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Comparação da patogenicidade das assemblages A e B de *Giardia duodenalis* sobre o intestino delgado de camundongos Swiss

Maringá
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Tese apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutora em Biociências e Fisiopatologia.
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“Não é sobre chegar no topo do mundo

e saber que venceu. É sobre escalar e

sentir que o caminho te fortaleceu...”

Ana Carolina Vilela Da Costa

Comparação da patogenicidade das *assemblages* A e B de *Giardia duodenalis* sobre o intestino delgado de camundongos Swiss

RESUMO

Giardia duodenalis é o protozoário parasito que mais acomete humanos e animais em todo o mundo. Esta espécie parasitária apresenta oito perfis genéticos distintos baseados no polimorfismo molecular. Tais perfis são denominados *assemblages* e são classificadas de A à H, sendo as *assemblages* A e B as mais encontradas nas infecções em humanos. As manifestações clínicas são variáveis entre indivíduos infectados com *G. duodenalis*, variando desde infecções subclínicas a casos severos. Em função da existência de perfis genéticos distintos de *G. duodenalis* e intensidades variáveis de sintomas em indivíduos infectados hipotetiza-se que a patogenicidade deste protozoário esteja relacionada à *assemblage*. Dessa forma o objetivo deste trabalho foi comparar a patogenicidade das infecções causadas pelas *assemblage*s A e B de *G. duodenalis* quanto à resposta do intestino delgado, parâmetros clínicos e comportamentais e leucometria em camundongos Swiss. No primeiro artigo "Assemblages A and B of *Giardia duodenalis* reduce enteric glial cells in the small intestine in mice" foi estudado o tempo de trânsito gastrointestinal, a túnica muscular e elementos do sistema nervoso entérico do duodeno e jejuno de camundongos infectados pelas *assemblages* A e B de *G. duodenalis*. Verificou-se que ambas as *assemblages* do parasito diminuíram o número de células da glia entérica nos plexos mientérico e submucoso, diminuíram a espessura da túnica muscular e alteraram a morfologia de neurônios. No segundo artigo "Comparative study of effects of assemblages AII and BIV of *Giardia duodenalis* on mucosa and microbiota of the small intestine in mice" foram avaliados leucometria, comportamento compatível com dor e ansiedade, microbiota intestinal e parâmetros histológicos do duodeno e jejuno de camundongos infectados pelas *assemblages* A e B de *G. duodenalis*. Ambas as *assemblages* promoveram modificações na composição da microbiota intestinal. A infecção pela *assemblage* A promoveu leucocitose, por aumento de polimorfonucleares, aumento dos linfócitos intraepiteliais (LIE) e dor nos animais, mostrando-se mais agressiva à mucosa intestinal e o duodeno o segmento mais afetado pela infecção. Conclui-se, com os artigos apresentados, que a *assemblage* do parasito é um parâmetro importante para a sintomatologia do hospedeiro. O perfil de infecção observado para a *assemblage* A refletiu em maior inflamação da mucosa, com

aumento de LIE e comportamento de dor nos animais. Além disso, a *assemblage* A promoveu mais alterações no plexo submucoso. Ao contrário, a *assemblage* B mostrou ser mais patogênica ao plexo mientérico e também reduziu a espessura da túnica muscular. A infecção pelas *assemblages* A e B de *G. duodenalis* apresentou patogenicidade distinta no modelo experimental adotado neste estudo, não foi observado diarreia e o duodeno foi o segmento mais afetado pela infecção.
Palavras-chave: Duodeno. Genótipo. Giardíase. Sistema nervoso entérico.

Comparison of pathogenicity of assemblages A and B of *Giardia duodenalis* on the small intestine of Swiss mice

ABSTRACT

Giardia duodenalis is the parasitic protozoan that most affects humans and animals around the world. This parasitic specie presents eight distinct genetic profiles based on molecular polymorphism. These profiles are called assemblages and are classified as A to H, with assemblages A and B being the most found in infections in humans. Clinical manifestations are variable among individuals infected with *G. duodenalis*, ranging from subclinical infections to severe cases. Due to the existence of distinct genetic profiles of *G. duodenalis* and variable intensities of symptoms in infected individuals, it is hypothesized that the pathogenicity of this protozoan is related to its assemblage. Thus, the aim of this work was to compare the pathogenicity of infections caused by assemblages A and B of *G. duodenalis* on small bowel response, clinical and behavioral parameters and leukometry in Swiss mice. In the first article "Assemblages A and B of *Giardia duodenalis* reduce enteric glial cells in the small intestine in mice" were studied the intestinal transit time, the muscle layer and elements of the enteric nervous system of the duodenum and jejunum of mice infected by assemblages A and B of *G. duodenalis*. It was observed that both assemblages of the parasite decreased the numbers of enteric glial cells in the myenteric and submucosal plexuses, decreased the thickness of the muscle layer and alter the morphology of neurons. The second article "Comparative study of effects of assemblages of *Giardia duodenalis* on mucosa and microbiota of the small intestine in mice" evaluated leucometry, behavior compatible with pain and anxiety, intestinal microbiota and histological parameters of the duodenum and jejunum of mice infected with assemblages A and B of *G. duodenalis*. Both assemblages promoted changes in the composition of the intestinal microbiota. The infection with assemblage A promoted leukocytosis by increased of polymorphonuclear cells, increased intraepithelial lymphocytes (IEL) and caused pain in animals, being more aggressive to the intestinal mucosa and the duodenum the organ most affected by the infection. It is concluded, with the articles presented, that the assemblage of the parasite is an important parameter for the symptomatology of the host. The infection profile observed for assemblage A reflected in increased inflammation of the mucosa, with increased of IEL and pain behavior in the animals. In

addition, assemblage A promoted more changes in submucosal plexus. Otherwise, assemblage B was shown to be more pathogenic to the myenteric plexus and also reduced the thickness of the muscle layer. Infection with assemblages A and B of *G. duodenalis* presented distinct pathogenicity in the experimental model adopted in this study, no diarrhea was observed and the duodenum was the segment most affected by the infection.

Keywords: Duodenum. Genotype. Giardiasis. Enteric nervous system.

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LISTA DE ABREVIATURAS E SIGLAS

AB	Alcian blue
ANOVA	Analysis of variance
CFU	Colony-forming units
CG	Control group
DPI	Days post infection
EGC	Enteric glial cell
EPM	Elevated plus maze
GALT	Tecido linfóide associado ao intestino
GIA	Infected with assemblage A
GIB	Infected with assemblage B
HE	Hematoxylin and eosin
LIE	Linfócitos intraepiteliais
MALT	Tecido linfóide associado à mucosa
MPO	Myeloperoxidase
OFT	Open field test
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered-saline
PCR	Reação em cadeira da polimerase
RFLP	Polimorfismo do fragmento de restrição
SEM	Standard error of mean
SNE	Sistema nervoso entérico
WC	Waist circumference

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SUMÁRIO

CAPÍTULO I

1.1 *Giardia spp.*: EPIDEMIOLOGIA E BIOLOGIA DO PARASITO

O gênero *Giardia* possui seis espécies, destas, somente *Giardia duodenalis* (também denominada *G. lamblia* ou *G. intestinalis*) infecta seres humanos e outros animais. As demais espécies infectam exclusivamente animais (1). Este parasito é um protozoário flagelado binucleado descrito pela primeira vez em 1681 por Antonie van Leeuwenhoek, que visualizou trofozoítos em suas próprias fezes durante um exame microscópico (1,2).

A infecção humana por *G. duodenalis* é muito comum em todo o mundo (3). Tal infecção é denominada giardíase e ocorre pela via fecal-oral direta, por meio das mãos ou objetos contendo cistos do parasito, ou indireta, pela ingestão de água e alimentos contaminados (4). Os animais também representam importante papel na transmissão desta doença e o potencial zoonótico de diversas espécies animais tem sido relatado (5-8). Em função de ser uma zoonose de fácil transmissão, a giardíase, na atualidade, ainda representa um grave problema de saúde pública (5,9).

O parasito se apresenta sob duas formas estruturalmente distintas: cistos e trofozoítos. Os primeiros correspondem à forma infectante, sendo resistentes às condições ambientais. Os trofozoítos são formas vegetativas móveis, que possuem flagelo e colonizam o intestino, sendo responsáveis pelas manifestações clínicas da doença (1,10).

A giardíase apresenta alta prevalência em todo o mundo, estima-se que anualmente 280 milhões de pessoas no mundo desenvolvam esta doença (11), e com estimativas de 500.000 novos casos anuais na Ásia, África e América Latina (12). Nos Estados Unidos mais de 15.000 casos da doença são notificados anualmente (3), no Brasil não é uma doença de notificação obrigatória, mas com base em estudos epidemiológicos populacionais são registradas prevalências de 19,5% no norte (13), 31,2% no nordeste (14), 18,2% a 27,3% no sudeste (15,16) e varia entre 1% e 19% no sul do Brasil (17,18).

Fatores relacionados ao local de moradia podem propiciar a transmissão da giardíase. Pesquisas relacionaram esta infecção com alguns fatores sócio-epidemiológicos: número de moradores e animais na residência; forma de higienização dos alimentos; educação sanitária; condições sanitárias de moradia, como água tratada e rede de esgoto; frequência de lavagem das mãos e condições educacionais e

socioeconômicas (19-22).

A infecção por G. *duodenalis* inicia quando os cistos do parasito são ingeridos, o ambiente ácido do estômago induz a excistação, na qual cada cisto dará origem a dois trofozoítos, que irão colonizar o intestino delgado do hospedeiro. Os trofozoítos se aderem aos enterócitos da mucosa intestinal por meio de seus discos ventrais, se multiplicam por fissão binária e, ao migrar para a porção inferior do trato gastrointestinal encontram um ambiente com diferentes níveis de acidez e concentrações de bile e colesterol. Tais condições estimulam o processo de encistamento no qual cistos serão novamente formados e liberados nas fezes do hospedeiro infectado (1,4,23).

Os trofozoítos de *G. duodenalis* são os responsáveis por desencadear as manifestações da doença por diversos mecanismos fisiopatológicos de agressão à mucosa do intestino delgado e ao Sistema Nervoso Entérico (SNE) (24).

1.2 DIVERSIDADE GENÉTICA DE *Giardia duodenalis*

Estudos moleculares utilizando reação em cadeira da polimerase (PCR), a partir da amplificação do gene glutamato desidrogenase (gdh) (25) ou β-giardina (26), associada à técnica de polimorfismo do fragmento de restrição (RFLP) possibilitaram a descoberta de diferentes *assemblages* genéticas de *G. duodenalis* (25-29) e confirmação do potencial zoonótico do parasito (30). As *assemblages* correspondem ao genótipo do parasito e até o momento oito *assemblages*, identificadas de A a H, foram descritas. As *assemblages* A e B são frequentemente encontradas em humanos, especialmente as sub-*assemblages* AI, AII, BIII e BIV (18,26). Entretanto, recentemente, parasitos da *assemblage* E foram identificados em infecções de crianças do Rio de Janeiro (31). No interior do estado do Paraná foi encontrado em humanos uma prevalência de 68,2% de *assemblage* A e 31,8% de *assemblage* B (32).

Em pesquisas de perfis genéticos de *G. duodenalis* de animais em diferentes regiões do mundo, foram isoladas as *assemblages* A, B, C e D em cães (18,28,33), B em macacos (28), A e E em bezerros (28), E em búfalos (26), F em gatos (28), G em roedores (34) e H em vertebrados marinhos (30). No Egito foi detectada transmissão zoonótica da *assemblage* C de cão para humano (30).

Além da afinidade por hospedeiro, as *assemblages* de *G. duodenalis* estão relacionadas a diferentes riscos zoonóticos, facilidade no diagnóstico com determinadas enzimas de restrição, prevalência em humanos e em diferentes regiões do mundo e dinâmica de transmissão (30). Existem suspeitas de que a *assemblage* de *G. duodenalis*

também possa estar relacionada com a patogenicidade da infecção. A relação entre a *assemblage* do parasito e a evolução clínica da giardíase não está completamente esclarecida. Há relatos de predomínio de *assemblage* A em pacientes sintomáticos (diarreia) (35) e B nos casos assintomáticos (36). Por outro lado, outros autores (33,37) não encontraram relação entre *assemblage* e manifestações clínicas. Deste modo, estudar as diferentes *assemblages* de *G. duodenalis* tem se tornado tão importante que trabalhos recentes (13,38) sugerem a divisão do gênero *Giardia* em duas espécies de acordo com sua variação gênica: *Giardia duodenalis* (*assemblage* A) e *Giardia enterica* (*assemblage* B).

1.3 INTESTINO DELGADO E SISTEMA NERVOSO ENTÉRICO

O intestino delgado é dividido em três segmentos: duodeno, jejuno e íleo. É o local final da digestão dos alimentos, absorção de nutrientes, secreção endócrina e exócrina e barreira imunológica. A mucosa do intestino delgado apresenta diversos elementos que aumentam sua superfície como as pregas, vilos e criptas. No epitélio do intestino delgado encontra-se células absortivas (enterócitos), células caliciformes, linfócitos intraepiteliais (LIE), células de Paneth e também as células enteroendócrinas. Os enterócitos têm como principal função absorver, por meio de transporte ativo, as moléculas nutrientes produzidas durante a digestão. Já as células caliciformes estão distribuídas entre os enterócitos e produzem mucinas, que originam o muco que protege e lubrifica o epitélio intestinal (39). Os LIE são em geral linfócitos T que captam informações antigênicas e modulam o crescimento epitelial, exercendo papel importante na tolerância imunológica (40). As células de Paneth estão localizadas no fundo das criptas e produzem peptídeos antimicrobianos que regulam a microbiota intestinal, bem como os fatores de crescimento envolvidos na manutenção de células tronco. As células enteroendócrinas representam cerca de 1% de todas as células epiteliais e produzem hormônios, os quais regulam diversas funções do epitélio intestinal (41).

O intestino também apresenta uma túnica muscular própria e, entre esta e a túnica mucosa encontra-se a tela submucosa. Além disso, entre a submucosa e a mucosa existe uma fina camada muscular, a *muscularis mucosa*. Tal camada corresponde a uma barreira física que separa regiões com quantidades opostas de células linfoides (40).

Além dos elementos descritos, o trato grastrointestinal, do qual faz parte o intestino, apresenta seu próprio sistema nervoso: o SNE. Localiza-se na parede intestinal, onde exerce funções de controle do trato gastrointestinal, como motilidade,

secreção e imunoregulação (42,43).

O SNE é formado por uma série de redes interligadas, os plexos, por neurônios e células da glia entérica. Dentre os plexos dois principais são ganglionados, o mientérico e o submucoso (Figura 1). O primeiro, conhecido antigamente como plexo de Auerbach, se estende por todo o trato gastrointestinal, é mais externo e fica localizado entre as camadas musculares longitudinal e circular da túnica muscular. Apresenta como principal função o controle da atividade muscular por meio da regulação do tônus da parede intestinal, do ritmo das contrações e da velocidade de condução das ondas excitatórias ao longo da parede do intestino. Já o plexo submucoso, anteriormente denominado plexo de Meissner, está localizado na tela submucosa. Este controla basicamente a secreção gastrointestinal e o fluxo sanguíneo local, por meio de sua ação na secreção e absorção local (43,44).

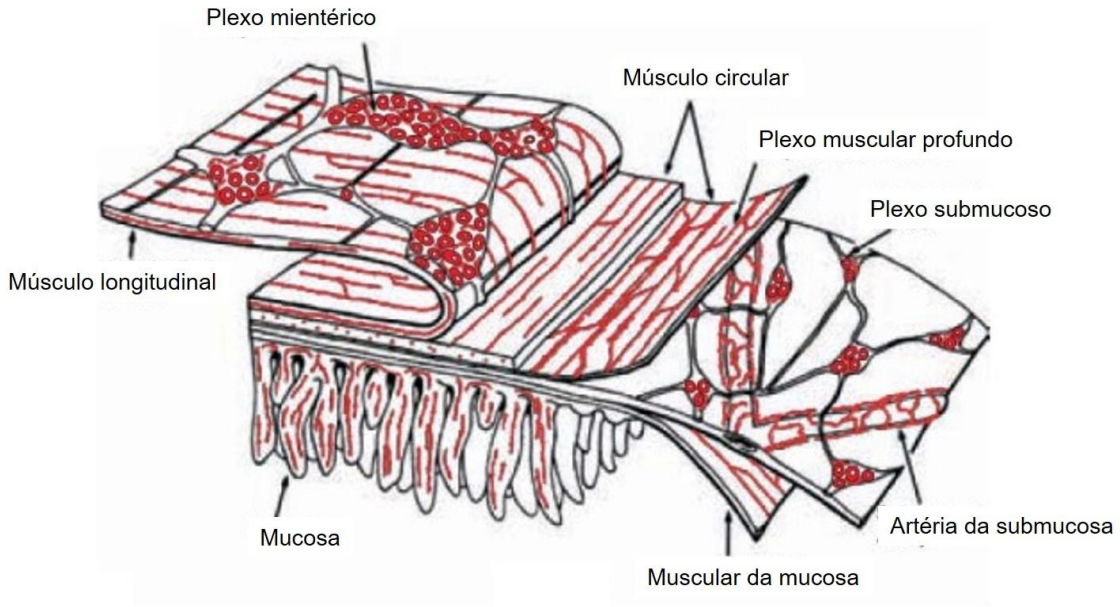

Figura 1. Plexos do SNE a partir de preparados de membrana.
FONTE: Adaptado de Furness (44).

Durante seu ciclo biológico, os trofozoítos de *Giardia spp.* têm tropismo por se alojar no intestino delgado do hospedeiro e até onde se sabe existe apenas dois estudos que apontam a possibilidade de interação de *G. duodenalis* com o SNE. Um relaciona o aumento na produção e liberação de neurotransmissores que alteram a permeabilidade intestinal ao cálcio (45) e outro a atividade da NO sintase neuronal (34) na eliminação da infecção por tal protozoário (46). O pequeno número de estudos tona as pesquisas

sobre a giardíase e alterações no SNE uma forma de contribuição para elucidação das variações envolvidas da patogenicidade diversa da infecção.

1.4 SISTEMA IMUNE DA MUCOSA INTESTINAL

A mucosa intestinal corresponde a uma fonte de acesso ao organismo e diariamente sofre ataques de antígenos oriundos da dieta e da microbiota. Assim, para realizar a defesa da mucosa existe o Tecido Linfóide Associado à Mucosa (MALT), organizado e estrategicamente localizado com células de captura, processamento e apresentação de antígenos, além de participar da produção de anticorpos e secreção de citocinas (40).

O MALT consiste de uma rede preenchida por linfócitos e macrófagos e pode ser dividido quanto à sua localização. No intestino está presente o Tecido Linfóide Associado ao Intestino (GALT) (Figura 2), o qual contém os LIE que representam uma das principais populações de células T do intestino delgado (20 LIE/100 células epiteliais). Os LIE correspondem principalmente a linfócitos T CD8+ (1) e são células pequenas (5 a 9 µm de diâmetro), de citoplasma claro, poucas organelas e núcleo central. Estão localizados entre as células epiteliais favorecendo seu papel inicial na imunidade das mucosas. Sua função de proteção do hospedeiro contra patógenos entéricos se deve às características de potente ação citolítica, capacidade imunorregulatória e sustentação da integridade epitelial (40,47).

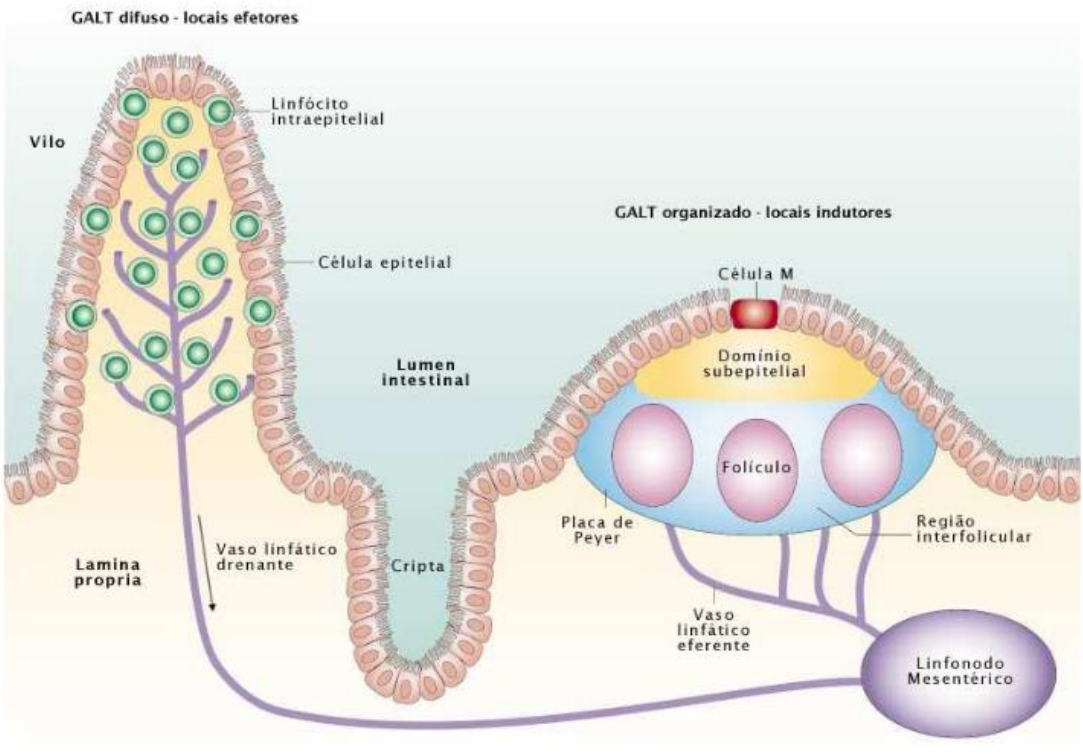

Figura 2. Tecido Linfóide Associado ao Intestino.
FONTE: Mowat (48).

Em algumas situações, como na infecção por *G. duodenalis*, a resposta imune pode ser alterada e reações inflamatórias ocorrem. Nestes casos ocorre um aumento na população de LIE e tais células vão produzir moléculas, como as integrinas, que visam a eliminação do patógeno (40). Dessa forma, quantificar os LIE pode ser uma forma de verificar a presença de inflamação intestinal em modelos murinos de infecção experimental por *G. duodenalis.*

1.5 MICROBIOTA INTESTINAL E GIARDÍASE

A microbiota intestinal corresponde a população de microrganismos que colonizam o intestino. Estima-se que neste ecossistema estejam presentes cerca de 1.800 gêneros e de 15.000 a 36.000 espécies de bactérias (49). As bactérias da microbiota intestinal contribuem com a modulação da resposta imune por meio da competição com patógenos por sítios de adesão, para fixação a captação de nutrientes, e produção de bacteriocinas. Ademais, a microbiota intestinal exerce efeitos sobre uma série de reações bioquímicas no hospedeiro (50).

Para a saúde do hospedeiro a microbiota intestinal precisa estar em equilíbrio,

pois assim seus componentes impedem que patógenos exerçam efeitos maléficos. Dessa forma, quando algo promove o desequilíbrio da microbiota, patógenos se desenvolvem promovendo diarreia, inflamação da mucosa e desordens na permeabilidade do intestino (50).

A ação de *G. duodenalis* no intestino delgado do hospedeiro pode levar a disbiose, que conceitualmente corresponde ao crescimento de espécies bacterianas não comuns a microbiota (51). Assim, a disbiose e as demais alterações na mucosa intestinal promovidas pelo parasito são responsáveis por disfunção na barreira epitelial podendo contribuir para a transposição microbiana e resposta inflamatória local (52) e também, por alterações denominadas "pós-giardíase" (52, 53).

A microbiota intestinal pode interferir na fisiopatologia da giardíase limitando o desenvolvimento parasitário por competição por recursos, liberação de fatores inibitórios, toxicidade direta e indução de resposta imune por reação cruzada. Assim, a microbiota não só apresenta um efeito anti-*Giardia* mas também protege e preserva a integridade do intestino durante a infecção (1).

Diversos estudos (50,52-56) investigaram alterações na microbiota intestinal em modelos experimentais de giardíase. Apesar das publicações já existentes, ainda não foram realizadas comparações da composição da microbiota intestinal em modelos de infecção pelas *assemblages* A e B de *G. duodenalis.* Já que variações na composição da microbiota poderiam explicar a variabilidade na patogenia e susceptibilidade à infecção (1).

1.6 FISIOPATOLOGIA DA GIARDÍASE

A giardíase pode se apresentar de forma assintomática ou sintomática, as manifestações clínicas surgem entre uma e duas semanas após a infecção e podem incluir vômito, diarreia, dor abdominal, perda de peso e sinais relacionados à má absorção, como deficiência de vitamina A e anemia ferropriva (57-59). A infecção também está associada à desnutrição, déficit de crescimento físico e desenvolvimento cognitivo em crianças (23,58). Evidências apontam que a giardíase implica no desenvolvimento de desordens crônicas no intestino, incluindo a Síndrome do Intestino Irritável e insuficiências nutricionais, por mecanismos que precisam ainda ser compreendidos (59,60).

G. duodenalis é tipicamente um parasito extracelular que não invade as células do intestino delgado, permanecendo aderido às microvilosidades na luz intestinal (61).

Tal aderência é realizada pelos trofozoítos na superfície do epitélio intestinal com auxílio do seu disco ventral, nesta interação estão envolvidas diversas substâncias como as lectinas de superfícies, proteínas giardinas, proteínas de superfície variante e cisteína proteases inespecíficas (60).

A adesão ao epitélio desencadeia uma série de eventos reponsáveis pela diarreia do hospedeiro. Dentre estes eventos pode-se destacar: apoptose de enterócitos, disfunção da barreira intestinal, ativação linfocitária, deficiência de dissacaridases, encurtamento das bordas em escova das microvilosidades com ou sem atrofia do vilo (58,60), hipersecreção de íons, má absorção de água, glicose e sódio, hiperplasia de cripta intestinal, aumento da produção de muco e hipermotilidade do intestino delgado (58,60,62).

Apesar de pouco elucidado há fatores do hospedeiro que também participam na patogênese da giardíase. O sistema imune da mucosa é extremamente complexo e capaz de reconhecer os patógenos e responder às infecções por mecanismos inatos e adaptativos, que atuam em sincronia (Figura 3) (1).

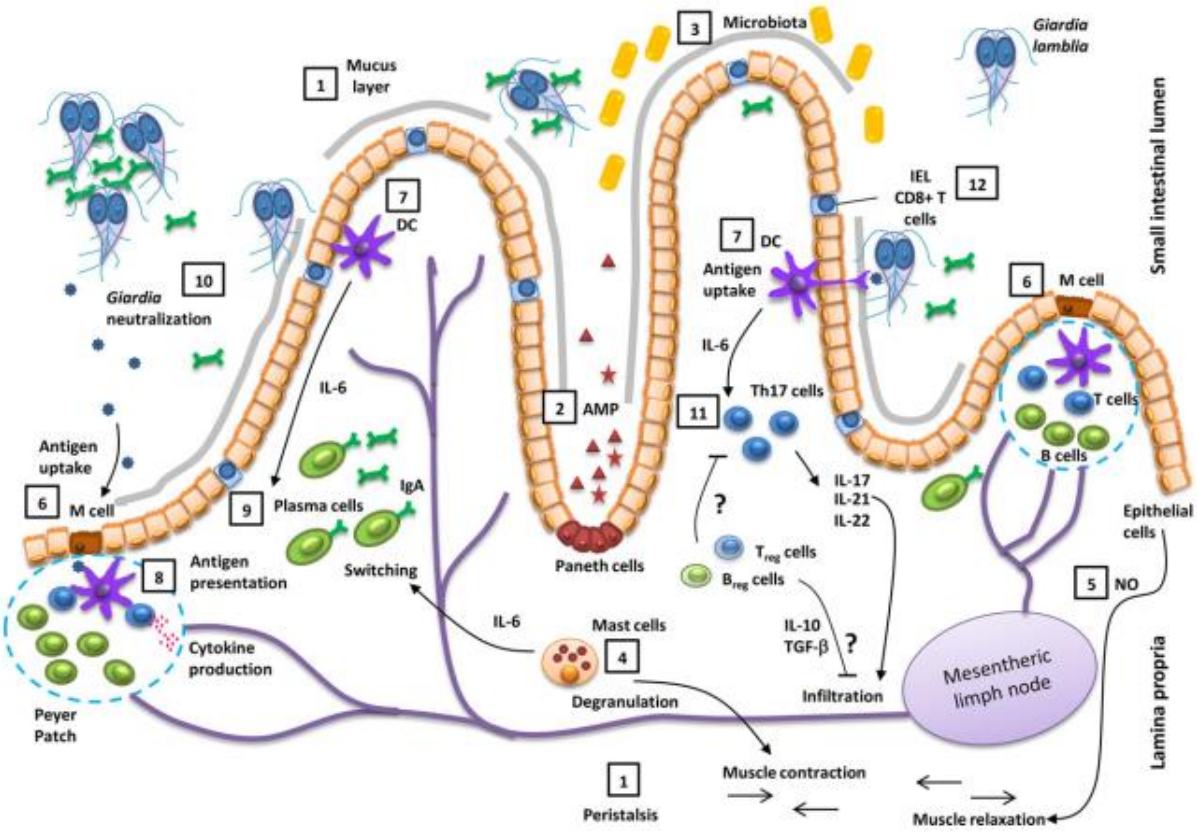

Figura 3. Mecanismos de defesa do hospedeiro contra a infecção por *G. duodenalis.* [1] Peristalse. [2] Camada de muco. [3] Microbiota. [4] Mastócitos. [5] Óxido nítrico. [6] Células M. [7] Células dendríticas. [8] Citocinas. [9] Interleucina 6 e plasmócitos. [10] Neutralização de *Giardia spp.* na luz intestinal pela IgA. [11] Th17. [12] LIE.
FONTE: Lopez-Romero et al. (1).

Como *G. duodenalis* não é um parasito invasivo ao epitélio, sua presença promove pouca inflamação intestinal. Os mecanismos de imunidade contra a colonização de patógenos envolvem a camada de muco da superfície epitelial, movimentos peristálticos, liberação de citocinas pró inflamatórias por mastócitos, produção de óxido nítrico, ativação de linfócitos T, liberação de IgA por células plasmáticas que migram até a lâmina própria, além da migração de LIE. Os neutrófilos também podem ser efetivos na resposta giardicida por meio de efeitos oxidantes, assim como a microbiota intestinal (1).

A partir dos mecanismos descritos, estudos que envolvam análises acerca dos três principais componentes envolvidos na resposta do hospedeiro à giardíase (microbiota, epitélio intestinal e muco) se tornam importantes na caracterização da

fisiopatologia das infecções por diferentes *assemblages* de *G. duodenalis.*

1.7 JUSTIFICATIVA

G. duodenalis é um dos protozoários parasitos que mais acomete humanos e animais de todo o mundo. Estima-se que a cada ano 280 milhões de pessoas se infectem, tantos nos países desenvolvidos quanto naqueles em desenvolvimento.

Esta espécie parasitária é considerada complexa por diversos autores por apresentar oito perfis genéticos distintos baseados no seu polimorfismo molecular. Tais perfis são denominados *assemblages* e são classificadas de A a H. Destas, as *assemblages* A e B são as mais frequentemente encontradas em humanos e as sub-*assemblages* AII e BIV as mais prevalentes no sul do Brasil.

As manifestações clínicas são variáveis entre indivíduos infectados com *G. duodenalis*, variando desde casos assintomáticos a casos severos da doença. Dentre as manifestações mais comuns estão: diarreia, náusea, vômito, flatulência e dor abdominal. Também há evidências de casos de síndrome do intestino irritável como complicação "pós-giardíase".

Em função da existência de perfis moleculares distintos de *G. duodenalis* e intensidades variáveis de sintomas em indivíduos infectados acredita-se que a patogenicidade deste protozoário possa estar relacionada à sua *assemblage*. Ainda não existem estudos que avaliaram se existem diferenças de virulência entre as *assemblages* mais frequentemente isoladas em humanos. É possível que estas diferenças possam ser a explicação do surgimento de portadores de giardíase que apresentam sintomatologia e aqueles que desenvolvem a doença de forma assintomática. Esta questão da sintomatologia ainda é um assunto que gera muitas controvérsias entre estudiosos do mundo todo, portanto, a patogenicidade de diferentes *assemblages* de *G. duodenalis* é um assunto de extrema importância para a saúde pública, já que esta infecção parasitária está entre as mais prevalentes do mundo e acomete principalmente a população de países subdesenvolvidos.

Até onde se sabe, os estudos que avaliaram a interação de *G. duodenalis* com o SNE não esclareceram as alterações que este parasito causa, assim como são escassas as pesquisas envolvendo microbiota intestinal, leucometria, parâmetros clínicos e comportamento de dor em modelos experimentais murinos. Diante deste contexto, compreender a patogenicidade comparativa de duas *assemblages* prevalentes em humanos e verificar a fisiopatologia de mudanças que venham ocorrer na morfologia

intestinal e na microbiota se justificam visando contribuir com o avanço do conhecimento na área.

1.8 OBJETIVOS

1.8.1 Objetivo geral

Comparar a patogenicidade da infecção pelas *assemblages* A e B de *G. duodenalis* quanto à resposta do intestino delgado, parâmetros clínicos e comportamentais e leucometria em camundongos Swiss.

1.8.2 Objetivos específicos

- Monitorar sinais clínicos e estimar o tempo de trânsito intestinal em camundongos infectados com as *assemblages* A e B de *G. duodenalis*;

- Comparar as *assemblages* A e B de *G. duodenalis* quanto à produção de óxido nítrico em muco e macerado tecidual;

- Diferenciar a inflamação induzida pelas *assemblages* A e B de *G. duodenalis* por meio da contagem de leucócitos em sangue, muco e fezes e contagem de linfócitos intraepiteliais;

- Identificar como as *assemblages* A e B de *G. duodenalis* promovem alteração intestinal por meio de análise morfométrica e contagem de células caliciformes em duodeno e jejuno;

- Estimar o dano ao sistema nervoso entérico promovido pelas *assemblages* A e B de *G. duodenalis* por meio da contagem de células da glia entérica e neurônios e avaliação da morfometria neuronal;

- Avaliar a dor manifestada na infecção pelas *assemblages* A e B de *G. duodenalis* com base em testes de comportamento exploratório;

- Conhecer as alterações na microbiota intestinal promovidas durante a infecção pelas *assemblages* A e B de *G. duodenalis*.

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CAPÍTULO II

Artigo 1: "Assemblages A and B of *Giardia duodenalis* reduce enteric glial cells in the small intestine in mice"

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Assemblages A and B of *Giardia duodenalis* reduce enteric glial cells in the small intestine in mice

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Abstract

Infection with *Giardia duodenalis* is the most common human parasitic disease worldwide. This infection may be related to significant changes in the enteric nervous system. Our goal was to evaluate the myenteric and submucosal plexus, the intestinal muscle layer, and gastrointestinal transit in mice that were infected with assemblages A and B of *Giardia duodenalis.* Swiss mice were infected with assemblages A and B of *G. duodenalis* for 15 days. Gastrointestinal transit time was evaluated before euthanasia. Duodenum and jejunum were removed for histological and immunohistochemical analyses. We observed a reduction of the enteric glial cell counting and decrease in the ratio of enteric glial cells to neurons. The number of neurons did not change, but morphological changes were observed in the duodenum and jejunum in both plexus, including an increase in the nuclear area and a reduction of cell bodies in the myenteric plexus and a decrease in the nuclear area in the submucosal plexus. A reduction of the thickness of the muscle layer was observed in the duodenum, with no significant differences in gastrointestinal transit time. Assemblages A and B of *G. duodenalis* decreased the number of enteric glial cells in the myenteric and submucosal plexus, decreased the thickness of the muscle layer, and altered the morphology of neurons.

Keywords: Duodenum; enteric nervous system; giardiasis.

Introduction

Infection with *Giardia duodenalis* is the most common human parasitic disease worldwide (El Basha et al. 2016). An estimated 280 million people have this disease annually (Ankarklev et al. 2012), and it was included in the list of neglected diseases by the World Health Organization in 2006 (Buret et al. 2015). In the United States, the incidence of giardiasis was reported to be 6.4 and 5.8 per 100,000 individuals in 2011 and 2012, respectively (Painter et al. 2015). In Brazil, the prevalence is 16.9% in the north (Nunes et al. 2016), 31.2% in the northeast (Mariano et al. 2015), 7.9% to 27.3% in the southeast (Pinheiro et al. 2011; David et al. 2015), and 1% (Casavechia et al. 2016) to 19% (Colli et al. 2015) in the south.

Variations in the symptomatology and evolution of the disease appear to be related to characteristics of the host (e.g., age and nutritional and immunological status) (Pestechian et al. 2014; Thompson and Ash 2016) and characteristics of the parasite (e.g., invasive potential and genetic assemblage) (Martínez-Gordillo et al. 2014; Pestechian et al. 2014). *G. duodenalis* can manifest as eight genetic assemblages (A-H). Assemblages A and B predominate in human infection in all the world (Atherton et al. 2013; Tamer et al. 2015; Matsuchita et al. 2017). Assemblage A can be subdivided into sub-assemblages AI and AII, and assemblage B can be subdivided into BIII and BIV. Brazil has a predominance of assemblages BIV in the southern region (Colli et al. 2015), but parasites with assemblage E have also been recently reported in children in Rio de Janeiro (Fantinatti et al. 2016).

The pathogenesis of giardiasis remains unclear since the parasite is classified as non-invasive and does not secrete known toxins (Ankarklev et al. 2010). The relationship between different assemblages of *G. duodenalis* and the clinical course of giardiasis is not fully understood. There are reports of a predominance of assemblage A in symptomatic patients (i.e., with diarrhea) (Aydin et al. 2004; Pestechian et al. 2014) and assemblage B in asymptomatic cases (Aydin et al. 2004).

In rodent models of infection, increases in intestinal motility and diarrhea have been attributed to changes in the enteric nervous system (ENS) (Halliez and Buret 2015) or an increase in cholecystokinin and mast cell degranulation (Li et al. 2007). These changes, however, have not been associated with different assemblages of the parasite or the role of enteric glial cells (EGCs). Intestinal motor control depends on the integrated action of neurons and EGCs (Khen-Dunlop et al. 2013). Determining the

response of these neural elements to infection is essential to elucidate the possible mechanism. In the present study, we compared the most prevalent parasite assemblages in human infection worldwide with regard to their ability to alter elements of myenteric and submucosal innervation.

The goal of the present study was to evaluate the myenteric and submucosal plexus, the intestinal muscle layer, and gastrointestinal transit in mice that were infected with assemblages A and B of *Giardia duodenalis*.

Materials and methods

Ethical aspects

The study was conducted based on the guidelines of the Sociedade Brasileira de Ciência em Animais de Laboratório and was approved by the Comissão de Ética em Experimentação Animal of the Centro Universitário Integrado (Statement no. 1070).

Inoculum

Cysts of *G. duodenalis* were obtained from the genotyped sample database of the Laboratório de Parasitologia Ambiental e de Alimentos, Universidade Estadual de Maringá, Brazil (accession numbers of GenBank KJ741310–KJ741313). The cysts were isolated from feces of residents of Ângulo, Paraná, in southern Brazil by Colli et al. (2015).

Experimental groups

Forty-two male Swiss mice aging 21 days old were obtained from the animal facility of the Universidade Estadual de Maringá and housed in the animal house of the Centro Universitário Integrado. The animals were randomly assigned into three groups (n:14): uninfected control group (CG) and animals infected with 1,000 cysts of *G. duodenalis* assemblages AII and BIV by gavage (GIA and GIB groups, respectively).

Groups of mice were housed in polypropylene cages with a wire grid floor to avoid contact with excreta. The animals were maintained in a 12 h/12 h light/dark cycle with controlled temperature and humidity. Food and water were provided *ad libitum*.

Confirmation of infection

Three days post infection (dpi), it was performed parasitological examination according to the method of Faust et al. (1939) Molecular techniques (polymerase chain

reaction and restriction fragment length polymorphism) (Colli et al. 2015) were performed to confirm infection with the appropriate assemblages of *G. duodenalis* and exclude the presence of *Giardia muris*.

Daily evaluation of clinical and physiological parameters

The experiment lasted 15 days. During this time period, the animals were observed daily for the presence or absence of diarrhea, and weight, body temperature, waist circumference (WC), and water and food consumption were recorded.

Weight was measured (in grams) using a semi-analytical balance. Temperature was measured (in ºC) using an infrared digital thermometer with a laser sight (model TI-890, Instrutherm). Waist circumference was measured (in centimeters) using a tape measure that was placed one finger above the animal's hind legs. Daily food and water consumption were calculated as the difference from the amount of food and water what was provided over 24 h.

Assessment of gastrointestinal transit time

To evaluate gastrointestinal transit time at 6 and 12 dpi, 100 µL of an aqueous solution that contained a non-absorbable marker (Carmim Red Hydro + ethylcellulose) was administered to each animal by gavage. Mice were observed continuously and gastrointestinal transit time was determined as the time between administration of the dye solution and elimination of the first red-colored stool pellet (Calcina et al. 2005).

Euthanasia

The animals were intraperitoneally anesthetized with xylazine® (10 mg/kg to 10%) and ketamine® (110 mg/kg to 10%) and then euthanized by intracardiac administration of KCl (2 mL).

Material collection and processing

The abdomen was cut-opened along the medial line to obtain the duodenum and proximal jejunum. A one centimeter ring was collected from each organ for histological processing, and a two centimeter ring was collected for immunohistochemistry. The segments were fixed in Bouin's solution, dehydrated, diaphanized, paraffin-embedded, cut transversely (4 µm, semi-serial), and stained with hematoxylin/eosin.

For immunohistochemistry, the segments were washed with phosphate-buffered saline (PBS; 0.1 M, pH 7.4) and fixed in 4% paraformaldehyde for 3 h. Then, each segment was opened along the mesenteric border and successively washed in PBS to remove excess fixative and stored in PBS with 0.08% sodium azide at 4°C. The segments were dissected under a stereomicroscope (Motic SMZ-140, Motic, Hong Kong, China) to obtain the whole mounts containing the myenteric and submucosal plexus.

Immunofluorescence

Whole mounts were submitted to immunohistochemistry for the detection of β-tubulin III protein (marker of neurons) and S-100β protein (marker of EGC). The whole mounts were washed twice for 5 min with 0.1 M PBS + 0.5% Triton and incubated in goat serum blocking solution + 2% bovine serum albumin for 1 h. Then, they were then incubated in a solution that contained chicken anti-β-tubulin III primary antibody (1:750, TUJ1, Aveslab) and rabbit anti-S-100β primary antibody (1:200, S2644, Sigma) at room temperature for 48 h. Afterward, they were washed 5 times with 0.1 M PBS + 0.5% Triton for 5 min and incubated with the following secondary antibodies for 2 h: Alexa Fluor goat anti-chicken antibody (1:750; A11039, Life Technologies) and Alexa Fluor 546 goat anti-rabbit antibody (1:200; A11010, Invitrogen). They were then washed three times in 0.1 M PBS for 5 min, extended on glass slides, and stored in the fridge.

Quantitative analysis

Images were captured using an Olympus FSX-100 microscope (Olympus, Tokyo, Japan) at 20x magnification and then transferred to a computer and analyzed using Image-Pro Plus software. Neurons and intraganglionic EGC that were present in 32 images of each mice were counted (Araújo et al. 2015). Based on this counting of neurons and EGCs in the ganglia, the ratio of the number of EGC to the number of neurons was calculated (EGCs : neurons).

Morphometric analysis

Using Image-Pro Plus software, the area of the nucleus and cell bodies of 100 myenteric and submucosal neurons of each mice was measured. The area of the

cytoplasm was calculated, and the ratio between nucleus and cell bodies area was obtained. Histological sections of the duodenum and jejunum were photographed using an Olympus BX50 microscope (Olympus, Tokyo, Japan) at 20× and 100× magnification for the muscle layer and enterocytes, respectively, using Image-Pro Plus software. Sixty-four measurements of the thickness of the muscle layer and eighty measurements of the height and width of enterocytes and their nuclei were performed for each organ for each animal.

Statistical analysis

The data had a normal distribution by the D'Agostinho-Pearson test and were expressed by mean ± standard error of mean (SEM). Results were compared between groups using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison post hoc test. The statistical analysis of the data was performed using Graph Pad Prism 5.01 software. For all statistic tests, p-values less than 0.05 were considered significant.

Results

The microscopic and molecular analyses revealed the presence of *G. duodenalis* in feces in all infected mice at 3 dpi. Diarrhea was not observed at any time during the study, with no changes in body weight or food and water intake.

A significant increase in body temperature was observed in the GIA and GIB groups at 14 dpi. The animals in the GIB group had a greater WC at 8 dpi compared with the control group.

Gastrointestinal transit time

No significant differences in gastrointestinal transit time were observed between the infected and control groups both at 6 and 12 dpi ($p > 0.05$).

Enteric Glial Cells

Figures 1 and 2 show that in the myenteric plexus, the GIB group presented a 56% reduction of the duodenal EGC compared with the control group ($p < 0.05$). Assemblage AII did not alter the number of myenteric glial cells. *G. duodenalis* assemblages A and B caused a reduction of duodenal and jejunal EGCs in the submucosal plexus (Fig. 1).

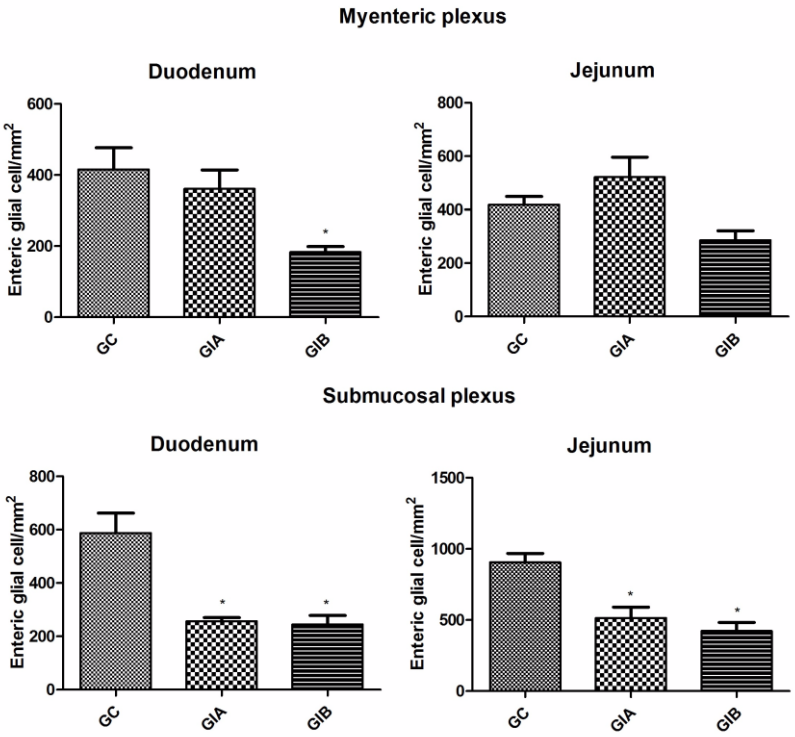

Fig. 1 Mean ± standard error of mean of the number of enteric glial cells in the myenteric and submucosal plexus of uninfected mice (control group [CG]) and mice infected with *Giardia duodenalis* assemblage A (GIA) and assemblage B (GIB). **p* < 0.05, significant difference compared with CG (ANOVA followed by Dunnett's test)

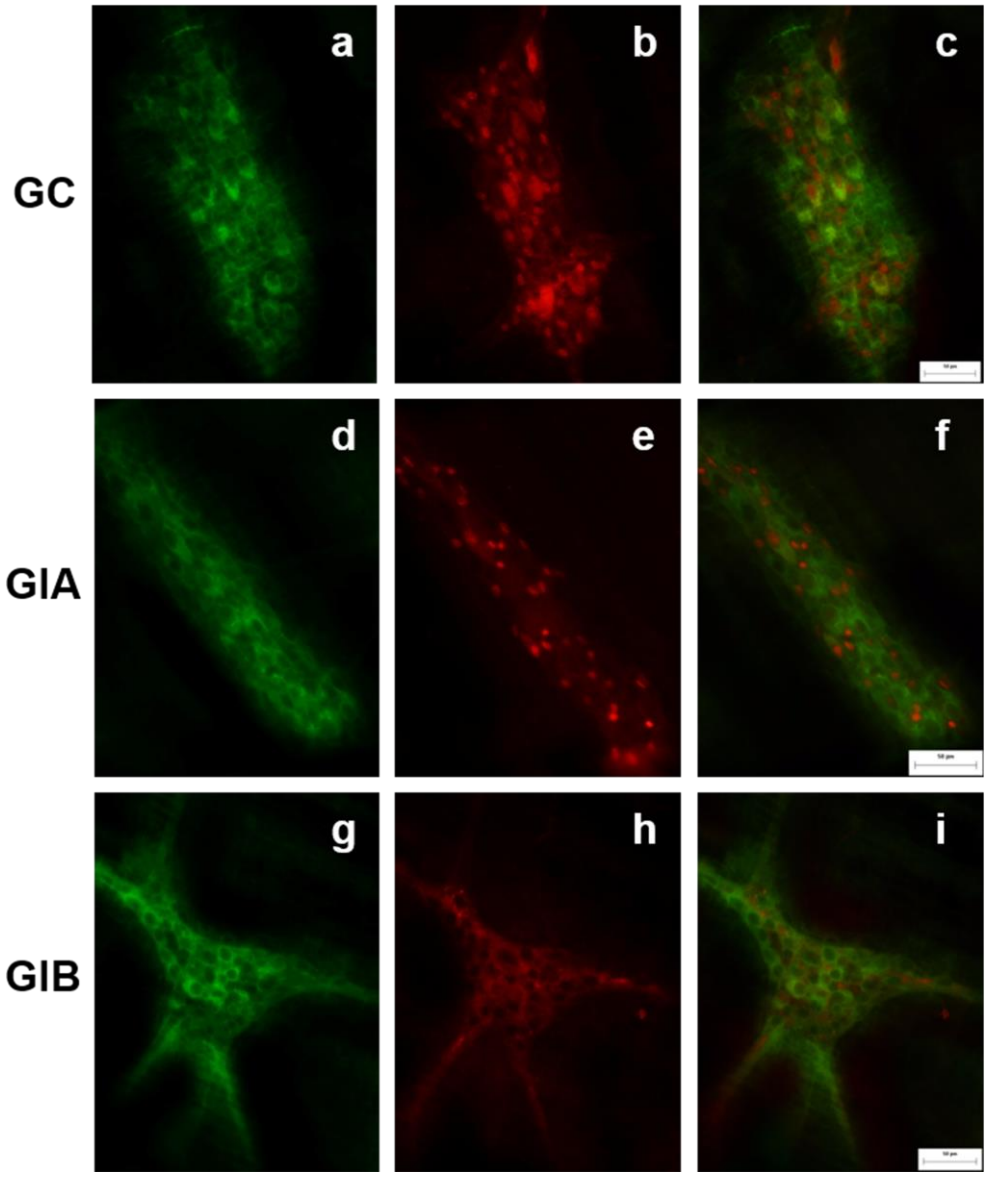

Fig. 2 Photomicrographs of myenteric ganglia of the duodenum of mice in the control group (CG) (a-c), GIA group (d-f), and GIB group (g-i). A significant reduction of the number of enteric glial cells was observed in the GIB group. β-tubulin (a, d, g). S-100β (b, e, h). Merge (c, f, i). Scale bar = 50 µm

Neurons

Assemblages AII and BIV of *G. duodenalis* did not cause the loss of myenteric or submucosal neurons during the time of infection (Table 1).

Table 1 Density of enteric neurons and ratio of enteric glial cells to neurons in the duodenum and jejunum of myenteric and submucosal plexus in uninfected mice and mice infected with assemblages A and B of *Giardia duodenalis*

	Myenteric plexus					
	Duodenum			Jejunum		
	CG	GIA	GIB	CG	GIA	GIB
Neurons/mm^2	166.8 ± 8.5^a	204.4 ± 17.7^a	186.3 ± 4.5^a	137 ± 14.9^a	169.6 ± 15.9^a	164.1 ± 11.6^a
Ratio enteric glial cells/neuron	2.39 ± 0.5^a	2.02 ± 0.5^a	1.07 ± 0.07^b	3.27 ± 0.5^a	3.64 ± 0.4^a	2.05 ± 0.3^b
	Submucosal plexus					
	Duodenum			Jejunum		
	CG	GIA	GIB	CG	GIA	GIB
Neurons/mm^2	261,2 ± 51,8^a	261,5 ± 27,4^a	251,4 ± 17,5^a	278,3 ± 22,2^a	262,3 ± 14,8^a	254 ± 16,0^a
Ratio enteric glial cells/neuron	2,61 ± 0,7^a	1,04 ± 0,1^b	1,06 ± 0,16^b	3,06 ± 0,3^a	2,01 ± 0,3^a	1,38 ± 0,1^b

The data are expressed as mean ± SEM. Different letters (a/b) in the same row indicate a significant difference between groups ($p < 0.05$, ANOVA followed by Dunnett's test).

Ratio of EGC to neurons

Infection by *G. duodenalis* reduced the number of EGCs but did not alter the number of neurons. Therefore, we observed a smaller EGC:neuron ratio in both plexus analyzed (Table 1).

Morphometry of neurons

Although assemblage AII did not cause any changes in the number of duodenal myenteric neurons, this assemblage increased the nuclear area of these cells by 23% relative to the control group ($p < 0.05$; Fig. 3). Infection by assemblages AII and BIV did not alter the cell body area of duodenal myenteric neurons ($p > 0.05$). In the jejunum of the myenteric plexus, cell bodies were significantly reduced by 17.2% and 15.8% in the GIA and GIB groups, respectively, compared with the control group. A 12.4% reduction of the nuclear area was observed in the GIB group compared with the control group. The cytoplasm area was reduced by 21.3% in the GIA group compared with the

control group. The nucleus : cell body ratio increased by 16% in the GIA group compared with the control group ($p < 0.05$; Fig. 3).

In the submucosal plexus, no significant variations in the morphometry of duodenum neurons was observed. In the jejunum, the nuclear area was reduced by 19.8% in the GIA group, the nucleus : cell body ratio decreased and the presence of intracellular vacuoles was observed in neurons in the GIA and GIB groups compared with the control group ($p < 0.05$; Fig. 3).

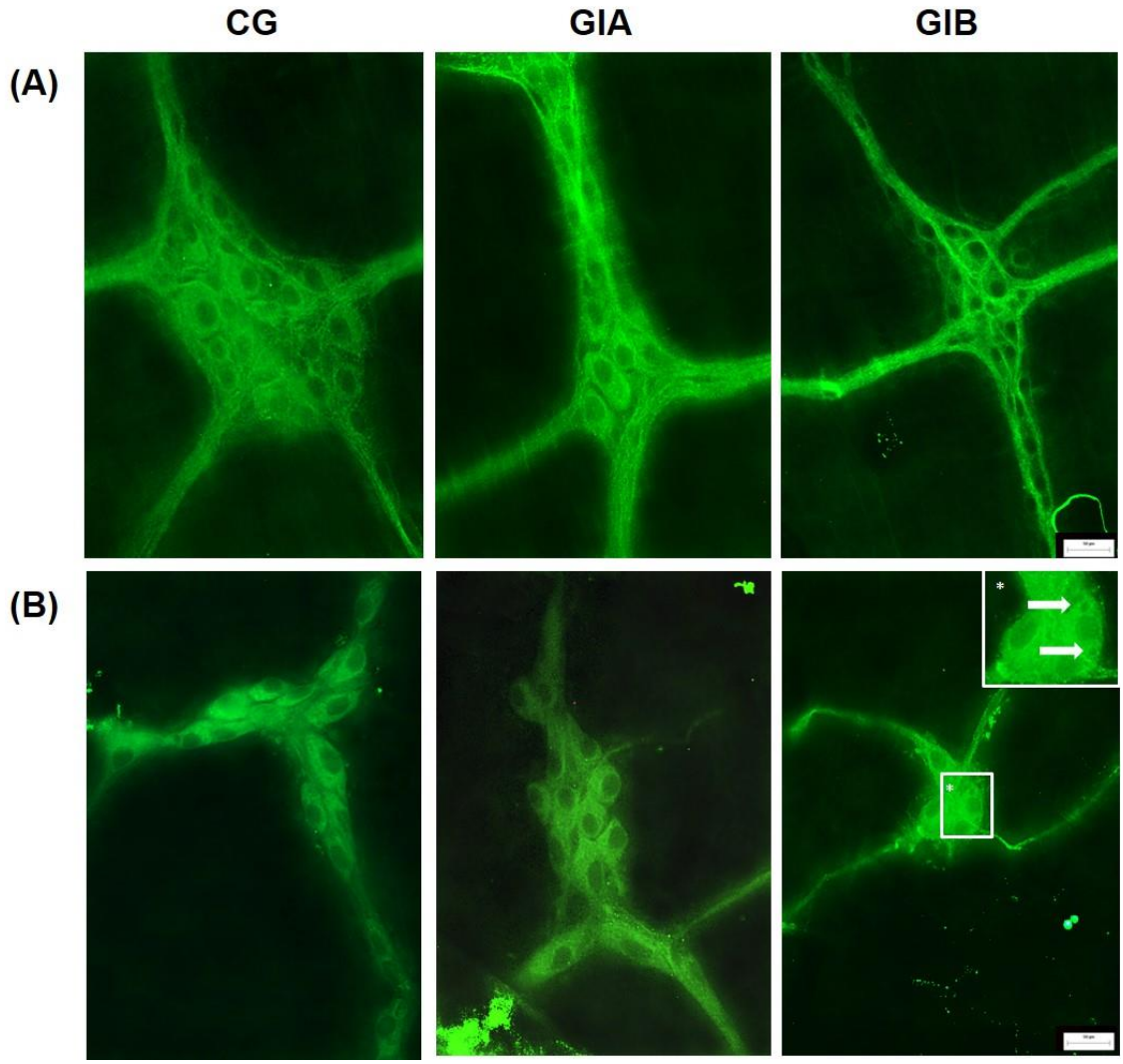

Fig. 3 Photomicrographs of myenteric (a) and submucosal (b) neurons in the jejunum in the control group (CG), GIA group, and GIB group, indicating variations in the morphometry of neurons. *Presence of intracellular vacuoles. Scale bar = 50 µm

Morphometry of the muscle layer and enterocytes

The muscle layer of the duodenum in the GIB group was 16.1% less thick compared with the control group ($p < 0.05$; Fig. 4). No difference was observed in the jejunal muscle layer between the infected and control groups.

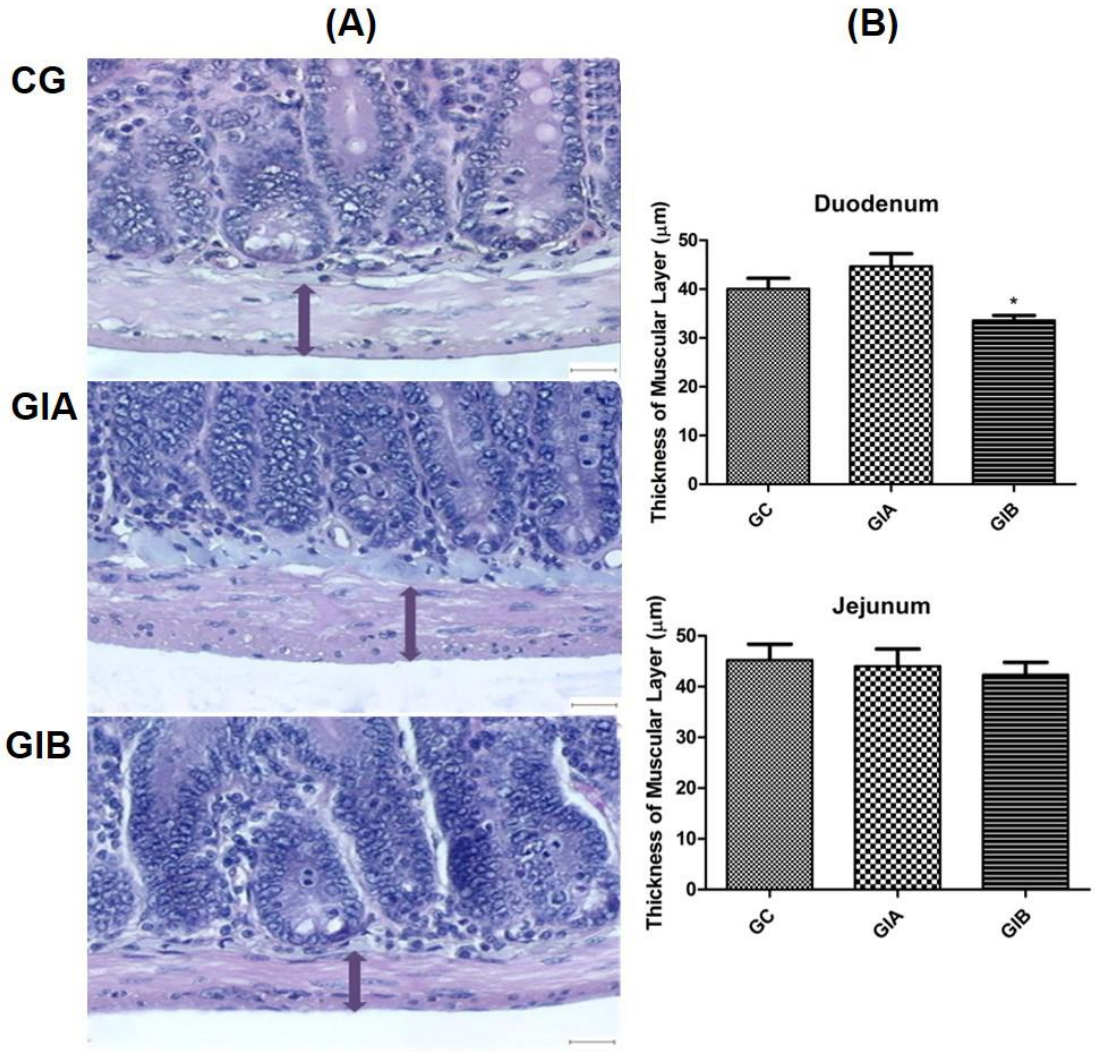

Fig. 4 Morphometry of the external muscle layer in uninfected mice (control group [CG]) and mice infected with *Giardia duodenalis* assemblage A (GIA) and assemblage B (GIB). (a) Photomicrographs showing a decrease in the thickness of the duodenal muscle layer in the GIB group (arrow). Hematoxylin/eosin staining (20× magnification). (b) Mean ± standard error of mean of the thickness of the duodenal and jejunal muscle layers. *$p < 0.05$, significant difference compared with CG (ANOVA followed by Dunnett's test)

In the duodenum, infection by assemblage A resulted in a significant 17.3% increase ($p < 0.05$) in the height of enterocytes (Table 2). In the jejunum, none of the

assemblages altered the morphometry of enterocytes or their nuclei compared with the control group.

Table 2 Height and width of enterocytes and their nuclei in the duodenum in uninfected mice (CG) and mice infected with assemblage A (GIA) and assemblage B (GIB) of *Giardia duodenalis*

Parameter (µm)	CG	GIA	GIB
Enterocyte height	24.1 ± 0.34^{a}	28.3 ± 1.04^{b}	26.1 ± 0.77^{a}
Enterocyte width	4.9 ± 0.09^{a}	4.8 ± 0.20^{a}	4.7 ± 0.14^{a}
Enterocyte nucleus height	7.2 ± 0.10^{a}	7.9 ± 0.26^{a}	7.7 ± 0.20^{a}
Enterocyte nucleus width	4.4 ± 0.07^{a}	4.4 ± 0.23^{a}	4.2 ± 0.15^{a}

The data are expressed as mean ± SEM. Different letters (a/b) in the same row indicate a significant difference between groups ($p < 0.05$, ANOVA followed by Dunnett's test).

Discussion

This is the first study that evaluated the response of the myenteric and submucosal plexus in mice that were infected with different assemblages of *G. duodenalis*. We found that both assemblages A and B of *G. duodenalis* reduced the number of EGCs and altered the morphology of neurons; only assemblage B reduced the thickness of the muscle layer.

Despite the absence of changes in gastrointestinal transit time that occurred with experimental infection in the present study, *G. duodenalis* infection reduced the number of EGCs. The EGC : neuron ratio decreased in the duodenum and jejunum and the function of these cells is essential for impulse transmission between neurons and between neurons and muscle cells. They also play an anti-apoptotic role and maintain and protect intestinal integrity (Grubišić and Gulbransen 2017).

The reduction of the number of EGCs may be related to the loss of cellular responsiveness to the marker that was used or related to phenotypic shift that is caused by exposure of EGC to inflammatory factors (Grubišić and Gulbransen 2017). Additionally, such a reduction in the acute phase of infection can cause chronic functional impairment of the intestine, which was also observed in other studies of *T.*

cruzi infection (Rassi et al. 2010). *T. cruzi* infection is usually associated with the initial loss of EGCs and the subsequent loss of neurons because of the lack of EGCs (Almeida-Leite et al. 2014). However, during the period of infection in the present study, the neuronal density was unaltered by the two assemblages. Studies that employ a longer duration of infection may observe a reduction of the neuronal population. Studies with other protozoa have reported decreases in neurons that were induced by direct or indirect actions of the parasite (Silveira et al. 2007; Araújo et al. 2015; Braga-Silva et al. 2016; Góis et al. 2016). Other studies (Creuzet et al. 1998; Parlog et al. 2015) reported that protozoa have a higher preference for EGC than for neurons.

The shortage of EGCs causes neuronal injury and may lead to neuronal death (Almeida-Leite et al. 2014). Thus, changes in the morphometry of the neurons that were measured, including the presence of intracellular vacuoles, may indicate neuronal dysfunction (Rogers-Cotrone et al. 2010), the mechanisms of which need to be investigated further. Injured cells have been hypothesized to form intracellular vacuoles in response to parasitic infection or the release of bacterial endotoxins, which may reveal pathological signs of cellular degeneration (Kumar et al. 2014).

The increase in the area of the nucleus of duodenal myenteric neurons in infected mice may reflect an increase in the metabolic activity of these cells that is caused by the reduction of EGC (Araújo et al. 2015). In the jejunum in the GIA group, significant reduction of the area of cell bodies and cytoplasm of neurons were found. The changes in the area of the nucleus of neurons in the present study may be a sign of cellular regeneration in response to lesions that are caused by giardiasis. During the process of cellular adaptation, the size of the nucleus increases. If this process fails, then nuclear retraction occurs, indicating that the cell is preparing to enter the process of cell death (Kumar et al. 2014). Morphological changes in neurons have been identified in viral (Bielefeldt-Ohmann et al. 2008), bacterial (Lucero et al. 2012) and parasitic infections (Odorizzi et al. 2010; Araújo et al. 2015; Parlog et al. 2015; Góis et al. 2016). However, such changes have not been reported for *G. duodenalis.*

The reduction of the population of EGCs and morphometric alterations in neurons are characteristics of cellular injury, which may have led to the reduction of the thickness of the muscle layer. A significant reduction of the thickness of the duodenal muscle layer was observed in the GIB group, where a reduction of the population of EGCs was also observed. Enteric glial cells produce growth factors that can act on smooth muscles (Steinkamp et al. 2012), and the lack of EGCs may have resulted in the

reduction of the thickness of the muscle layer that was observed in the present study. Inflammatory and immunological alterations can trigger such complications as irritable bowel syndrome, a functional disorder that is seen in giardiasis (Dizdar et al. 2007; Kim and Chang 2012; Grover et al. 2014; Wensaas et al. 2016).

Conclusions

In summary, both assemblages A and B of *G. duodenalis* reduced the number of EGC and altered the morphology of neurons and only assemblage B reduced the thickness of the muscle layer. These findings suggest that assemblage B is more pathogenic to duodenum of Swiss mice.

Conflict of interest

The authors declare that they have no conflict of interest.

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Artigo 2: "Comparative study of effects of assemblages AII and BIV of *Giardia duodenalis* on mucosa and microbiota of the small intestine in mice"

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Comparative study of effects of assemblages AII and BIV of *Giardia duodenalis* on mucosa and microbiota of the small intestine in mice

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ABSTRACT

Giardiasis is one of the major causes of diarrhea worldwide and its symptoms vary in intensity, which can be attributed to different parasite assemblages. The goal of the present study was to compare the effects of infection that was caused by assemblages AII and BIV of *Giardia duodenalis* on the response of the small intestine, microbiota, and behavioral parameters in mice. Swiss mice were infected with assemblages AII and BIV of *G. duodenalis* for 15 days. Leucometry, pain, intestinal microbiota and histological parameters of the duodenum and jejunum were evaluated in all of the experimental groups. Both assemblages modified the composition of the intestinal microbiota. Infection with assemblage AII promoted leukocytosis, reflected by increases in polymorphonuclear cells, intraepithelial lymphocytes and pain-related behavior, indicating that this was the more aggressive assemblage with regard to its effects on the intestinal mucosa and duodenum. The specific assemblage of the parasite is an important parameter that affects symptomatology in the host.

Keywords: Genotype, Duodenum, Giardiasis.

1. Introduction

Giardiasis is caused by *Giardia* spp infection and is one of the major causes of diarrhea [1] in both developed and developing countries [2,3]. The pathogenesis of giardiasis involves the adherence of *Giardia duodenalis* trophozoites to the mucosa of the small intestine, which promotes villous atrophy and brush border shortening [4]. This parasite can cause epithelial barrier dysfunction, with consequent malabsorption [5]. Among the signs and symptoms of giardiasis are abdominal pain, nausea, vomiting, flatulence, and aqueous diarrhea [5,6]. This infection was recently associated with chronic manifestations, such as irritable bowel syndrome, which may arise even after the eradication of infection [6].

A balanced microbiota is capable of inhibiting the development of pathogenic microorganisms [7], but the presence of *G. duodenalis* can lead to dysbiosis with consequent intestinal disorders, referred to as "post-giardiasis" alterations [8,9]. Dysbiosis and other changes in the intestinal mucosa are responsible for dysfunction of the epithelial barrier and may contribute to microbial transposition and a local inflammatory response [8].

The symptoms of giardiasis present variable intensities that are attributable to both host and parasite factors. *G. duodenalis* can manifest as eight genetic assemblages (A-H). Assemblages A and B are the most prevalent in humans, and sub-assemblages AII and BIV are the most prevalent in southern Brazil [10-12]. Studies that have attempted to relate the symptomatology of giardiasis to specific parasite assemblages are scarce, and the results are controversial. The goal of the present study was to compare the effects of infection that was caused by assemblages AII and BIV of *Giardia duodenalis* on the response of the small intestine, microbiota, and behavioral parameters in mice.

2. Materials and Methods

The study adhered to the guidelines of the Sociedade Brasileira de Ciência em Animais de Laboratório and was approved by the Ethics Committee on Animal Experimentation of the Centro Universitário Integrado, Brazil (statement no. 1070).

2.1. Inoculum and Experimental groups

Cysts of *G. duodenalis w*ere obtained from the genotyped sample database of the Laboratory of Environmental Parasitology and Food, Universidade Estadual de Maringá, Brazil (accession numbers of GenBank KJ741310–KJ741313). The cysts were concentrated from positive feces for *G. duodenalis* of residents of Ângulo, Paraná, in southern Brazil, and purified through the sucrose gradient technique [13-15].

Forty-two male Swiss mice, 21 days old, were obtained from the animal facility of the Universidade Estadual de Maringá. The animals were randomly assigned to three groups: uninfected control group (CG) and animals infected with 1,000 cysts of *G. duodenalis* assemblages AII and BIV by gavage (GIA and GIB groups, respectively).

Three days postinfection (dpi) and on the day of euthanasia (15 dpi), parasitological examination was performed according to the method of Faust et al. [16]. Molecular techniques (polymerase chain reaction and restriction fragment length polymorphism) [11] were performed to confirm infection by assemblages AII and BIV of *G. duodenalis* and exclude an infection by *G. muris*.. All of the analyses were performed in a blinded fashion.

The groups of mice were housed in polypropylene cages with a wire grid floor to avoid contact with excreta. The animals were maintained on a 12 h/12 h light/dark cycle with controlled temperature and humidity. Food and water were provided *ad libitum*.

2.2. Course of experimental infection

The experiment lasted 15 days. During this time period, the animals were observed daily for the presence or absence of diarrhea. At 0, 7, 14 dpi, tests of exploratory behavior were performed, and blood was collected to determine total and differential leukocyte counts. Intestinal mucus and feces were collected at the end of the experiment (15 dpi) to determine total leukocyte count. After removing the intestine, the duodenum mucus was gently scraped with a sterile spatula, and a pellet of freshly discarded feces was collected.

2.3. Leukocyte count

Blood was collected from the caudal vein and placed in tubes that contained ethylenediaminetetraacetic acid. For total leukocyte count, the blood sample was diluted in hemolyzing solution in a 1:20 ratio, and leukocytes were quantified in a Neubauer chamber using four lateral quadrants. For differential leukocyte counts in blood and

leukocyte counts in feces and mucus, it was made analysis in smear with May-Grünwald-Giemsa staining [17].

2.4. Subjective pain assessment

The subjective pain assessment was performed using tests of exploratory behavior in the elevated plus maze (EPM) and open field test (OFT). These tests were previously validated by Pellow et al. [18] and Archer [19], respectively. These tests were used because pain can be defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (International Association for the Study of Pain) [20]. Additionally, animals with pain exhibit an increase in exploratory behavior [21]. The subjective pain assessments were always performed at the same time and by the same researcher.

2.4.1. Elevated plus maze

Each animal underwent a single 5-min session on each day of evaluation (0, 7, and 14 dpi). The session began when the researcher placed the animal in the center of the maze, facing one of the closed arms [21]. The time spent on the open and closed arms of the maze was recorded using X-Plo-Rat 3.3 software [22]. After each animal was tested, the apparatus was cleaned with 70% ethanol.

2.4.2. Open field test

The animals were placed in the center of the arena [23]. The sessions lasted 5-min each. After each session (0, 7, and 14 dpi), the apparatus was cleaned with 70% ethanol. The following parameters were analyzed: number of squares crossed (with all four paws), number of rears, time spent grooming (in seconds) and number of evacuations.

2.5. Euthanasia and material collection

The animals were intraperitoneally anesthetized with xylazine (10 mg/kg) and ketamine (110 mg/kg) and then euthanized by intracardial administration of KCl (2 mL) at 36 days of age (15 dpi). Necropsy was performed aseptically along the medial line to obtain the duodenum and proximal jejunum. A 1-cm ring was collected from each segment for histological processing. Jejunum fragment was collected for microbiological analysis, determination of myeloperoxidase (MPO) activity and

determination of nitrite concentration. Duodenum fragment was collected for counting leucocytes in intestinal mucus.

2.6. Determination of myeloperoxidase activity

Myeloperoxidase activity was assayed using homogenate supernatants of the jejunum according to Bradley & Priebat [24]. The segment was weighed, and a 20× volume of potassium phosphate buffer (pH 6.0) was added that contained 0.5% hexadecyl trimethyl ammonium bromide (1 mL/50 mg of tissue) in a Potter homogenizer. The homogenate was shaken in a vortex mixer and centrifuged for 15 min at 5,000 rotations per minute (rpm) at 25ºC. Aliquots (10 µL) of the supernatant were added to each well of a 96-well microplate with 200 µl of the buffer solution [*O*-dianisidine dihydrochloride (16.7 mg), double-distilled water (90 ml), potassium phosphate buffer (10 mL) and 1% H_2O_2 (50 µL)]. After 5 min, the reaction was stopped by the addition of sodium acetate. Myeloperoxidase activity was determined by reading absorbance at 450 nm using a microplate spectrophotometer (Spectra Max Plus), recorded at 15-s intervals for 2 min. All of the tests were performed in duplicate.

2.7. Determination of nitrite concentration

Nitrite concentration was determined in mucus and tissue fragments of the jejunum. Mucus samples that adhered to the mucosa of the jejunum were removed with a sterile spatula, and jejunum samples were macerated in sterile phosphate-buffered saline (PBS), both maintained at -20°C until analysis.

The samples were centrifuged at 1500 rpm for 10 min, and the supernatant was used to determine the nitrite concentration using the Griess reaction [25], in which 50 µL of the supernatant was incubated with the same amount of Griess solution (phosphoric acid, sulfanilamide, and N-1-naphthalylethylenediamide). A 96-well microplate and enzyme-linked immunosorbent assay reader (570 nm absorbance) were used. The tests were performed in duplicate.

2.8. Microbiological analyses

A fragment of the jejunum was opened longitudinally, and the luminal contents were removed under aseptic conditions, diluted in 500 µL of 0.9% sterile physiological solution, weighed, and stored in a sterile Eppendorf tube. The solution was seeded in blood agar in duplicate and incubated at 37°C for 48 h. Colony-forming units (CFUs)

were converted into grams of luminal content (CFU/g), thus representing superficial bacterial counts [8].

The CFUs were subjected to Gram staining [26] and transferred to Rogosa agar, Mac Conkey agar, and bile esculin agar to isolate *Lactobacillus* spp, enterobacteria and *Enterococcus* spp, respectively. The cultures were incubated at 37°C for 48 h, with the exception of Rogosa agar, which was incubated for 72 h and under anaerobic conditions according to the manufacturer's instructions. All of the microbiological analyses were performed in duplicate.

2.9. Bacterial identification

2.9.1. *Lactobacillus* spp

The CFUs from Rogosa agar were counted, subjected to Gram staining [26], and analyzed in the catalase assay to confirm the bacterial genus.

2.9.2. Enterobacteria

The CFUs from Mac Conkey agar were counted and subjected to Gram staining [26]. Those that presented lactose fermentation were used for the tests: oxidase research, seeding in citrate agar and triple iron acid agar. These tests were used to verify the following bacterial characteristics: the fermentation of glucose, lactose, and sucrose and the production of gas and H_2S. All of the material was incubated at 37°C for 24 h.

2.9.3. *Enterococcus* spp

Streptococcal colonies from blood agar were seeded on bile esculin agar and incubated at 37°C for 48 h. *Enterococcus* spp was identified based on the visualization of esculin hydrolysis, observed as a blackened color that was acquired by the agar.

2.10. Histological processing

Segments of the duodenum and jejunum were fixed in Bouin's solution, dehydrated, diaphanized, paraffin-embedded, cut transversely (4 μm, semi-serial), and stained with hematoxylin/eosin (HE) to investigate the morphometry of the mucosa, submucosa, villi, and crypts and quantify intraepithelial lymphocytes (IELs).

We also prepared slides for periodic acid-Schiff (PAS) staining to detect neutral mucins and labile sialomucins (PAS+), Alcian blue (AB; pH 2.5) staining (AB 2.5+) to

detect sialomucins and sulphomucins, and AB (pH 1.0) staining (AB 1.0+) to detect sulphomucins. All of the analyses were performed in a blinded fashion.

2.11. Quantification of intraepithelial lymphocytes and goblet cells

Four slides from each segment were divided into four quadrants, thus generating 16 images from the mucosa for each mouse. In each specimen, IELs were counted in 2500 epithelial cells to calculate the proportion of IELs/100 epithelial cells. This procedure allowed determination of the number of goblet cells/100 epithelial cells. We used sections that were stained with PAS, AB (pH 2.5), and AB (pH 1.0).

2.12. Morphometric analysis of the intestinal wall

We used HE-stained sections to morphometrically analyze the intestinal wall. Images were taken with a Pro series 3CCD digital camera that was coupled to an Olympus BX50 optical microscope. The width and height of the villi and crypts and mucosa were measured using a 4× objective. The submucosa was measured using a 20× objective. Four images of each section were obtained. Sixteen measurements were obtained per animal for each parameter of the intestinal wall using ImagePro Plus software (Media Cybernetics) [27-28].

2.13. Statistical analysis

The data are expressed as the mean ± standard error of mean (SEM). Results were compared between groups using one-way analysis of variance (ANOVA) and Dunnett's multiple-comparison *post hoc* test. The statistical analysis was performed using GraphPad Prism 5.01 software. For all of the statistical tests, values of $p < 0.05$ were considered statistically significant.

3. Results

The microscopic analyses revealed the presence of *G. duodenalis* in feces in all of the infected mice at three dpi and on the day of euthanasia (15 dpi). The glutamate dehydrogenase gene was amplified in all samples of infected mice and the sequencing confirmed the infection by assemblages AII and BIV of *G. duodenalis* in GIA and GIB groups, respectively, and excluded a contamination by *G. muris.*

No diarrhea was observed at any time during the study.

3.1. Leukocyte count

No significant amounts of leukocytes were found in the feces or in the intestinal mucus in any of the groups in the present study. In blood, 141.5% and 180.6% increases in the total leukocyte count were observed in the GIA and GIB group, respectively, at 14 dpi compared with the CG ($p < 0.0001$). At 14 dpi, a significant increase in the number of polymorphonuclear leukocytes was observed in the GIA group compared with the control group (Fig. 1), reflected by a 112.6% increase in neutrophils. A decrease in mononuclear cells was also observed in the GIA group, reflected by a 12.1% decrease in lymphocytes compared with the control group ($p < 0.05$). Monocyte, basophil, and eosinophil counts were not significantly different between groups.

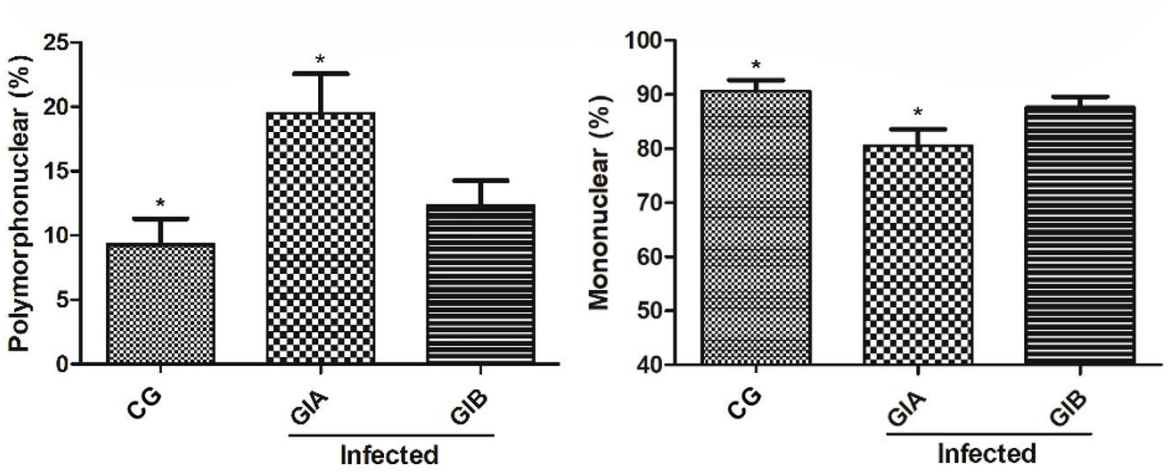

Fig. 1. Percentage of polymorphonuclear and mononuclear leukocytes counted in blood in uninfected mice (control group [CG]) and mice infected with *Giardia duodenalis* assemblage A (GIA) and assemblage B (GIB), 14 dpi. *$p < 0.05$, significant difference compared with CG (ANOVA followed by Dunnett's test).

3.2. Subjective pain assessment

In the EPM, an increase in time spent on the open arms was observed in the GIA group ($p < 0.05$) at 7 dpi. At 14 dpi, no difference was observed between groups. In the OFT, no differences in the behavioral parameters were observed between groups at 7 dpi. At 14 dpi, a 164% increase in the number of evacuations was observed in the GIA group, compared with CG ($p < 0.05$).

3.3. Determination of myeloperoxidase activity and nitrite concentration

No difference in MPO activity in the jejunum was observed between infected animals and the control group. Nitrite concentration in the intestinal mucus was not significantly different between groups, but in the jejunum fragment, a 55.4% decrease in nitrite concentration was observed in the GIA group compared with the control group ($p < 0.05$).

3.4. Microbiological analysis

Bacterial growth (CFU/g) in blood agar was not significantly different between groups ($p > 0.05$). Morphotinorial characteristics, revealed by Gram staining and complementary tests, were used to identify *Lactobacillus* spp, *Enterococcus* spp, and enterobacteria (Fig. 2A). In the infected groups, a decrease in the amount of *Lactobacillus* spp and increase in *Enterococcus* spp and enterobacteria were observed. The biochemical tests allowed the classification of enterobacteria according to their fermentation profile for glucose, lactose, and sucrose and gas/H_2S production (Fig. 2B). The fermentation profile also changed with infection to the enterobacteria.

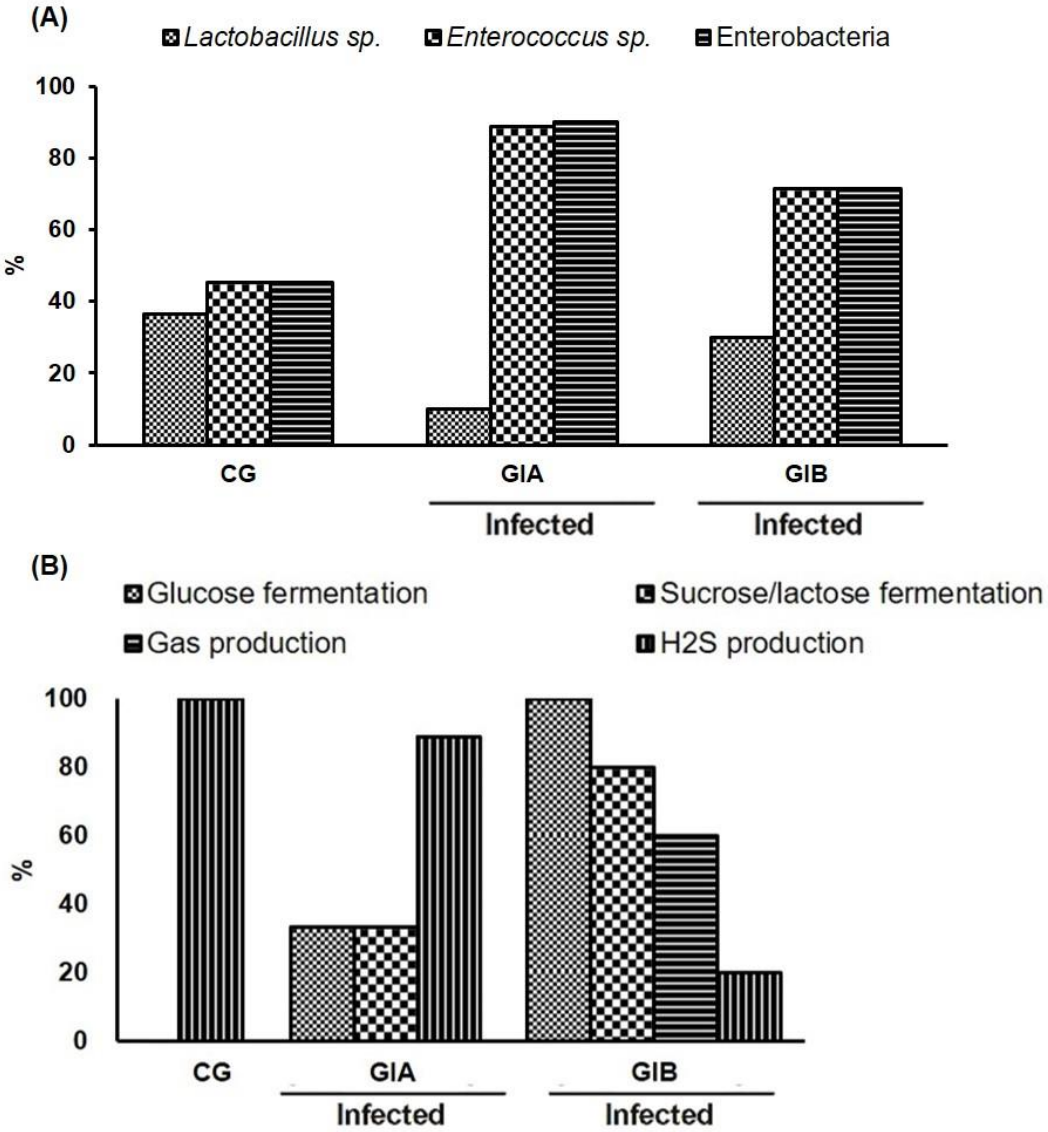

Fig. 2. (A) Growth rate (%) of *Lactobacillus* spp, *Enterococcus* spp, and enterobacteria. (B) Biochemical characteristics (%) of enterobacteria isolated in jejunal mucus in uninfected mice (control group [CG]) and mice infected with *Giardia duodenalis* assemblage A (GIA) and assemblage B (GIB).

3.5. Quantification of intraepithelial lymphocytes and goblet cells and morphometric analysis of the intestinal wall

The quantification of IELs indicated that only assemblage A increased these cells in the duodenum and jejunum (Fig. 3).

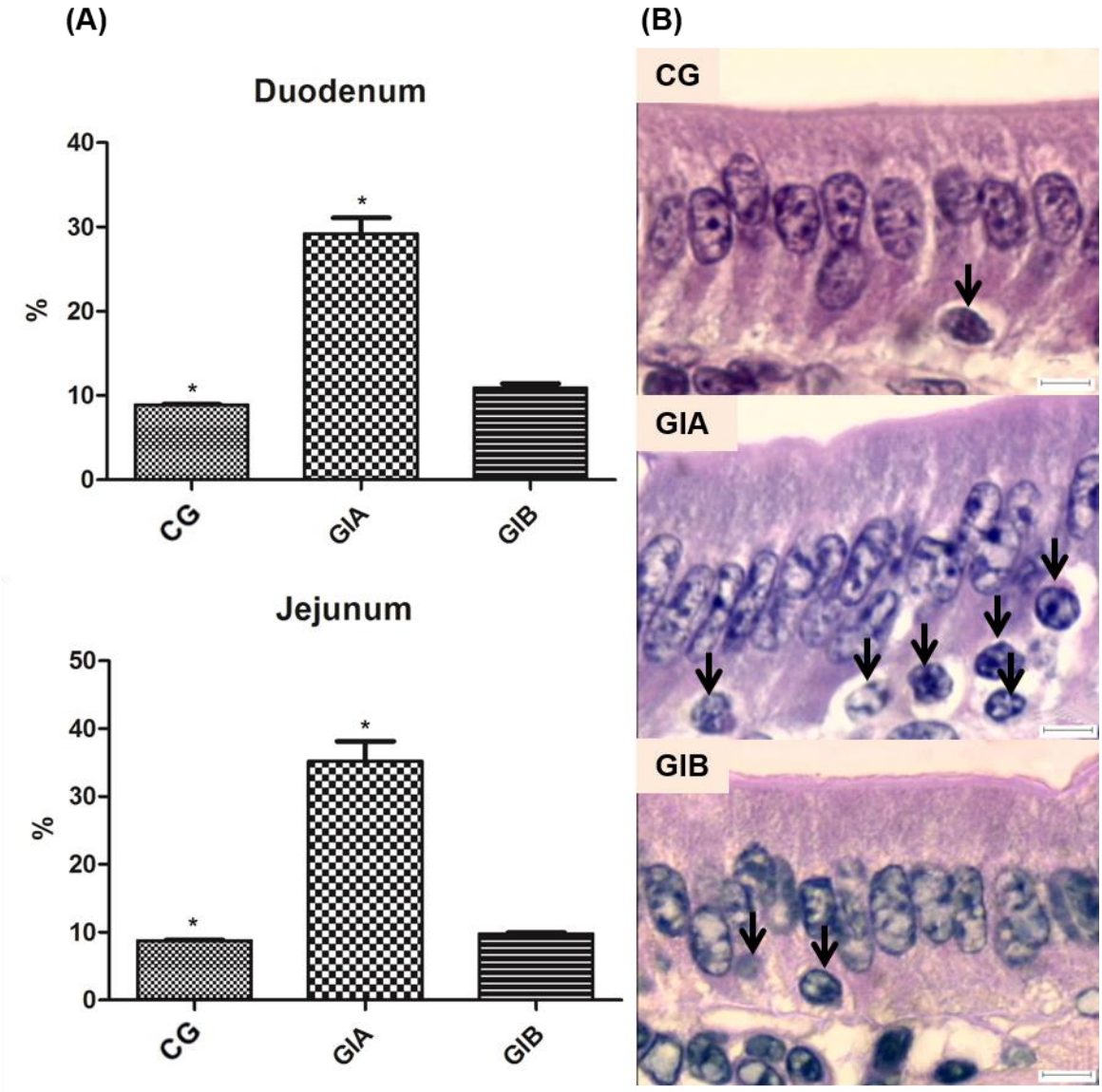

Fig. 3. Proportion of IELs/100 epithelial cells (%) in the duodenum and jejunum in uninfected mice (control group [CG]) and mice infected with *Giardia duodenalis* assemblage A (GIA) and assemblage B (GIB). (A) Percentage of IELs in the duodenum and jejunum. (B) Photomicrography showing increase in IELs (arrows) in the jejunum in the GIA group. HE, Scale bar = 5 µm. *$p < 0.05$, significant difference compared with CG (ANOVA followed by Dunnett's test).

Infection with assemblage A of *G. duodenalis* reduced neutral mucin producer (goblet cell) counts, whereas assemblage B increased the number of acidic mucin (AB 2.5+) producers in the duodenum. In the jejunum, assemblage B increased neutral goblet cells and reduced acidic goblet cells (AB 1.0+; Fig. 4).

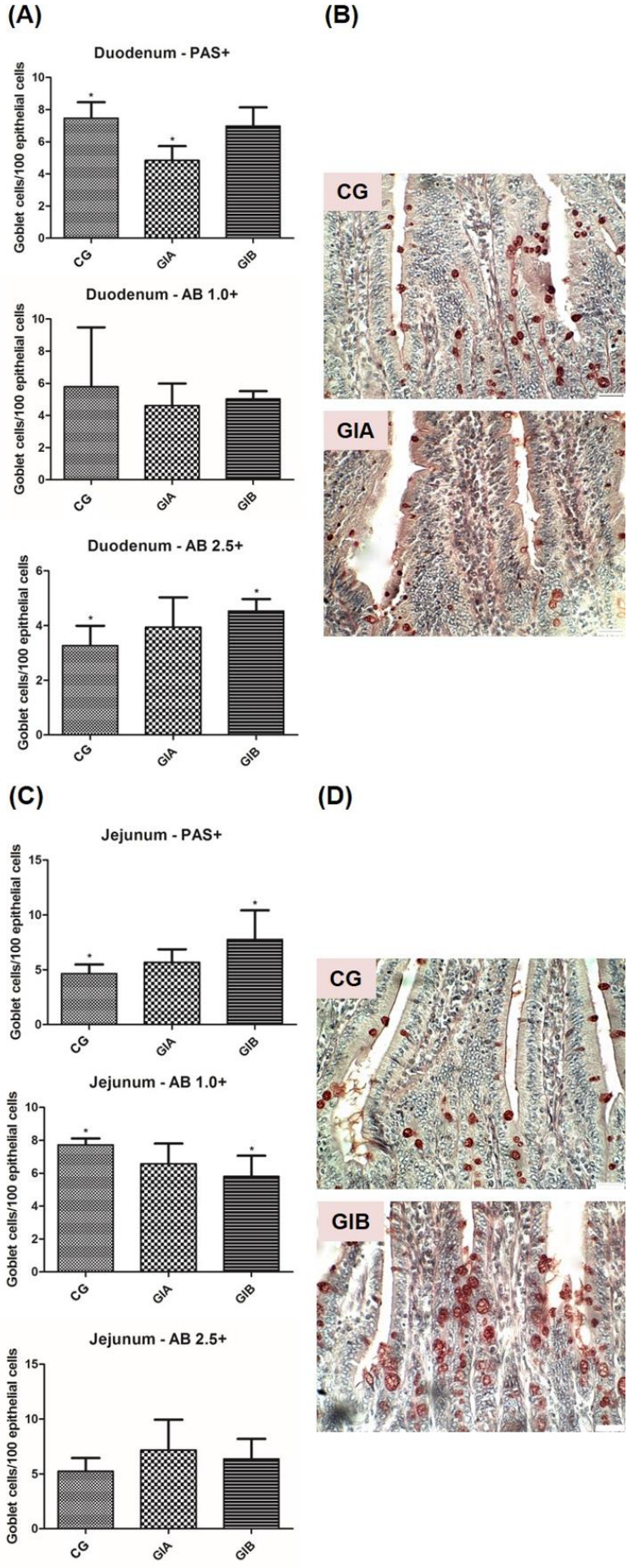
(A)
Duodenum - PAS+
Goblet cells/100 epithelial cells
CG
GIA
GIB
Duodenum - AB 1.0+
Goblet cells/100 epithelial cells
CG
GIA
GIB
Duodenum - AB 2.5+
Goblet cells/100 epithelial cells
CG
GIA
GIB
(B)
CG
GIA
(C)
Jejunum - PAS+
Goblet cells/100 epithelial cells
CG
GIA
GIB
Jejunum - AB 1.0+
Goblet cells/100 epithelial cells
CG
GIA
GIB
Jejunum - AB 2.5+
Goblet cells/100 epithelial cells
CG
GIA
GIB
(D)
CG
GIB

Fig. 4. Proportion of goblet cells/100 epithelial cells (%) in the duodenum and jejunum in uninfected mice (control group [CG]) and mice infected with *Giardia duodenalis* assemblage A (GIA) and assemblage B (GIB). (A) Percentage of goblet cells in the duodenum. (B) Photomicrography showing decrease in goblet cells (PAS+) in the duodenum in the GIA group. Scale bar = 20 µm. (C) Percentage of goblet cells in the jejunum. (D) Photomicrography showing increase in goblet cells (PAS+) in the jejunum in the GIB group. PAS, Scale bar = 20 µm. *$p < 0.05$, significant difference compared with CG (ANOVA followed by Dunnett's test).

In the duodenum, infection with assemblage B caused a 10.7% decrease in the mucosal thickness and 11.5% decrease in the submucosal thickness (Table 1). Assemblage A caused a significant 17.7% increase ($p < 0.05$) in villus width (Fig. 5). In the jejunum, neither assemblage altered the morphometry of the parameters analyzed compared with the control group.

Table 1. Thickness of the mucosa and submucosa of the duodenum in uninfected mice (control group [CG]) and mice infected with assemblage A (GIA) and assemblage B (GIB) of *Giardia duodenalis*.

Parameter (µm)	CG	GIA	GIB
Mucosa	196,7 ± 6,1[a]	197,6 ±6,3[a]	175,7 ± 4,8[b]
Submucosa	160,6 ± 4,9[a]	152,9 ± 5,0[a]	142,1 ± 4,6[b]

The data are expressed as mean ± SEM. Different letters in the same row indicate a significant difference compared with CG ($p< 0.05$, ANOVA followed by Dunnett's test).

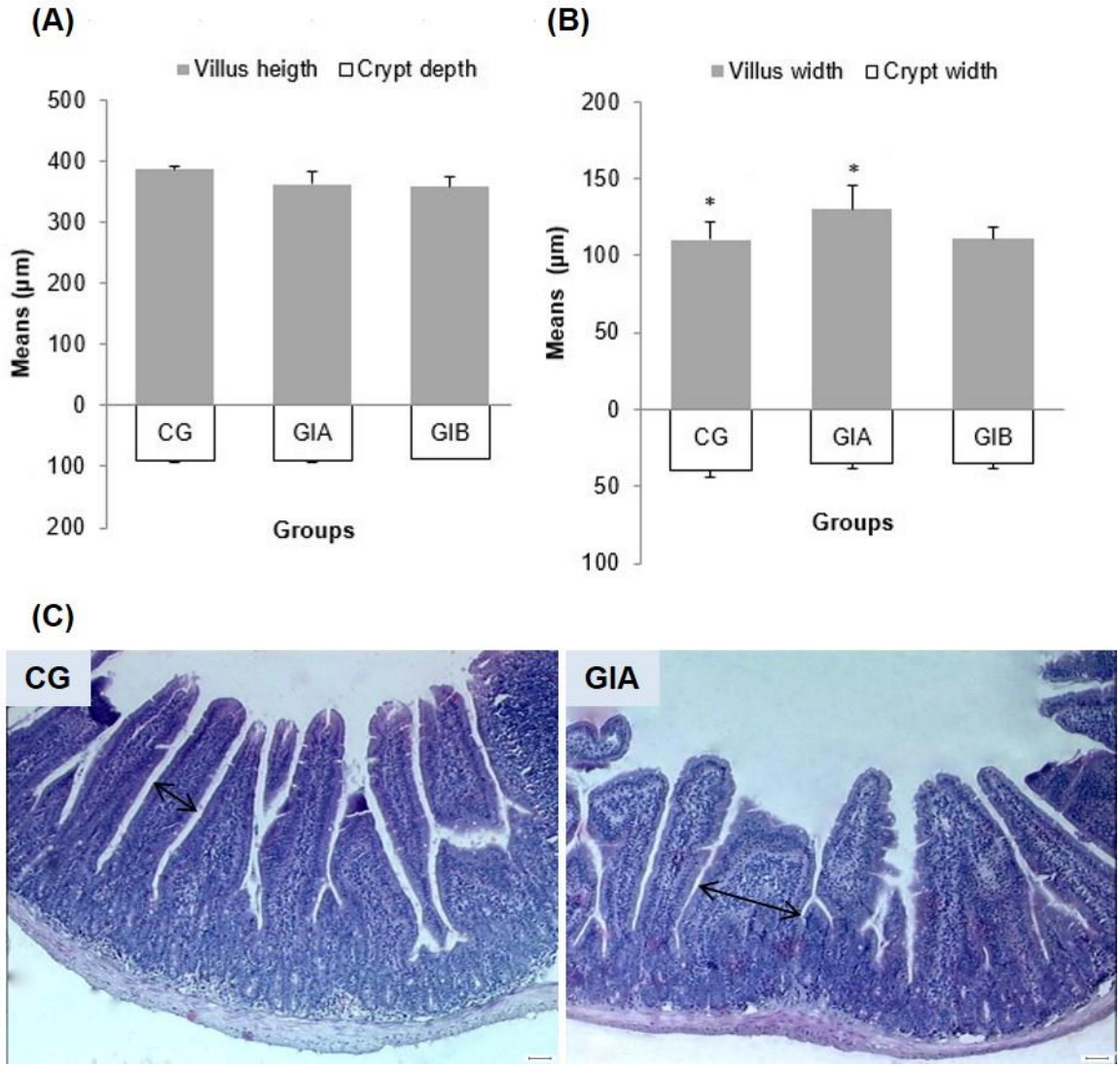

Fig. 5. Morphometry of the epithelium of the duodenum in uninfected mice (control group [CG]) and mice infected with *Giardia duodenalis* assemblage A (GIA) and assemblage B (GIB). (A) Villus height and crypt depth. (B) Villus and crypt width. (C) Photomicrography showing increase in the villus width in the GIA group (arrow). HE, Scale bar = 50 µm. *$p < 0.05$, significant difference compared with CG (ANOVA followed by Dunnett's test).

4. Discussion

The genotype of *G. duodenalis* is a determining factor for the course of infection and its impact on the gastrointestinal tract of the host. Despite divergent descriptions in the literature [29-33], the present study found that assemblage A was more pathogenic with regard to tissue lesions and microbiota alterations, and the duodenum was the most affected segment.

Blood leukocytosis that was detected after 14 dpi was attributed to an increase in polymorphonuclear leukocytes (neutrophils) in animals that were infected with assemblage A of *G. duodenalis*. Polymorphonuclear leukocytes are present in acute inflammatory bowel responses, usually associated with bacterial infection [34]. During the period of infection, the migration of neutrophils to the jejunum was not detected, in which we found no increase in MPO activity and no leucocytes in the mucus or feces, possibly because of the relatively short period of observation. Although the mechanism of these effects is unknown, neutrophils may participate in the immune response that occurs in giardiasis [35].

Although leukocytosis occurred, blood lymphopenia was observed in the GIA group after 14 dpi. Such a reduction was previously observed in a male patient with persistent giardiasis [36]. Lymphocytes participate in the response to *G. duodenalis* [37]. A reduction of lymphocytes can be explained by the dynamics of migration to the site of infection. A greater lymphocyte concentration was found in the lamina propria (i.e., larger villus width), with an increase in IELs. These cells participate in the development of chronic giardiasis in humans and animals [35,37].

Symptomatic giardiasis is characterized by discomfort and abdominal pain [29,37] and inflammatory bowel disease [38]. The present study evaluated pain in mice based on tests of exploratory behavior in the EPM and OFT [20,39-45]. In the EPM, we observed an increase in the time spent on the open arms at 7 dpi in the GIA group, reflecting anxiety-like and pain-related behavior [21,46], which was not detectable at 14 dpi. In the OFT at 7 dpi, no behavioral changes were observed. At 14 dpi, the GIA group exhibited a greater number of evacuations, although the other behavioral parameters did not change (i.e., number of squares crossed, number of rears, and time spent grooming). In this experimental model, we found that only assemblage A altered pain-related and anxiety-like behavior, predominately in the early acute phase of infection.

Three main components are involved in the pathogenesis of giardiasis: (1) microbiota, (2) intestinal epithelium, and (3) mucus [47]. Infection with both assemblages caused changes in the composition of the intestinal microbiota. According to the literature, such changes in composition are sufficient to promote the translocation of bacteria into the epithelium [6,8,48] and may characterize dysbiosis. This can subsequently impair the function of the epithelial barrier and contribute to inflammation of the intestinal mucosa [9,49]. These changes are responsible for initiating a sequence

of events that culminates in the postinfectious symptomatology of giardiasis [50]. Additionally, *G. duodenalis* infection may alter the gene expression of commensal bacteria, inducing their virulence [9]. Lopez-Romero et al. [35] argued that different assemblages may cause distinct responses in the host because parasites interact in different ways with an individual's microbiota and activate the innate immune response. The present study was the first to find alterations of the composition of microbiota that are caused by assemblages AII and BIV.

Among the epithelial alterations, we observed an increase in IELs in the GIA group. The greater migration of lymphocytes to the epithelium has been described in the pathophysiology of giardiasis [50,51] and verified in other experimental models [4,52-54]. Intraepithelial lymphocytes correspond mainly to CD8+ T-lymphocytes that are located between epithelial cells [35]. Among their functions is protection of the host from enteric pathogens [54]. Epithelial barrier cells are able to produce nitric oxide, which is an important cytostatic agent of *G. duodenalis* [35]. The indirect detection of nitric oxide indicated no changes in the mucus of the jejunum in infected animals but was reduced in the mucosa of the jejunum in animals that were infected with assemblage A. Notably, at 15 dpi, we confirmed the presence of *G. duodenalis* cysts in the feces of infected animals. The concentration of nitric oxide may have increased before 15 dpi and may have contributed to the cytostatic control of trophozoites, promoting their encystation. The detection only of *G. duodenalis* cysts indicates a stable infection in an immunocompetent host [55]. Therefore, our experimental model simulates the reality of most *G. duodenalis* infections, in which the balance between parasitic pathogenesis and the immune response keeps immunocompetent patients asymptomatic.

In the present study, infection with different assemblages caused different alterations of the composition of the mucus and intestinal segments. Mucus participates in the innate immune response of giardiasis [35] and serves as support and nutrition for the normal microbiota [56,57]. Therefore, changes in mucus composition may be responsible for changes in the composition of microbiota. In the duodenum, the mucus became more fluid, with a decrease in neutral mucins and increase in acidic mucins. *G. duodenalis* presents tropism by the duodenum [58,59], and this segment is known to have a scarce bacterial count in the microbiota [8,50,59]. Therefore, in the duodenum, the parasite exerts direct actions on the wall of the mucosa (i.e., adhesion), in which a poor microbiota and more fluid mucus, promoted by an increase in acidic goblet cells

(AB 2.5+), favors the interaction of the protozoan with the duodenum. The duodenum, when exposed, responds to infection by increasing the production of IELs. The increase in IELs may induce malabsorption syndrome and brush border injury, which is also related to chronic giardiasis in many patients [35].

We found that the mucus in the jejunum became dense in the GIB group and remained unchanged in the GIA group. Despite these differences, animals in both groups exhibited changes in the microbiota and an increase in IELs. Additionally, the reduction of the production of acidic mucins may have contributed to the decrease in the number of *Lactobacillus* spp in the jejunum, in which these bacteria prefer acidic environments [57]. Changes in mucus composition may enable bacterial transposition, thus contributing to the local inflammatory response that was detected. Dysbiosis and an imbalance of homeostasis can be aggravated by direct breakdown of the mucus barrier through degradation of mucin by the enteropathogen [50,60]. Lymphocytes play a central role in regulating this process, which causes pathology of the organ [50]. These changes were insufficient to alter the morphology of the jejunum. However, the duodenum was responsive to the actions of the parasite. In the duodenum, we observed reductions of the thickness of the mucosa and submucosa in the GIB group. These histopathological changes reinforce tropism by the segment and contradict the tendencies of nomenclature changes for the parasite of assemblage B that according to recent studies would no longer be called "*duodenalis*" and would be identified as *Giardia enterica* [61,62].

The reduction of villus height is one of the intestinal changes that has been described in the literature [63-65], which was not observed in the present study, thus corroborating other studies [66,67]. In animals that were infected with assemblage A of *G. duodenalis*, an increase in villus width was observed in the duodenum, possibly because of lymphocyte migration to the lamina propria [35].

The present study has some limitations. We evaluated only the acute phase of infection and investigations of the microbiota do not contemplate molecular techniques for the best detailing of its composition. Nonetheless, the present study is the first that compared the pathophysiology of murine infection with assemblages AII and BIV of *G. duodenalis*. We also performed leucometry, conducted behavioral tests to assess pain, and analyzed the microbiota and such intestinal parameters as the morphometry and quantification of IELs and goblet cells. Our findings may be beneficial for developing

appropriate experimental models for drug testing and understanding their therapeutic effects in infections that are caused by genotypically distinct parasites.

5. Conclusions

We hypothesized that different assemblages of *G. duodenalis* induce different immunopathological changes and tissue damage in the host. Both assemblages of the parasite altered the composition of the intestinal microbiota. Assemblage AII was more aggressive than assemblage BIV with regard to its effects on the duodenum mucosa and pain-related behavior in Swiss mice.

Conflict of interest

The authors have no conflicts of interest.

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CAPÍTULO III

3.1 CONCLUSÕES

A infecção pelas *assemblages* A e B de *G. duodenalis* apresentou patogenicidade distinta no modelo experimental adotado neste estudo e a diarreia, manifestação mais associada à giardíase, não foi provocada por nenhuma das *assemblages*. Na maioria das variáveis analisadas o segmento mais afetado pela infecção foi o duodeno o que nos faz concluir que a classificação da espécie como *Giardia duodenalis* permanece adequada, pelo menos para as espécies mais frequentes na infecção pelo ser humano.

Ambas as *assemblages* provocaram alterações sistêmicas nos animais como aumento da temperatura corporal e dos leucócitos sanguíneos. Também alteraram de forma semelhante a composição da microbiota intestinal, com redução na população de *Lactobacillus sp.* e aumento de *Enterococcus sp.* e enterobactérias. Tal perfil bacteriano da microbiota provavelmente foi influenciado pelas mudanças na secreção de mucinas ácidas e neutras, evidenciadas na contagem de células caliciformes. Além disso, foi comum às *assemblages* A e B a redução na contagem de células da glia entérica do duodeno e alterações na morfologia dos neurônios.

O perfil de infecção observado para a *assemblage* A refletiu em maior inflamação da mucosa, com aumento de LIE e comportamento de dor nos animais. O aumento dos LIE possivelmente influenciou no aumento da largura do vilo em razão do alargamento da lâmina própria. Além disso, a *assemblage* A promoveu mais alterações no plexo submucoso. Do contrário, a *assemblage* B se mostrou ser mais patogênica ao plexo mientérico e também reduziu a espessura da túnica muscular.

De forma geral, concluímos que a *assemblage* A mostrou-se mais patogênica no modelo de roedores. Merece destaque que mesmo com ausência de diarreia a giardíase provocada, que poderia ser considerada assintomática, foi suficiente para promover alterações importantes na estrutura da parede intestinal e da microbiota. São necessários novos estudos para acompanhar de forma crônica esta infecção por ambas as *assemblages*.

3.2 PERSPECTIVAS FUTURAS

A presente investigação propôs pela primeira vez comparar a patogenicidade das infecções pelas *assemblages* A e B de *G. duodenalis* sob vários aspectos e, partir dos resultados obtidos, entende-se que para o desenvolvimento desta linha de pesquisa seja interessante investigar os mesmos aspectos avaliados em fases mais tardias da infecção.

Além disso, as análises da microbiota intestinal deverão contemplar técnicas moleculares, as quais permitirão o detalhamento dos diversos gêneros e espécies bacterianas. Explorar a resposta imunológica envolvida na infecção por meio da dosagem de citocinas inflamatórias permitirá conhecer as vias utilizadas pelo hospedeiro para combater as diferentes *assemblages* do parasito.

Quanto aos estudos em relação ao SNE, sugere-se que diferentes subpopulações de neurônios sejam avaliadas, tanto para o plexo mientérico quanto para o submucoso, assim como o uso de outros marcadores gliais devem ser incentivados. Assim como ensaios adicionais de viabilidade ou morte celular para compreender o motivo da redução na quantidade das células da glia entérica.

Estes passos não só contribuirão para o entendimento acerca da fisiopatologia dos diferentes padrões de giardíase relatados, mas também beneficiarão o desenvolvimento de novos modelos experimentais para ensaios clínicos que visem compreender os efeitos terapêuticos dos fármacos nas infecções por parasitos geneticamente distintos.

ANEXOS

ANEXO 1 – CERTIFICADO DE APROVAÇÃO ÉTICA

INTEGRADO
FACULDADE
CAMPO MOURÃO/PR

COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

(CEEA)

CERTIFICADO

Certificamos que o projeto intitulado "**AVALIAÇÃO DA PATOGENICIDADE DAS ASSEMBLAGENS A E B DE *Giardia duodenalis***" sob responsabilidade da Professora Mariana Felgueira Pavanelli encontra-se de acordo com os princípios Éticos em Experimentação Animal, tendo sido aprovado pela CEEA em 22/10/2014 sob o protocolo 1070.

Campo Mourão, 22 de outubro de 2014

CEEA - Comissão de Ética em Experimentação Animal
Faculdade Integrado de Campo Mourão - PR

Profa. Dra. Daniele Maggioni Chefer
Vice-Presidente da CEEA, Faculdade Integrado
Curso de Medicina Veterinária

ANEXO 2 – INSTRUÇÕES PARA AUTORES (REVISTA PARASITOLOGY RESEARCH)

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Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

- Disclosure of potential conflicts of interest
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ANEXO 3 – INSTRUÇÕES PARA AUTORES (REVISTA BIOMEDICINE & PHARMACOTHERAPY)

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[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
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[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
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[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
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