria* and *C. albiceps* all had significant ($p < 0.001$) antibacterial activity against *E. coli* from 8 h to 20 h after incubation compared with the control group (Fig. 1). Significant antibacterial activity was also recorded against *S. marcescens* and *S. aureus* but was only tested with *C. putoria* and *C. megacephala* NES (Figs. 2 and 3).

The dynamics of bacterial inhibition is similar with all three species and their growth contrasts the *E. coli* control (Fig. 1). Initially, for the control without any NES, there is a characteristic lag period of 4 h before bacterial growth begins and rapidly enters the log phase to follow a normal sigmoidal bacterial growth curve (Fig. 1). With the three NES experimental groups from the three calliphorids, however, growth inhibition occurs almost completely throughout the experiment and fails to recover by the end of 20 h incubation (Fig. 1). There is no significant difference between the three species NES and the penicillin control at any time. Fig. 1 is derived from three experiments each for *C. megacephala* and *C. putoria*, but from only 1 experiment for *C. albiceps.*

The dynamics of growth of *S. aureus*, following incubation with either the NES from *C. megacephala* or *C. putoria*, are similar (Fig. 2). Both NESs showed strong inhibition of bacterial growth, which became significant ($p < 0.001$) in comparison with the controls at 6 h. In addition, there was little bacterial growth in the NES wells up to 18 h for *C. megacephala* and none at all for *C. putoria* throughout the experiment. Antibacterial activity was so strong that there was no significant difference between the NES from both insect species in comparison with the antibiotic control, ampicillin, at any time.

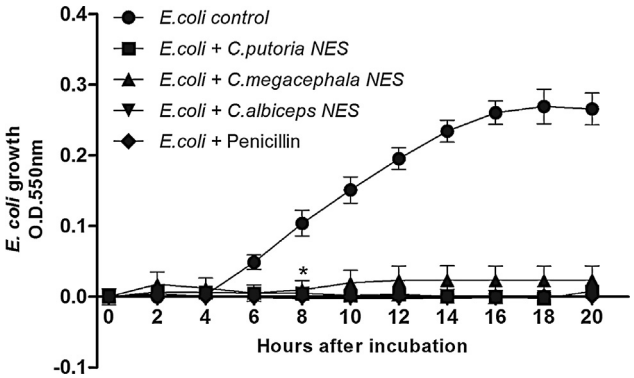

Fig. 1. Antibacterial activity of native excretion/secretion (NES) from the maggots of three calliphorid species, *C. putoria, C. megacephala* and *C. albiceps* against *E. coli* over 20 h. Each point represents 3 experiments in triplicate wells ($n = 9$). Bars are SEMs and * indicates significance differences at <0.001 between *E. coli* control and *E. coli* + NES of *C. putoria*, *C. megacephala* and *C. albiceps* from 8 h to the end of experiment at 20 h incubation.

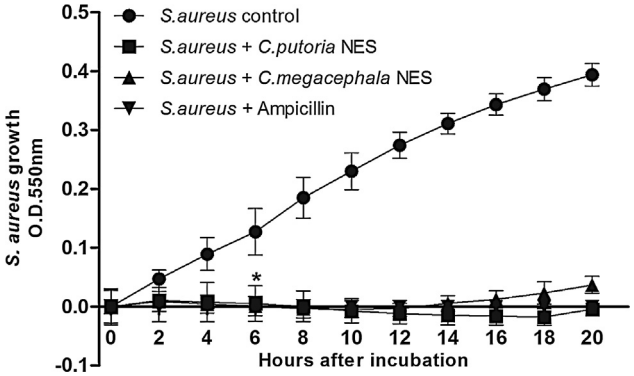

Fig. 2. Antibacterial activity of native excretion/secretion (NES) from the maggots of *C. megacephala* and *C. putoria* against *S. aureus* over 20 h. Each point represents 3 experiments in triplicate wells ($n = 9$). Bars are SEMs and * indicates significance differences at <0.001 between *S. aureus* control and *S. aureus* + NES of *C. putoria* and *C. megacephala* from 6 h to the end of experiment at 20 h incubation.

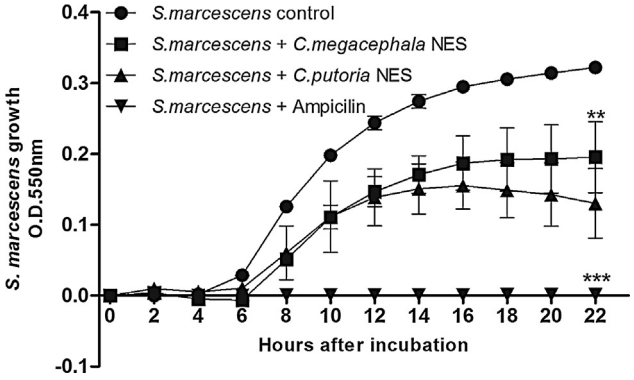

Fig. 3. Antibacterial activity of native excretion/secretion (NES) from the maggots of *C. megacephala* and *C. putoria* against *S. marcescens.* Each point represent 3 experiments in triplicate wells ($n = 9$). Bars are SEMs. * Indicates significance difference ($p < 0.05$) between both NES groups and the control at 12 h of incubation, ** indicates significance difference ($p < 0.01$) between both NES groups and the control at 22 h of incubation, *** indicates significance difference ($p < 0.001$) between the control group and ampicillin at 22 h incubation.

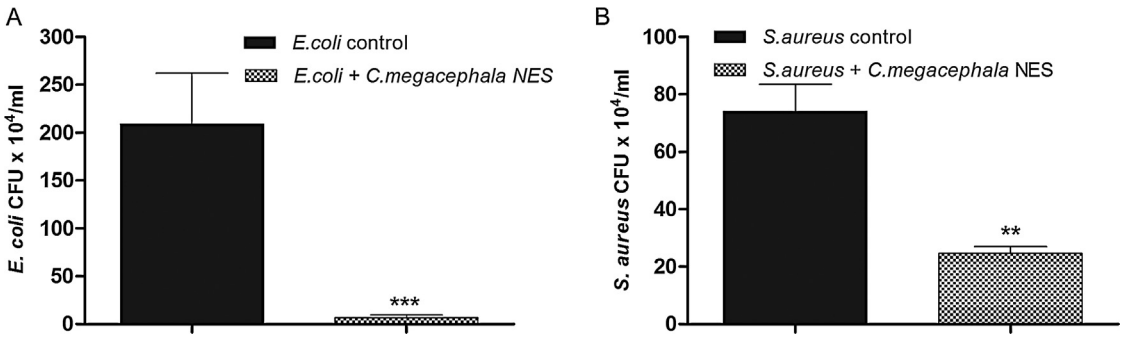

Fig. 4. Bacterial colony forming unit (CFUs) of *E. coli* and *S. aureus* 20 h after incubation with *C. megacephala* native excretion/secretion (NES). Controls groups were bacteria incubated without NES. Bars are SEMs. ** indicates significance at <0.01 and *** indicates significance at <0.001.

In addition, the growth of the *S. aureus* controls lacked the characteristic lag period seen with *E. coli* (Fig. 1) but, even so, the bacteria were still effectively inhibited by both NES (Fig. 2).

The dynamics of growth of *S. marcescens* following incubation with NES of *C. megacephala* and *C. putoria* are similar, but the antibacterial activity only became significantly different from the control group after 12 h incubation ($p < 0.05$) and until the end of the experiment at 22 h ($p < 0.01$) (Fig. 3).

In addition, the bacterial control growth by 22 h was also significantly higher ($p < 0.001$) than in the ampicillin control. Thus, there is significant antibacterial activity against *S. marcescens* but less than that recorded against both *E. coli* and *S. aureus*.

3.2. Colony-forming (CFU) units

In CFU counts of actual bacterial growth for *E. coli* and *S. aureus* in the *C. megacephala* NES after 20 h show the effectiveness of the inhibition of *E. coli* by the NES in comparison with the control ($p < 0.001$) (Fig. 4). In addition, although there was significant inhibition of *S. aureus* ($p < 0.01$), there was more growth of the bacteria than was apparent from the TB assay (see Fig. 2).

3.3. Physicochemical properties

Preliminary physiochemical properties of the antibacterial factors present in the NES of *C. megacephala* were also examined and showed that heating, freeze–thawing or trypsin treatments, all failed to inhibit the antibacterial activity of the NES against *E. coli* (Fig. 5). The heat-treated, freeze–thawed, trypsin-treated NES, as well as the untreated NES and the penicillin control, were significantly different to the *E. coli* control from 10 h ($p < 0.001$), and remained so until the end of the experiment at 20 h. The NES from all three calliphorid species also retained some significant activity after storage at −20 °C for up to 2 months although activity varied from one batch to another.

3.4. Effect of lipopolysaccharide (LPS) on NES activity

Finally, results of the effect of pre-incubating the *C. megacephala* NES with *E. coli* LPS resulted in significant inhibition ($p < 0.001$) of the antibacterial properties of the NES against *E. coli* in comparison with untreated NES after 20 h incubation (Fig. 6). The antimicrobial activity of both NES groups was significantly different to the control group from 10 h to 20 h incubation.

4. Discussion

The results of this study indicate that calliphorids possess one or more antibacterial factors capable of inhibiting both Gram-positive and Gram-negative bacteria *in vitro*. The NES from both

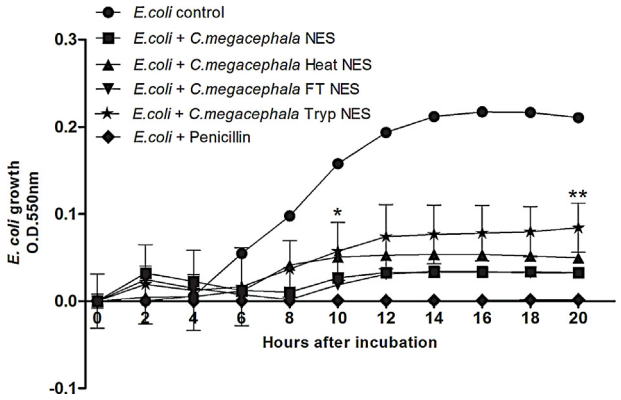

Fig. 5. Antibacterial activity of native excretion/secretion (NES) from maggots of *C. megacephala* over 20 h against *E. coli* following heating for 1 h at 100 °C (Heat NES) or 10 cycles of freezing and thawing (FT NES) or after pre-incubation with trypsin (Tryp NES) for 24 h at 37 °C. Results from 2 experiments ($n = 6$). Bars are SEMs and asterisks indicate significance at $p < 0.001$. * Difference between the control group and the group subjected to freezing/thawing cycles, heating and treated with trypsin at 10 h. ** Difference between the control and all experimental groups at 20 h.

C. megacephala and *C. putoria* showed high antibacterial activity against *E. coli* and *S. aureus*. Potent antibacterial activity against *E. coli* and *S. aureus* has also been recorded in the NES of other Calliphoridae species (Bexfield et al., 2004; Barnes et al., 2010). In contrast, the NES activity from *C. megacephala* and *C. putoria* tested against *S. marcescens* was less potent than with the other bacteria tested. It is well know that *S. marcescens* possess an intrinsic and

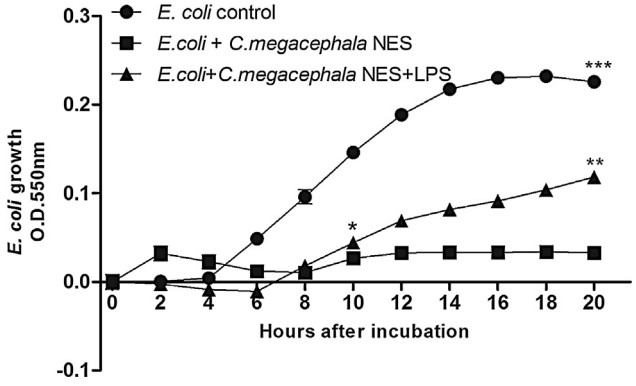

Fig. 6. Antibacterial activity of native excretion/secretion (NES) from maggots of *C. megacephala* against *E. coli* with and without pre-incubation of NES with LPS. Each point represent 2 experiments in triplicate wells ($n = 6$). Bars are SEMs and asterisks indicate significance at $p < 0.001$. * Difference between the control and the two NES experimental groups at 10 h. ** Difference between the *C. megacephala* untreated NES group and *C. megacephala* NES LPS treated group. *** Difference between the control and the two NES experimental groups at 20 h.

acquired potential resistance to a range of antimicrobial agents, including an extended-spectrum beta-lactamase or metallo beta-lactamase (Mahlen, 2011; Iguchi et al., 2014). These factors may cause the lower antibacterial activity against *S. marcescens*.

In addition, at least in *C. megacephala*, the antimicrobial factors are resistant to heating, freezing and trypsin digestion. The presence of such antimicrobial factors in insects has previously been extensively reported with over 400 different antimicrobial peptides detected to date (Ratcliffe et al., 2011, 2014). Many of these, however, are unstable and susceptible to temperature, pH, salt concentration and protease activity, which, together with other factors, have hindered their development as new drugs (Kang et al., 2012). The stability of the calliphorid antibacterial factors in the present study is encouraging for their further investigation as potential new drugs.

Throughout this study, the activity and storage properties of the harvested NES preparations varied considerably. On occasions, all activity was lost, despite the fact that reagents and protocols were identical for all experiments. One possible contributor to this variability was LPS from bacterial contaminants. The NES were filter sterilised soon after harvesting, however, they were collected from non-sterile insects and CFU counts showed that, prior to filtration, the NES contained bacteria that would have released LPS and other products which filtration would have failed to remove. These products could have potentially interacted with the NES antibacterial factors and inhibited their activity (see, Ratcliffe et al., 2011). This hypothesis was tested by pre-incubating the *C. megacephala* NES with *E. coli* LPS which resulted in significant inhibition of the NES antimicrobial activity against *E. coli* after 20 h incubation. It is known that many insect antimicrobial peptides are cationic and bind to negatively charged targets, such as LPS, by electrochemical interactions (Carter and Hurd, 2010; Ratcliffe et al., 2011), and this may have accounted for the inhibition of NES activity recorded. Attempts to produce active NES samples, however, without LPS contamination by using sterile insects failed. However, loss in activity of NES of both sterile and non-sterile larvae has previously been reported in other laboratories with experiments using *L. sericata* and may be correlated with both seasonal variations and undetermined differences in rearing protocols (Ratcliffe, unpublished).

As far as we are aware, our previous presentation (Stahl et al., 2012, Abst XL Ann Meeting Brazil Biochem Mol Biol Soc), Sahalan et al. (2007) and Díaz-Roa et al., 2014 are the first reports of antibacterial factors in Neotropical calliphorids although other Neotropical insects, such as triatomine bugs, have been shown to produce a range of antimicrobial peptides (Lopez et al., 2003; Ursic-Bedoya et al., 2011; Waniek et al., 2009; Vieira et al., 2014). In addition, in other biozones, muscid or calliphorid flies, such as *L. sericata*, are at present the subject of intense investigation. The reason for the interest in calliphorids results from the discovery, in this group, of a range of antimicrobial and anti-tumour factors, with different molecular masses, including lucifensins (Andersen et al., 2010; Čeřovský et al., 2010; El Shazely et al., 2013; Čeřovský and Bem, 2014), lucimycin (Pöppel et al., 2014) and seraticin (Bexfield et al., 2004, 2008), as well as two alloferons (Chernysh et al., 2012; Chernysh and Kozuharova, 2013). The latter molecules have been shown to have antiviral, anti-tumour and immunoregulatory functions and are rapidly being developed as new drugs. The calliphorids are thus one of the most promising group of insects for drug discovery and development.

The results of the present experiments give only a limited indication of the nature of the antibacterial factors detected. The fact that heating, freeze thawing or trypsin treatments failed to inhibit the antibacterial activity of the NES against *E. coli* suggests either that the antimicrobial factors involved are non-proteinaceous and/or more likely that the bacterial inhibition, as in *L. sericata* (Ratcliffe et al., 2014), derives from the presence of several antimicrobial factors in the NES. Therefore, the determination of the minimum inhibitory protein concentrations in *C. megacephala* NES would not produce relevant results and this is also confirmed by the great variations in activity from different batches of NES. Preliminary physicochemical properties tests with *C. putoria* NES, however, resulted in antibacterial activity inhibition against *E. coli* possibly indicating the protein nature of the antibacterial factors detected in this NES, and derived from antimicrobial peptides (data not shown).

Due to the biodiversity of calliphorids, with over 1000 species recorded throughout the World (Kutty et al., 2010), and due their scavenging of microbial infested carrion and dung, then it is likely that the Neotropical species produce novel antimicrobial factors.

In conclusion, the NES of three calliphorid species have been shown to have high antibacterial activity against both Gram-positive and Gram-negative bacteria. Following additional isolation and characterisation, these factors could potentially yield new pharmaceutical drugs.

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