UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Caracterização de vesículas extracelulares e tráfego de Antígeno B no contexto da infecção pela forma larval de *Echinococcus* spp.

Tese de doutorado

Edileuza Danieli da Silva

Porto Alegre, agosto de 2018

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como requisito parcial para a obtenção do título de Doutor em Ciências

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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

3'UTR	região 3' não traduzida, do inglês 3' *untranslated region*
ACN	acetonitrila
Ag5	Antígeno 5
AgB	Antígeno B
AgB8/1	subunidade 1 do Antígeno B
AgB8/2	subunidade 2 do Antígeno B
AgB8/3	subunidade 3 do Antígeno B
AgB8/4	subunidade 4 do Antígeno B
AgB8/5	subunidade 5 do Antígeno B
BHT	3,5-di-terc-butil-4-hidroxitolueno
BN-PAGE	do inglês, *blue native polyacrylamide gel electrophoresis*
BSA	albumina sérica bovina, do inglês *bovine serum albumine*
cDNA	DNA complementar
CHD	hidatidose cística, do inglês *cystic hydatid disease*
Ctx-B	subunidade B da toxina colérica
DALY	do inglês, *disability-adjusted life years*
DAPI	4 ',6-diamino-2-fenilindol
DDA/SEAPI	Departamento de Defesa Agropecuária/Secretaria da Agricultura, Pecuária e Irrigação
DMEM	*Dulbecco's Modified Eagle's medium*
DNA	ácido desoxirribonucleico, do inglês *deoxyribonucleic acid*
dNTP	desoxirribonucleotídeos
DTT	ditiotreitol
ELISA	do inglês, *enzyme-linked immunosorbent assay*
EVs	vesículas extracelulares, do inglês *extracellular vesicles*
EV/I	conjunto de amostras de vesículas extracelulares individuais
EV/P	conjunto de amostras de vesículas extracelulares constituído de *pools*
FABP	proteína de ligação a ácidos graxos, do inglês *fatty acid binding protein*
HDL	lipoproteína de alta densidade, do inglês *high density lipoprotein*
HEPES	ácido 4-(2-hidroxietil)piperazina-1-etanosulfonico
HLBP	do inglês, *hydrophobic ligand binding protein*
HSP	proteína de choque térmico, do inglês *heat shock protein*
IFNγ	interferon gama
IgG	Imunoglobulina G
IL-10	interleucina 10
KCl	cloreto de potássio
kDa	quilodalton
LC-MS	espectrometria de massas acoplada a cromatografia líquida
LDL	lipoproteína de baixa densidade, do inglês *low density lipoprotein*
LPS	lipopolissacarídeo
MEM	*Eagle's Minimum Essential Medium*
$MgCl_2$	cloreto de magnésio

MHC	complexo principal de histocompatibilidade, do inglês *major histocompatibility complex*
miRNA	microRNA
mRNA	RNA mensageiro
MS	espectrometria de massas, do inglês *mass spectrometry*
MTT	brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio
MWCO	do inglês, *molecular weight cut off*
NaCl	cloreto de sódio
NCBI	*National Center for Biotechnology Information*
NMWL	do inglês, *nominal molecular weight limit*
NTA	do inglês, *nanoparticle tracking analysis*
PAIR	do inglês, *puncture, aspiration, injection, re-aspiration*
PBS	tampão fosfato-salino, do inglês *phosphate buffered saline*
pH	potencial hidrogeniônico
PVDF	do inglês, *polyvinylidene difluoride*
RNA	ácido ribonucleico, do inglês *ribonucleic acid*
RPMI	*Roswell Park Memorial Institute*
rRNA	RNA ribossômico
RT-PCR	do inglês, *reverse transcriptase-polymerase chain reaction*
SDS	dodecil sulfato de sódio, do inglês *sodium dodecil sulfate*
SDS-PAGE	eletroforese em gel de poliacrilamida com SDS, do inglês SDS-*polyacrilamide gel electrophoresis*
SPGG	Secretaria de Planejamento, Governança e Gestão
TEM	microscopia eletrônica de transmissão, do inglês *transmission electron microscopy*
TFA	ácido trifluoroacético
Tfn	transferrina
Th1	linfócito T auxiliar do tipo 1
Th2	linfócito T auxiliar do tipo 2
TLR4	receptor do tipo Toll 4
TNFα	fator de necrose tumoral alfa
T_{reg}	linfócito T regulatório
UPLC	cromatografia líquida de ultra performance

RESUMO

Echinococcus granulosus e *Echinococcus ortleppi* são agentes etiológicos da hidatidose cística. A doença é decorrente do desenvolvimento do estágio larval, o cisto hidático, no interior das vísceras do hospedeiro, principalmente fígado e pulmões. Os cistos hidáticos tem uma estrutura unilocular e preenchida por fluido, que contém os produtos de excreção/secreção do parasito. Os produtos de excreção/secreção têm um importante papel na interação parasito-hospedeiro e constituem potenciais alvos para diagnóstico, drogas antiparasitárias e vacinas. Diversos estudos têm ressaltado a importância de vesículas extracelulares (EVs) na comunicação intercelular de helmintos parasitas. O Antígeno B (AgB) é um produto de excreção/secreção cujo envolvimento na imunoevasão e captura/transporte de lipídios é conhecido em *Echinococcus* spp. O objetivo deste estudo foi investigar a relação parasito-hospedeiro no contexto desses dois importantes produtos de excreção/secreção de *Echinococcus* spp.: as vesículas extracelulares e o Antígeno B. Combinando ensaios de inibição de endocitose e co-localização, nós encontramos que a endocitose mediada por *rafts* lipídicas é a principal via envolvida na internalização de AgB por células *in vitro*. A via de endocitose mediada por vesículas de clatrina também pode contribuir para a internalização do AgB, porém em taxas menores. EVs de *E. granulosus* e *E. ortleppi* foram isoladas de líquido hidático e sobrenadantes de cultura de protoescólices por ultracentrifugação. A presença de EVs nas preparações foi confirmada por microscopia eletrônica de transmissão e NTA (*nanoparticle tracking analysis*), que mostraram a maioria das EVs com um diâmetro em torno de 100 nm. Uma linhagem de hepatócitos (RH) foi capaz de internalizar as EVs de *Echinococcus* spp., confirmando seu potencial para interação com o hospedeiro na hidatidose cística. A análise proteômica mostrou que a variabilidade no conteúdo de EVs entre cistos da mesma espécie é um traço comum em *E. granulosus* e *E. ortleppi*. Na comparação entre as duas espécies foi observada maior diversidade de proteínas em *E. granulosus.* As proteínas identificadas podem ser essenciais para a sobrevivência de *Echinococcus* spp., e algumas, como enolase, 14-3-3 e Antígeno 5, já foram sugeridas tendo papel nas interações parasito-hospedeiro. Adicionalmente, nós detectamos mRNAs nas preparações, indicando que essa classe de biomoléculas também integra as EVs de *Echinococcus* spp. O entendimento dos mecanismos associados às atividades do AgB e dos agentes moleculares nas EVs levará a uma melhor compreensão da relação *Echinococcus*-hospedeiro.

ABSTRACT

Echinococcus granulosus and *Echinococcus ortleppi* are aetiological agents of cystic hydatid disease (CHD). The disease is caused by the development of the larval stage, the hydatid cyst, at visceral sites, mainly liver and lungs. Hydatid cysts are fluid-filled, unilocular structures that contains the parasite excretory/secretory products. The excretory/secretory products have an important role in the host-parasite interplay and constitute potential targets for diagnosis, anti-parasitic drugs and vaccines. Several reports have highlighted the importance of extracellular vesicles (EVs) in helminth parasites intercellular communication. Antigen B (AgB) is an excretory/secretory product known to be enrolled in both immunoevasion and lipid uptake/transport in *Echinococcus* spp. The objective of this study was to investigate host-parasite interplay in the context of these two important *Echinococcus* spp. excretory/secretory products: extracellular vesicles and Antigen B. By combining endocytosis inhibition and colocalization assays, we found that raft-mediated endocytosis is the major pathway involved in AgB uptake by cells *in vitro*. Clathrin-mediated endocytosis might also contribute to AgB internalization, but in a lesser extent way. *E. granulosus* and *E. ortleppi* EVs were isolated from hydatid fluid and protoscolex culture supernatant by ultracentrifugation. The presence of EVs in the preparation was confirmed by transmission electron microscopy and nanoparticle tracking analysis, that showed most of EVs are around 100 nm in diameter. A hepatocyte cell line (RH) was able to internalize *Echinococcus* spp. EVs, confirming their potential for host interaction in CHD. The proteomic analysis showed that variability in the protein content of EVs is a common trait among cysts from the same species in both *E. granulosus* and *E. ortleppi.* In the comparative analysis of the two species it was observed a higher diversity of proteins in *E. granulosus* EVs. The identified proteins could be essential for *Echinococcus* spp. survival and some, such as enolase, 14-3-3 and Antigen 5, had already been suggested playing a role in host-parasite interactions. Additionally, we detected mRNAs in the preparations, indicating that this class of biomolecules is also integrant of *Echinococcus* spp. EVs. The understanding of the mechanisms associated to AgB activities and the molecular players in the EVs will lead to a better comprehension of *Echinococcus*-host relationship.

1 INTRODUÇÃO

1.1 A hidatidose cística

A hidatidose cística é a doença decorrente da infecção com a forma larval (metacestódeo ou cisto hidático) de espécies pertencentes ao complexo *Echinococcus granulosus sensu lato.* Este complexo compreende espécies cuja forma larval patogênica tem como característica comum a estrutura semelhante a uma bexiga, com uma parede delimitando uma cavidade unilocular preenchida por líquido e contendo formas pré-adultas (Fig. 1). Além disso, algumas linhagens do complexo eram consideradas genótipos de *E. granulosus* e agora foram elevadas a espécies. As espécies diferenciam-se principalmente pela especificidade em relação aos hospedeiros, taxa de desenvolvimento e características morfológicas do estágio adulto (THOMPSON, 2017).

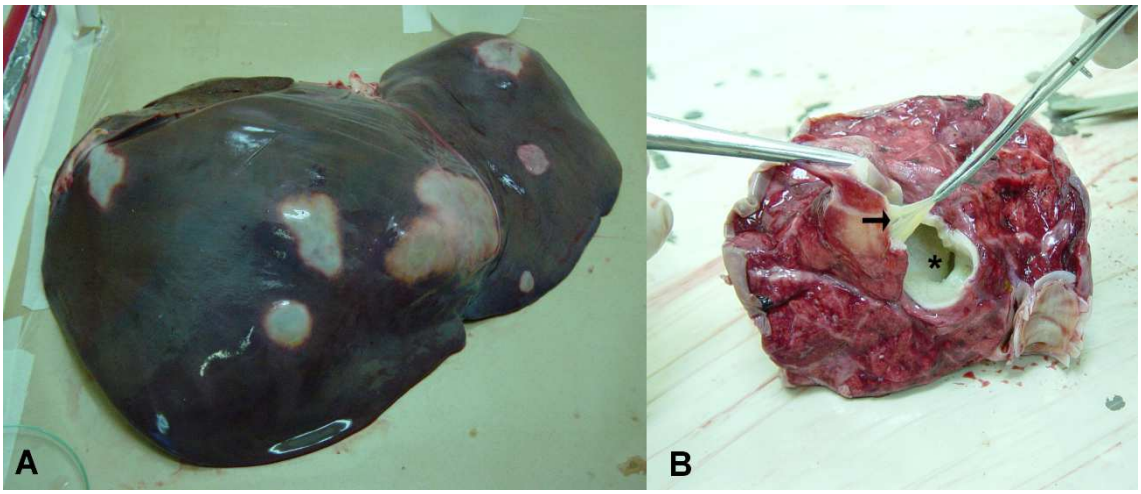

Figura 1. Cistos hidáticos bovinos. (A) Fígado infectado com diversos cistos hidáticos. (B) Pulmão contendo um cisto hidático aberto mostrando a cavidade interna (∗) e a parede do cisto (→).

Atualmente o complexo *E. granulosus s.l.* é composto por 6 espécies: *Echinococcus granulosus sensu stricto, Echinococcus ortleppi, Echinococcus equinus, Echinococcus canadensis, Echinococcus intermedius* e *Echinococcus felidis,* mas constantes revisões são feitas em virtude de novos estudos genéticos e moleculares (LYMBERY, 2017; THOMPSON, 2017). O *E. granulosus s.s.* (ou simplesmente *E. granulosus*) é a espécie mais relevante do ponto de vista epidemiológico, pois está distribuída mundialmente, tem uma alta prevalência nos rebanhos e é responsável pela grande maioria dos casos em humanos.

Desde 2010, a hidatidose cística está incluída na lista das Doenças Tropicais Negligenciadas da Organização Mundial da Saúde

(http://www.who.int/neglected_diseases/diseases/en/). Em seres humanos, estima-se cerca de 188.000 novos casos por ano, resultando em perdas aproximadas de 184.000 DALY (*disability adjusted life years*) (TORGERSON *et al*., 2015). Se levada em consideração a subnotificação, as perdas podem chegar a 1 milhão de DALY, com prejuízos anuais da ordem de US$ 760 milhões decorrentes de gastos com tratamento e perdas associadas ao tempo em que um indivíduo enfermo fica incapacitado (BATTELLI, 2009; TORGERSON; MACPHERSON, 2011). Na pecuária, os prejuízos anuais determinados pela infecção de bovinos, ovinos e suínos com cistos hidáticos somam mais de US$ 2 bilhões em todo o mundo (BATTELLI, 2009; BUDKE; DEPLAZES; TORGERSON, 2006).

Na América do Sul, a hidatidose cística ocorre com alta prevalência em parte da Argentina (Patagônia, pampas e costa), Bolívia (sudeste), Brasil (sul), Chile (vale central e sul), Peru e Uruguai (ALVAREZ ROJAS; ROMIG; LIGHTOWLERS, 2014; CUCHER *et al*., 2016; DEPLAZES *et al*., 2017) (Fig. 2).

No Brasil, os municípios gaúchos das regiões de fronteira com Uruguai e Argentina são reconhecidos como aqueles nos quais a hidatidose humana é mais prevalente, especialmente entre indivíduos com histórico de residência em áreas rurais (DE LA RUE, 2008; FARIAS *et al*., 2004). As principais espécies que ocorrem nessa região são *E. granulosus* e *E. ortleppi*, associadas, principalmente, a criação extensiva de ovinos e bovinos, onde o uso de cães para o manejo e o hábito de alimentá-los com vísceras cruas perpetua o ciclo de vida do parasito (DE LA RUE, 2008). O contato direto com cães infectados ou o consumo de vegetais ou água contaminados com suas fezes são importantes meios de transmissão ao ser humano. Em reconhecimento à importância da doença para a saúde pública do Rio Grande do Sul, o governo estabeleceu a notificação compulsória de casos de hidatidose humana e a cobertura de seu tratamento pelo SUS em âmbito estadual a partir de março de 2010 (Portaria 203/2010 de 17/03/2010).

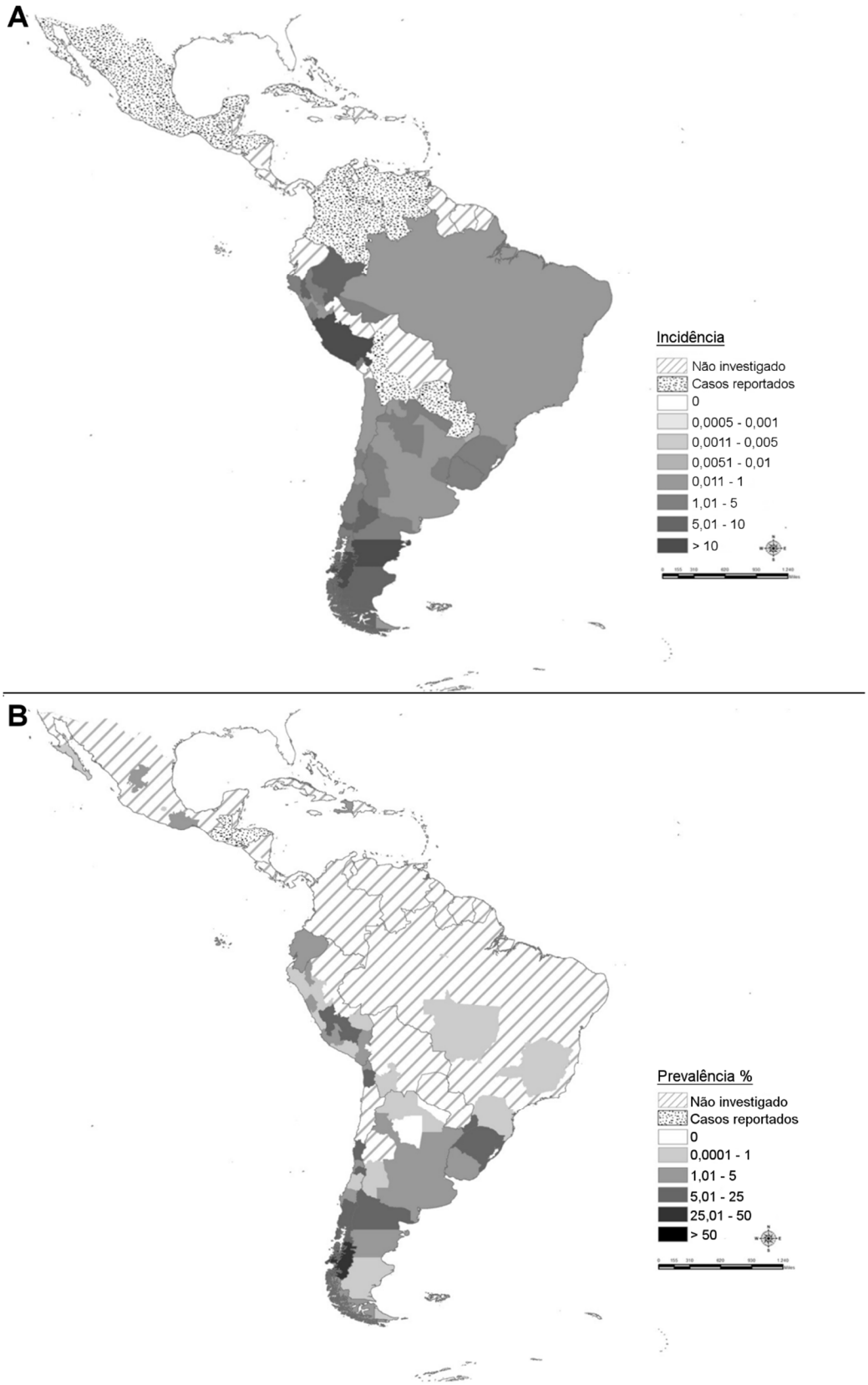

Figura 2. Distribuição da hidatidose cística na América do Sul e na América Central conforme levantamento de 2017. (A) Incidência em humanos. (B) Prevalência nos hospedeiros intermediários domésticos (ovinos, bovinos e suínos). Adaptado de Deplazes *et al*. (2017).

O Rio Grande do Sul possui um rebanho ovino de aproximadamente 4 milhões de cabeças e um rebanho bovino de aproximadamente 14 milhões de cabeças. Esses valores colocam o Rio Grande do Sul como 1º e 6º maior rebanho ovino e bovino, respectivamente, do Brasil (Atlas Socioeconômico do RS/SPGG). E a relevância da hidatidose cística para a pecuária gaúcha é evidenciada por dados coletados de abatedouros de inspeção federal, que registraram uma prevalência entre bovinos de 12 a 16% e entre ovinos de 3 a 19% (DE LA RUE *et al*., 2011). Os abatedouros de inspeção estadual registraram, em 2015, lesões por hidatidose em 26% dos bovinos abatidos (dados da Seção de Epidemiologia e Estatística, DDA/SEAPI). Os prejuízos na pecuária estão associados à redução na quantidade e na qualidade de carne, leite e/ou lã, à redução da fertilidade e da natalidade, à condenação de vísceras infectadas e aos custos de destruição destas vísceras (BENNER *et al*., 2010).

Nos estágios iniciais a infecção é geralmente assintomática, mas o cisto hidático pode induzir algum evento patológico e se tornar sintomático à medida que vai crescendo. Em função do crescimento lento do cisto, o hospedeiro o tolera na maior parte do tempo e comumente as manifestações clínicas da hidatidose cística tem relação com alterações no corpo por causa da localização anatômica ou do grande volume dos cistos, com pressão mecânica exercida em tecidos próximos, ou com ruptura da parede do cisto que gera algum fenômeno alérgico e disseminação da infecção (MANDAL; MANDAL, 2012; SIRACUSANO *et al*., 2012a). Tanto o crescimento lento como a falta de sintomas durante a maior parte da infecção dificultam o diagnóstico precoce da hidatidose cística (MORO; SCHANTZ, 2009).

O diagnóstico da hidatidose cística em humanos baseia-se principalmente em técnicas de imagens, como ultrassonografia e tomografia computadorizada, e confirmação por testes imunodiagnósticos como ELISA e *imunoblot* (ECKERT; DEPLAZES, 2004). Os métodos de imagem, porém, possuem baixa sensibilidade para a detecção de cistos pequenos e por isso são uma desvantagem quando o diagnóstico precoce é fundamental para o sucesso do tratamento (MCMANUS *et al*., 2012). Em estágios iniciais pode ser usado o diagnóstico sorológico, pois mesmo em uma infecção assintomática o hospedeiro pode produzir uma resposta humoral.

O tratamento da hidatidose cística em humanos pode ser quimioterápico ou cirúrgico, podendo as intervenções cirúrgicas envolver a remoção completa do cisto ou pela técnica chamada PAIR (do inglês *puncture, aspiration, injection*, *re-aspiration*) (CROMPTON; PETERS, 2010; MCMANUS *et al*., 2003). Em casos onde não é possível

a intervenção cirúrgica, os quimioterápicos permanecem como a única opção disponível, sendo os mais utilizados os benzimidazóis (albendazol, mebendazol) e praziquantel, capazes de eliminar o metacestódeo e protoescólices, porém com efetividade limitada (~60%). A eficácia dos benzimidazóis é reduzida em cistos grandes (>10 cm) devido à alta quantidade de líquido hidático. Além disso, o albendazol é teratogênico em ratos e coelhos e pode gerar toxicidade em tratamentos a longo prazo (BRUNETTI; KERN; VUITTON, 2010). Em *Echinococcus multilocularis*, uma espécie relacionada causadora da hidatidose alveolar, foi observado que o efeito do albendazol no metacestódeo é mais parasitostático do que parasiticida (REUTER *et al*., 2004), além da droga causar problemas de tolerância e hepatotoxicidade.

1.2 Biologia e ciclo de vida de *Echinococcus granulosus* e *Echinococcus ortleppi*

Organismos do gênero *Echinococcus* são parasitas obrigatórios pertencentes à classe Cestoda, família Taeniidae, cujo ciclo de vida é dependente de dois hospedeiros mamíferos, incluindo o homem em algumas espécies. *E. granulosus* e *E. ortleppi* são espécies endêmicas no sul do Brasil e relevantes do ponto de vista tanto econômico, devido as infecções nos rebanhos, quanto de saúde pública, em função das infecções em humanos.

Os ciclos de vida de *E. granulosus* e de *E. ortleppi* são muito semelhantes e predominantemente domésticos, envolvendo o cão e ungulados criados para consumo. O verme adulto vive no intestino delgado do hospedeiro definitivo (canídeo), onde libera proglótides grávidas contendo ovos. Os ovos embrionados, contento a oncosfera, são dispersados no ambiente juntamente com as fezes do animal. Após a ingestão dos ovos pelos hospedeiros intermediários (ungulados ou o ser humano), as oncosferas ativadas penetram a parede intestinal, chegando a circulação sanguínea ou linfática. As oncosferas são carregadas pela circulação até as vísceras, principalmente fígado e pulmões, onde se estabelecem e se desenvolvem no cisto hidático (metacestódeo). O cisto produz no seu interior os protoescólices, que são formas pré-adultas do parasito. Ao se alimentar de vísceras contendo cistos hidáticos, o hospedeiro definitivo ingere os protoescólices que então se desenvolvem no verme adulto (THOMPSON, 2017) (Fig 3).

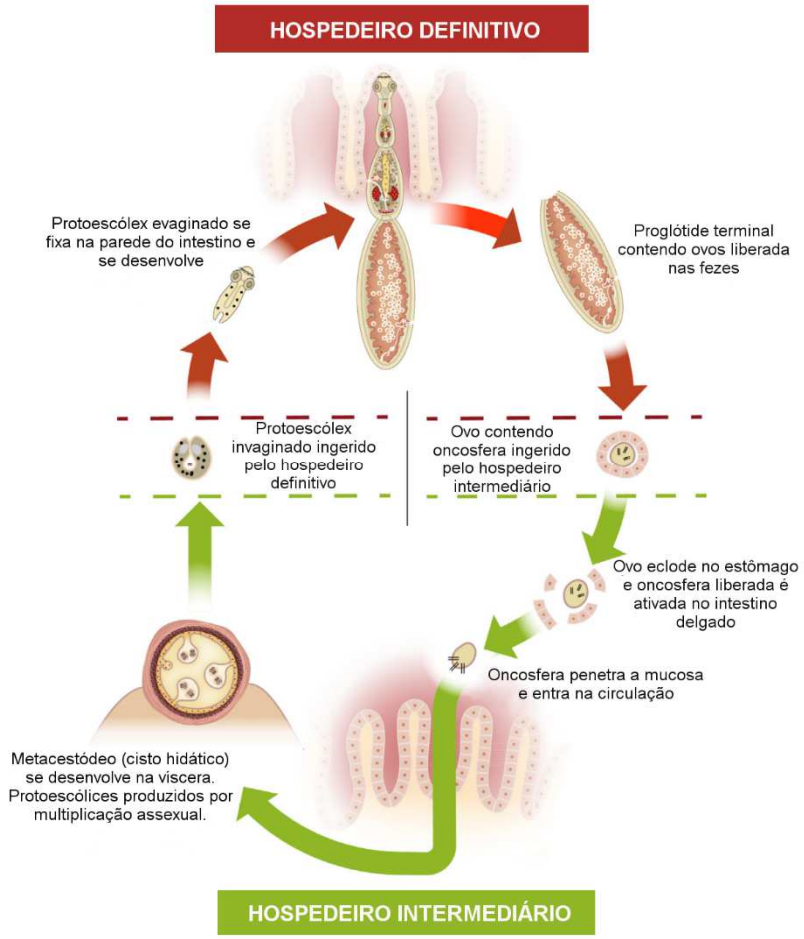

Figura 3. Ciclo biológico de *E. granulosus* e *E. ortleppi*. Adaptado de Thompson *et al*. (2017).

Por muito tempo *E. ortleppi* foi considerado um genótipo (G5) dentro da espécie *E. granulosus*, mas estudos de caracterização molecular mostraram que G5 era geneticamente distinto de G1 (*E. granulosus*), fazendo com que fosse elevado à espécie (NAKAO *et al*., 2013; THOMPSON, 2008). Algumas diferenças que podem ser destacadas são: a especificidade de *E. granulosus* à ovelha e de *E. ortleppi* ao boi como hospedeiros intermediários; diferenças morfológicas nas formas adultas e o crescimento e maturação mais rápido do adulto de *E. ortleppi* (THOMPSON; KUMARATILAKE; ECKERT, 1984). Cabe ressaltar que a forma larval desses parasitos é capaz de infectar outros mamíferos. *E. granulosus*, por exemplo, é amplamente descrito infectando o rebanho bovino, porém, quando comparado com o hospedeiro típico, o cisto frequentemente apresenta menor viabilidade e fertilidade, ou desencadeia um processo inflamatório que não se resolve (BALBINOTTI *et al*., 2012; DÍAZ *et al*., 2011; ROMIG *et al*., 2017).

O cisto hidático, ou metacestódeo, é a fase do ciclo mais estudada em função da sua patogenicidade. A estrutura do cisto hidático consiste em uma parede externa, formada por duas camadas: a camada germinativa, mais interna, e a camada laminar, mais externa. A camada germinativa é o tecido mais ativo do metacestódeo, ela dá origem à camada laminar na face externa, e às cápsulas prolígeras com os protoescólices na face interna. Além disso, as células da camada germinativa e os protoescólices secretam os componentes do fluido que preenche o cisto, o líquido hidático (DÍAZ *et al*., 2015a; TAMAROZZI *et al*., 2016). Externamente à parede do cisto ocorre a formação de uma camada adventícia, que corresponde a um tecido fibroso produzido pelo hospedeiro em resposta à presença do parasito (Fig. 4).

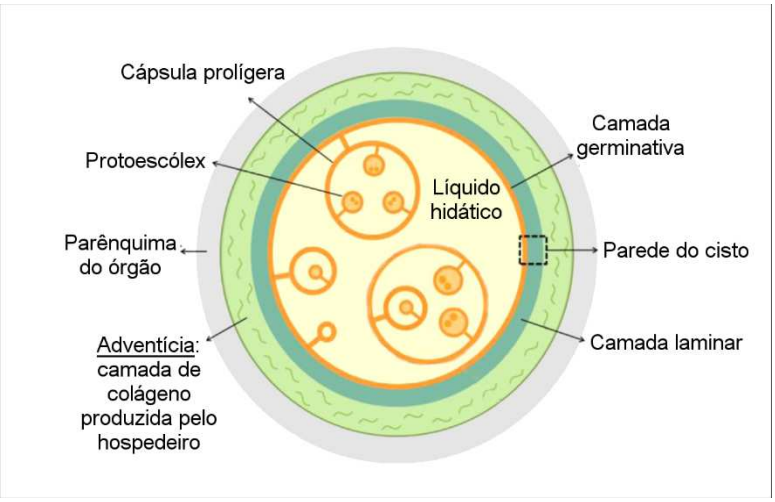

Figura 4. Diagrama estrutural de um cisto hidático. A parede do cisto delimita uma cavidade interna preenchida por líquido hidático e que contém as cápsulas prolígeras e os protoescólices, formas infectivas ao hospedeiro definitivo. Em resposta à presença do parasito o hospedeiro intermediário produz uma camada de colágeno que circunda o cisto hidático. Adaptado de Silva-Álvarez *et al*. (2015b).

O desenvolvimento do metacestódeo no interior dos órgãos propicia um contato muito próximo dos tecidos parasitários e do hospedeiro. Por um lado, isso pode facilitar a assimilação de nutrientes pelo parasito, mas por outro, resulta em uma alta exposição ao sistema de defesas do hospedeiro. Associa-se a isso a longa persistência do cisto hidático no hospedeiro intermediário e tem-se uma interface parasito-hospedeiro de constantes interações e cujos mecanismos moleculares são de grande interesse científico. A elucidação de mecanismos de aquisição de compostos do hospedeiro e de proteção frente à resposta imune tem sido foco de muitos estudos, pois pode ajudar no

desenvolvimento de novas formas de tratamento da hidatidose cística indicando alvos para intervenção.

1.2.1 Produtos de excreção/secreção nas interações Echinococcus-*hospedeiro*

O desfecho das interações parasito-hospedeiro tem uma forte influência na sobrevivência ou eliminação do cisto hidático. O estabelecimento do metacestódeo nos órgãos internos desencadeia uma resposta do hospedeiro buscando eliminar o parasito, ou ao menos inativá-lo. O *Echinococcus* por sua vez produz alguns fatores para interferir na resposta do hospedeiro e se proteger, e outros para sequestrar moléculas essenciais a sua sobrevivência. Em função disso, o líquido hidático contém uma miscelânea de compostos, secretados tanto pelo parasito como pelo hospedeiro, potencialmente atuando em interações. Essa característica faz do líquido hidático a principal fonte de material para estudo da interface *Echinococcus*-hospedeiro.

Inicialmente os estudos estavam voltados para a caracterização imunológica do líquido hidático de *E. granulosus*, e evidenciou-se proteínas como o Antígeno B (AgB) e o Antígeno 5 (Ag5) como seus principais componentes antigênicos, capazes de induzir alta resposta humoral na hidatidose cística (para revisão, ver DÍAZ *et al*., 2015a). Estudos mais recentes utilizando abordagens proteômicas corroboram os dados que o AgB e o Ag5 são as proteínas majoritárias no líquido hidático (AZIZ *et al*., 2011; MONTEIRO *et al*., 2010).

Com a disponibilidade de metodologias proteômicas, tem-se avançado bastante na identificação das proteínas que compõem o líquido hidático, principalmente de *E. granulosus*. A análise do repertório total de proteínas por espectrometria de massas permitiu identificar outras proteínas com papel imunomodulatório e potencial vacinal, como por exemplo, paramiosina, tetraspanina e ciclofilina, bem como proteínas associadas a outros processos vitais, como ferritina, HSP (do inglês, *heat shock protein*), tioredoxina peroxidase e FABP (do inglês, *fatty acid binding protein*) (AZIZ *et al*., 2011; MONTEIRO *et al*., 2010; SANTOS *et al*., 2016).

O conjunto de proteínas no líquido hidático mostra-se bastante diversificado, e em análises de ontologia aparecem termos como atividade catalítica, processos metabólicos, resposta a estímulo, atividade transportadora, entre outros (AZIZ *et al*., 2011; SANTOS *et al*., 2016). Aziz *et al*. (2011) analisaram líquido hidático de cistos de diferentes hospedeiros (bovinos, ovinos e humanos), e encontraram diferenças que podem estar relacionadas com o contexto específico em que cada metacestódeo se desenvolveu. Esses

dados fornecem uma ideia do quão complexa deve ser a interação *Echinococcus*-hospedeiro.

A ampliação do repertório de moléculas conhecidas ajuda a elucidar mecanismos moleculares atuando na interface parasito-hospedeiro e a compreender melhor as estratégias de sobrevivência de *Echinococcus* spp. Além de conhecer os fatores importantes presentes no líquido hidático, é necessário investigar suas atividades biológicas no contexto da hidatidose cística tentando encontrar mecanismos de interação que possam servir para o desenvolvimento de novos métodos terapêuticos.

1.2.1.1 Antígeno B

O Antígeno B (AgB) é uma das proteínas mais abundantes e imunogênicas do líquido hidático (MUSIANI *et al*., 1978) e sua participação em mecanismos de sobrevivência do parasito é evidenciada pelas suas funções imunomodulatória e de transporte de lipídios. O AgB faz parte da família de lipoproteínas HLBPs (do inglês, *hydrophobic ligand binding proteins*) específica de cestódeos. HLBPs são conhecidas por sua estrutura oligomérica de alta massa molecular, composta por subunidades de 7-11 kDa ricas em α-hélices (ALVITE; ESTEVES, 2012; LEE *et al*., 2007). O AgB isolado do líquido hidático apresenta uma massa molecular variando de 160-220 kDa correspondente a partículas formadas por subunidades proteicas menores, de aproximadamente 8 kDa, organizadas de maneira a acomodar uma variedade de lipídios oriundos do hospedeiro, que, por sua vez, perfazem até 50% da massa total da partícula (LIGHTOWLERS *et al*., 1989; OBAL *et al*., 2012; ORIOL; ORIOL, 1975). Os lipídios associados à partícula de AgB são de uma ampla variedade, tanto neutros como polares, e dentre as principais classes lipídicas encontradas estão triacilglicerídeos, esteróis e fosfolipídios (OBAL *et al*., 2012).

Organismos da classe Cestoda, como o gênero *Echinococcus,* têm diversas rotas de biossíntese reduzidas, pois podem obter os produtos dessas rotas diretamente do hospedeiro. É o caso dos compostos lipídicos presentes na partícula do AgB, cujas enzimas necessárias para síntese não são codificadas pelo genoma de *E. granulosus,* enquanto enzimas de modificação são codificadas (TSAI *et al*., 2013; ZHENG *et al*., 2013). Considerando a importância desses compostos para atividades metabólicas básicas e manutenção de membranas celulares, é provável que os mecanismos que atuam no sequestro e transporte de lipídios sejam essenciais para a sobrevivência de *Echinococcus* spp.

As subunidades do AgB são codificadas por uma família multigênica (CHEMALE *et al*., 2001) e cinco subunidades distintas são conhecidas. A contribuição de cada subunidade na composição final e função da partícula do AgB ainda não foi totalmente elucidada. As subunidades do AgB isoladamente têm capacidade de oligomerização, embora com graus de compactação variáveis (MONTEIRO *et al*., 2007; SILVA-ÁLVAREZ *et al*., 2015b). Modelos estruturais das subunidades do AgB mostram uma distribuição de cargas na superfície das moléculas que corroboram um processo de oligomerização eletrostaticamente direcionado (MONTEIRO; ZAHA; FERREIRA, 2008; SILVA-ÁLVAREZ *et al*., 2015a). Os lipídios associados influenciam o tamanho dos oligômeros formados, porém não são imprescindíveis, pois a oligomerização também ocorre com as subunidades deslipidadas (SILVA-ÁLVAREZ *et al*., 2015b). Contudo, a estrutura das partículas nativas de AgB deve ser determinada pela junção de todos esses fatores, interações eletrostáticas das subunidades proteicas e associação a lipídios, que formam um centro hidrofóbico recoberto por uma superfície hidrofílica, permitindo assim a formação de uma molécula de alta massa molecular estável (OBAL *et al*., 2012).

A expressão das diferentes subunidades do AgB parece ser modulada ao longo do desenvolvimento do cisto hidático e dos estágios do ciclo de vida do parasito (ESPÍNOLA; FERREIRA; ZAHA, 2014; TSAI *et al*., 2013; ZHANG *et al*., 2010). Estudos proteômicos mostram que as subunidades encontram-se em abundâncias diferentes no líquido hidático e também que o perfil quantitativo das subunidades varia entre cistos de diferentes hospedeiros (AZIZ *et al*., 2011; FOLLE *et al*., 2017; MONTEIRO *et al*., 2012). Isso sugere papéis diferentes para as subunidades do AgB na biologia de *Echinococcus* spp. Silva-Álvarez *et al*. (2015a), usando as subunidades AgB8/2 e AgB8/3 recombinantes deslipidadas, mostraram que as mesmas ligam lipídios de forma seletiva, sendo capazes de ligar ácidos graxos de 16 e 18 carbonos, mas não colesterol. Entretanto, a presença de colesterol no AgB nativo sugere que outra(s) subunidade(s) teria(m) afinidade por essa molécula. Esse mesmo trabalho reportou a transferência de um análogo de ácido graxo para membranas fosfolipídicas artificiais pelas subunidades recombinantes AgB8/2 e AgB8/3. Esses achados indicam que o AgB estaria envolvido na assimilação de compostos lipídicos no tecido hospedeiro e posterior transporte e distribuição para os tecidos do parasito.

Outro aspecto que chama atenção em relação ao AgB é a sua antigenicidade. Já no início da infecção com a fase larval são detectados anticorpos anti-AgB circulantes, indicando que o AgB alcança o lado externo do cisto hidático e interage com o tecido do

hospedeiro (ZHANG *et al*., 2003). Estudos de interação AgB-sistema imune, descrevem diversas atividades imunomodulatórias para o AgB. Riganò *et al*. (2001) analisaram a produção de citocinas em resposta ao estímulo com AgB em células mononucleares do sangue periférico obtidas de pacientes com hidatidose cística ativa e inativa. Os autores observaram um perfil de citocinas predominante Th1 em pacientes com a doença inativa, enquanto que um perfil predominante Th2 foi observado em pacientes com a doença ativa, por isso foi sugerido que o AgB polariza para uma resposta predominante Th2 não-protetora. Similarmente, células dendríticas sentinelas (imaturas) estimuladas com AgB polarizam linfócitos T para uma resposta Th2 (RIGANÒ *et al*., 2007). Siracusano *et al*. (2008b) detectaram taxas de apoptose aumentadas em células de pacientes com hidatidose cística. Virginio *et al*. (2007) observaram que neutrófilos ativados tem sua produção de H_2O_2 reduzida após exposição ao AgB.

1.2.1.2 Vesículas extracelulares

As vesículas extracelulares (EVs) são um mecanismo ubíquo de secreção, presente nos três grandes reinos da vida, e que têm papel central na comunicação intercelular. A produção de EVs é um processo normal da fisiologia das células, no entanto, ele pode ser modificado de inúmeras formas por estímulos externos, como por exemplo uma infecção. Em resposta a algum estímulo as células podem aumentar a produção de EVs, alterar o conteúdo de moléculas das EVs e produzir subtipos diferentes. De Jong *et al*. (2012) mostraram alterações no proteoma e no transcritoma de EVs secretadas por células endoteliais *in vitro*, após exposição a estressores como hipóxia e alta concentração de glicose. Em *Leishmania donovani* foi demonstrado que a incubação de promastigotas em condições que simulam condições da infecção (37 °C e pH 5,5) induzem liberação de maior quantidade de EVs e também modificam sua composição proteica (SILVERMAN *et al*., 2010). Ji *et al*. (2013) compararam o perfil proteômico de EVs de células de câncer coloretal metastático e não-metastático e encontraram um enriquecimento de fatores de metástase e transdução de sinal nas EVs das células metastáticas.

O termo vesícula extracelular se refere a estruturas arredondadas delimitadas por membrana e que carregam uma variedade de biomoléculas destinadas a funções extracelulares ou a outras células. As EVs podem ser classificadas em dois tipos principais: i) exossomos, que se originam no interior da célula, no sistema endossomal, e em geral possuem um diâmetro de até 100 nm; e ii) microvesículas, que se originam por

"brotamento" da membrana plasmática e possuem 100-1000 nm de diâmetro. Porém, para cada tipo de EV, diversos subtipos podem ser originados, que variam principalmente em tamanho, densidade, biogênese e moléculas componentes. Entretanto, muitas características se sobrepõem entre os subtipos tornando o isolamento dos mesmos uma tarefa complexa e por isso costuma-se trabalhar com o conjunto total de EVs, especialmente em caracterizações iniciais.

EVs podem carregar qualquer tipo de biomolécula, como proteínas, ácidos nucléicos, lipídios e metabólitos. Isso provavelmente contribui para a diversidade de estratégias moleculares mediadas por EVs que têm sido descritas em organismos parasitas de diferentes grupos taxonômicos. Nos estágios iniciais da infecção com *Leishmania,* esse parasito secreta EVs direcionadas para as células do hospedeiro, onde as proteínas contidas nas EVs modificam vias de sinalização facilitando a infecção e sobrevivência do parasito (SILVERMAN; REINER, 2012). Em *Trypanosoma brucei* foi descrito um mecanismo de troca de fatores de virulência entre os parasitos via EVs. Esses fatores melhoram a resistência à resposta imune inata do hospedeiro e causam anemia no hospedeiro (SZEMPRUCH *et al*., 2016). Eritrócitos infectados com *Plasmodium falciparum* secretam EVs contendo material genético e proteínas parasitárias que são transferidas para outros eritrócitos infectados. Essas EVs causaram um aumento na transição para as formas sexuais (gametócito) do parasito, sugerindo que se trata de uma forma de comunicação voltada para a indução da gametocitogênese (REGEV-RUDZKI *et al*., 2013).

Em um estudo pioneiro em platelmintos, Marcilla *et al*. (2012) mostraram a presença de EVs em produtos de secreção/excreção de duas espécies de trematódeos, *Fasciola hepatica* e *Echinostoma caproni*, e que essas EVs podem ser internalizadas por células do hospedeiro. O conjunto de proteínas identificadas nas EVs desses helmintos corresponde a aproximadamente 50% das proteínas previamente identificadas no secretoma total, indicando que as EVs compõem uma parcela importante do secretoma. Posteriormente, a secreção de EVs foi demonstrada em inúmeras espécies de helmintos parasitas. O metacestódeo de *E. multilocularis* produz EVs que suprimem a produção de óxido nítrico e de citocinas pró-inflamatórias em macrófagos *in vitro* (ZHENG *et al*., 2017). EVs de *Schistosoma japonicum* contêm uma alta proporção de proteínas de ligação a ácidos nucléicos e do sistema ubiquitina-proteassomo, ambos passíveis de intervenção farmacológica, e também antígenos de superfície, que são candidatos a vacinas, ressaltando o potencial dos componentes de EVs como alvo terapêutico (ZHU *et al*.,

2016a). Também foi demonstrado em *S. japonicum* que ovos em cultura liberam EVs que são internalizadas por uma linhagem celular de hepatócitos (ZHU *et al*., 2016b). Isso indica que os parasitos podem usar as EVs na comunicação com o hospedeiro nos diferentes contextos encontrados ao longo do ciclo de vida.

RNAs regulatórios, principalmente miRNAs, têm sido amplamente descritos compondo o repertório de moléculas de EVs de helmintos e um grande potencial para exercer atividade sobre alvos no hospedeiro tem sido sugerido. Por exemplo, dentre os RNAs que compõem as EVs do nematódeo *Heligmosomoides polygyrus*, estão miRNAs que possuem sítio alvo na porção 3'UTR do gene *dusp1* de camundongo e uma análise por microarranjo mostrou esse gene regulado negativamente em células incubadas com exossomos de *H. polygyrus* (BUCK *et al*., 2014). DUSP1 é uma fosfatase cuja deficiência parece resultar em uma expressão sustentada de IL-10, que previne uma resposta inflamatória (HAMMER *et al*., 2006).

Descrições de repertórios de miRNAs vesiculares foram feitas para diversos helmintos, como *Dicrocoelium dendriticum*, *S. japonicum*, *Brugia malayi*, *Mesocestoides corti*, *Taenia crassiceps*, entre outros (ANCAROLA *et al*., 2017; BERNAL *et al*., 2014; ZAMANIAN *et al*., 2015; ZHU *et al*., 2016a). O estudo desses repertórios ajuda a elucidar mecanismos pelos quais os parasitos podem regular a expressão gênica do hospedeiro, moldando o ambiente a seu favor, e também a identificar biomarcadores para diagnóstico das infecções, que poderiam ser coletados de fluidos corporais, já que as EVs podem alcançar diferentes partes no organismo do hospedeiro. Um estudo em *S. japonicum* mostrou a identificação de miRNAs circulantes específicos do parasito em infecções em coelhos (CHENG *et al*., 2013). Esse trabalho utilizou como fonte dos miRNAS o plasma total dos coelhos, mas é possível que essas identificações possam ser refinadas isolando-se as EVs do plasma, o que permitiria identificar moléculas menos representadas.

A liberação de EVs pelo hospedeiro também é descrita na literatura como um mecanismo de defesa frente a um invasor. A infecção com *Cryptosporidium parvum* desencadeia a sinalização via TLR4 que culmina na liberação de grande quantidade de exossomos pelo epitélio biliar. Os exossomos liberados contêm peptídeos com atividade antimicrobiana e a exposição de esporozoítos de *C. parvum* a esses exossomos diminui sua viabilidade e infectividade (HU *et al*., 2013).

2 JUSTIFICATIVA E OBJETIVOS

A hidatidose cística é uma doença de distribuição global, que determina prejuízos significativos à saúde pública e à economia de muitos países (BENNER *et al*., 2010; CARDONA; CARMENA, 2013; TORGERSON; MACPHERSON, 2011). Apesar do impacto socioeconômico causado pela hidatidose, as ferramentas disponíveis para o seu controle ainda são bastante limitadas. Diversos aspectos básicos da biologia do gênero *Echinococcus* e de outros helmintos parasitos ainda são pouco conhecidos e isso reflete em limitações para as estratégias de tratamento da hidatidose cística e de outras helmintíases.

Atualmente o tratamento farmacológico utilizado na clínica é a terapia com benzimidazóis, sendo a primeira escolha o albendazol sozinho ou combinado com praziquantel. Contudo, problemas quanto à eficácia e segurança desses compostos têm sido reportados. Em relação a vacinas, poucas formulações foram testadas em animais e as taxas de proteção são variáveis (LARRIEU *et al*., 2015; POURSEIF *et al*., 2018). Há, portanto, carência de estratégias terapêuticas para um melhor manejo da hidatidose cística.

Organismos como o *E. granulosus* e o *E. ortleppi*, capazes de causar uma infecção crônica, por vezes assintomática e com uma resposta imune controlada, e que utilizam o hospedeiro como fonte de recursos sem causar sua morte, empregam mecanismos moleculares para sua sobrevivência que são finamente regulados e versáteis. A disponibilização de conhecimento sobre a biologia de *Echinococcus* spp. e, especificamente, dos mecanismos moleculares presentes na interface parasito-hospedeiro, tem impacto direto na identificação de potenciais alvos vacinais, farmacológicos e para diagnóstico.

Os produtos de excreção/secreção de *Echinococcus* spp. são um material interessante para estudo, pois podem entrar em contato com tecidos e fatores do hospedeiro e, por conseguinte, acessíveis a drogas e vacinas. O AgB é uma proteína com evidências de interação com os tecidos do hospedeiro, tanto pela resposta humoral que elicita como por apresentar lipídios originários do hospedeiro associados ao motivo proteico. Entretanto, pouco se sabe sobre que tipo de interação é necessário que ocorra entre a proteína e as células do hospedeiro para que o AgB exerça seu papel biológico. A determinação de formas de interação do AgB com as células do hospedeiro é um importante passo para o entendimento da função dessa proteína no contexto dos

mecanismos empregados pelo parasito para interagir, modular e captar nutrientes no tecido hospedeiro.

EVs desempenham um papel essencial na infecção por diferentes organismos parasitas, atuando como veículos para moléculas efetoras produzidas pelos parasitos para facilitar seu estabelecimento e sobrevivência. Considerando os múltiplos processos em que as EVs podem atuar, e ainda mais a possibilidade de serem meios de comunicação entre espécies distintas, a caracterização molecular de EVs de *E. granulosus* e *E. ortleppi* é essencial para a compreensão do complexo cenário molecular na interface parasito-hospedeiro. A comparação entre espécies capazes de infectar os mesmos hospedeiros é importante para ressaltar similaridades e diferenças associadas à infecção que ajudarão no planejamento de estudos epidemiológicos e estratégias de intervenção.

2.1 Objetivo geral

O objetivo principal deste trabalho é investigar mecanismos de interação parasito-hospedeiro envolvendo o Antígeno B e a produção de vesículas extracelulares em infecções bovinas com *Echinococcus* spp.

2.1.1 Objetivos específicos

- Elucidar mecanismos de interação do AgB de *E. granulosus* com células de mamíferos em cultura.
- Identificar vias de endocitose associadas à internalização do AgB de *E. granulosus* por células de mamífero em cultura.
- Isolar e caracterizar morfologicamente as EVs secretadas por *E. granulous* e *E. ortleppi* em líquido hidático e sobrenadante de cultivo de protoescólices.
- Analisar a interação de EVs de *E. granulous* e *E. ortleppi* com células de mamífero em cultura.
- Analisar o conteúdo proteico de EVs de *E. granulous* e *E. ortleppi.*
- Investigar a presença de RNAs específicos em EVs de *E. granulous* e *E. ortleppi*.

CAPÍTULO I

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Antigen B from *Echinococcus granulosus* enters mammalian cells by endocytic pathways

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Antigen B from *Echinococcus granulosus* enters mammalian cells by endocytic pathways

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Abstract

Background

Cystic hydatid disease is a zoonosis caused by the larval stage (hydatid) of *Echinococcus granulosus* (Cestoda, Taeniidae). The hydatid develops in the viscera of intermediate host as a unilocular structure filled by the hydatid fluid, which contains parasitic excretory/secretory products. The lipoprotein Antigen B (AgB) is the major component of *E. granulosus* metacestode hydatid fluid. Functionally, AgB has been implicated in immunomodulation and lipid transport. However, the mechanisms underlying AgB functions are not completely known.

Methodology/Principal findings

In this study, we investigated AgB interactions with different mammalian cell types and the pathways involved in its internalization. AgB uptake was observed in four different cell lines, NIH-3T3, A549, J774 and RH. Inhibition of caveolae/raft-mediated endocytosis causes about 50 and 69% decrease in AgB internalization by RH and A549 cells, respectively. Interestingly, AgB colocalized with the raft endocytic marker, but also showed a partial colocalization with the clathrin endocytic marker. Finally, AgB colocalized with an endolysosomal tracker, providing evidence for a possible AgB destination after endocytosis.

Conclusions/Significance

The results indicate that caveolae/raft-mediated endocytosis is the main route to AgB internalization, and that a clathrin-mediated entry may also occur at a lower frequency. A possible fate for AgB after endocytosis seems to be the endolysosomal system. Cellular internalization and further access to subcellular compartments could be a requirement for AgB functions as a lipid carrier and/or immunomodulatory molecule, contributing to create a more permissive microenvironment to metacestode development and survival.

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Author summary

Antigen B (AgB) is an oligomeric lipoprotein highly abundant in *Echinococcus granulosus* hydatid fluid. AgB has already been characterized as an immunomodulatory protein, capable of inducing a permissive immune response to parasite development. Also, an important role in lipid acquisition is attributed to AgB, because it has been found associated to different classes of host lipids. However, the mechanisms of interaction employed by AgB to perform its functions remain undetermined. In this study, we demonstrate that mammalian cells are able to internalize *E. granulosus* AgB in culture and found that specific mechanisms of endocytosis are involved. Our results extend the understanding of AgB biological role indicating cellular internalization as a mechanism of interaction, which in turn, may represent a target to intervention.

Introduction

Cystic hydatid disease (CHD), caused by the larval stage (hydatid or metacestode) of parasites belonging to the *Echinococcus granulosus sensu lato* (s.l.) complex, is a zoonosis of worldwide occurrence, with a considerable medical and economic impact [1]. CHD is endemic or hyperendemic in South America, especially in Argentina, Southern Brazil, Uruguay, Chile and mountainous regions of Peru and Bolivia [2]. In 2010, the World Health Organization added CHD to its list of Neglected Tropical Diseases (http://www.who.int/neglected_diseases/diseases/en/). *Echinococcus granulosus sensu stricto*, or simply *Echinococcus granulosus*, is one of the cryptic species of the *E. granulosus* s.l. complex and is the species most widely distributed worldwide. Also, *E. granulosus* is responsible for most cases of human CHD infections [3].

The adult tapeworm lives in the small intestine of a definitive canid host, and the larval stage develops in the viscera of a wide range of mammal species, including humans. *E. granulosus* life cycle is predominantly domestic, where dogs are the definitive hosts and ungulates are the intermediate hosts [4]. The metacestode is a fluid-filled, unilocular cyst that is classified as fertile when protoescoleces are present in the lumen. Protoescoleces are the pre-adults, infective to the definitive host, which remain quiescent and immersed in the hydatid fluid (HF), which is a complex mixture of molecules of both host and parasite origin. The excretory/secretory products of the metacestode are of special relevance for the host-parasite relationship, as they have a greater potential to interact with host proteins and cells.

Antigen B (AgB) is a lipoprotein, highly abundant and the major immunodominant protein among the excretory/secretory metacestode products in *E. granulosus* HF. AgB belongs to the group of hydrophobic ligand binding proteins (HLBPs), a cestode protein family whose members are known by their high abundance and immunogenicity, and by their oligomeric structure, comprising 7–10 kDa α-helix rich subunits [5–7]. AgB oligomers have been observed predominantly in the molecular mass range of 150–230 kDa, but aggregates with higher molecular masses have also been detected [8,9]. The AgB oligomeric structure comprises 8 kDa subunits (AgB8/1 to AgB8/5) encoded by a multigene family [10], which are differentially expressed among *E. granulosus* life-cycle stages, metacestode tissues and individuals [8,11,12]. The subunits AgB8/1 to AgB8/4 have been identified compounding the proteic moiety of *E. granulosus* native AgB from bovine and human HF samples. AgB8/1 was the most represented subunit in both bovine and human samples. AgB8/3 had distinct relative abundancy between the different hosts, it was the less abundant in bovine samples and it was found in relative high abundance in human samples [8]. A recent study with AgB particles obtained from fertile HF

of swine origin, infected by *Echinococcus canadensis*, a closely related specie, also found AgB8/1 as the most represented subunit. Additionally, the authors found AgB8/4, AgB8/3 and AgB8/5, while AgB8/2 was not detected [13].

It has been demonstrated that delipidated AgB is able to bind hydrophobic compounds *in vitro* [14]. The lipid moiety associated with AgB represent 40–50% of the total particle mass and is constituted by different lipid classes, from hydrophobic lipids, like sterol esters and triacylglycerides, to a variety of phospholipids [9,13]. Moreover, delipidated recombinant AgB8/2 and AgB8/3 subunits were capable of transferring fatty acids analogues to artificial phospholipid membranes [15]. *E. granulosus* genome lacks sequences for several key enzymes for fatty acid and cholesterol synthesis, thus the parasite is incapable of synthesizing these compounds *de novo* [16,17]. Hydatid viability relies on the sequestration and utilization of host lipids, and AgB might be involved in lipid uptake from host tissue and its transport to the parasite, by stabilizing insoluble lipids into a lipoproteic particle [9].

Helminths are said to elicit a modified type 2 response, which consists in Th2 profile with an anti-inflammatory component, probably involving T_{reg} stimulation [18]. AgB roles in the modulation of both innate and adaptive immunity have been proposed. It has been described that neutrophils have both the recruitment inhibited and hydrogen peroxide production decreased by AgB [19,20]. Immature dendritic cells stimulated with AgB matured and primed lymphocytes towards the type 2 response [21]. Also, a Th2 polarization in the Th1/Th2 cytokine ratios was observed in AgB-stimulated peripheral blood mononuclear cells from patients with active CHD [22].

Considering the two main roles attributed to AgB, immunomodulation and lipid transport, it is reasonable to consider that a direct interaction with host cells and tissues should occur. In fact, it was recently demonstrated that AgB binds to macrophages and the plasma membrane of inflammatory monocytes, inducing a non-inflammatory phenotype in macrophages [23]. However, little is known about the molecular details of AgB-cell interaction and whether AgB interacts with non-immune cells, or even enters into the cell.

In the present work, we investigated the ability of HF-purified AgB to enter into different mammalian cell types *in vitro*, and the mechanisms involved in AgB internalization. Immunopurified AgB was incubated with four distinct cell lines representative of different cell types, namely hepatocytes, fibroblasts, macrophages, and lung epithelial cells. We demonstrated the entry of AgB into the cytoplasm of all studied cell lines. Moreover, we provided evidence that the endocytic pathways are involved in AgB internalization by cells, with caveolae/raft-mediated endocytosis being the prevailing one.

Methods

Biological material

Hydatids were obtained from bovine viscera of naturally infected animals slaughtered in the routine work of a local abattoir (São Leopoldo, Brazil). Animal slaughtering was conducted according to Brazilian laws and under supervision of the *Serviço de Inspeção Federal* (Brazilian Sanitary Authority) of the Brazilian *Ministério da Agricultura, Pecuária e Abastecimento*. *Echinococcus* spp contaminated viscera, identified during mandatory meat inspection, were donated by the abattoir for use in this work. HF was removed by punction and aspiration from individual fertile cysts and kept at -80˚C until use. Protoescoleces were collected for parasite genotyping and species determination [24]. Only cysts belonging to *E. granulosus* (G1 strain) were used in this work.

Immunoblot

Aliquots of 100 μl of individual *E. granulosus* HF samples were resolved on SDS-PAGE 12% and electrophoretically transferred onto a nitrocellulose membrane. A pool of rabbit

polyclonal antibodies raised against each recombinant AgB subunit (AgB8/1 to 5) were used at 1:70.000 dilution as primary antibody. The pool of antibodies corresponds to the IgG fraction of each subunit specific serum purified using HiTrap ProteinG HP columns (GE Healthcare), according to the manufacturer's protocol. A horseradish peroxidase-conjugated goat anti-rabbit IgG (GE Healthcare) diluted at 1:7.000 was used as the secondary antibody. Blots were developed using the chemiluminescent reagent ECL Plus (Pierce, ThermoScientific) and imaged in VersaDoc system (BioRad). HF samples with higher AgB content were used for the protein purification step (S1A Fig).

E. granulosus AgB purification

AgB purification was carried out following the protocol described by Oriol *et al.* [25], with some modifications. Briefly, parasite proteins from individual *E. granulosus* HF were precipitated by sodium acetate (5 mM, pH 5.0) and the resultant material was resuspended in phosphate-buffered saline (PBS) containing 20 μM 3,5-di-tert-butyl–4-hydroxytoluene (BHT). The HF parasite enriched fraction, obtained from individual cysts, was subjected to immunoaffinity chromatography using primary IgG antibodies against the recombinant forms of AgB8/1, AgB8/2 and AgB8/4 (see "Immunoblot" section). Antibodies were separately coupled to cyanogen bromide-activated Sepharose 4B resin (GE Healthcare) and the previously prepared HF material was passed through the columns. Bound AgB from each column was eluted with 100 mM tris-glycine pH 2.5, pooled together to reconstitute the original HF sample, then dialyzed against PBS/BHT and concentrated on Amicon Ultra-15 centrifugal filter device, MWCO 3 kDa (Millipore). Resultant purified AgB from individual cysts was kept separated and analyzed on SDS-PAGE 12% (S1B Fig). AgB concentration was determined using a Qubit quantitation fluorometer and Quant-iT reagents (Life Technologies).

Blue Native PAGE (BN-PAGE)

BN–PAGE was carried out using the NativePAGE Novex Bis–Tris Gel System (Invitrogen) according to the manufacturer's specifications. Precast NativePAGE Novex 4–16% Bis–Tris gels were run at 150 V for 1.5–2 h in 50 mM BisTris/Tricine pH 6.8 running buffer, where 0.02% Coomassie G-250 was added to the cathode buffer. Protein samples (10 μg) were mixed with the sample buffer provided. Gels were stained also according to the NativePAGE Novex Bis–Tris Gel System instructions. Briefly, the gel was firstly placed in fix solution (40% methanol, 10% acetic acid), then in destain solution (8% acetic acid) until desired background was obtained.

Cell cultures

A549 (human lung adenocarcinoma, European Collection of Authenticated Cell Cultures: ECACC 86012804), J774.A1 (mouse macrophages, *Banco de Células do Rio de Janeiro*: BCRJ code 0121), NIH-3T3 (mouse fibroblasts, American Type Culture Collection: ATCC CRL-1658) and RH (rat Reuber hepatoma, ATCC CRL-1600) cells were cultivated in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified environment at 37˚C. J774.A1 culture media was also supplemented with MEM non-essential amino acid solution, 2 mM glutamine, 10 mM HEPES and 1 mM sodium pyruvate. All cells lines were free from mycoplasma contamination.

AgB internalization assays

NIH-3T3, A549, J774.A1 and RH cells were grown on sterile glass coverslips in 35 mm Petri dishes until 70–80% of confluency. Cell media was changed to serum-free medium and the

cells were then incubated with 40 μg/ml of AgB for 4 h at 37˚C, or 4˚C. Controls were incubated with equal volume of PBS/BHT. Unbound protein was then removed by three washes with cold PBS and cells were fixed in 4% paraformaldehyde/PBS at room temperature for 15 min.

In all microscopy preparations, a pool of the same antibodies used for AgB purification (anti-AgB8/1, anti-AgB8/2 and anti-AgB8/4) was used as primary antibody for detection of AgB. Fixed cells were permeabilized with 0.2% Triton X-100/PBS and unspecific sites were blocked with 5% BSA in PBS-T (PBS with 0.05% Tween-20). After, cells were incubated overnight at 4˚C with the primary antibodies (1:500) and then for 1 h with 1:200 diluted Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Molecular Probes) at room temperature. Nuclei were stained with 100 nM 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Actin was stained with 50 nM Alexa Fluor 594-conjugated phalloidin (Molecular Probes). Cells were imaged using a LSM 710 Zeiss confocal microscope.

The fluorophore CM-DiI (Molecular Probes) was used to directly label AgB particle, because it has affinity to the lipidic compounds associated to the protein. DiI-labelled AgB was used to analyze internalization without cells fixation. AgB was labelled with 5 μM CM-DiI (Molecular Probes) for 1 h at room temperature. Dye excess was washing out with 5-fold the original PBS volume on Amicon Ultra-0.5 centrifugal filter devices, NMWL 100 kDa (Millipore). RH cells were incubated with 40 μg/ml of DiI-labelled AgB for 4 h at 37˚C, washed three times with cold PBS, and immediately analyzed using an Olympus FluoView 1000 confocal microscope.

Endocytosis inhibition assays

RH and A549 cell monolayers were grown until 70–80% of confluency on sterile glass coverslips in six-well tissue culture plates. After changing the cell media to serum-free DMEM, cells were pre-treated with endocytosis inhibitors for 30 min at 37˚C. A pilot test, where cells were incubated with different concentrations of the inhibitors, was conducted to determine the best concentration to be used. The highest concentration where >80% of the cells remained attached and with little morphological alterations was chosen.

Genistein (Santa Cruz Biotechnology) was used at 100 μg/ml concentration and chlorpormazine (Santa Cruz Biotechnology) at 5 μg/ml. AgB was then added at 40 μg/ml and after incubation at 37˚C for 1.5 h, the unbound proteins were removed by acidic stripping (0.5 M NaCl, 0.5% acetic acid, pH 3.0) and three washes with cold PBS. Cells were fixed and prepared for microscopy as described above. Cells were imaged using an Olympus FluoView 1000 confocal microscope. Immunofluorescence intensity normalized by cell area was assessed with ImageJ software [26]. Image analysis was done on two (A549) or three (RH) independent experiments, where three microscopy fields were counted for each experiment (100–300 cells/experiment).

Colocalization assays

RH cell monolayers were grown until 70–80% of confluency on sterile glass coverslips in 6-well tissue culture plates. Cell media was replaced to serum-free DMEM containing 40 μg/ml AgB and the distribution of internalized protein was compared with that of different endocytic markers following up to 1.5 h incubation at 37˚C. Endogenous transferrin receptors were labeled with 50 μg/ml Alexa Fluor 633-conjugated transferrin (Tfn) (Molecular Probes), added in the last 45 min. Alexa Fluor 555-conjugated cholera toxin subunit B (Ctx-B) (Molecular Probes) at 1 μg/ml concentration was added in the last 15 min of incubation. Adsorbed and unbound proteins were removed by acidic stripping (0.5 M NaCl, 0.5% acetic acid, pH 3.0)

and three washes with cold PBS. Cells were prepared for microscopy and imaged as described for endocytosis assay.

To analyze colocalization of AgB with the endolysosomal system, RH cells were incubated with 40 μg/ml DiI-labelled AgB (described in "AgB internalization assays" section) and 1.5 μM Lysosensor Green DND-189 (Molecular Probes). After 1.5 h incubation at 37˚C, cell monolayers were washed three times with cold PBS and immediately analyzed by confocal microscopy.

Colocalization of AgB with Ctx-B, Tfn or Lysosensor was assessed using JaCoP plugin from ImageJ software [27]. Image analysis was done for two independent experiments.

MTT reduction assays

A549 and RH cells were plated onto 96-well plates at a density of 10^4 cells/well. AgB were added to the cell media at 2.5–40 μg/ml final concentrations. After 24 h incubation, 0.5 mg/ml of MTT solution in PBS was added to each well and incubated for a further 4 h. To solubilize formazan, 100 μl of cell lysis buffer (16% SDS, 40% N,N-dimethylformamide, 2% acetic acid, pH 4.7) was added to each well and the samples were incubated overnight at 37˚C in a humidified incubator. Absorbance values of formazan were determined at 595 nm with an automatic microplate reader (Bio-Rad, model 550). Analysis was done for five independent experiments.

Statistics

A Kolmogorov-Smirnov was applied to verify the normality of the data. Statistical significance was analyzed by unpaired Student's t-test using the GraphPad Prism 6.0 software. Data are expressed as mean ± SEM and p values of less than 0.05 were considered statistically significant.

Results

AgB is internalized by mammalian cells in culture

The ability of AgB particles to interact with and to be internalized by mammalian cells was investigated using AgB immunopurified from *E. granulosus* HF. The native AgB is able to form particles with variable masses, thus the AgB preparation is probably heterogeneous in size. To have some clue on the predominant particles present in our AgB samples, a BN-PAGE analysis was carried out. The presence of a major band corresponding to ~160 kDa, as well as, a band corresponding to higher molecular mass and a smear, which could be resultant of particles with variable molecular masses confirm that our AgB preparation fits the information on literature regarding the molecular mass expected for native AgB (S1C Fig).

Immunopurified AgB was added to the culture medium of NIH-3T3, A549, RH or J774 cells and internalization was evaluated after 4 h of incubation at 37˚C using an immunofluorescence assay. Cells were prepared for confocal microscopy by labelling AgB with polyclonal antibodies against AgB8/1, 2 and 4 subunits and a secondary anti-rabbit IgG conjugated to Alexa Fluor 488. AgB signals were detected in the four cell lines tested, and no differences in extension of AgB internalization was observed, thus suggesting that AgB is able to interact with mammalian cells by a mechanism independent of cellular type. No signals were detected in the cells without AgB (Fig 1).

To confirm the internal localization of AgB in the cells, the lipoprotein was labelled with DiI and incubated with RH cells in the same way as before. However, the analysis on confocal microscope was conducted right after incubation had been finished, without cell fixation. The intermediate sections from confocal z-stacks showed higher AgB signal than top or bottom sections, confirming that AgB was inside cells and not just adsorbed to cell membrane (S2 Fig).

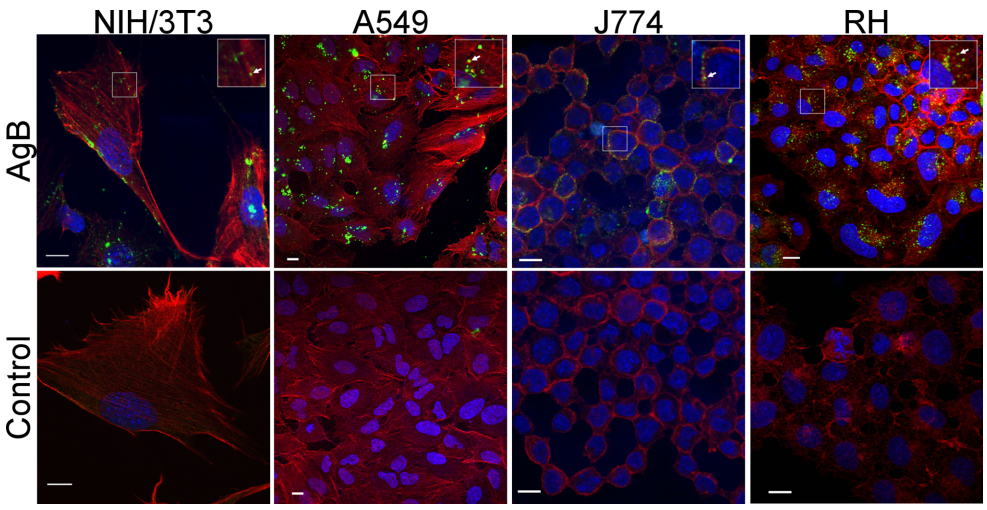

Fig 1. *E. granulosus* AgB uptake by mammalian cells in culture. Immunofluorescence assay was performed on NIH-3T3, A549, J774 and RH cells exposed to 40 μg/ml AgB for 4 h, and mock treated cells (Control). AgB was labeled with polyclonal antibodies against AgB8/1, 2 and 4 subunits and an Alexa Fluor 488-conjugated secondary antibody (green). Nuclei and cytoskeleton were stained with DAPI (blue) and Alexa Fluor 594-conjugated phalloidin (red), respectively. Images are median optical sections from z-stacks obtained by confocal microscopy. Inserts correspond to two-fold digital magnification of boxed areas. Arrows indicate vesicular-like distribution of internalized AgB. Scale bar, 10 μm.

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AgB was detected in the cell cytoplasm, but not in the nucleus. In addition, Fig 1 and S2 Fig show vesicular-like distribution of AgB in the cytoplasm of the cell lines analyzed, indicating an internalization through endocytosis. Supporting this idea, AgB internalization does not occur when RH cells were incubated at low temperature (S3 Fig), a condition known to interfere in endocytosis-dependent cellular internalization [28].

Endocytic pathways involved in AgB internalization

Having established that AgB could access the cytoplasmic compartment of mammalian cells, we then investigated which endocytic pathway could be responsible for this AgB uptake by RH and A549 cells. These two cell lines were chosen to perform the following experiments in an attempt to simulate the natural situation, as liver and lungs are the primary organs infected by *E. granulosus*.

Genistein, a tyrosine kinase inhibitor that prevents caveoale/raft-mediated endocytosis, was used to treat RH cells; and after 30 min AgB were added to the culture media and left to incubate for another 1.5 h at 37˚C. We found that internalization of AgB was inhibited by ~50% in RH cells treated with genistein (Fig 2A and 2B). The same inhibition assay was carried out with A549 cells and we found very similar results, where AgB uptake was inhibited by ~69% (Fig 2A and 2B). The results were statistically significant for both cell lines (Fig 2B).

In order to test whether other endocytic pathways could be involved in AgB uptake by RH cells, the inhibition assay was performed using chlorpromazine, an inhibitor of clathrin-mediated endocytosis. After chlorpromazine treatment, internalization of AgB was reduced by ~13%, however this was not statistically significant ($p = 0.39$) (Fig 2C and 2D). The higher inhibition of AgB internalization in the genistein treatment indicates that caveolae/raft-mediated endocytosis is the major pathway associated with AgB uptake.

The specificity of the inhibitors was confirmed using established endocytic markers as controls. Internalization of transferrin (Tfn), which undergoes clathrin-mediated endocytosis, was

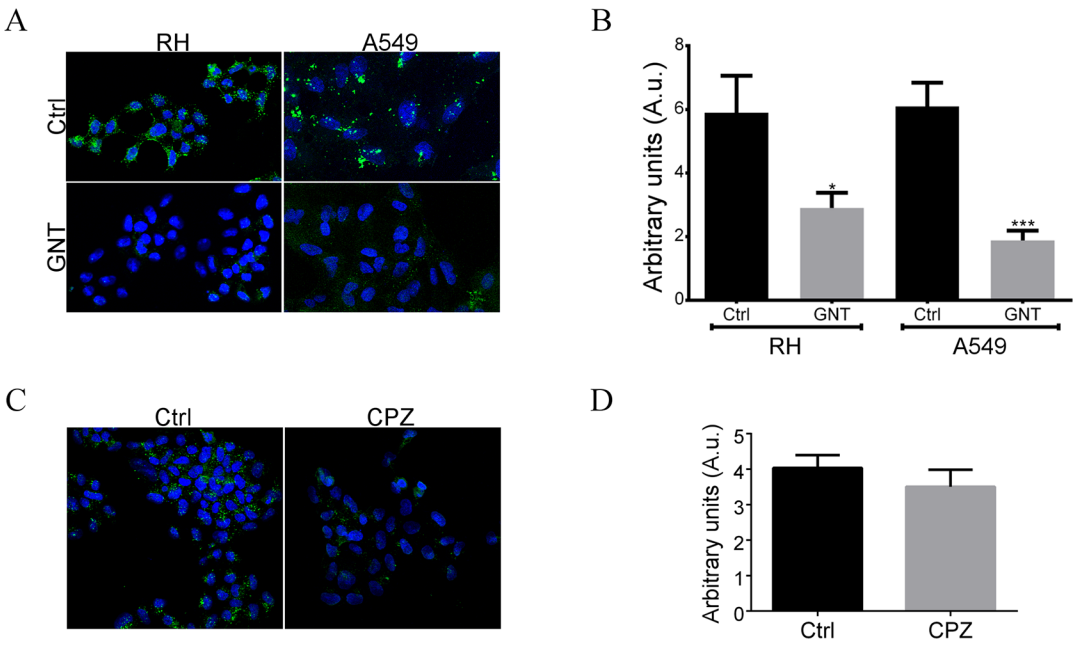

Fig 2. Caveolae/raft-mediated endocytosis is the main route involved in *E. granulosus* AgB internalization by A549 and RH cells. Indicated cells were incubated in medium alone (Ctrl), with 100 μg/ml genistein (GNT), or with 5 μg/ml chlorpromazine (CPZ) for 30 min and then exposed to 40 μg/ml AgB for 1.5 h. AgB was detected using anti-AgB polyclonal antibodies followed by anti-rabbit IgG Alexa Fluor 488 conjugated antibody (green). Cell nuclei were labelled with DAPI (blue). *A* and *B*, Inhibition of caveolae/raft-mediated endocytosis by genistein reduces AgB internalization. *C* and *D*, inhibition of clathrin-mediated endocytosis pathway by chlorpromazine does not cause a significant decrease in uptake of AgB. The quantitative data presented in B and D are measurements from three experiments with RH cells and two with A549 cells. Arbitrary units correspond to immunofluorescence intensity calculated as total immunofluorescence in the cell divided by the area of the cell. Error bars indicate SEM. $^{*}p = 0.037$, $^{***}p = 0.0004$ according to Student's t-test.

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affected only by chlorpromazine. In contrast, genistein only reduced internalization of cholera toxin subunit B (Ctx-B), which undergoes caveolae/raft-mediated endocytosis (S4 Fig).

In a complementary approach to inhibition assays, the distribution of internalized AgB was compared with that of established endocytic markers, Tfn and Ctx-B. RH cells were cultivated in the presence of AgB for 1.5 h, with either Tfn or Ctx-B being added in the last 45 and 15 min of incubation, respectively. Results were analyzed by confocal microscopy and the level of colocalization between the two fluorophores and, consequently, the two proteins, was determined according to Pearson's correlation coefficient. The results indicated that AgB was colocalized with Ctx-B (Pearson's coefficient = 0.64 ± 0.02) (Fig 3), which is in accordance with our previous results that caveolae/raft-mediated endocytosis is involved in AgB uptake. Interestingly, AgB seemed partially colocalized with Tfn (Pearson's coefficient = 0.49 ± 0.04), suggesting that in some degree AgB could be internalized by clathrin-mediated endocytosis.

Altogether, the above findings provide evidence that AgB entry into mammalian cells occurs mainly via caveolae/raft-mediated endocytosis, although it could also occur by clathrin-mediated endocytosis in a lesser extent.

AgB is delivered to the endolysosomal system after internalization

Lysosensor dyes are acidotropic probes that accumulate in acidic organelles in live cells and are used to track the endolysosomal system. To have some clue on AgB fate after endocytosis we sought to check whether AgB signal colocalized with late endosomes/lysosomes by using the Lysosensor DND-189 probe. Incubation of RH cells with DiI-labelled AgB and Lysosensor

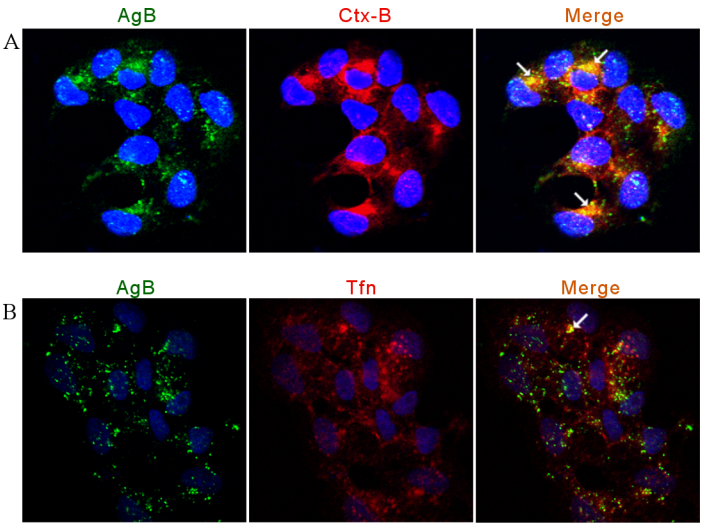

Fig 3. Internalized *E. granulosus* AgB colocalizes with protein endocytic markers in RH cells. Alexa Fluor 633-conjugated Tfn was used as a marker of clathrin-mediated endocytosis and Alexa Fluor 555-conjugated Ctx-B was used as a marker of caveolae/raft-mediated endocytosis. Confocal microscopy images of RH cells incubated with 40 μg/ml AgB and 1 μg/ml Ctx-B (A) or 50 μg/ml Tfn (B) are presented. AgB was detected using polyclonal antibodies against AgB8/1, 2 and 4 subunits and a secondary anti-rabbit IgG Alexa Fluor 488 conjugated antibody (green). Arrows indicate colocalization points. Cell nuclei were labeled with DAPI (blue). The endocytic markers are shown in red.

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was carried out at 37˚C for 1.5 h and colocalization was assessed in live cells using confocal microscopy. A considerable proportion of internalized AgB was found colocalizing with late endosomes/lysosomes (Pearson's coefficient = 0.77 ± 0.008) (Fig 4). This result indicates that AgB trafficks in acidified compartments of endolysosomal system after uptake by cells.

AgB does not induce cellular toxicity

To further investigate the possible effects of AgB internalization by mammalian cells, A549 and RH cells were incubated with different concentrations of AgB (2.5–40 μg/ml) for 24 h. Alterations of cell physiological state were then evaluated by MTT reduction assays. Both cell lines did not present any significant decrease in their ability to metabolize MTT in presence of AgB (Fig 5). AgB do not have a cytotoxic effect on cells, so this interaction is probably part of the mechanisms underlying AgB function in host-parasite interplay.

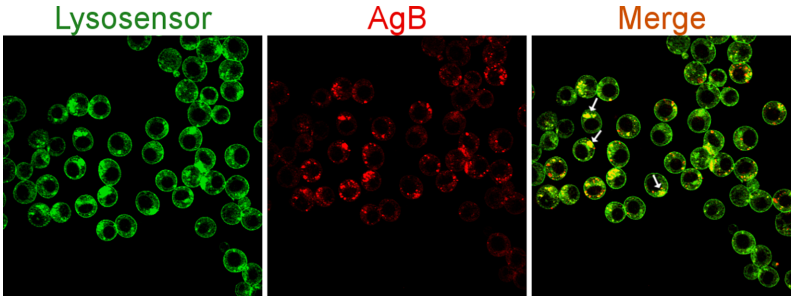

Fig 4. *E. granulsous* AgB reaches the endolysosomal system after endocytosis by RH cells. DiI-labelled AgB (red) at 40 μg/ml and Lysosensor DND-189 (green) at 1.5 μM were added to the cell culture media and left incubate for 1.5 h. Median sections from z-stacks of confocal images are shown. Arrows indicate colocalization points.

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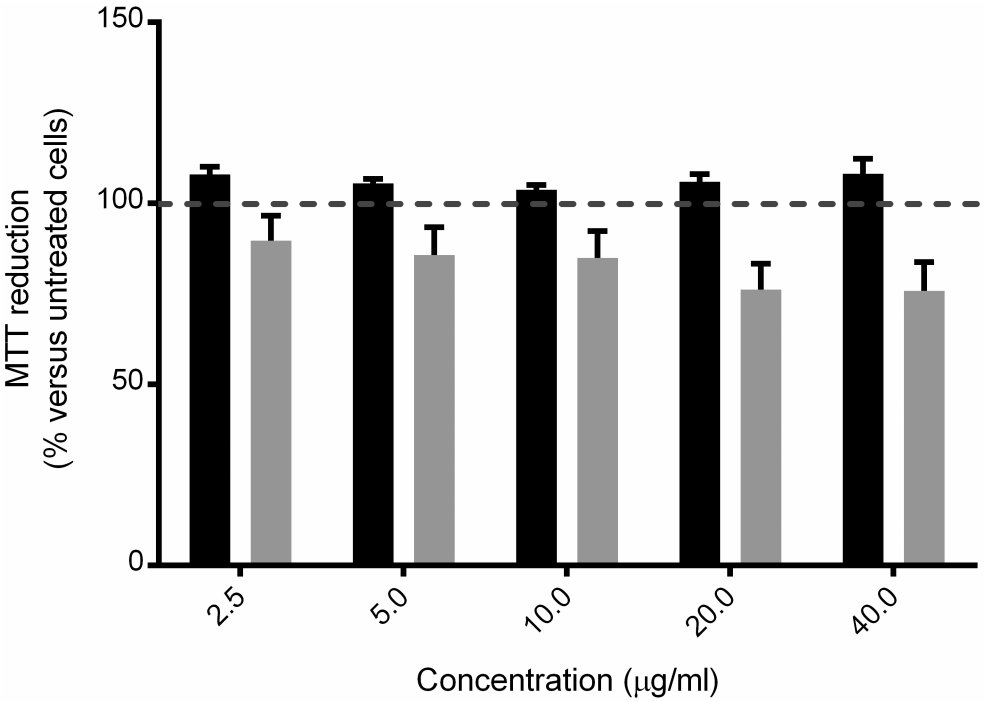

Fig 5. MTT reduction assay. A549 (black bars) and RH (gray bars) cells were treated for 24 h with the indicated concentrations of *E. granulosus* AgB. Cell viability is expressed as percentage of MTT reduction measured for untreated cells and assumed as 100% (horizontal dashed line). Error bars correspond to the SEM values of five independent experiments.

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Discussion

A long-term growth is characteristic of the chronic infection caused by *E. granulosus* metacestodes, and to allow that, different molecular mechanisms are employed by the parasite to ensure its survival and development in the host microenvironment. In an attempt to evade the host's immune response and absorb nutrients from the host, the parasite secretes several molecules. AgB is the major antigen in HF and has been implicated in immunomodulation processes, as well as in lipid uptake and transport [9,29].

AgB has been studied for a long time and it is known to generate a strong humoral response and to modulate host immune response, which is in agreement with the idea that AgB is secreted towards the outside of the hydatid cyst [29,30]. Moreover, there are many studies reporting AgB effects on immune cells that support a direct AgB-cell interaction in host tissue [21,23]. However, whether this interaction occurs with other cell types and what underlying mechanisms are involved in this process, are still unclear. We hypothesized that AgB may interact with host tissue cells surrounding the hydatid cyst to interfere with host cell homeostasis, facilitating nutrient acquisition and immune evasion. In this study, we demonstrated that *E. granulosus* AgB is taken up by mammalian cells *in vitro* by endocytosis. Since AgB internalization seems to be independent of the cell type, it most likely occurs by a ubiquitous mechanism. Thus, we further evaluate the involvement of specific endocytic pathways on AgB internalization.

Clathrin-mediated endocytosis is the best-understood internalization pathway and refers to intake of receptors and their bound ligands through vesicles which are coated by the protein clathrin [31]. Among the molecules known to be internalized by this pathway is the Tfn

receptor, hence Tfn was used as a marker for clathrin-mediated endocytosis [32]. Besides the clathrin-mediated pathway, lipid rafts domains, and its subdomain caveolae, are important contributors to endocytosis processes. Caveolae/raft domains are heterogeneous membrane domains enriched in sphingolipids and cholesterol, and are involved in the endocytosis of various receptors and ligands with a multitude of mechanisms and regulation factors [33]. Ctx-B is a molecule that binds to glycosphingolipid GM1 on caveolae and non-caveolar rafts to be subsequently internalized, so we used it as a marker for caveolae/raft-mediated endocytosis [34]. Chlorpromazine and genistein were used in this study to inhibit clathrin- and caveolae/raft-mediated endocytosis pathways, respectively.

Treatment with genistein was able to significantly decrease AgB uptake by RH and A549 cells. Accordingly, we found that AgB colocalizes with Ctx-B in RH cells. We observed a partial colocalization of AgB with Tfn and the inhibition assay with chlorpromazine showed only a slight, not significant, decrease in AgB internalization. It is possible that clathrin-mediated endocytosis accounts for just a small part of AgB uptake, making difficult the detection of a difference after inhibition by chlorpromazine. Taken together, our results are consistent with the idea that the endocytosis process is required for AgB entry into mammalian cells. Indeed, caveoale/raft-mediated endocytosis is most likely the main pathway involved in AgB uptake by cells. However, a minor role for clathrin-mediated endocytosis in AgB internalization cannot be excluded.

It was proposed that AgB binds to macrophages and monocytes plasma membranes through a lipoprotein receptor; however no specific receptor could be determined [23]. As a lipoprotein, AgB may resemble the structure of some host lipoproteins, then tricking cell receptors to be internalized [9]. Our findings are in agreement with this idea because some lipoprotein receptors, such as lectin-like oxidized LDL receptor-1 (LOX-1), LDL receptor-related protein 6 (LRP6), and scavenger receptors CD36 and CD204 use caveolae/raft-mediated pathways for endocytosis [35–38]. Considering our results, it is also possible that more than one receptor might be involved in AgB binding, so that a higher efficiency of internalization is obtained. Alternatively, the receptor might undergo endocytosis by both pathways upon AgB binding. This regulatory mechanism involving different endocytic routes has been observed with LRP6, in which the receptor is internalized by caveolae to promote Wnt/β-catenin signaling transduction, whereas the clathrin route leads to LRP6 degradation [39].

In some molecules, structural and physicochemical features have central roles in interaction with membrane rafts. An amphipatic helix present in the tyrosine kinase interacting protein (Tip) from *Herpesvirus saimiri* has been shown target Tip to lipid rafts with cholesterol being important to the interaction [40]. Studies on nanoparticles (NP) uptake and dynamics have implicate surface characteristics on raft association and resultant NP mechanism of endocytosis. Lipidic NPs show higher affinity for caveolae as higher their lipophilicity [41], and NP probes designed with cationic surface are preferentially internalized by clathrin coated pits, while zwitterionic-lipophilic nanoprobes are preferentially internalized by lipid rafts [42]. AgB subunits are helix rich lipophilic proteins and predicted as amphiphilic molecules by *in silico* models [9,14,43]. These properties might promote AgB association with caveolaes/rafts lipidic components, which in turn could act as receptors and trigger the endocytosis process upon AgB binding.

AgB uptake did not induce toxicity to cells according to our MTT assay, therefore internalization is more likely part of the mechanisms underlying AgB roles during *E. granulosus* metacestode infection. *E. granulosus* genome lacks genes coding for several key enzymes involved in fatty acid and cholesterol synthesis [16,17]. On the other hand, *E. granulosus* AgB and other lipid binding proteins, like FABPs, are produced and secreted by the parasite to acquire lipidic compounds from host and supply parasite's needs [44]. However, the mechanisms implicated

in lipid capture by AgB are unknown. Since lipids are essential for metacestode survival and development, the understanding of molecular mechanisms employed by the parasites to acquire these host macromolecules will provide potential targets for therapeutic discovery efforts.

AgB interaction with cells from the host tissue surrounding the hydatid could be a suitable scenario to get lipids from biological membranes or inner cell storages. A similar scenario has been described for the *Taenia solium* metacestode, where HLBPs were able to translocate lipid analogs to parasite's tissues, and also colocalize with lipid droplets in the granuloma surrounding the metacestode [6]. The mechanism of internalization of a lipoprotein to get lipids has been described in mammals too, where apolipoprotein A-I (apoA-I) binds macrophage (foam cells) and is internalized. The lipidated apoA-I particle is probably resecreted resulting in an efflux of cholesterol from the foam cell [45].

The oligomeric nature of AgB and its subunit composition could also have some relation with uptake by cells. Recombinant AgB subunits (AgB8/2 and AgB8/3), tested separately, were able to bind fatty acids analogs, but not cholesterol analogs. Cholesterol is found associated to the native particles, so AgB subunits could have selective capacity to bind lipids [15]. Similarly, AgB subunits could participate differently in the internalization process, for example, by varying in affinity for the ligand on the cell membrane. AgB particle is composed by a dozen subunits, but the actual proportion of each one into the final particle is unknown. It is possible that some subunits have major roles in lipid binding, while others are crucial to AgB-cell interaction. Future works testing the subunits individually are necessary to establish whether there is preference in cell association and internalization regarding each AgB subunit.

An endocytosed protein has many possible destinations inside a cell, including route to the trans-Golgi network, to the late endosomes and lysosomes for degradation or to recycling endosomes that bring part of cargo back to the plasma membrane [46]. A possible destination for AgB identified in this work was the acidic organelles in endolysosomal system, i.e., the late endosomes, the lysosomes and the product of their fusion, the endolysosomes. These components form the degradative branch of endocytic pathway, so as consequence of internalization, AgB could be degraded. However, there are intersects between the endolysosomal system and both the secretory pathway and retrograde transport to other organelles [34]. Multivesicular bodies (MVBs), for example, are derived from late endosomes and generate exosomes, that in turn, are released to extracellular milieu upon fusion of MVBs with the plasma membrane [47]. The MVBs could be a route of escape from degradation and redirection to secretion as exosome cargo [48]. However, we cannot exclude the possibility that the consequences of AgB presence in the endolysosomal system may be different according to the target cell. A more deep investigation is necessary to clarify if AgB goes to lysosome for degradation, or if it could escape and follow to secretory pathways with further exocytosis and return to parasitic tissues. Additionally, AgB-cell interaction may be a mechanism used by the parasite to create a more permissive microenvironment for metacestode development and survival. AgB presence in cytoplasm could interfere with cell metabolism, generating molecules and/or signals beneficial to the parasite.

Previous work showed AgB binding to macrophages induces a non-inflammatory phenotype, probably resultant of signaling through cell receptors [23]. In this work, we found that macrophages could also internalize AgB particles, expanding the possibilities on how AgB could modulate the immune response and influence the signalization towards non-inflammatory or alternative pathways. After endocytosis, AgB may access different cell compartments and interfere in cell processes related to immune activity as phagocytosis, antigen presentation, cytokine secretion, vesicles trafficking, etc. Similar mechanisms of immunoevasion were found in others helminth parasites. In *Fasciola hepatica* a cathelicidin-like protein was

described to bind lipid rafts, and after internalization, to divert macrophages function by suppressing lysosomal activity and, consequently, interfering with antigen presentation [49]. Sm16 of *Schistosoma mansoni*, an oligomeric protein with membrane binding capacity, is quickly uptaken by macrophages, but after that is retained in early endosomes, delaying traffic to lysosomes and processing [50,51]. Retention in endosomes might mediate sequestration of essential TLR signaling machinery blocking stimulation of specific TLRs [51].

Like AgB, cestode HLBPs are involved in parasite lipid homeostasis and immunological process [6,52]. Thus, further investigations on the cellular and molecular effect of HLBPs on host cells are important steps to improve the understanding of the parasites biology and disease progression. It will be important to analyze AgB interaction with host-derived cells to confirm biological significance of caveolae/raft-mediated AgB endocytosis. Likewise, elucidating how the molecules sequestered by HLBPs become available to parasites cells will help to identify potential targets for the treatment and control of cestodiasis.

Supporting information

S1 Fig. *E. granulosus* AgB purification. (A) Immunoblot to confirm AgB presence in HF samples. An aliquot of 100 μl from each collected hydatid fluid was resolved on 12% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (lanes 1 to 8). A pool of anti-AgB subunit purified IgGs was used as the primary antibodies. (B) 12% SDS-PAGE analysis illustrating four independent AgB purifications, named 1 to 4. HF refers to hydatid fluid after precipitation by sodium acetate (5 mM, pH 5.0), resuspended in PBS containing 20 μM BHT. AgB refers to the samples eluted after immunoaffinity chromatography and concentrated to 1 ml final on Amicon Ultra-15 device. Aliquots of 10 μl were applied in the gel for both HF and AgB samples. The typical ladder-like pattern of native AgB is observed. Arrow indicates monomeric AgB. Arrowheads indicate AgB multimers. (C) BN-PAGE of immunopurified AgB. Samples (10 μg) from two independent purifications (1 and 2) were resolved on 4–20% BisTris/Tricine polyacrylamide gels and stained with Coomassie blue. The migration of molecular mass markers is indicated on the left of the gel. Protein markers were bovine thyroglobulin (669 kDa), bovine gamma-globulin (158 kDa) and bovine albumin (66 kDa).
(TIFF)

S2 Fig. *E. granulosus* AgB uptake by RH cells. Cells were incubated with 40 μg/ml of DiI-labelled AgB for 4 h at 37˚C. Images were acquired on a confocal microscope without cell fixation. Three different sections and orthogonal views (XZ and XY) for the intermediate section (middle panel) are shown. White lines indicate position of orthogonal views in XY plane. Scale bar, 10 μm.
(TIFF)

S3 Fig. Endocytosis-mediated mechanisms are involved in AgB internalization. RH cells were exposed to AgB at 4˚C for 4h, then fixed with paraformaldehyde. AgB was labelled with antibodies against subunits AgB8/1, 2 and 4 and an Alexa Fluor 488-conjugated secondary antibody (green). Nuclei and cytoskeleton were stained with DAPI (blue) and Alexa Fluor 594-conjugated phalloidin (red), respectively. Scale bar, 10 μm.
(TIFF)

S4 Fig. Effects of genistein and chlorpromazine treatment on Ctx-B and Tfn internalization. RH cells were treated with 100 μg/ml genistein or 5 μg/ml chlorpromazine 30 min prior to addition of Alexa Fluor 555-conjugated Ctx-B or Alexa Fluor 633-conjugated Tfn. Cells were fixed in 4% paraformaldehyde and nuclei were stained with DAPI (blue). Ctx-B and Tfn

are shown in red. Scale bar 10 μm.
(TIF)

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Supporting information

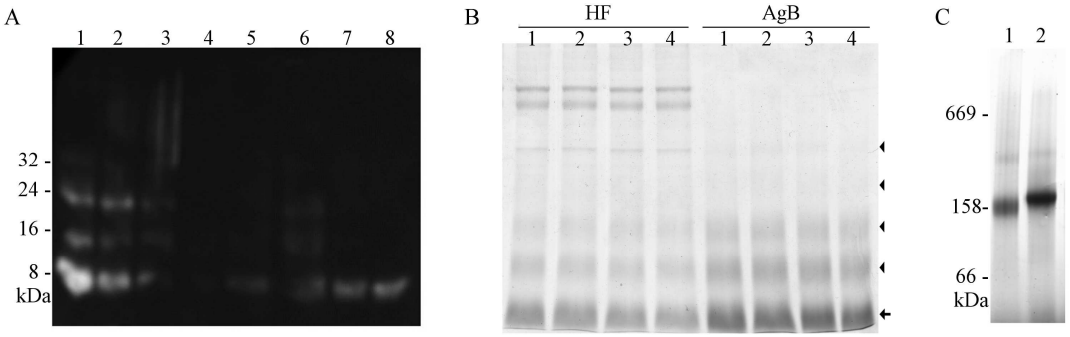

Fig S1. *E. granulosus* AgB purification. (A) Immunoblot to confirm AgB presence in HF samples. An aliquot of 100 µl from each collected hydatid fluid was resolved on 12% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (lanes 1 to 8). A pool of anti-AgB subunit purified IgGs was used as the primary antibodies. (B) 12% SDS-PAGE analysis illustrating four independent AgB purifications, named 1 to 4. HF refers to hydatid fluid after precipitation by sodium acetate (5 mM, pH 5.0), resuspended in PBS containing 20 µM BHT. AgB refers to the samples eluted after immunoaffinity chromatography and concentrated to 1 ml final on Amicon Ultra-15 device. Aliquots of 10 µl were applied in the gel for both HF and AgB samples. The typical ladder-like pattern of native AgB is observed. Arrow indicates monomeric AgB. Arrowheads indicate AgB multimers. (C) BN-PAGE of immunopurified AgB. Samples (10 µg) from two independent purifications (1 and 2) were resolved on 4–20% BisTris/Tricine polyacrylamide gels and stained with Coomassie blue. The migration of molecular mass markers is indicated on the left of the gel. Protein markers were bovine thyroglobulin (669 kDa), bovine gamma-globulin (158 kDa) and bovine albumin (66 kDa).

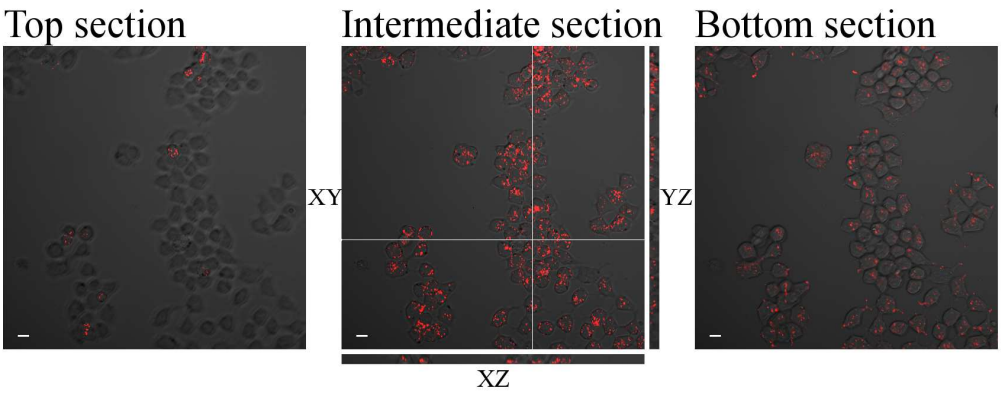

Fig S2. Cells were incubated with 40 µg/ml of DiI-labelled AgB for 4 h at 37°C. Images were acquired on a confocal microscope without cell fixation. Three different sections and orthogonal views (XZ and XY) for the intermediate section (middle panel) are shown. White lines indicate position of orthogonal views in XY plane. Scale bar, 10 µm.

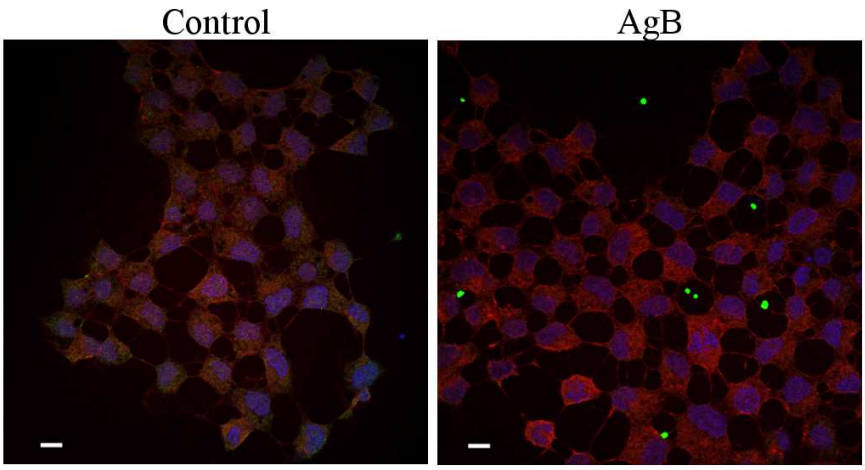

Fig S3. Endocytosis-mediated mechanisms are involved in AgB internalization. RH cells were exposed to AgB at 4°C for 4h, then fixed with paraformaldehyde. AgB was labelled with antibodies against subunits AgB8/1, 2 and 4 and an Alexa Fluor 488-conjugated secondary antibody (green). Nuclei and cytoskeleton were stained with DAPI (blue) and Alexa Fluor 594-conjugated phalloidin (red), respectively. Scale bar, 10 µm.

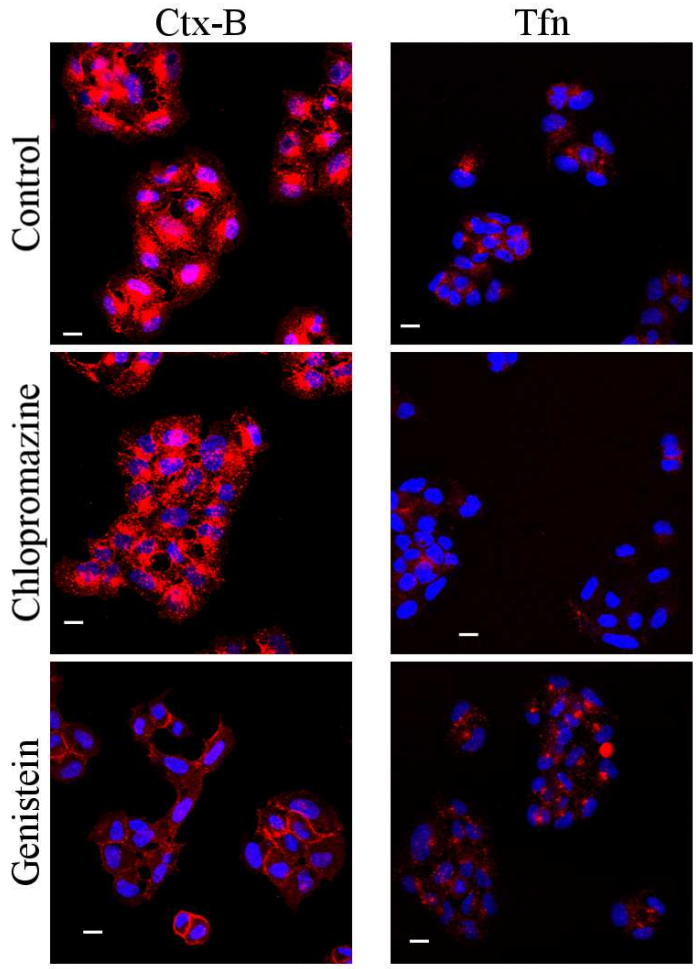

Fig S4. Effects of genistein and chlorpromazine treatment on Ctx-B and Tfn internalization. RH cells were treated with 100 µg/ml genistein or 5 µg/ml chlorpromazine 30 min prior to addition of Alexa Fluor 555-conjugated Ctx-B or Alexa Fluor 633-conjugated Tfn. Cells were fixed in 4% paraformaldehyde and nuclei were stained with DAPI (blue). Ctx-B and Tfn are shown in red. Scale bar 10 µm.

CAPÍTULO II

Artigo a ser submetido para publicação

Isolation of extracellular vesicles released by *Echinococcus* spp. parasites and identification of cargo molecules

Edileuza Danieli da Silva, Maria Eduarda Battistella, Guilherme Brzoskowski dos Santos, Karina Mariante Monteiro, Martin Cancela, Henrique Bunselmeyer Ferreira, Arnaldo Zaha

*Tabelas suplementares deste capítulo estão disponíveis no link abaixo:
https://drive.google.com/drive/folders/1YTNNh5eu8VZxgRrdtD6uU6UKTmbjOXkc

Isolation of extracellular vesicles released by *Echinococcus* spp. parasites and identification of cargo molecules

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ABSTRACT

Cystic hydatid disease (CHD), or hydatidosis, is a parasitic disease caused by the larval stage (hydatid) of *Echinococcus granulosus* and *Echinococcus ortleppi*. A long-term growth is characteristic of the chronic infection caused by *Echinococcus* spp. metacestodes, suggesting that diverse molecular mechanisms are employed by the parasite to ensure its survival and development in the host microenvironment. The hydatid cyst is filled by the hydatid fluid, which contains secretory products from the parasite as well as from the host. Secretory products have an important role in the host-parasite interplay, because they participate in processes such as immunoevasion, nutrient uptake and cell communication. Recent reports have highlighted the importance of secreted extracellular vesicles (EVs) in these processes in helminth parasites. In this study, a comparative analysis of *E. granulosus* and *E. ortleppi* EVs is provided. The EVs population isolated from hydatid fluid by ultracentrifugation, besides showing variable sizes, were enriched in exosome-like vesicles (diameter around 100 nm). *Echinococcus* spp. EVs were internalized by a hepatocyte cell line (RH) *in vitro,* confirming the EV potential of host interaction in CHD. The proteomic analysis of *Echinococcus* spp. EVs revealed proteins mainly related to metabolic processes, binding and catalytic activity, which could be essential for parasite infection. Additionally, we detected by RT-PCR mRNAs of Antigen B, cyclophillin and glyceraldehyde-3-phosphate dehydrogenase, indicating that this class of biomolecules are also integrant of *Echinococcus* spp. EVs. The presence of EVs in the secretory products of *E. granulosus* and *E. ortleppi* point to an important role for these structures in host-parasite communication. The study of the EVs cargo molecules will help to shed some light in the intricate molecular mechanisms acting at host-parasite interface.

Keywords: *Echinococcus* spp., hydatid cyst, secretory products, extracellular vesicles.

INTRODUCTION

Echinococcus granulosus and *Echinococcus ortleppi* are aetiological agents of the cystic hydatid disease (CHD), a neglected tropical disease that imposes an economic burden in developing countries associated to the losses in husbandry and medical treatment of infected people (1). *E. granulosus* is globally distributed and the most frequent species causing human CHD (2). South America is an endemic or hyperendemic region for *E. granulosus* and *E. ortleppi*, especially in southern Brazil, Uruguay and Argentina (3). The pastoral activities in those regions have great economic importance and the high prevalence of these species in the livestock represents a threat in terms of both veterinary and public health (4).

E. granulosus and *E. ortleppi* are cestode parasites with a life cycle dependent of two hosts. The adult stage is a tapeworm that lives in the intestine of canids, the definitive hosts, producing eggs that are released in the feces. Infection of intermediate hosts, mainly livestock species and humans, occurs after ingestion of embryonated eggs. Eggs contain the oncosphere, which actively penetrates the intestinal wall, and travels via blood and lymphatic vessels to the target sites, mostly lungs and liver. CHD results from the larval stage (metacestode or hydatid) development into the viscera of the intermediate host. The metacestode develops as a unilocular bladder-like structure that contains protoscoleces, the pre-adult forms infective to the definitive host. The hydatid fluid (HF), which is rich in products secreted by both the parasite and the host, fills the metacestode internal cavity.

The identification of key factors produced and secreted by *Echinococcus* spp. during CHD is essential to the development of new therapeutics, by the identification of potential intervention targets. Characterization of *E. granulosus* HF proteins has been achieved by proteomic approaches in different studies (5–7), and all indicate that HF is a complex mixture resultant of an active host-parasite cross-talk. Recently, a work from our group found a surprising proportion of proteins (35%) without signal peptide, or other known pattern of secretion, among the parasitic proteins present in HF from fertile cysts (containing protoscoleces). In addition, it was evidenced that extracellular vesicles (EVs) are part of *E. granulosus* HF, what would explain the presence of such leaderless proteins among the excretory/secretory products (6).

EVs are membrane-delimited rounded structures that carry biomolecules and could deliver them to cells other than that of the origin. It is accepted that EVs are heterogeneous in size and composition, but two main group are commonly described,

exosomes and microvesicles. Exosomes originate from intraluminal vesicles of multivesicular bodies and have a diameter range of ~ 30-100 nm, while microvesicles are up to 1 μm and originate following direct outward budding of cell membrane (8).

It has been demonstrated that inter-cellular communication by EVs is a ubiquitous mechanism, and the production of EVs has emerged as an important secretory pathway in parasitic biology (9). Increasing evidences show that EVs secretion is part of host-parasites interplay, where EVs are able to shuttle parasitic factors into host cells and to alter host physiological processes (10–13). EVs secreted by the liver fluke *Opisthorchis viverrini* induce cholangiocytes to adopt a tumorigenic phenotype (13). *Echinococcus multilocularis*, another important species from *Echinococcus* genera, secretes EVs that exert regulatory effects upon macrophages *in vitro* (12). Recently, a characterization of protein content of EVs isolated from *E. granulosus* HF collected from sheep hydatid cysts was published (14). The authors report a high abundance of peptidases, oxidoreductases and lipid binding proteins in their exosomal preparation, and suggest exosomes could be considered as one of the main transporters of virulence factors in *E. granulosus.* EVs are important vehicles to nucleic acids too, especially regulatory RNAs. miRNAs are the nucleic acids most commonly found enclosed in EVs and there is evidence that host transcripts could be targeted by them (10, 15).

E. granulosus and *E. ortleppi* are phylogenetically related species and have some sympatric distribution in South America. The molecular characterization of related species is essential to highlight the main genes, proteins or others factors associated to their survival strategies. The knowledge of the shared and/or exclusive molecular mecanisms employed by related organisms might help to design more effective control strategies. In the present study, we collected bovine fertile hydatid cysts from *E. granulosus* and *E. ortleppi* and comparatively analyze the EVs present in the HF and secreted by protoscoleces cultivated *in vitro*. EVs morphological characteristics were determined, protein profiles and some RNA cargo were identified, and evidences for *Echinococcus* spp. EVs interaction with host cells are shown.

METHODS

Biological material

Viscera containing hydatid cysts were obtained from naturally infected cattle at a local abattoir (São Leopoldo, Rio Grande do Sul, Brazil). Animals were slaughtered in the abattoir routine work, in conformity to Brazilian sanitary authority laws. After meat

inspection, condemned infected viscera were taken to the laboratory to dissecation. HF was obtained by punction and aspiration of hydatid cyst, and then inspected for the presence (fertile condition) or absence (infertile condition) of protoscoleces. Only material obtained from fertile cysts containing protoscoleces with viability over 80% was used in this work.

A sample containing ~20 µl of protoscoleces from each selected hydatid cyst was used for species determination as previously described (16).

Protoscoleces *in vitro* cultivation

Protoscoleces collected from *E. granulosus* and *E. ortleppi* fertile cysts, showing >80% viability, were washed in sterile PBS containing antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). To induce an activated state, protoscoleces were treated with 2 mg/mL pepsin for 20–30 min at pH 2 to mimic the contact with the digestive enzymes of the definitive host. After pepsin treatment, the evaginated protoscoleces were kept in RPMI-1640 (Sigma-Aldrich) supplemented with 10% exosome-depleted fetal bovine serum (Gibco) for 3 days, with daily changes of the medium. The removed culture supernatant from every day was saved, and at the end of cultivation, pooled and subjected to EVs isolation procedure.

Extracellular vesicles isolation

E. granulosus and *E. ortleppi* EVs were isolated by ultracentrifugation essentially as previously described (17). Firstly, HF and protoscolex culture supernatant were clarified by sequential steps of low speed centrifugation: 500 *x g* for 10 min and 2,000 *x g* for 20 min at 4 °C. Then, denaturated proteins and particles were removed by centrifugation at 10,000 *x g* for 45 min at 4 °C. The supernatant was collected and filtered through 0.22 µm membrane filter (Millipore). The filtrate was ultracentrifuged at 120,000 *x g* for 130 min at 4 °C in Type 50.2 Ti rotor in a Beckmann Coulter Optima-90K ultracentrifuge. The sedimented material was completely ressuspended in 3 ml of PBS and ultracentrifuged again at 120,000 *x g* for 70 min. From this point, the isolated EVs were used as described specifically to each experiment.

Immunoblot

Immunoblot was carried out with 25 µg of proteins from HF EVs, total HF and protoscolex total extract, all originated from individual hydatids. EVs extract was

prepared by ressuspending the ultracentrifugation pellet in 50-100 µl PBS. Total HF samples correspond to material prior ultracentrifugation. To prepare total extract, protoscoleces in 50 mM Tris-HCl pH 8.0, 150mM NaCl and 1% Triton X-100 were sonicated five times for 30s with 1 min intervals. The resultant lysate was centrifuged at 14,000 rpm, 4 °C for 30 min. For all samples, the protein content was estimated using Micro BCA protein assay kit (Thermo Scientific).

Samples were resolved by 12% SDS-PAGE and transferred onto PVDF membranes (Hybond, GE Healthcare). The membranes were blocked for 16 h with 5% skimmed milk in PBS-T (PBS containing 0.1% Tween-20). Rabbit polyclonal anti-rEg14-3-3.1/3 (diluted 1:20,000 v/v) or anti-rEgEnolase (diluted 1:5,000 v/v), described elsewhere (18, 19), were used as primary antibodies. Horseradish peroxidase (HRP)-labeled anti-rabbit IgG (ECL, GE Healthcare) was used as secondary antibody (1:7,000 v/v dilution). Antigen−antibody complexes were detected with the ECL Plus Western Blotting Substrate (Pierce) and imaged using the VersaDoc imaging system (Bio-Rad).

Transmission electron microscopy (TEM)

Immediately after ultracentrifugation, EVs isolated from individual HF or protoscolex culture supernatant were ressuspended in 50 µl PBS and fixed in 2% paraformaldehyde for 15 min at room temperature. Formvar/carbon grids (300 mesh) were deposited onto drops of fixed EVs for 20 min. Grids were washed by passages on Milli-Q water drops, post-fixed in 2.5% glutaraldehyde, washed again and negatively stained in 2% uranyl acetate. Images were recorded on electron microscope JEOL JEM 1200 EXll.

Immunogold

After paraformaldehyde fixing step, EVs from *E. granulosus* HF were permeabilized in 0.01% triton X-100 and blocked with 5% bovine serum albumin (BSA). Proteins were immunolabelled by incubating the grids with anti-rEgEnolase (1:30 v/v dilution) or anti-rEg14-3-3.1/3 (1:80 v/v dilution) at room temperature for 3 h. After washing with PBS, the grids were incubated with 15 nm gold-conjugated Protein G (1:20 v/v dilution) (Electron Microscopy Sciences) at room temperature for 1.5 h. Samples were post-fixed in 2% glutaraldehyde, contrasted and analyzed as described above.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was carried out with the ZetaView equipment (Particle Metrix) to ascertain size distribution of individual *E. granulsous* and *E. ortleppi* HF EV preparations. In NTA, the Brownian motion of particles in solution is related back to particle sizes and numbers, allowing better resolution of vesicle size and concentration. Two EV preparations in 1 ml PBS solution from each species was analyzed. For each sample, videos with at least 10 positions were taken and analyzed using the ZetaView software with default settings. The minimum size was 5 nm, temperature was 21 °C and conductivity was 4000 μS/cm. Results represent the mean of a representative video acquired for a given sample.

Extracellular vesicles interaction with mammalian cells

Cell culture

RH (rat Reuber hepatoma) cells were cultivated in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified environment at 37 °C. For the interaction assay, RH cells were grown on sterile glass coverslips in 35 mm Petri dishes containing 1.5 ml of media.

Extracellular vesicles labelling

Echinococcus spp. isolated EVs were ressupended in 100 μl PBS and protein content was estimated using Micro BCA protein assay kit (Thermo Scientific). Samples of EVs, equivalent to 30-75 μg of protein, were labelled with 5 μM CM-DiI dye (Invitrogen) for 1 h at room temperature. DiI is a lipophilic fluorescent dye, thus used to label the vesicles membrane. EVs were washed in PBS with centrifugation at 120,000g for 90 min at 4 °C to remove unbound DiI.

Interaction assay

DiI-labeled EVs were added to the culture medium of RH cells and incubated for 4 h. Cells were then washed in cold PBS and immediately imaged, or fixed in 4% paraformaldehyde, stained with 100 nM 4’,6-diamidino-2-phenylindole (DAPI) (Thermo Scientific) for 20 minutes and then imaged. The images were acquired using a confocal microscope (Olympus FV1000).

Mass spectrometry (MS)

EV samples selection

MS analysis of proteins from EVs isolated from HF was performed in two sets of samples, so-called EV isolates (EV/I) and EV pools (EV/P). EV/I set consisted of six biological replicates for each species, i.e., each EV preparation was obtained from an individual hydatid and separately analyzed by MS. At least 40 μg protein estimated content was prepared for MS analysis. For the EV/P set, three EV preparations (15 μg each) from individual hydatids were mixed to make a pool. Three pools for each species were generated and separately subjected to MS analysis.

EVs isolated from protoscolex culture supernatant were also subjected to MS analysis. However, the protein content in *E. ortleppi* samples was too low, precluding further analysis. Then, a unique sample of protoscolex EVs from *E. granulosus,* that yielded 100 μg protein content, was analyzed with technical duplicates.

For all samples, the protein content estimation was carried out with the Micro BCA protein assay kit (Thermo Scientific).

Trypsin digestion and MS^{E} *analysis*

EV samples in PBS were mixed with equal volume of 0.2% RapiGest SF (Waters) and sonicated at 30 W setting, in 3 cycles of 30 s pulse and 1 min interval. Proteins were reduced and alkylated in 5 mM DTT (dithiotreitol) and 10 mM iodoacetamide, respectively. Tryptic digestion was carried out overnight at 37 °C using 1:30 proportion of Trypsin Gold, Mass Spectrometry Grade (Promega). Trifluoroacetic acid (TFA) was added to the samples and RapiGest SF was removed according to the procedure specified in the manufacturer's manual. The peptide mixtures were desalted in OASIS HLB Cartridges (Waters) and lyophilized using a vacuum lyophilizer.

Tryptic digested samples were analyzed by LC-MS^{E} using a nanoACQUITY UPLC and a Xevo G2-XS Q-Tof mass spectrometer (Waters) with a low-flow probe at the source. Lyophilized samples were resuspended in 0.1% formic acid and separated on an Acquity UPLC BEH C18, 1.7 µm, 2.1 × 50 mm analytical column (Waters). To ensure that all samples in a defined set (EV/I or EV/P) were injected with the same amount into the columns, stoichiometric measurements based on scouting runs of the integrated total ion account (TIC) were performed prior to analysis. Peptides were eluted from the column at a flow rate of 8 µl/min, using a 90 min linear gradient from 7 to 85% water/ACN 0.1% formic acid. All data were collected in data-independent MS^{E} mode of acquisition. The

MS survey scan was set to 0.5 s and recorded from 50 to 2000 m/z. MS/MS scans were acquired from 50 to 2000 m/z, and scan time was set to 1 s.

Database searching and quantification

For proper spectra processing and database searching conditions, the Protein Lynx Global Server v.3.0.3 (PLGS) (Waters) was used. Peptide and fragment ion tolerances were determined automatically (approximately 10 and 20 ppm, respectively) by the program and the maximum allowed missed cleavages by trypsin were up to one. Carbamidomethylation of cysteines was specified as a fixed modification, whereas the oxidation of methionine was specified as a variable modification. Protein searches were performed against the deduced amino acid sequences from the *E. granulosus* genome assembly (PRJEB121) version WBPS9, available at WormBase ParaSite (http://parasite.wormbase.org) or the *Bos taurus* protein sequences obtained from UniProt/Swiss-Prot. The databases used were reversed and appended to their original database to assess the false discovery rate (FDR) during identification. FDR was set to 4% at the protein level of the identification algorithm. Results were filtered to a minimum of 2 peptides per protein.

For relative protein quantitation of EV/P samples, the PLGS v.3.0.3 software was used with the IdentityE algorithm using the Top3 methodology. The intensity of each peptide was normalized against the intensity value of peptides of the protein Antigen 5 (EgrG_000184900), which was the protein identified in all EV/P samples (*E. granulosus* and *E. ortleppi*) with a coefficient of variation of zero. The quantitative results were additionally filtered to include only proteins detected in at least 2 out of 3 replicates from each species. Protein relative abundances were presented by the PLGS as a probability (P) of up- and down-regulation in calculated ratios among the two species. A protein was considered up-regulated when $P > 0.95$ and down-regulated when $P < 0.05$.

Extracellular vesicles RNA analysis

mRNA extraction and cDNA synthesis

Ultracentrifugated EVs from *E. granulosus* and *E. ortleppi* HF were directly ressuspended in 1 ml TRIzol (Invitrogen). Aliquots of 1 mL of the HF pre and pos ultracentrifugation were lyophilized and ressuspended in 1 ml TRIzol. The samples in TRIzol were frozen in liquid nitrogen and kept at -80°C until the RNA extraction.

Total RNA was extracted following the manufacturer's instructions. Isolated RNA was treated with RNase-free DNase I (Sigma-Aldrich) to remove genomic DNA, and then used to first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen) and oligo $(dT)_{18}$ as anchor primer. Each RNA sample was divided in two parts, one half was used to effectively synthesize cDNA and other half was used for RT(-) reaction, where no reverse transcriptase was added.

RT-PCR analysis

Transcripts of genes coding for Antigen B subunits (*AgB1 to 5),* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and cyclophillin (*CYP-1*) were amplified with a set of primers previously described (20). Synthesized cDNA (1 μL) was used as template for PCR in the following reaction mixture: 0.2 μM of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs, PCR Buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1 U Platinum Taq DNA polymerase (Invitrogen), and MilliQ water in a final reaction volume of 25 μL. The RT(-) reaction was included to confirm the absence of genomic DNA. The RT-PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles with denaturation at 94 °C for 1 min, annealing at 60 °C for 45 s and extension at 72 °C for 1 min, and at the end, a final extension step at 72 °C for 10 min. The amplified products were analyzed by electrophoresis in 2% agarose gels.

RESULTS

Extracellular vesicles isolation and morphological characterization

E. granulosus and *E. ortleppi* HF and protoscolex culture supernatant were subjected to a differential centrifugation protocol to isolate EVs. The isolated material was prepared to analysis by TEM and images shown the presence of spherical structures characteristic of EVs (Fig 1). Most of observed vesicles showed diameters around 100 nm, in accordance with the expected size for exosome-like vesicles. However, in some images, vesicles around 50 nm or larger than 150 nm are noticeable (Fig 1 and S1).

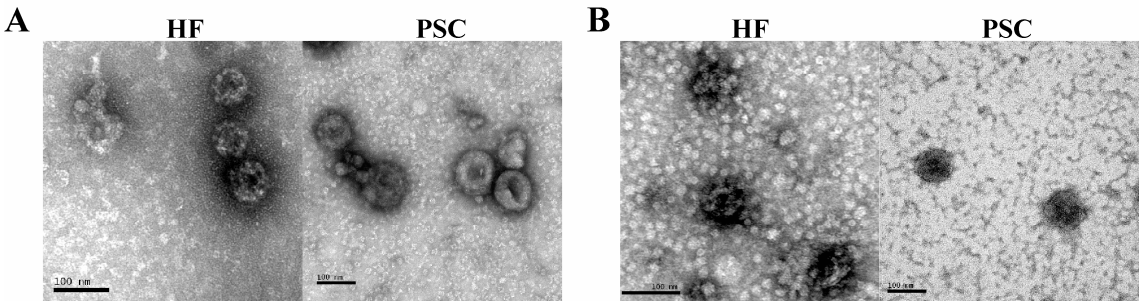

Figure 1. TEM analysis of *Echinococcus* spp. extracellular vesicles. EVs isolated from *E. granulosus* (A) and *E. ortleppi* (B) hydatid fluid (HF) and protoscolex culture supernatant (PSC), were adsorbed onto formvar/carbon grids and contrasted with uranyl acetate. The scale bar indicates 100 nm.

A NTA was performed to estimate the size distribution within EV preparations from *E. granulosus* and *E. ortleppi* HF. In agreement with TEM analysis, different diameter sizes were detected, including small ones like 30 nm, and diameters higher than 200 nm. The major subpopulations of vesicles were estimated by NTA with a mean diameter of 100 nm for *E. granulosus* and 118 nm for *E. ortleppi*, indicative of an enrichment of exosome-like EVs in our preparations, especially in *E. granulosus* (Fig 2). The most represented diameter of 118 nm in *E. ortleppi* might indicate a significant production of microvesicles in this species, however, because is a minimal difference, future investigations using fractionation by density gradient are necessary to clear this issue. Altogether, TEM and NTA results indicate that *Echinococcus* spp. isolated material probably contain different types of EVs, including exosomes and microvesicles.

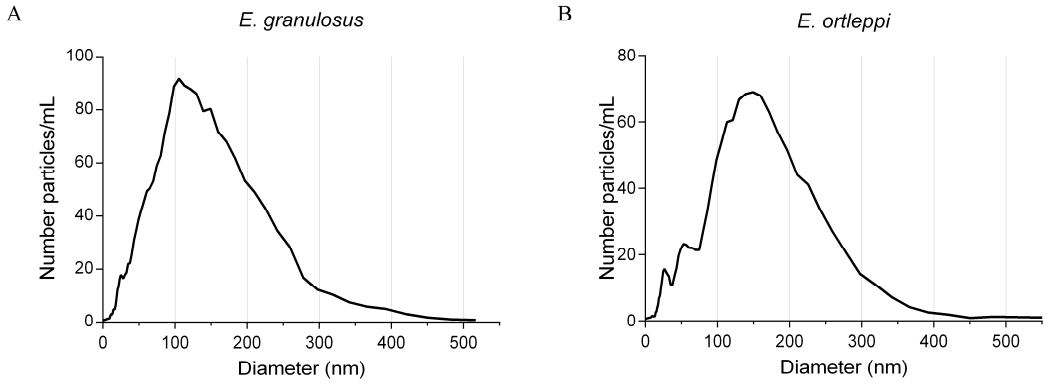

Figure 2. Nanoparticle tracking analysis of extracellular vesicles from (A) *E. granulosus* and (B) *E. ortleppi* HF.

Enolase and 14-3-3 proteins are extracellular vesicle cargoes in *E. granulosus* and *E. ortleppi*

Previous studies revealed the presence of proteins lacking signal peptide among *E. granulosus* secreted proteins, and our group has studied some of them in detail, such as enolase and 14-3-3 proteins (18, 19). The presence of enolase and 14-3-3 proteins in EV samples from *E. granulosus* has been demonstrated (6). Here, we performed immunoblot assays with EVs from both species *E. granulosus* and *E. ortleppi* in parallel. In that way, we demonstrate enolase and 14-3-3 proteins are also present in *E. ortleppi* EVs (Fig 3A).

In order to demonstrate unequivocally that enolase and 14-3-3 are secreted into EVs, the immunogold technique was applied in *E. granulosus* samples. EVs were fixed and permeabilized, then incubated with primary antibodies specific to *E. granulosus* enolase or 14-3-3.1/3 and protein G gold-conjugated. TEM images obtained show the gold particles associated to vesicles (Fig 3B).

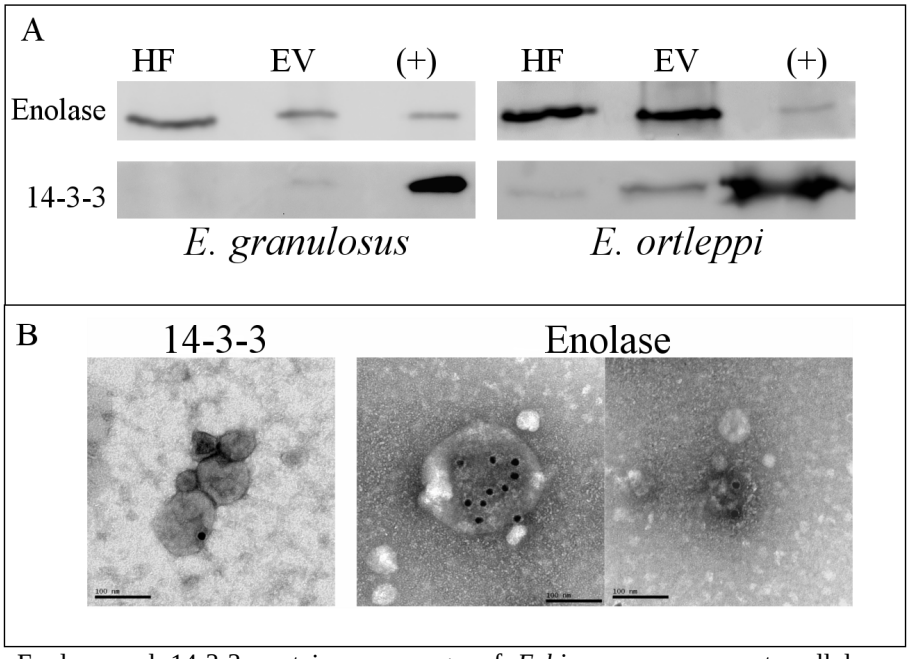

Figure 3. Enolase and 14-3-3 proteins are cargo of *Echinococcus* spp. extracellular vesicles. (A) Immunoblot using anti-rEgEnolase and anti-rEg14-3-3.1/3 to probe extracellular vesicles (EV) and hydatid fluid (HF) extracts obtained from a cyst of the indicated species. Positive controls (+) are protoscolex extracts. (B) TEM images showing immunolabelled extracellular vesicles from *E. granulosus.* Labelling was done by using anti-rEgEnolase and anti-rEg14-3-3.1/3 as primary antibodies and protein G gold-conjugated (15 nm) as secondary. The scale bar indicates 100 nm.

Enolase immunogold labelling was observed in EVs of different types, exosome- and microvesicle-like, whereas 14-3-3.1/3 immunogold labelling was detected only in exosome-like EVs. These results agree with the idea that EVs might be the secretory

pathway to enolase and 14-3-3, but also suggest that a driving processes may exist to include the proteins in different types of EVs.

E. granulosus and *E. ortleppi* extracellular vesicles are internalized by hepatocytes

EVs are involved in inter-cellular communication and are able to reach tissues at long distances. Therefore, parasitic EVs could carry biomolecules and deliver them to host cells. Hence, we evaluate interaction of *E. granulosus* and *E. ortleppi* EVs with a hepatocyte cell line (RH). EVs were isolated from HF and labelled with 5 µM CM-DiI. RH cells were incubated with DiI-labelled EVs at 37 °C for 4 h, then washed and fixed. Further imaging by confocal microscopy showed EVs presence in cell cytoplasm in close proximity to the nucleus, indicative of internalization by endocytic mechanisms (Fig 4).

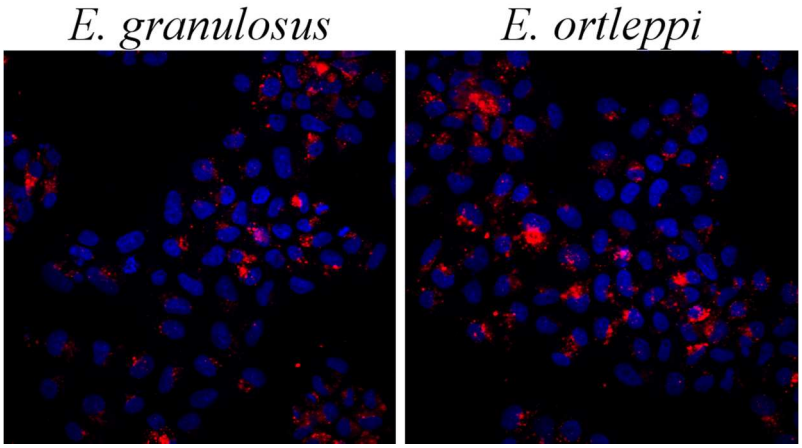

Figure 4. *Echinococcus* spp. EVs are internalized by RH cells *in vitro.* EVs isolated from hydatid fluid were labelled with the cell tracker CM-DiI. Then, DiI-labelled EVs were incubated with RH cells for 4 h. Cells were further fixed with paraformaldehyde and the nuclei were stained with DAPI. Images correspond to median *z* sections obtained by confocal microscopy at 600x magnification.

To verify whether EVs were in fact internalized by RH cells or just associated to the membranes, the interaction assay was repeated and the cells were imaged without the fixing step to preserve cell structure. In addition, because we cannot exclude the possibility that host EVs are also present in HF, the EVs isolated from *E. granulosus* protoscolex culture supernatant were used too. By analyzing different *z* planes of confocal series, bottom, median and top, we observed a higher intensity in median sections, confirming that both HF and protoscolex EVs were inside the cells (Fig S2). Thus, we confirm that the parasite's EVs are internalized by RH cells. These results suggest that *Echinococcus* spp. EVs might interact with host cells in CHD.

Protein profiles of EVs isolated from *E. granulosus* and *E. ortleppi* HF

In this study, we applied LC-MS^E to identify the repertoire of EVs protein in *E. granulosus* and *E. ortleppi* natural bovine infection, and provide a comparative analysis of these EV protein repertoires.

Firstly, we described the EV proteins for *E. granulosus* and *E. ortleppi* through the analysis of samples obtained from six individual metacestodes for each species, the EV/I samples. Overall, we were able to identify 42 and 40 non-redundant proteins in *E. granulosus* and *E. ortleppi* EVs, respectively. Nevertheless, for both species we found very variable intraspecific protein profiles by comparing the individual EV/I samples, i.e., the biological replicates. In fact, a high proportion of proteins, 69% in *E. granulosus* dataset and 62.5% in *E. ortleppi* dataset, were identified only in one EV/I sample (Tables 1 and 2). We believe the observed variability among biological replicates is naturally occurring in the isolated EVs and it is not a limitation in the MS design.

Considering only proteins identified in at least 3 out of 6 samples in each species, Antigen 5, cathepsin D lysosomal aspartyl protease, ferritin heavy chain, polyubiquitin, poly(u) specific endoribonuclease and tetraspanin were found in both *E. granulosus* and *E. ortleppi*. An expressed protein was exclusively identified in *E. granulosus,* while basement membrane specific heparan sulfate and N acetylated alpha linked acidic dipeptidase proteins were exclusively identified in *E. ortleppi* (Tables 1 and 2).

Table 1. Parasite proteins identified in *E. granulosus* extracellular vesicles.

Protein details		EV/I Samples					
Gene ID	Description	EGR1 n = 10	EGR2 n = 10	EGR3 n = 13	EGR4 n = 9	EGR5 n = 9	EGR6 n = 19
EgrG_000184900	Antigen 5	■	■	■	■	■	■
EgrG_000970500	Cathepsin D lysosomal aspartyl protease	■		■	■	■	■
EgrG_000315600	Expressed protein			■	■	■	■
EgrG_000382200	Ferritin heavy chain			■	■	■	■
EgrG_000516500	Polyubiquitin		■		■	■	■
EgrG_001132700	Poly(u) specific endoribonuclease	■		■			■
EgrG_000355700	Tetraspanin		■			■	■
EgrG_000355800	Tetraspanin TSP1		■				■
EgrG_000190400	Actin-3				■		■
EgrG_000472800	Histone H2B (inferred by orthology to a *S. mansoni* protein)		■				■
EgrG_000142500	Major vault protein (inferred by orthology to a human protein)		■			■	
EgrG_000453900	Syndecan binding protein syntenin					■	■
EgrG_000704400	Lysosomal alpha mannosidase	■			■		
EgrG_000789900	Beta mannosidase	■					
EgrG_000880000	Laminin subunit beta	■					
EgrG_001132400	Laminin	■					
EgrG_000143500	Lysosomal alpha glucosidase	■					
EgrG_000343000	Neurogenic locus notch protein	■					
EgrG_000672500	U6 snRNA associated Sm protein LSm4	■					
EgrG_000946800	Dynein light chain		■				
EgrG_001061900	Expressed conserved protein		■				
EgrG_001110500	Expressed protein		■				
EgrG_001029500	N/A		■				
EgrG_000530400	Amiloride sensitive amine oxidase			■			
EgrG_000701800	Basement membrane specific heparan sulfate			■			
EgrG_000144300	Collagen alpha 1V chain			■			
EgrG_000144350	Collagen alpha 1(IV) chain			■			
EgrG_002018100	N/A			■			
EgrG_000887700	Ribosomal protein L40			■			
EgrG_000381200	Tapeworm specific antigen B (AgB8/1)			■			
EgrG_000381400	Tapeworm specific antigen B (AgB8/4)			■			
EgrG_000057700	Prominin protein				■		
EgrG_000834300	Tetraspanin family protein 16 invertebrate				■		
EgrG_000721900	Expressed protein					■	
EgrG_001032250	Aminotransferase class III; Ornithine aminotransferase						■
EgrG_000244000	Annexin						■
EgrG_000724500	Expressed protein						■
EgrG_000927700	Histone H2A						■
EgrG_000355000	Tetraspanin						■
EgrG_000791700	Thioredoxin peroxidase						■
EgrG_000413200	Tubulin alpha 1C chain						■
EgrG_000955100	Tubulin beta 2C chain						■

Gene ID for the Wormbase ParaSite, protein description and the number (n) of non-redundant proteins identified in each biological replicate (EGR1 to 6) are listed. Filled cells means identification in the corresponding biological replicate. N/A: Non-annotated.

Table 2. Parasite proteins identified in *E. ortleppi* extracellular vesicles.

Protein details		EV/I Samples					
Gene ID	Description	EOR1 n = 6	EOR2 n = 12	EOR3 n = 6	EOR4 n = 15	EOR5 n = 27	EOR6 n = 6
EgrG_000184900	Antigen 5	■	■	■	■	■	■
EgrG_000970500	Cathepsin D lysosomal aspartyl protease	■	■	■	■	■	
EgrG_000516500	Polyubiquitin	■	■	■		■	
EgrG_000355700	Tetraspanin	■	■	■		■	
EgrG_000701800	Basement membrane specific heparan sulfate				■	■	■
EgrG_001132700	Poly(u) specific endoribonuclease				■	■	■
EgrG_000908900	N acetylated alpha linked acidic dipeptidase	■			■	■	
EgrG_000382200	Ferritin heavy chain			■	■	■	
EgrG_000144300	Collagen alpha 1V chain				■	■	
EgrG_000704400	Lysosomal alpha mannosidase				■	■	
EgrG_000355800	Tetraspanin TSP1				■	■	
EgrG_001169400	Iron:zinc purple acid phosphatase protein				■	■	
EgrG_000315600	Expressed protein			■	■		
EgrG_000381200	Tapeworm specific antigen B (AgB8/1)					■	■
EgrG_001061900	Expressed conserved protein	■	■				
EgrG_000406900	Actin-1		■				
EgrG_000244000	Annexin		■				
EgrG_000756700	Expressed conserved protein		■				
EgrG_001018600	Expressed conserved protein		■				
EgrG_000485800	H17g protein tegumental antigen		■				
EgrG_001085400	Heat shock 70 kDa protein 4		■				
EgrG_000624400	Phospholipid scramblase 3		■				
EgrG_000194000	Glycogenin 1				■		
EgrG_000142500	Major vault protein (inferred by orthology to a human protein)				■		
EgrG_000085400	Mastin				■		
EgrG_000144400	Abnormal EMBroygenesis family member emb 9					■	
EgrG_000061200	Actin					■	
EgrG_000524700	BRO1 domain containing protein BROX					■	
EgrG_000144350	Collagen alpha 1(IV) chain					■	
EgrG_000596300	Expressed conserved protein					■	
EgrG_000198700	Four and a half LIM domains protein 3					■	
EgrG_000684200	Lipid transport protein N terminal					■	
EgrG_000217900	Lysyl oxidase					■	
EgrG_001003150	N/A					■	
EgrG_000920600	Peptidyl-prolyl cis-trans isomerase (inferred by orthology to a *S. mansoni* protein)					■	
EgrG_001011700	Protein FAM188A					■	
EgrG_000381400	Tapeworm specific antigen B (AgB8/4)					■	
EgrG_000666500	Thioredoxin fold					■	
EgrG_002019000	Universal stress protein					■	
EgrG_000849600	Proteinase inhibitor I25 cystatin						■

Gene ID for the Wormbase ParaSite, protein description and the number (n) of non-redundant proteins identified in each biological replicate (EOR1 to 6) are listed. Filled cells means identification in the corresponding biological replicate. N/A: Non-annotated.

Gene ontology (GO) terms were assigned to the proteins identified in *E. granulosus* and *E. ortleppi* EV/I sample sets, based on sequence similarity using Blast2GO. The assigned ontologies at level 2 were very similar between the two species. In the molecular function category, the terms binding and catalytic activity gathered the largest number of sequences. In the biological process category, the terms with most sequences associated were metabolic process, single-organism process and cellular process. Protein categorization according to cellular component showed a larger number of sequences in cell and membrane categories, probably reflecting the intracellular biogenesis of EVs (Fig 5).

Looking into level 3 of the above mentioned GO categories we can notice, in both *E. granulosus* and *E. ortleppi,* some subcategories potentially involved in uptake and metabolism of nutrients and protection from host immune defenses: ion binding, hydrolase activity and organic substance metabolic process (Tables S1 and S2). Proteins in ion binding subcategory included: ferritin heavy chain, lysosomal alpha mannosidase and annexin, all present in the two species, also, amiloride sensitive amine oxidase and aminotransferase class III in *E. granulosus,* and iron:zinc purple acid phosphatase protein, heat shock 70 kDa protein 4 and lysyl oxidase in *E. ortleppi*. Proteins in hydrolase activity subcategory included: lysosomal alpha mannosidase, cathepsin D lysosomal aspartyl protease and poly(u) specific endoribonuclease, present in the two species, also, beta mannosidase and lysosomal alpha glucosidase in *E. granulosus,* and mastin and iron:zinc purple acid phosphatase protein in *E. ortleppi.* Proteins in organic substance metabolic process subcategory included: Antigen 5, lysosomal alpha mannosidase and polyubiquitin, present in the two species, also, amiloride sensitive amine oxidase, beta mannosidase and aminotransferase class III in *E. granulosus,* and peptidyl-prolyl cis-trans isomerase and proteinase inhibitor I25 cystatin in *E. ortleppi.*

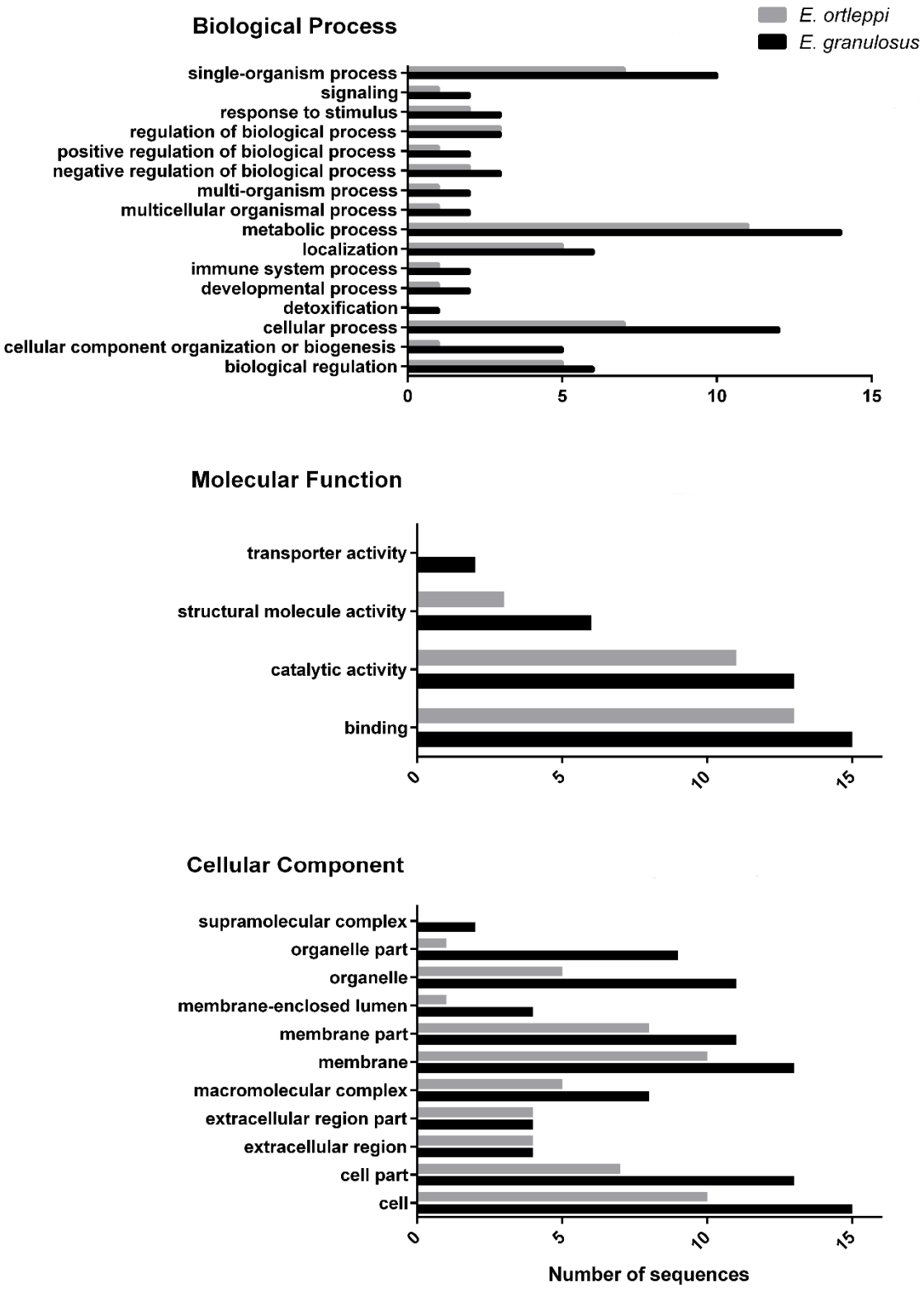

Figure 5. Gene Ontology analysis of parasite proteins identified in *E. granulosus* and *E. ortleppi* extracellular vesicles. EVs were isolated from six individual hydatid cysts for each species and the proteins were identified by mass spectrometry. Blast2GO tool was used to retrieve the ontologies based on sequence similarity to the NCBI databank. The GO categories at level 2 for each major category: biological process, molecular function and cellular component, are indicated in the graphs.

HF contains host-derived molecules, so we reasoned that bovine EVs could also be present in our samples. Then MS results of EV/I sample sets were searched against a *B. taurus* protein database, allowing the identification of 36 proteins in *E. granulosus* EV/I set and 7 proteins in *E. ortleppi* EV/I set (Tables S3 and S4). The variation among the isolated samples (biological replicates) was even more pronounced than for the *Echinococcus* proteins. In fact, one unique sample of *E. granulosus* allowed the identification of 31 proteins, and from those, just 9 proteins were shared with some other sample. In the other five *E. granulosus* samples, the number of proteins identified was much lower, ranging from 0 to 7 (Table S3). Regarding the seven host proteins identified in *E. ortleppi* samples, only one protein was found in more than one sample (Table S4).

In order to compare the EV protein repertoires of *E. granulosus* and *E. ortleppi*, and to define confidently the differentially abundant proteins, we used a new set of samples in a different approach. Pools of isolated EVs were prepared, the EV/P samples, and only proteins identified in at least two out of three EV/P samples for each species were included in the comparative analysis. A group of 41 different parasite proteins was identified, where 11 proteins were shared between the two species, 28 proteins were exclusive of *E. granulosus*, and only 2 proteins were exclusive of *E. ortleppi* (Fig 6 and Table S5). The bovine proteins identified in the EV/P sample sets are described in Suplementary Table S6, but they were not analyzed because no protein met the inclusion criteria.

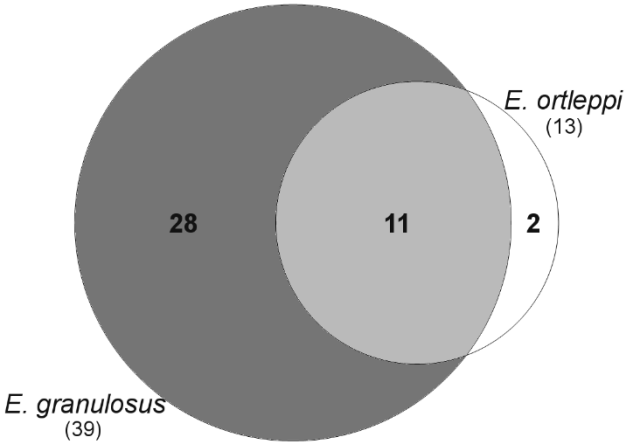

Figure 6. Venn diagram of parasite proteins identified in pools of extracellular vesicles isolated from *E. granulosus* and *E. ortleppi* hydatid fluid.

Amongst the *Echinococcus* spp. shared proteins, 8 were found with higher relative abundance in *E. granulosus*: basement membrane specific heparan sulfate, cathepsin D

lysosomal aspartyl protease, lysosomal alpha mannosidase, ferritin heavy chain, iron:zinc purple acid phosphatase protein, poly(u) specific endoribonuclease, tetraspanin and an expressed protein. The remaining 3 proteins, Antigen 5, collagen alpha 1(IV) chain and collagen alpha 1V chain, were similarly abundant in both species (Table 3).

Table 3. Analysis of relative abundance of *E. ortleppi* (EO) and *E. granulosus* (EG) shared proteins.

Gene ID	Description	Score	EO:EG Ratio	Variance	Log_2 Ratio	EO:EG P[a]
EgrG_000184900	Antigen 5[b]	57227.38	-	-	-	-
EgrG_000701800	Basement membrane specific heparan sulfate	14168.82	0.844	1.07	-0.25	0.00
EgrG_000970500	Cathepsin D lysosomal aspartyl protease	12469.94	0.684	1.15	-0.55	0.00
EgrG_000144350	Collagen alpha 1(IV) chain	3584.11	1.116	1.17	0.14	0.89
EgrG_000144300	Collagen alpha 1V chain	5164.52	0.905	1.14	-0.14	0.11
EgrG_000315600	Expressed protein	11655.54	0.278	1.60	-1.85	0.00
EgrG_000382200	Ferritin heavy chain	20280.66	0.038	1.16	-4.73	0.00
EgrG_001169400	Iron:zinc purple acid phosphatase protein	2491.62	0.741	1.39	-0.43	0.04
EgrG_000704400	Lysosomal alpha mannosidase	3388.69	0.361	1.23	-1.47	0.00
EgrG_001132700	Poly(u) specific endoribonuclease	2517.64	0.527	1.40	-0.92	0.00
EgrG_000355700	Tetraspanin	11237.37	0.244	1.17	-2.03	0.00

Gene ID for the Wormbase ParaSite, protein description, PLGS score, ratio (*E. ortleppi*/*E. granulosus*) and its respective variance and Log_2, and the probability (P) of up or down regulation are listed.
[a]A $P \leq 0.05$ identifies proteins less abundant in *E. ortleppi* than in *E. granulosus*, whereas a $P \geq 0.95$ identifies proteins more abundant.
[b]Protein present in all samples of *E. granulosus* and *E. ortleppi*, with zero variance. It was used to normalize the data.

Protein profile of EVs isolated from culture supernatant of *E. granulosus* protoscoleces

Protoscoleces obtained from one *E. granulosus* and one *E. ortleppi* hydatid cyst were treated with pepsin and kept at 37 °C for 3 days with daily changes of the medium. The removed culture supernatant from every day was pooled and subjected to EVs isolation procedure. Unfortunately, the yield of EVs from *E. ortleppi* culture supernatant was too low, impairing the use of that sample for the proteomic analysis. Therefore, the LC-MS^E analysis was conducted only for the *E. granulosus* sample, where 90 non-redundant proteins were identified (Table S7). Qualitative comparisons of EV protein repertoire from protoscoleces with that of the HF (i.e., EV/I plus EV/P unique identifications) show only a partial overlap. Of the 132 total unique proteins, 15 (11.3%) were shared between *E. granulosus* protoscolex and HF EVs (Fig S3). This observation suggests that EV protein composition is modulated along *E. granulosus* life cycle.

The gene ontology analysis (level 2) by Blast2GO showed a protein categorization very similar to that of HF EVs (Fig 7). In molecular function category, the majority of proteins were associated to the terms binding and catalytic activity. Regarding to the proteins assigned under biological process category, most of sequences were associated to single-organism process, cellular process and localization terms. As expected, the assignments to cellular component category showed many sequences associated to cell and membrane terms. Altogether, these findings suggest that the EVs protein composition is modified along *E. granulosus* life cycle, but the main molecular and biological functions played by them are quite similar.

Analyzing GO subcategories (level 3), it can be noticed that the terms establishment of localization, regulation of cellular process and cellular response to stimulus gather a large number of sequences (Table S8). The assignments to those subcategories included proteins potentially involved in EVs biogenesis, such as: Ras and Rab proteins, syntaxin binding protein 1, vacuolar protein sorting associated protein 4A, vesicle associated membrane protein and ATP binding cassette subfamily B. In addition, proteins that might be related to protoscolex establishment and development were also associated to those subcategories, such as: phospholipase D, fatty acid-binding protein homolog 1, phospholipid transporting ATPase IIB, solute carrier family 5, anion exchange protein 3 and neutral amino acid transporter A.

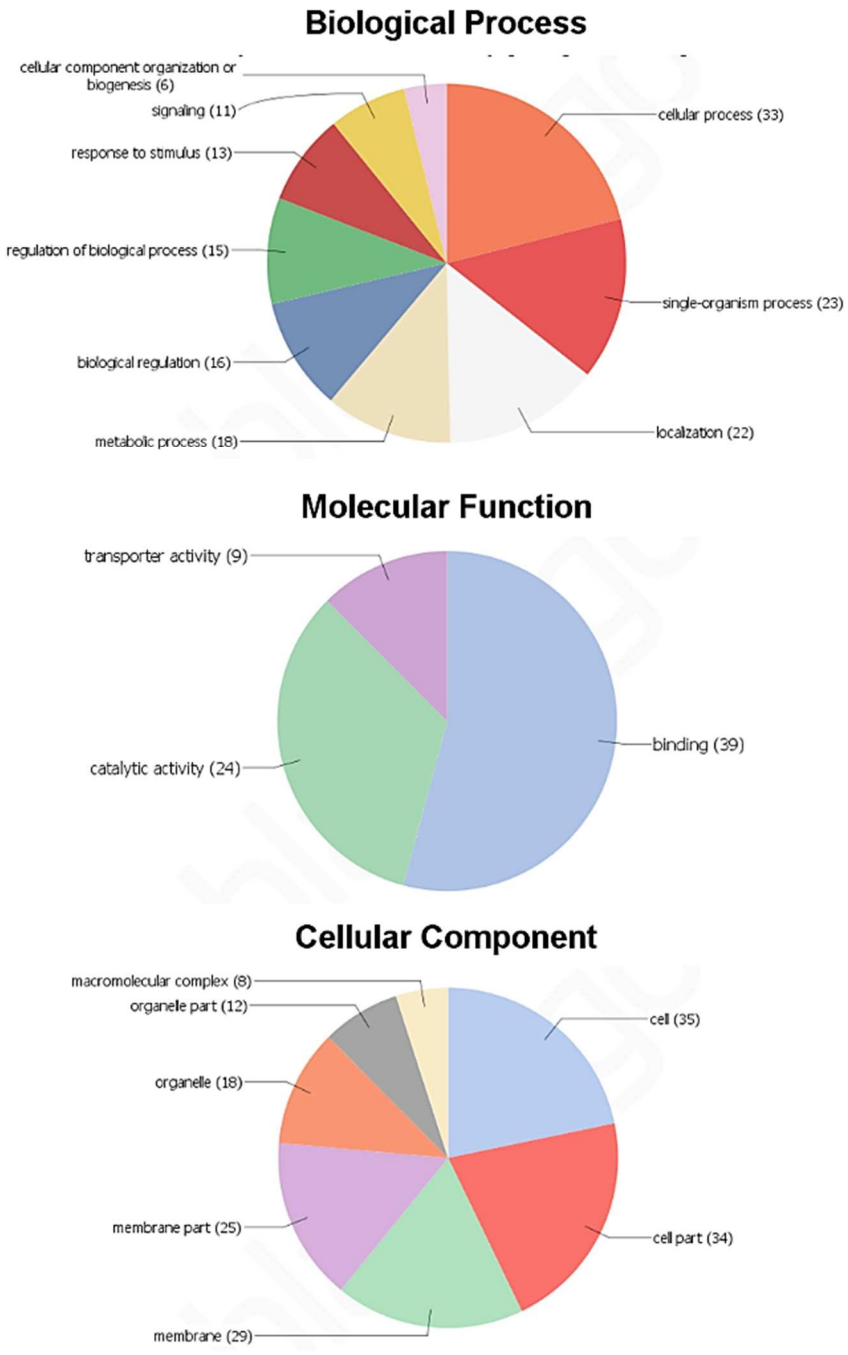

Figure 7. Gene Ontology analysis of proteins identified in extracellular vesicles from *E. granulosus* protoscoleces. EVs were isolated from a protoscolex culture supernatant and the proteins were identified by mass spectrometry in technical duplicates. Blast2GO tool was used to retrieve the ontologies based on sequence similarity to the NCBI databank. The GO categories at level 2 for each major category: biological process, molecular function and cellular component, are indicated in the graph.

E. granulosus and *E. ortleppi* extracellular vesicles contain mRNA

An important feature of EVs is the ability to transport different classes of biomolecules. Therefore, we next ask whether RNA is present in *E. granulosus* and *E. ortleppi* EVs. The EVs isolated by ultracentrifugation were ressuspendend in TRIzol and

proceeded to total RNA isolation. Quantification by Qubit fluorometer or Nanodrop, however, were not able to detect RNA in our final samples. Although the presence of regulatory RNAs and mRNAs in EVs are well established (15, 21), most studies report absence or minor amounts of ribosomal RNA (8). Then, we rationalized that recovered RNA could be in too low quantity to be detected by those quantification techniques, and decided to search specific mRNAs by RT-PCR. First-strand cDNA was synthetized from total isolated RNA and PCR was carried out using primers for genes with expression patterns known in *E. granulosus* metacestode: *AgB* gene family, *GAPDH* and *CYP-1*. *E. granulosus* and *E. ortleppi* samples showed equivalency regarding positive amplification, that was obtained for all genes except *AgB5* (Fig 8). *AgB5* has already been described as less expressed in *E. granulosus* metacestode stage (20, 22).

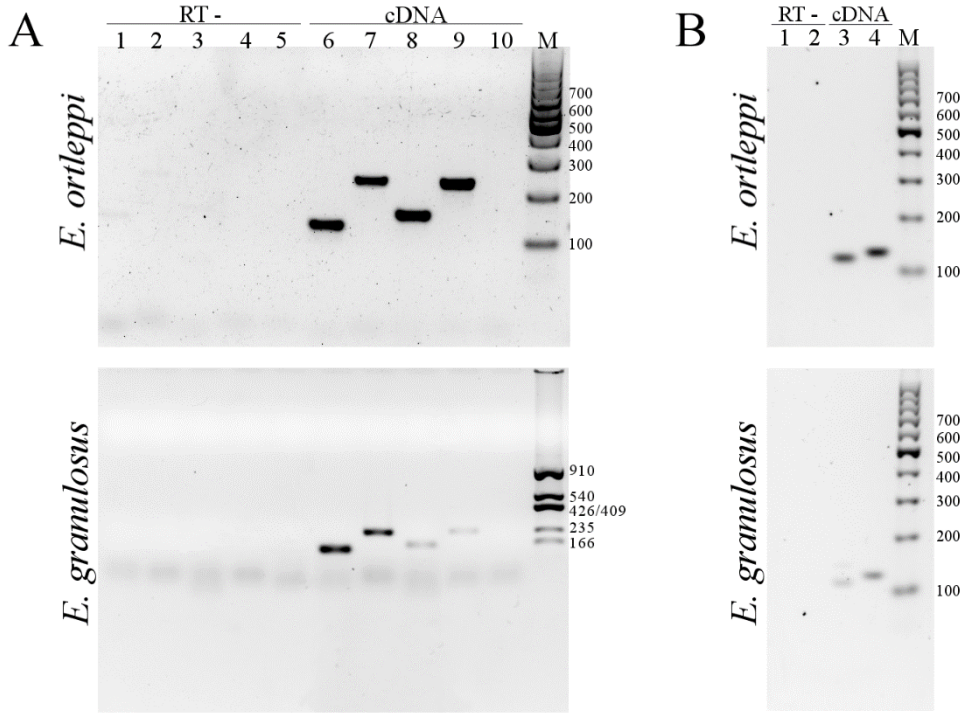

Figure 8. *E. granulosus* and *E. ortleppi* extracellular vesicles contain mRNA. Total RNA was extracted from HF extracellular vesicles and transcripts of *AgB1-5*, *CYP-1* and *GAPDH* genes were identified by RT-PCR. PCR was performed with synthesized cDNA and the respective RT- reaction. (A) Amplification products of *AgB1* (lanes 1 and 6), *AgB2* (lanes 2 and 7), *AgB3* (lanes 3 and 8), *AgB4* (lanes 4 and 9) and *AgB5* (lanes 5 and 10). (B) Amplification products of *CYP-1* (lanes 1 and 3) and *GAPDH* (lanes 2 and 4). M: molecular weight; size in base pairs is indicated to the right.

RNA isolation, cDNA synthesis and RT-PCR were also performed with complete HF (pre-ultracentrifugation material) and supernatant after ultracentrifugation. *AgB1*, *AgB3* and *AgB4* gene transcripts were detected in complete HF of both species, whereas *AgB2* transcripts were detected only in complete HF of *E. granulosus* (Fig S4A and B).

CYP-1 and *GAPDH* did not produce visible amplification in complete HF samples (Fig S4C and D).

Even without a visible amplification for *AgB2* in *E. ortleppi* HF, and *CYP-1* and *GAPDH* in both *E. granulosus* and *E. ortleppi* HF, it was possible to detect the amplification in the corresponding EV sample, suggesting an enrichment of mRNAs in EV preparations. So, the amplification of *AgB2*, *CYP-1* and *GAPDH* in EV samples can be achieved because of the increase in the relative proportion of these mRNAs resultant of EVs isolation. Additionally, no amplification was observed for any gene tested in the samples from supernatant after ultracentrifugation for both species (Fig S4). The depletion of tested mRNAs from HF after the ultracentrifugation procedure to isolate vesicles supports the idea that these mRNAs are associated to EVs.

DISCUSSION

Extracellular vesicles have been found involved in a plethora of activities, from physiological to pathological processes. In parasitic helminths, the presence of EVs generates many possibilities in terms of host physiology modulation and it opens up an important field of study at host-parasite interface. EVs could transport factors involved in immunomodulation, cell communication, nutrient uptake, etc., contributing to the establishment of chronic infections that are characteristic in *Echinococcus* genera. Additionally, EVs are interesting to therapeutic intervention, because their components have potential to spread throughout the host organism and could be targeted to new drugs and vaccines.

Previous works, in separate, have detected EVs in HF from cattle and sheep hydatids (6, 14), but, regarding the EVs detected in the cyst from cattle, no extensive characterization was done. In this work, the EVs were isolated from hydatids of natural bovine infections with two CHD aetiological agents, *E. granulosus* and *E. ortleppi*. Then, EVs were subjected to a comparative analysis according to morphological and compositional features.

The morphological evaluation, by TEM and NTA, showed that EVs population of both *E. granulosus* and *E. ortleppi* are constituted by a multitude of sizes, but is predominantly composed by vesicles around 100 nm in diameter. Thus in concordance with other flatworms EVs described in the literature (13, 15, 23).

Further, we demonstrate that proteins potentially involved in host-parasite interactions are secreted in EVs. Previous works by our group have addressed the

significance of enolase and 14-3-3 proteins in *E. granulosus* biology (18, 19). Enolase and 14-3-3 have been found among the parasite's secretory products, but the mechanism of secretion was unknown. By using *Echinococcus* specific antibodies in immunoblot and immunogold assays, we show that enolase and 14-3-3.1/3 are secreted by EVs in *E. granulosus* and *E. ortleppi*. In fact, as in the study of Teichmann and co-workers (2015), our immunoblot did not detect 14-3-3.1/3 proteins in complete HF from *E. granulosus.* However, 14-3-3.1/3 were detected in the EV sample, probably as a result of the increase in the relative proportion of these proteins after EV isolation, corroborating that 14-3-3.1/3 are EVs protein cargo.

Interestingly, immunolocalization studies found enolase and the four isoforms of 14-3-3 associated to tegument of protoscoleces, also 14-3-3.4 and enolase were detected in laminated layer of the cyst wall (18, 19). The laminated layer is an acellular, carbohydrate-rich sheath delimiting metacestode structure and in close contact with host tissue. Likewise, when protoscoleces are ingested by the definitive host, the tegument is in direct contact with the host organism. Secretion of enolase and 14-3-3 in EVs could aid in their functions by protecting them from degradation by host proteases. Furthermore, these findings indicate EVs may be secreted towards outside parasite's tissues to deliver molecules acting in interactive processes with the host.

Interaction and modulation of host responses by parasitic EVs is reported for different parasites. *Echinostoma caproni* EVs are incorporated into intestinal cells in a time-dependent and metabolically dependent manner, most likely by endocytosis (23). EVs secreted by the carcinogenic liver fluke *Opistorchis viverrini* are promptly uptaken by cholangiocytes and induce cell proliferation (13). An important virulence factor of *Leishmania major*, GP63, is detected in the cytoplasm of host cell after interaction with EVs (24). Here, we demonstrate that EVs secreted by the metacestode into hydatid fluid, and by protoscoleces in culture, are internalized by a hepatocyte cell line. Internalization was observed at cell cytoplasm with concentration of the fluorescent puncta in perinuclear region, suggesting that EVs may enter cells through endocytic pathway (25). The perinuclear area usually contains the recycling endosomes that connect endocytic and exocytic pathways by receiving internalized molecules from early endosomes, and returning them to the plasma membrane, or driving them to another appropriate cell compartment (26). Following endocytosis by host cells, the *Echinococcus* spp. EV cargo might be delivered to any cell organelle or subcellular compartment to exert its biological role.

We identify in EVs from *E. granulosus* and/or *E. ortleppi*, proteins or protein families that have been also described as component of EVs in other studies with platyhelminths (15, 23, 27), including a study with EVs from *E. granulosus* hydatids infecting sheeps (14). This supports the denomination of these proteins as EV cargo. Among these proteins are membrane proteins (prominin, tetraspanin, ATP binding cassette subfamily B and basement membrane specific heparan sulfate), structural/cytoskeleton proteins (actin and tubulin), extracellular matrix constituents or ligands (laminin, collagen alpha 1 IV and V), hydrolases (alkaline phosphatase, phospholipase D, beta mannosidase, lysosomal alpha mannosidase and lysosomal alpha glucosidase) and regulatory/signaling proteins (14-3-3, anoctamin, major vault protein and abnormal EMBryogenesis family member emb 9).

Few proteins related to EV biogenesis and trafficking were identified in the HF datasets, just annexins, syntenin and BROX. It is worth mentioning that some proteins related to EV biogenesis were actually found in one EV/P sample, thus outside the criteria for protein inclusion on that dataset. On the other hand, in EVs from protoscoleces this group of proteins appears quite diversified, and included also several Rab and Ras proteins, IST1, vesicle associated membrane protein, syntaxin binding protein 1, endophilin A3, charged multivesicular body proteins and vacuolar protein sorting associated proteins.

Antigen 5 was by far the protein most uniformly identified in the analysis of HF datasets, it was found in every EV/I and EV/P sample, indicating an important role for this protein in EV function and metacestode biology. Antigen 5 is one of the main components of hydatid cyst fluid, being widely used in confirmation of CHD by serodiagnostic methods (28, 29). It is a glycoprotein composed by 2 disulphide-bridged subunits, of 38 kDa and 22–24 kDa, encoded by a single gene. The amino acid sequence of the 38 kDa subunit shows similarity to that of serine proteases of the trypsin family (30). The smaller subunit binds calcium and possesses a glycosaminoglycan-binding motif that appears to be responsible for the observed binding of Antigen 5 to heparin. This domain may provide interaction with cell surface and the extracellular matrix (31). Antigen 5 is localized mostly extracellularly and it has been rarely found within cells, but when it occurred, it is associated with small vesicles and internal membranes (32). The presence of Antigen 5 in the cell vesicular system agrees with our findings that this protein is an EV cargo because the exosome formation process occurs in the vesicular/endomembrane system.

Interestingly, peptidases (N acetylated alpha linked acidic dipeptidase, cathepsin D and L) were identified in HF datasets but not in protoscolex dataset. In fact, cathepsin D lysosomal aspartyl protease was one of the proteins consistently identified in HF datasets from both *E. granulosus* and *E. ortleppi*. Proteases are important virulence factors expressed by several parasites and are mainly employed to facilitate invasion process and degrade host factors in order to obtain nutrients or to counteract immune response. The cathepsin D-like peptidase has been often discussed as an antiparasitic intervention strategy (33). Cathepsin D aspartyl protease plays an integral role in blood degradation in *Schistosoma mansoni* providing amino acids to the parasite (34). In addition, *Schistosoma japonicum* recombinant cathepsin D was able to cleave human proteins IgG, complement C3 and albumin *in vitro* (35). Vaccination with recombinant *S. japonicum* cathepsin D induces partial protection in mice by reducing worm burden (36). Additionally, it has been demonstrated that cathepsin D increases the activity of secreted plasminogen activators in breast cancer, which in turn would contribute to trigger a proteolytic cascade facilitating cancer cell invasion and metastasis (37). The interaction of parasites with fibrinolytic system has a proposed role in infection. It is reported that proteins secreted by pathogenic organisms are able to directly activate plasminogen or bind to it facilitating conversion to plasmin by the endogenous plasminogen activator system of the host. Proteolytically active plasmin can degrade components of extracellular matrices and help the parasites to penetrate and migrate through tissues (38).

A possible explanation for the difference observed in the HF and protoscoleces dataset regarding the presence of proteases could be the final location of the metacestode and the adult worm in host's body. The larval stage could employ proteases to facilitate the migration through tissues until it reaches the liver and lungs. Once the parasite is established, the proteases might act in the remodeling of the surrounding tissues to facilitate growing, and making amino acids available to the metacestode nutritional requirements. Meanwhile, protoscoleces are orally ingested and passively reach the intestine, where it can fixate and develop into the adult worm. At the intestine, plenty of nutrients are available as result of the host's digestive enzymes activity. In this way, the proteases would be more required by the larval stage (39–41).

N acetylated alpha linked acidic dipeptidase (NAALADase: EC 3.4.17.21; also known as glutamate carboxypeptidase II and folate hydrolase) was exclusively identified in *E. ortleppi* EV/I samples in our study. NAALADase is better characterized in human nervous tissues, where it modulates glutamatergic neurotransmission through its

carboxypeptidase activity against neuropeptide NAAG (N-acetylaspartylglutamate) (42, 43). However, NAALADase is also found in non-neural tissues, where presents folate hydrolase activity against polyglutamates (44). The biological role for NAALADase in *E. ortleppi* excretory/secretory products is unknown. Whether the folate hydrolase activity could be used by *E. ortleppi* to obtain folate derivatives, which are necessary to purine and thymidine synthesis, or whether the carboxypeptidase activity would be employed to provide glutamate to the parasite needs further investigation.

The analysis of the EV/I datasets showed a high variability in the protein profile in both *E. granulosus* and *E. ortleppi*. Even though many proteomic studies use the identification in at least two samples as criteria to validate an identification, we choose to show the proteins identified in only one EV/I sample too. Those proteins appeared in great proportion although we used six individual samples, thus it may be an inherent feature of *Echinococcus* spp EVs. The nature of the material used to isolate the EVs in this work (i.e. hydatid cysts obtained from natural infections) could be related to the high variability in the protein content observed among biological replicates. Each hydatid corresponds to an individual organism, dealing with the host response and developing in a specific context, so the EVs secreted may reflect this context. Moreover, it is not possible to know the age of the metacestode in these infections, and the EV composition might change along parasite growing. The variable protein profile we found indicates the EV content in these species is highly dynamic and it could be modulated along the course of the infection.

The study of individual samples could reveal subtle details of the organisms, and these individual attributes are important to the complete understanding of the parasite's biology and the infection dynamic. However, the analysis of pools of samples it is necessary to define the commonly found molecules, which constitute the core of the model. By using pools of new EV samples, we attempted to establish the most common proteins in *E. granulosus* and *E. ortleppi* EVs (the EV/P datasets). *E. granulosus* HF derived EVs seemed to be more diverse in their protein repertoire than *E. ortleppi* EVs. Even the proteins shared between the two species have a tendency to be more abundant in *E.granulosus.* This suggest that even though being species closely related genetically, *E. granulosus* and *E. ortleppi* might employ different molecular players to deal in the host-parasite interface. It is said that *E. ortleppi* is better adapted to bovines as intermediate host, and maybe the lower diversity in EV protein content is a trait resultant from this more specialized relationship (45). Nevertheless, EVs also contain nucleic

acids, especially miRNAs, which has been found in different parasitic EVs and probably have key roles in host-parasite relationship (10, 46, 47). A previous work has detected miRNAs in EVs of the cestodes *Taenia crassiceps* and *Mesocestoides corti* (15) however, the RNA content of *Echinococcus* sp EVs was not characterized yet. *E. ortleppi* could use miRNAs as the main regulatory molecule, so its EVs would be richer in miRNAs in detriment of the proteins, what would explain our comparative results where proteins in *E. ortleppi* EVs were less abundant, as well as, less diversified than in *E. granulosus* EVs. It would be interesting to investigate whether the RNA diversity in EVs from these parasites present an inverted pattern from that we observe for the proteins.

The presence of mRNAs in EVs from *E. granulosus* and *E. ortleppi* was investigated in this work by RT-PCR with gene-specific primers. Transcripts for the *AgB* gene family, *GAPDH* and *CYP-1* were detected in both *E. ortleppi* and *E. granulosus* HF EVs. However, it was not possible establish if these molecules are selected to be incorporated into EVs or if represent a "snapshot" of the transcriptional moment of the metacestode. In addition, another crucial question arises: what is the function of this EV mRNAs? Considering EVs are potentially internalized by host cells, mRNAs residing in EVs could regulate or serve as templates for protein production (21). Translation of exogenous exosomal mRNA by recipient cells have already been demonstrated in a mouse-human model of exosome interaction (48). Therefore, an interesting hypothesis is that the host cell machinery could translate the parasite mRNAs, and then the coded proteins would be promptly located at host's tissue, where they could exert their role. A new, comprehensive work will be necessary to elucidate both questions, if the mRNAs are selected to be incorporated in EVs and if they could be translated by host cells.

Parasitic platyhelminthes employ a great diversity of strategies to infect their hosts and survive inside a body that is constantly trying to eliminate them. Recently EVs have been added to the repertoire of mechanisms by which parasitic platyhelminthes surpass host-protective responses. EVs carry several molecules potentially able to interact with host cells and adjust the microenvironment to benefit the parasites. *Echinococcus* spp causes a chronic infection that can persist in the intermediate host for many years. The noticeable variability in the EVs protein content detected in this study could reflect the *Echinococcus* spp ability of adjustment to the changes faced throughout the persistence of the infection. The use of more sensitive techniques to refine the findings described in this work will aggregate information and help to elucidate the *Echinococcus* spp EVs main factors and their biological roles. The identification of molecular players associated

to EVs may lead to the discovery of valuable targets to therapeutic intervention that would help to reduce the health and economic problems associated with CHD.

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Supplementary figures

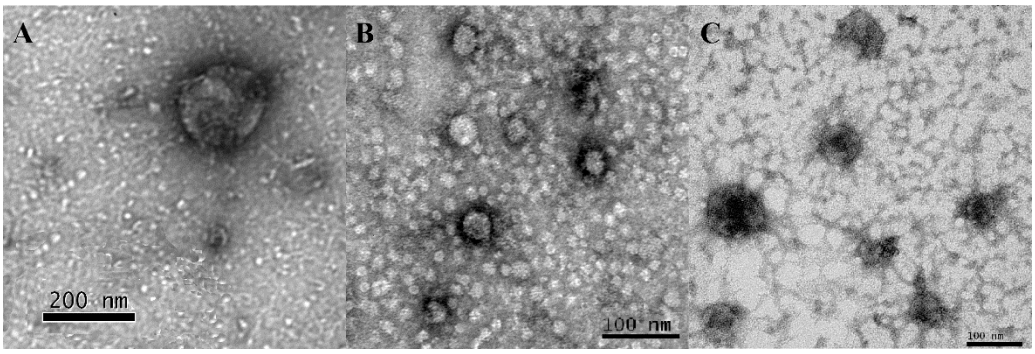

Figure S1. TEM analysis showing variable sizes of *Echinococcus* spp. extracellular vesicles. EVs from *E. granulosus* HF (**A**), *E. ortleppi* HF (**B**) and *E. ortleppi* protoscolex culture supernatant (**C**) were adsorbed onto formvar/carbon grids, contrasted with uranyl acetate and imaged in an electronic microscope JEOL JEM 1200.

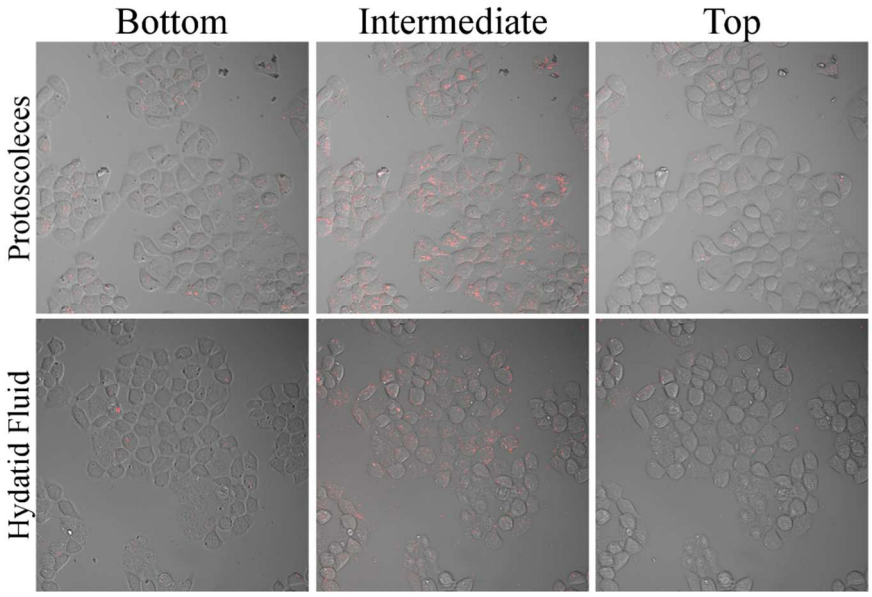

Figure S2. *E. granulosus* EVs uptake by RH cells. Cells were incubated with DiI-labelled EVs for 4 h at 37°C. Images were acquired on a confocal microscope without cell fixation, at 400x magnification. Three different sections of a *z* series are shown.

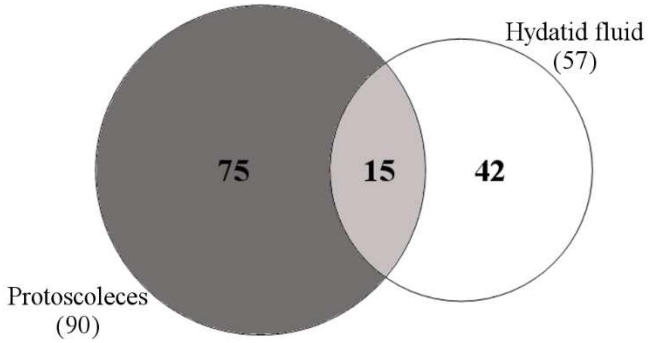

Figure S3. Venn diagram of protein identified in extracellular vesicles isolated from *E. granulosus* HF and protoscolex culture supernatant. The 57 proteins in hydatid fluid correspond to the grouping of identifications from EV/I and EV/P samples.

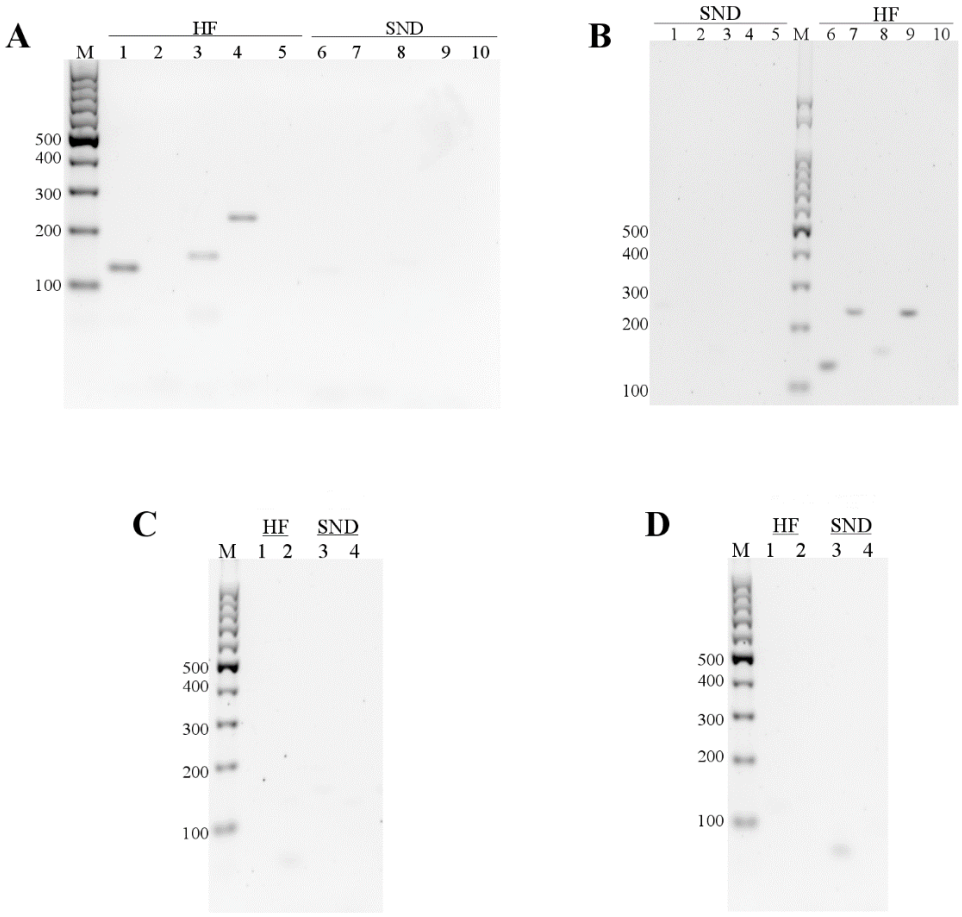

Figure S4. Analysis of mRNA presence in the material pre (HF) and pos (SND) ultracentrifugation procedure to separate the EVs. **A** and **B,** RT-PCR for *AgB1* (lanes 1 and 6), *AgB2* (lanes 2 and 7), *AgB3* (lanes 3 and 8), *AgB4* (lanes 4 and 9) and *AgB5* (lanes 5 and 10) genes. **C** and **D,** RT-PCR for *CYP-1* (lanes 1 and 3) and *GAPDH* (lanes 2 and 4). **A** and **C** correspond to reactions using *E. ortleppi* samples. **B** and **D** correspond to reactions using *E. granulosus* samples. M: molecular weight; size in base pairs is indicated to the left.

3 DISCUSSÃO GERAL E CONCLUSÕES

O estágio larval de *Echinococcus* spp. apresenta uma marcante capacidade de persistência no hospedeiro, portanto, diferentes mecanismos moleculares de interação parasito-hospedeiro devem estar constantemente atuando. Embora o cisto hidático seja protegido externamente pela camada laminar, existe passagem de fatores do parasito para o hospedeiro e vice-versa. Além disso, o desenvolvimento do metacestódeo no interior de órgãos do hospedeiro intermediário cria um microambiente de interação parasito-hospedeiro, o qual deve ser precisamente regulado. O estudo da biologia básica de *Echinococcus* spp. é necessário para revelar os elementos que participam ativamente dos mecanismos moleculares na interface parasito-hospedeiro e, por conseguinte, tem papel central na sobrevivência do metacestódeo e persistência da infecção. Muitos desses elementos compõem o fluido que preenche o cisto, o líquido hidático, e constituem o conjunto de produtos de excreção/secreção do parasito.

Neste trabalho, buscou-se contribuir para a elucidação de mecanismos moleculares atuantes na interface parasito-hospedeiro, gerando conhecimento acerca da biologia de *E. granulosus* e *E. ortleppi*, no contexto da infecção com suas formas larvais. Dois importantes produtos de excreção/secreção presentes no líquido hidático foram os alvos do estudo: vesículas extracelulares (EVs) e o Antígeno B (AgB).

A presença de EVs no líquido hidático de *E. granulosus* foi demonstrada recentemente (SANTOS *et al*., 2016; SILES-LUCAS *et al*., 2017), expondo um novo e complexo campo a ser explorado. Como mecanismo de comunicação intercelular, as EVs estão envolvidas em inúmeros processos fisiológicos, e também patológicos, tendo a capacidade de transferir informação tanto para células diferentes daquelas de origem quanto para células de outro organismo. Essas características são muito interessantes do ponto de vista da interação parasito-hospedeiro na hidatidose cística, pois fatores produzidos pelo parasito poderiam ser direcionados para células do hospedeiro por meio de EVs, onde também estariam protegidos de degradação. Além disso, variados mecanismos de interação parasito-hospedeiro podem ser facilitados pela secreção de EVs em virtude da possibilidade de transporte de diferentes populações de biomoléculas. De fato, é o que tem sido demonstrado em diversos trabalhos recentes, onde EVs produzidas por helmintos parasitas interagem e são internalizadas por células dos hospedeiros, transferindo fatores de virulência, modulando a resposta imune e regulando a expressão gênica do hospedeiro (BUCK *et al*., 2014; CHAIYADET *et al*., 2015; EICHENBERGER; SOTILLO; LOUKAS, 2018; ZHU *et al*., 2016a).

Em *E. caproni*, a injeção de EVs do parasito em camundongos Balb/c induz uma resposta balanceada Th2/T regulatória, que reduz a patologia associada à infecção em desafios subsequentes e favorece a sobrevivência do parasito (TRELIS *et al*., 2016). Tanto EVs liberadas pela filária *Brugia malayi* como EVs de *S. japonicum* interagem com uma linhagem de macrófagos, *in vitro*, e estimulam a via de ativação clássica (pró-inflamatória) (WANG *et al*., 2015; ZAMANIAN *et al*., 2015). A administração de EVs do nematoide *Heligmosomoides polygyrus* em camundongos suprimiu a resposta inata do tipo 2 e a eosinofilia induzida por um alergeno (BUCK *et al*., 2014). Além disso, os autores mostraram que miRNAs contidos nas EVs de *H. polygyrus* reprimem uma fosfatase (DUSP1), que é um regulador chave para a resposta inflamatória contra ligantes de receptores tipo Toll. A internalização de EVs de *Opistorchis viverrini* por colangiócitos induz mudanças na expressão de proteínas associadas a endocitose, reparo tecidual e câncer (CHAIYADET *et al*., 2015).

A importância de identificar os componentes de EVs de *Echinococcus* spp. é evidente, e no caso de *E. granulosus* e *E. ortleppi*, que no Brasil possuem distribuição geográfica sobreposta, pode gerar conhecimentos essenciais para a caracterização da patogenia causada por essas espécies. A análise de EVs de *E. granulosus* e *E. ortleppi* pode ajudar a identificar tanto marcadores moleculares específicos de cada espécie como alvos terapêuticos comuns a ambas e a conhecer melhor suas estratégias de sobrevivência no organismo hospedeiro. Em uma publicação prévia do nosso grupo, foram detectadas EVs em líquido hidático de cistos bovinos de *E. granulosus*, porém a caracterização de seu conteúdo não foi realizada (SANTOS *et al*., 2016). Durante o desenvolvimento deste trabalho foi publicado por outro grupo de pesquisa a caracterização proteômica de EVs de cistos ovinos de *E. granulosus* (SILES-LUCAS *et al*., 2017). Aqui, foi feita a análise em paralelo de EVs das principais espécies causadoras da hidatidose cística no sul do Brasil, *E. granulosus* e *E. ortleppi*, utilizando-se material oriundo de infecções naturais em bovinos. De modo geral, os resultados deste trabalho concordam com os resultados de Siles-Lucas *et al*. (2017), no entanto é preciso destacar que este último produziu um número maior de identificações, que pode ser tanto decorrente do procedimento experimental ou equipamento utilizados, como pelo fato do material biológico ser de origem ovina. O desenvolvimento do cisto hidático em diferentes hospedeiros possivelmente influencia o repertório de proteínas de EVs, podendo gerar um conjunto maior quando em ovinos.

A análise das proteínas que compõem as EVs de líquido hidático de *E. granulosus* e *E. ortleppi* mostrou uma grande variabilidade entre diferentes amostras biológicas (amostras EV/I). Isso sugere que as EVs têm um repertório dinâmico de proteínas, que pode se ajustar ao longo da infecção conforme as necessidades do parasito. Mesmo assim, algumas proteínas foram constantemente identificadas entre as diferentes amostras analisadas, entre elas: Antígeno 5 (Ag5), catepsina D lisossomal, endoribonuclease poli(U) específica e tetraspanina. Essas proteínas são potenciais marcadores de EVs de *E. granulosus* e *E. ortleppi,* e sua recorrente identificação indica uma função relacionada a processos constitutivos de sobrevivência do parasito, que por sua vez, as tornam também alvos interessantes para drogas e vacinas.

O Ag5, juntamente com o AgB, é uma proteína imunogênica encontrada em grande quantidade no líquido hidático (MUSIANI *et al*., 1978). Sabe-se que o Ag5 possui homologia com serino proteases da família da tripsina, contudo não foi detectada atividade catalítica nem ligação a inibidores de proteases, de modo que a função biológica do Ag5 permanece desconhecida (LORENZO *et al*., 2003). Portanto, futuros estudos para a elucidação da função do Ag5 são necessários e contribuirão para a definição de possíveis estratégias utilizando essa proteína como alvo de drogas ou antígeno vacinal.

A presença da aspártico protease catepsina D é interessante pois essa classe de proteínas já foi proposta como alvo terapêutico em virtude de resultados obtidos com outros parasitos, incluindo protozoários, nematódeos e platelmintos (SOJKA *et al*., 2016). A catepsina D de *S. japonicum* foi utilizada na imunização de camundongos, o que reduziu a carga parasitária dos animais em até 38% (VERITY; MCMANUS; BRINDLEY, 2001). Para *Necator americanus* uma formulação vacinal contendo uma aspártico protease recombinante está sendo testada em humanos (HOTEZ *et al*., 2016).

Tetraspaninas são proteínas transmembrana encontradas com frequência associadas à EVs devido ao seu papel na biogênese e tráfego das mesmas (ANDREU; YÁÑEZ-MÓ, 2014; YÁÑEZ-MÓ *et al*., 2015). As tetraspaninas atuam no recrutamento das moléculas cargo dos exossomos por meio de microdomínios, denominados TEM (do inglês, *tetraspanin-enriched microdomain*), presentes nas membranas celulares (ANDREU; YÁÑEZ-MÓ, 2014). Também já foi demonstrado que as tetraspaninas presentes na membrana de EVs são importantes para a interação destas com células alvo (CHAIYADET *et al*., 2015; NAZARENKO *et al*., 2010). Utilizando um antissoro contra uma tetraspanina recombinante de *O. viverrini*, Chaiyadet *et al*. (2015) conseguiram bloquear a internalização de EVs deste parasito por colangiócitos. Sendo assim, o

bloqueio de tetraspaninas é uma estratégia plausível para inibir a interação de EVs com células alvo, e consequentemente, interferir no efeito das EVs. Tetraspaninas também têm sido avaliadas quanto ao potencial vacinal. Em *E. multilocularis* foi demonstrado que duas (TSP1 e TSP3) das sete tetraspaninas encontradas nesse organismo tem potencial para uso como vacina (DANG *et al*., 2009, 2012). Em *Schistosoma mansoni,* a vacinação de camundongos com TSP1 e TSP2 causou significante redução da carga parasitária e da quantidade de ovos depositados no fígado (TRAN *et al*., 2006).

A relevância biológica da endoribonuclease poli(U) específica em platelmintos ainda é desconhecida. Mas em relação às endoribonucleases de forma geral, sabe-se que são fundamentais para processamento de extremidades 3' de mRNAs, maturação de rRNAs e nas vias de interferência por RNA (ARRAIANO *et al*., 2013). Também há indícios de atividades relacionadas ao remodelamento de estruturas celulares e à regulação da resposta imune (AKIRA, 2013; POE *et al*., 2014; SCHWARZ; BLOWER, 2014).

A participação de EVs em mecanismos de sobrevivência de parasitos é bastante clara, e em *Echinococcus* sp. isso é corroborado pelo fato de proteínas que vem sendo determinadas como componentes de EVs já terem sido anteriormente relacionadas com interações parasito-hospedeiro. O estudo dos componentes de EVs em *E. granulosus* e *E. ortleppi* é necessário para conhecer de maneira completa os mecanismos atuantes na interface com o hospedeiro, ajudando no desenvolvimento racional de abordagens para controle e tratamento da hidatidose cística.

O conteúdo proteico de EVs de *E. granulosus* e *E. ortleppi* foi analisado comparativamente em uma abordagem complementar. Foram utilizados *pools* de EVs (amostras EV/P) obtidas de líquido hidático com o objetivo de identificar as proteínas predominantes em cada espécie, bem como comparar a abundância daquelas compartilhadas. Os resultados mostraram uma maior diversidade de proteínas em EVs de *E. granulosus,* com um número superior de proteínas exclusivas. Além disso, a maioria das proteínas compartilhadas entre as duas espécies foram encontradas em maior abundância em *E. granulosus*. Isso poderia ser o reflexo das diferentes estratégias de interação com o hospedeiro, bem como de defesa frente ao sistema imune do mesmo, empregadas pelas duas espécies. EVs transportam diferentes classes de moléculas, e talvez as EVs de *E. ortleppi* tenham um conteúdo mais elevado de outras moléculas (RNA, por exemplo) em detrimento das proteínas. Em função disso, os níveis de proteínas presentes nas EVs de *E. ortleppi* seriam menores do que nas EVs de *E. granulosus.* Nesse

caso, uma identificação mais abrangente do conjunto de proteínas nas EVs de *E. ortleppi* requer a utilização de equipamentos mais sensíveis, que permitem identificar proteínas em baixas quantidades.

Nesse trabalho também foi feita a identificação de proteínas constituintes de EVs secretadas por protoescólices em cultura. Em função do pouco rendimento ao final do isolamento de EVs, só foi possível a utilização de uma amostra de *E. granulosus* para esta análise. O conjunto de proteínas identificadas em EVs de protoescólices mostrou diferenças em relação aquele das EVs de líquido hidático, dentre as principais estão a ausência de proteínas de matriz extracelular e proteases, e a presença de inúmeras GTPases e demais proteínas envolvidas na biogênese e tráfego vesicular. É provável que as EVs produzidas sejam modificadas como resultado da adaptação aos diferentes hospedeiros e modos de vida ao longo do ciclo, e o ajuste do conteúdo de EVs quando da passagem para um novo estágio e hospedeiro ajudaria o *Echinococcus* a continuar modulando a interface parasito-hospedeiro no novo contexto de interações. Contudo, é preciso ressaltar que as EVs de protoescólices foram obtidas de cultivos *in vitro*, onde a pressão do hospedeiro para eliminar o parasito está ausente. Sendo assim, é possível que a composição proteica de EVs de protoescólices analisadas nesse trabalho também tenha sido modificada em virtude das condições de cultivo.

Embora o estudo de EVs tenha se intensificado e expandido em diferentes tipos de organismos, ainda é em relação às EVs de mamíferos que se tem maior conhecimento, em especial, envolvendo células imunes e tumorais. Mudanças na composição de EVs em resposta a mudanças nas condições do meio/ambiente têm sido amplamente reportadas. Células dendríticas e endoteliais tem seu conteúdo de EVs modificado em resposta a sinais inflamatórios como LPS, TNFα e IFNγ (SEGURA *et al*., 2005). Células endoteliais modificam a composição de EVs após sofrerem hipóxia (DE JONG *et al*., 2012). Após a ativação, células dendríticas secretam exossomos que carreiam MHC de classe II e são transferidos para o linfócito T interagente (BUSCHOW *et al*., 2009).

Outro fator que pode influenciar a composição das EVs é sua biogênese, tanto o processo em si quanto a célula de origem. A formação de exossomos, por exemplo, ocorre nos corpos multivesiculares e existem evidências que populações heterogêneas de corpos multivesiculares coexistem em uma única célula e poderiam produzir subpopulações diferentes de exossomos (COLOMBO; RAPOSO; THÉRY, 2014; KOWAL; TKACH; THÉRY, 2014). O metacestódeo é um organismo complexo do ponto de vista da sua estrutura, ele possui tanto tecidos larvais (camada germinativa) quanto pré-adultos

(protoescólices) e ambos podem dar origem às EVs encontradas no líquido hidático. Entretanto é razoável supor que a camada germinativa é o maior contribuinte para a produção das EVs do líquido hidático, pois é o tecido mais ativo no metacestódeo e que poderia secretá-las também para fora do cisto. Nesse caso, as EVs isoladas de líquido hidático e de sobrenadante de cultivo de protoescólices seriam produtos de tipos celulares distintos, da camada germinativa e do protoescólex, respectivamente, o que, por sua vez, geraria variação na composição.

Em virtude de sua reduzida capacidade de biossíntese, parasitos têm como mecanismo essencial de sobrevivência a obtenção a partir do hospedeiro de diversos nutrientes e substratos necessários para a manutenção de processos fisiológicos básicos (BARRETT, 2009; TSAI *et al*., 2013; ZHENG *et al*., 2013). Lipídios estão entre as moléculas que o *Echinococcus* spp. assimila do hospedeiro, consequentemente, mecanismos especializados de captura, estabilização e transporte de lipídios dos tecidos do hospedeiro para o parasito são bem desenvolvidos. Dentre os fatores com função relacionada a assimilação de lipídios está o AgB, lipoproteína do grupo das HLBPs cujo papel biológico sugerido é de sequestro de lipídios do hospedeiro e distribuição para o parasito. A demonstração feita por Silva-Álvarez *et al*. (2015b) da capacidade de algumas subunidades do AgB de transferir compostos lipídicos para membranas artificiais representam um avanço para a compreensão das atividades do AgB, indicando que ele teria capacidade de transferir os compostos sequestrados dos tecidos hospedeiros para as membranas do parasito. No entanto, isso ainda precisa ser demonstrado *in vivo*. Também permanece indefinido o mecanismo pelo qual as moléculas lipídicas da partícula de AgB são a ele integradas.

A partícula de AgB obtida do líquido hidático apresenta até 50% de sua massa composta por lipídios de variadas classes originários do hospedeiro, o que requer uma estrutura muito bem organizada para acomodar todas essas moléculas. Por isso, foi proposto que as partículas de AgB apresentam similaridade estrutural com lipoproteínas séricas de mamíferos, especialmente o HDL (OBAL *et al*., 2012; SILVA-ÁLVAREZ *et al*., 2015a). Sendo assim, é possível que, apesar de não haver relação evolutiva entre o AgB e as lipoproteínas de mamíferos, exista similaridade funcional, oriunda da necessidade de *Echinococcus* spp. obter compostos lipídicos de forma eficiente.

Igualmente importante para a sobrevivência do parasito é evitar a eliminação pelo sistema de defesas do hospedeiro (SIRACUSANO *et al*., 2008a), e sabe-se que o AgB de *E. granulosus* desempenha um papel importante na modulação da resposta imune do

hospedeiro (GOTTSTEIN *et al*., 2017; SIRACUSANO *et al*., 2008b, 2012b). Diversos estudos investigaram os efeitos do AgB sobre diferentes tipos celulares que compõem o sistema imune, como macrófagos, células dendríticas e linfócitos (RIGANÒ *et al*., 2007; SILVA-ÁLVAREZ *et al*., 2016; SIRACUSANO *et al*., 2008b). A partir desses estudos, uma atividade imunomoduladora propensa a gerar um ambiente anti-inflamatório foi sugerida para o AgB (SILVA-ÁLVAREZ *et al*., 2016; SIRACUSANO *et al*., 2012b).

A importância do AgB na biologia de *Echinococcus* spp. é reconhecida, e existem fortes indícios das funções que ele desempenha. Entretanto a continuidade dos estudos com AgB é necessária, pois pode contribuir para detalhar seus mecanismos de ação, o que por sua vez poderia promover o desenvolvimento de fármacos específicos contra essa proteína. Uma possível interação do AgB com células não imunes, as quais também estão presentes no tecido circundando o metacestódeo e, portanto, também poderiam ser alvo do AgB, nunca foi avaliada. Neste trabalho foi demonstrado que além de interagir com uma linhagem celular de macrófagos (J774), o AgB também interage com tipos celulares de natureza não imune: fibroblastos (NIH/3T3), hepatócitos (RH) e células epiteliais pulmonares (A549).

Ao investigar mais detalhadamente a interação com as linhagens A549 e RH, que estariam mais próximas da situação biológica, foi encontrado que o AgB é internalizado por endocitose, sendo a endocitose via *rafts* lipídicas a principal via utilizada. A internalização do AgB por células de mamíferos é relevante no contexto da hidatidose cística, pois pode representar um passo essencial na sua função como molécula carreadora de lipídios. Uma HLBP de *Taenia solium* (TsM 150 kDa) é capaz de ligar compostos lipídicos do meio externo e transportá-los através da membrana do metacestódeo (LEE *et al*., 2007). Os autores também mostraram a co-localização da TsM 150 kDa com gotas lipídicas do granuloma do hospedeiro, em tecidos suínos infectados com *T. solium*, indicando uma possível fonte para os compostos captados por essa proteína *in vivo* (LEE *et al*., 2007). O AgB poderia carrear lipídios por um mecanismo semelhante, de modo que a endocitose permitiria ao AgB acessar as reservas lipídicas intracelulares, nas gotas lipídicas por exemplo. Contudo, não se pode excluir a hipótese que o AgB interage com células não imunes do hospedeiro para induzir alguma mudança fisiológica em favor do parasito, atuando como uma molécula sinalizadora, à semelhança do que ocorre em células imunes, onde o AgB é capaz de induzir uma resposta predominante do tipo Th2 associada à infecção crônica (SIRACUSANO *et al*., 2012b).

Outra questão intrigante e ainda sem resposta em relação ao AgB é de como se dá o tráfego entre os tecidos do parasito e do hospedeiro. A porção externa da parede do cisto é composta pela camada laminar, uma camada acelular, rica em carboidratos, e que dá sustentação e proteção a camada mais interna, a germinativa (DÍAZ *et al*., 2015b). Embora seja um tecido relativamente rígido, a camada laminar não é impermeável, sendo possível que o AgB seja produzido pela camada germinativa e secretado para fora do cisto, para posteriormente retornar com os lipídios associados. Interessantemente, neste trabalho foram encontrados mRNAs das subunidades do AgB no conteúdo de EVs de *E. granulosus* e *E. ortleppi*. Esse resultado sugere outra possibilidade para o tráfego do AgB, onde o mRNA seria secretado para fora do cisto por meio de EVs, estas seriam internalizadas por células do hospedeiro que traduziriam o mRNA. O AgB seria produzido já no tecido onde deveria executar sua função. Visto que todas as subunidades do AgB possuem peptídeo sinal para secreção, o AgB poderia ser secretado pela via clássica, podendo interagir com as proteínas séricas do hospedeiro e/ou ser endocitado por outras células. O contato com o tecido hospedeiro durante esses processos permitiria o sequestro dos compostos lipídicos e a partícula de AgB carregada poderia então ser absorvida pelo metacestódeo. A tradução de mRNAs de EVs por células receptoras já foi demonstrada em mamíferos (SKOG *et al*., 2008; VALADI *et al*., 2007). Estudos futuros investigando se os mRNAs secretados em EVs de *Echinococcus* spp. são traduzidos por células receptoras são essenciais para a verificação dessa hipótese. Adicionalmente, foram identificadas as subunidades 1 e 4 do AgB em EVs de *E. ortleppi* (amostras EV/I), bem como a subunidade 4 em EVs de *E. granulosus* (amostras EV/P). Como uma proteína importante para a biologia do *Echinococcus* spp., o AgB poderia ser incorporado nas EVs do parasito, essa forma de secreção pode ser importante para o AgB atingir maior distância no organismo do hospedeiro. Por outro lado, o AgB poderia estar em EVs de *Bos taurus*, onde as EVs seriam o meio do AgB retornar para o cisto hidático após ser produzido no tecido hospedeiro.

A presença de proteínas de origem do hospedeiro no líquido hidático é um fato conhecido e também diz respeito ao balanço da interação parasito-hospedeiro e seu desfecho (AZIZ *et al*., 2011; COLTORTI; VARELA-DÍAZ, 1972; MONTEIRO *et al*., 2010; SANTOS *et al*., 2016). Analisando EVs isoladas de cistos hidáticos ovinos, Siles-Lucas *et al*. (2017) encontraram também proteínas do hospedeiro, sugerindo que, assim como as proteínas livres, as EVs do hospedeiro poderiam ter acesso ao interior do cisto hidático. Nossa análise de EVs obtidas de líquido hidático bovino (amostras EV/I)

também encontrou proteínas do hospedeiro. Não podemos descartar totalmente a possibilidade de que as proteínas bovinas estejam contidas nas EVs do parasito. Supondo que o parasito utilize essas proteínas para suprir alguma necessidade, elas poderiam ter sido sequestradas pelas células da camada germinativa e secretadas para o interior do cisto através de EVs, podendo então ser utilizadas pelos protoescólices quiescentes. Porém, a produção de EVs por mamíferos em resposta a uma infecção é amplamente descrita (HASSANI; OLIVIER, 2013; HU *et al*., 2013; MONTANER *et al*., 2014). Dentre as proteínas bovinas identificadas encontram-se algumas comumente associadas às EVs, inclusive com envolvimento em sua biogênese, como HSP, anexina, GTPase e tetraspanina. Baseado nisso, é possível que o *B. taurus* produza EVs como uma forma de resposta à infecção com o *Echinococcus*, e que essas EVs possam acessar o interior do metacestódeo.

De forma geral identificou-se um número menor de proteínas bovinas nas amostras de *E. ortleppi* em relação a *E. granulosus.* Apesar de sua capacidade de infectar uma ampla gama de mamíferos, as espécies do gênero *Echinococcus* possuem hospedeiros intermediários para os quais estão mais adaptadas. O *E. ortleppi* é melhor adaptado ao *B. taurus*, enquanto que *E. granulosus* é melhor adaptado a *Ovis aries*, o que pode significar que o metacestódeo de *E. ortleppi* é menos susceptível a penetração de EVs do *B. taurus* do que *E. granulosus*. Entretanto, é necessário salientar que, em *E. granulosus*, ~61% das identificações foram exclusivas de uma única amostra biológica, ao passo que outra amostra não teve nenhuma proteína bovina identificada. Então também é possível que outro fator como idade ou estágio de desenvolvimento do metacestódeo esteja associado a maior ou menor presença de EVs do hospedeiro no líquido hidático de *E. granulosus*.

Santos *et al*. (2016) mostraram que a representatividade de proteínas bovinas no repertório de proteínas totais do líquido hidático de *E. granulosus* está associada a condição de fertilidade do metacestódeo. Cistos inférteis (sem protoescólices) apresentam maior número de proteínas do hospedeiro e menor número de proteínas do parasito, enquanto que em cistos férteis (com protoescólices) ocorre o inverso. As amostras utilizadas neste trabalho eram de cistos férteis com protoescólices apresentando alta viabilidade, e a presença de maior quantidade de EVs do hospedeiro no líquido hidático de um determinado cisto pode significar que, nesse caso específico o balanço das interações esteja tendendo em favor do hospedeiro.

Cestódeos parasitas dependem de complexos mecanismos de comunicação célula-célula e de interação com seus hospedeiros para regular adequadamente o desenvolvimento, a homeostase e as funções fisiológicas. As EVs e o AgB são produtos de secreção de *Echinococcus* spp. com potencial de participar ativamente na interface parasito-hospedeiro visando alcançar tal regulação. As EVs de *E. granulosus* e *E. ortleppi* carregam proteínas e RNAs e são internalizadas por hepatócitos *in vitro*. Logo, esses parasitos podem empregar EVs para interferir em diferentes processos nas células do hospedeiro, podendo inclusive atingir células distantes do local de estabelecimento do metacestódeo. O sequestro e transporte de lipídios ao qual o AgB está associado é essencial para o parasito suprir necessidades metabólicas e manter a integridade das membranas celulares. A endocitose do AgB por diferentes linhagens celulares *in vitro*, sugere um possível mecanismo de contato com as células do hospedeiro, onde a proteína poderia obter compostos lipídicos.

Estratégias terapêuticas inovadoras contra a hidatidose podem ser desenvolvidas tendo como alvo esses produtos de secreção, especialmente considerando *a)* a essencialidade da obtenção de lipídios, *b)* os potenciais antígenos vacinais nas EVs e seu alcance dentro do organismo do hospedeiro, e *c)* as diversas atividades biológicas dos componentes de EVs que poderiam ser bloqueadas.

4 PERSPECTIVAS

As principais perspectivas que surgem com este trabalho são: i) a identificação do receptor na célula de mamífero associado à endocitose do AgB; ii) a análise de possíveis efeitos do AgB na sinalização e metabolismo da célula, iii) a análise do destino do AgB após a endocitose, se degradado, re-secretado ou direcionado à algum subcompartimento celular, iv) a identificação do repertório de RNAs em EVs, v) a verificação da tradução dos mRNAs de EVs por células do hospedeiro. Além disso, estudos funcionais das principais proteínas encontradas em EVs são essenciais para o entendimento do papel biológico dessas estruturas.

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ANEXO I

Curriculum Vitae - 2018

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Formação acadêmica/titulação

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Título: Caracterização de vesículas extracelulares e tráfego de Antígeno B no contexto da infecção pela forma larval de *Echinococcus* spp.
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2012 - 2014 Mestrado em Biologia Celular e Molecular pelo Programa de Pós-Graduação em Biologia Celular e Molecular.
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Título: Avaliação dos efeitos do Antígeno B de *Echinococcus granulosus* sobre células de mamíferos em cultura, Ano de obtenção: 2014
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2006 - 2011 Graduação em Licenciatura em Ciências Biológicas.
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Título: A afetividade na sala de aula
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Formação complementar

2009 - 2009 Extensão universitária em Bioinformática no Desenvolvimento de Vacinas. (Carga horária: 20h).
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Producão

Produção bibliográfica

Artigos completos publicados em periódicos

1. **DA SILVA, EDILEUZA DANIELI**; CANCELA, MARTIN; MONTEIRO, KARINA MARIANTE; FERREIRA, HENRIQUE BUNSELMEYER; ZAHA, ARNALDO
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4. COSTA, CAROLINE B.; MONTEIRO, KARINA M.; TEICHMANN, ALINE; **DA SILVA, EDILEUZA D.**; LORENZATTO, KARINA R.; CANCELA, MARTÍN; PAES, JÉSSICA A.; BENITZ, ANDRÉ DE N. D.; CASTILLO, ESTELA; MARGIS, ROGÉRIO; ZAHA, ARNALDO; FERREIRA, HENRIQUE B.
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