UNIVERSIDADE FEDERAL DE ALFENAS

THIAGO CAETANO ANDRADE BELO

Ivermectin-induced bacterial gut dysbiosis does not increase susceptibility to *Pseudomonas aeruginosa* lung infection

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Interação patógeno-hospedeiro.

Orientador: Prof. Dr. Leonardo Augusto de Almeida

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RESUMO

A manutenção da microbiota intestinal é essencial para o equilíbrio fisiológico, metabólico e imunitário, além de influenciar no estado saúde-doença. Alguns estudos sugeriram a utilização da ivermectina para o tratamento da Covid-19 e mesmo sendo posteriormente refutada por estudos e rejeitado por agências de controle de medicamentos em todo o mundo, o seu uso permaneceu e foi incentivado por diversos segmentos do governo e saúde. O objetivo deste trabalho foi avaliar a influência do uso oral de ivermectina sobre a microbiota bacteriana intestinal e quais são os efeitos desta disbiose frente a pneumonia oportunista causada por *Pseudomonas aeruginosa* em modelo murino. Para isso, camundongos C57BL/6 foram submetidos ao tratamento consecutivo com PBS ou ivermectina por gavagem. Não houveram diferenças significativas no peso dos animais e da ração consumida durante o período experimental. Porém, observou-se o aumento da umidade e consistência disforme das fezes do grupo tratado com ivermectina. Através de análise metagenômica do DNA total das fezes, foi observada a diminuição dos filos *Bacteroidetes*, *Firmicute*s, *Proteobacteria* e *Tenericutes* e o aumento do filo *Verrucomicrobia* nos animais tratados com ivermectina, em comparação ao grupo PBS. Ademais, o conteúdo cecal dos animais tratados com ivermectina apresentou ser mais imunoestimulatório em macrófagos derivados da medula óssea murina pelo aumento de marcação da molécula CD86 na membrana dessas células quando analisados por imunofluorescência, além do aumento na secreção de IL-6 e diminuição de IL-10, quantificado por ELISA. A organização histopatológica cecal dos animais tratados com ivermectina apresentou-se alterado, além do tratamento com ivermectina induzir danos no tecido hepático e aumentar a expressão de citocinas pró e anti-inflamatórias no fígado. Ao serem desafiados com *P. aeruginosa*, não houve susceptibilidade aumentada à infecção nos animais disbióticos, apresentando semelhança entre os grupos tratados com PBS ou ivermectina e infectados na recuperação de bactérias viáveis no pulmão, fígado, baço e rim, análises histopatológicas e expressão de citocinas no pulmão ou secreção de citocinas pró ou anti-inflamatórias de esplenócitos cultivados de animais infectados e reestimulados com *P. aeruginosa.* Foi observado uma extensão nos danos hepáticos e aumento na expressão de citocinas pró e anti-inflamatórias em grupos tratados com ivermectina e desafiados com *P. aeruginosa*. É possível concluir que o uso contínuo de ivermectina não acarretou maior suscetibilidade ou resistência à *P. aeruginosa*, apesar do efeito desse fármaco sobre a microbiota intestinal dos animais tratados.

Palavras-chave: Disbiose intestinal, Ivermectina, Infecção pulmonar, *Pseudomonas aeruginosa*.

ABSTRACT

The maintenance of gut microbiota is essential for a physiological, metabolic, immune balance and to influence the health-disease state. Some studies have suggested the use of ivermectin for Covid-19 treatment and even though it was later refuted by studies and rejected by drug control agencies around the world, its use remained and was encouraged by various segments of government and health. The objective of this work was to evaluate the influence of oral ivermectin use on the bacterial gut microbiota and what are the effects of this gut dysbiosis in Pseudomonas aeruginosa opportunistic pneumonia in mice. For this, C57BL/6 isogenic mice were treated for 7 consecutive days with PBS or ivermectin by gavage. There were no significant differences in the mice's weight and the feed consumed during the experimental period. However, there was an increase in feces moisture and uneven consistency in the ivermectin-treated group. Through metagenomic analysis of the feces' total DNA, it was observed a decrease in the phyla Bacteroidetes, Firmicutes, Proteobacteria, and Tenericutes and an increase in the phylum Verrucomicrobia in mice ivermectin treated, compared to the PBS group. Furthermore, the cecal content of ivermectin-treated mice showed to be more immunostimulatory in macrophages derived from murine bone marrow due to the increase in CD86 molecules labeling in the membrane of these cells when analyzed by immunofluorescence, in addition to the increase in IL-6 secretion and decrease in IL-10, quantified by ELISA. The cecal tissue organization of ivermectin-treated mice was altered, in addition to the ivermectin treatment induced liver tissue damage and increased the expression of pro and anti-inflammatory cytokines in the liver. When mice were infected with P. aeruginosa, there was no increased susceptibility to infection in gut-dysbiotic mice, showing similarity between the PBS-treated and ivermectin-treated groups and infected in the viable recovery bacteria in the lung, liver, spleen, and kidney, histopathological analysis and expression of cytokines in the lung or secretion of pro- or anti-inflammatory cytokines from cultivated splenocytes from animals infected and restimulated with P. aeruginosa. Therefore, an extension in liver damage and up-regulation in the expression of pro-and anti-inflammatory cytokines were observed treated with ivermectin-treated and infected with P. aeruginosa group. It is possible to conclude that the ivermectin's continuous usage did not lead to a greater susceptibility or resistance to P. aeruginosa, despite the effect of this drug on the gut microbiota of mice-treated.

Keywords: *Gut dysbiosis, Ivermectin, Lung infection, Pseudomonas aeruginosa.*

LISTA DE ABREVIATURAS E SIGLAS

BMDM- Macrófagos derivados da medula óssea

CFTR- Regulador de condutância transmembranar de fibrose cística

DPOC- Doença pulmonar obstrutiva crônica

FDA- *Food and Drug Administration*

GABA- Ácido gama-aminobutírico

HIV- Vírus da imunodeficiência humana

HKPa- *Heat-killed Pseudomonas aeruginosa*

IL-1β- Interleucina 1 beta

IL6- Interleucina 6

IL10- Interleucina 10

IVM- Ivermectina

OMS- Organização Mundial da Saúde

PAC- Pneumonia adquirida na comunidade

PBS- Tampão salino-fosfato

RdRp- RNA polimerase dependente de RNA

RT-PCR- Transcriptase Reversa - Reação em Cadeia da Polimerase

SARS-CoV2- Síndrome Respiratória Aguda Grave - Coronavírus 2

UFC- Unidade formadora de colônia

UTI- Unidade de tratamento intensivo

SUMÁRIO

1 INTRODUÇÃO

A microbiota intestinal humana é composta por aproximadamente 10^{13} de diferentes espécies bacterianas, sendo essencial para manter um equilíbrio homeostático fisiológico, metabólico, imunitário e influenciar no estado saúde-doença do organismo humano, por meio de *cross-talk* entre hospedeiro e microbiota, protegendo-o de doenças relacionadas ou acentuadas pelo desiquilíbrio do microbioma (PETERSON *et al*., 2015; BECATTINI; TAUR; PAMER, 2016; SOMMER *et al*., 2017). Desde o nascimento, o trato gastrointestinal é prontamente colonizado por microrganismos, no qual irá interagir com o hospedeiro até a vida adulta, estabelecendo relações coordenadas imprescindíveis para a saúde do ser humano (GOMAA, 2020; YATSUNENKO *et al*., 2012). Composta por microrganismos autóctones ou alóctones, também conhecidos como indígenas ou transitórios, respectivamente, os quatro filos bacterianos mais presentes na microbiota intestinal são *Actinobacteria*, *Proteobacteria, Bacteroidetes* e *Firmicutes*, sendo os dois últimos representantes de 90% da microbiota intestinal (VILLANUEVA-MILLÁN; PEREZ-MATUTE; OTEO, 2015; QIN *et al*., 2010). A partir do momento no qual ocorre um desequilíbrio da microbiota intestinal, é estabelecido um quadro disbiose gastrointestinal, podendo ser incitada diversas formas, como, por exemplo, dietas desequilibradas, drogas e fármacos (ROSA *et al*., 2020; RINNINELLA *et al*., 2019; ENGEN *et al*., 2015).

O eixo bidirecional intestino-pulmão têm sido amplamente estudado nos últimos anos e vêm mostrando-se fundamental o papel do equilíbrio de microbiota bacteriana intestinal na homeostase pulmonar, no qual produtos do metabolismo microbiano podem ser refletidos em alteração da imunidade pulmonar via corrente sanguínea, e vice-versa, favorecendo a progressão de doenças pulmonares em organismos disbióticos (DUMAS *et al*., 2018). Gauguet e colaboradores (2015) verificaram, por exemplo, a importância da microbiota intestinal na regulação da imunidade efetora do perfil Th17 relacionada a presença de bactérias filamentosas segmentadas no trato gastrointestinal, no qual camundongos sem presença deste grupo bacteriano apresentaram quadros pneumônicos mais graves quando desafiados com *Staphylococcus aureus*.

Demonstrado a importância da microbiota intestinal na homeostase pulmonar e os impactos negativos que um quadro disbótico traz para o eixo pulmão-intestino, desconhece-se o impacto que muitos medicamentos acarretam sobre a microbiota intestinal, como a ivermectina, e quais são os seus reflexos no sistema imunológico em processos infecciosos pulmonares.

A ivermectina é um fármaco antiparasitário endectocida de alta eficácia e com atividade em amplo espectro, utilizada no tratamento de diversas parasitoses humanas e veterinárias, contando com uma extensa margem de segurança (OMURA; CRUMP, 2017). A ivermectina atua nos parasitas por meio da interrupção dos canais de cloro controlados por ligantes, em especial por glutamato, além de efeitos nos receptores do ácido γ-aminobutírico (GABA), paralisando atividades musculares e bomba faríngea por meio da hiperpolarização da membrana neuronal, resultando na interrupção da neurotransmissão entre as células nervosas e musculares, perfazendo assim a morte dos parasitas (CULLY *et al*., 1994; YATES; PORTILLO; WOLSTENHOLME, 2006; LIANG; GILLAN; DEVANEY, 2017). Ademais, a ivermectina é um fármaco seguro, já que não atravessa a barreira hematoencefálica de vertebrados, não atingindo o sistema nervoso central onde concentram-se a totalidade de receptores do GABA, além dos vertebrados não possuírem canais de cloreto controlados por glutamato (CRUMP, 2017; WOLSTENHOLME; ROGERS, 2006).

Nos últimos anos, a ivermectina vem sendo sugerida para outros fins, e trabalhos publicados demonstram atividades além da antiparasitária. Em estudos desenvolvidos por Wasgstaff e colaboradores (2012), foi verificado *in vitro* que a ivermectina é capaz de inibir em amplo espectro a importação nuclear mediada pela importina α/β1 sem interferir em outras vias de importação, e combater com eficácia vírus de RNA, como o HIV-1 e o vírus da dengue. Com o surgimento e disseminação acentuada do SARS-CoV-2 em 2019, diversos pesquisadores sugeriram a utilização da ivermectina como possível opção de tratamento da Covid-19 por reposicionamento farmacológico, pois estudos anteriores com proteínas do SARS-CoV demonstraram um papel fundamental da importina α/β1 durante a infecção viral no transporte nucleocitoplasmático das proteínas do nucleocapsídeo (WULAN *et al*., 2015). Também foi verificado que a proteína viral acessória ORF6 do SARS-CoV atua antagonizando o fator de transcrição STAT1, agindo no sequestro da importina α/β1 do reticulo endoplasmático rugoso e complexo de Golgi (FRIEMAN *et al*., 2007). Caly e colaboradores (2020) demonstraram *in vitro* que, a adição de ivermectina em células Vero-hSLAM, na concentração de 5 μM, houve redução em 24 horas de 93% de RNA presente no sobrenadante, relativo aos vírions liberados, e 99,8% de RNA viral associado a células, relativo aos vírions não empacotados, reduzindo em aproximadamente 5.000 vezes o RNA viral em amostras tratadas com ivermectina por 48 horas, sem toxicidade celular.

Desta forma, diversos segmentos da sociedade, governo e saúde com o intuito de prevenção e tratamento dos infectados com o SARS-CoV-2, adotaram o uso da ivermectina, mesmo sem estudos com combinações *in vivo*, ensaios clínicos, ensaios controlados

randomizados ou estudos de dose-resposta realizados (KAUR *et al*., 2021; HEIDARY; GHAREBAGHI, 2020; CHOUDHARY; SHARMA, 2020). Por incentivo de órgãos governamentais, a ivermectina entrou no senso comum no Brasil e em diversos países como possível prevenção da Covid-19, sendo este amplamente distribuído à população, como aconteceu em Itajaí, município do estado de Santa Catarina, que contou com uma distribuição de 2,5 milhões de comprimidos de ivermectina à população, de acordo com portal de notícias Globo G1, em 31 de março de 2021.

Apesar de aparentar ser um fármaco promissor, foi verificado que a dose de ivermectina aprovada pelo *Food and Drug Administration* (FDA) não é a ideal para o tratamento da Covid-19, já que a dose utilizada para obter o resultado de inibição de 50% (IC50) do SARS-CoV-2 *in vitro*, pelos estudos de Caly e colaboradores (2020), é 35 vezes maior do que a concentração plasmática máxima da dose aprovada após a administração oral em jejum e utilizando a dose aprovada de ivermectina, em modelos farmacocinéticos populacionais, não atingem o IC50, mesmo em níveis 10 vezes maiores (SCHMITH; ZHOU; LOHMER, 2020). Em um estudo clínico randomizado, Mohan e colaboradores (2021) verificaram que a dose única de ivermectina em pacientes com Covid-19 leve e moderado não aumentou significativamente a negatividade do RT-PCR ou a diminuição da carga viral após cinco dias de administração em comparação com o placebo.

Desta forma, devido ao aumento do uso indevido de ivermectina pela população, grupos de pesquisas vêm sugerindo o efeito que este uso prolongado conduziria sobre a microbiota intestinal e quais os impactos sistêmicos desta disbiose ao organismo (DICKS; DEANE; GROBBELAAR, 2022). Diversos estudos têm demonstrado que a disbiose intestinal aumenta a susceptibilidade para infecções pulmonares oportunistas pelo desbalanço no eixo pulmão-intestino, e um dos microrganismos de maior notoriedade no ambiente clínico é a bactéria extracelular *Pseudomonas aeruginosa* (ROSA *et al*., 2020; DESSEIN *et al*., 2020).

Descrita pela primeira vez em 1882 por Carle Gessard, a *Pseudomonas aeruginosa* é o agente etiológico para diversas patologias oportunistas, como infecções pulmonares (GELLATLY; HANCOCK, 2013), sendo considerada em 2017 uma das bactérias com maior risco à vida e listada pela Organização Mundial da Saúde como um dos microrganismos prioritários para a pesquisa e desenvolvimento de novas drogas antimicrobianas (OMS, 2017). Pertencente à família *Pseudomonadaceae* e com estrutura celular cilíndrica, a *Pseudomonas aeruginosa* é ubíqua, podendo ser isolado em diversos ambientes como solo, ambientes aquáticos e organismos vivos. Essa espécie bacteriana é caracterizada por ser Gram negativa, oxidase positiva, aeróbia facultativa, não formadora de esporos, não fermentadora de glicose,

possuindo um grande arsenal de fatores de virulência (WILSON, PANDELY, 2021; DIGGLE; WHITELEY, 2020; MIELKO *et al*., 2019; WU *et al*., 2015).

Uma das patologias no qual a infecção por *Pseudomonas aeruginosa* é prevalente, e frequentemente observada com caráter crônico, é a fibrose cística, doença pulmonar monogênica autossômica grave ocasionada pela mutação do gene *CFTR*, no qual o portador possui um acúmulo de muco nos pulmões, devido à ausência de proteína transmembrana nas células epiteliais, ocasionando a deficiência no transporte iônico entre membranas (ROSSI *et al*., 2020; SCOTT, 2013). Além dessa, pacientes nosocomiais em unidades de terapia intensiva (UTIs), portadores de demais doenças crônicas, como a doença pulmonar obstrutiva crônica (DPOC), e imunocomprometidos, como, por exemplo, pacientes oncológicos e portadores de HIV/AIDS, são os mais propensos a desenvolverem complicações com a infecção de *P. aeruginosa*, além deste agente etiológico ser o mais comum em pneumonias adquirida na comunidade (PAC), hospitais e associada à ventilação mecânica (FERNÁNDEZ-BARAT *et al*., 2017; FUJITANI *et al*., 2011).

Uma condição preocupante relacionada à infecção pela *P. aeruginosa* são os fatores de resistência intrínsecos, adaptativos ou adquiridos à antibióticos e a formação de biofilmes, dificultando o tratamento dos infectados, conduzindo organismo a infecções recorrentes e insuficiência pulmonar (PANG *et al*., 2019; SHARMA *et al*., 2014). Em um estudo de isolados clínicos de *P. aeruginosa* no Rio de Janeiro, entre os anos de 1995 e 2015, relatado por Santos e colaboradores (2019), foi verificado um aumento da resistência a antimicrobianos, em especial aos carbapenêmicos, fluoroquinolonas e aminoglicosídeos. A capacidade de formar biofilmes deste microrganismo, constituído por bactéria, matriz de substâncias poliméricas extracelulares, polissacarídeos, DNA extracelular, proteínas e lipídios, favorece a sua estadia recorrente em ambientes clínicos, como UTIs, protegendo a cepa de estresses e possibilitando sua permanência constantes em ambientes bióticos ou abióticos através da comunicação via transdução de sinal interconectadas *quorum sensing* (MORADALI; GHODS; REHM, 2017; GHAFOOR; HAY; REHM, 2011, ZHONG *et al*., 2020; THI; WIBOWO; REHM, 2020).

Portanto, averiguada a emergência da *P. aeruginosa*, sua importância para a saúde pública e seus impactos ao hospedeiro, esse trabalho hipotetiza que alterações na microbiota bacteriana intestinal causada pelo uso contínuo de ivermectina poderia acarretar também em uma resposta imune prejudicada contra a *P. aeruginosa*. Pouco se sabe do impacto da ivermectina na microbiota bacteriana intestinal, pois a literatura atual não dispõe de informações à cerca da ivermectina como indutor de disbiose e seu impacto no agravamento de infecções desencadeada por microrganismos oportunistas. Desta forma, caracterizar a

microbiota bacteriana intestinal em camundongos que receberam uso continuo de ivermectina e verificar se esta disbiose relaciona-se com o agravamento de infecções pulmonares oportunistas, como em quadros pneumônicos agudos pela bactéria emergente *Pseudomonas aeruginosa*, possibilitará uma melhor compreensão existente no eixo bidirecional intestino-pulmão, além de demonstrar às instituições promotoras de uso de medicamentos os impactos negativos do uso de fármacos contra patologias que já foram verificadas ineficácia clínica. Assim, o objetivo geral desse trabalho foi verificar e avaliar como o uso contínuo da ivermectina, amplamente utilizada durante a pandemia causada pelo novo coronavírus, afeta a microbiota bacteriana intestinal em modelo murino e quais são os efeitos desta disbiose intestinal frente à infecção pulmonar pela estirpe virulenta da bactéria oportunista *Pseudomonas aeruginosa* PA14.

CAPITULO 1:

ARTIGO: Ivermectin-induced bacterial gut dysbiosis does not increase susceptibility to *Pseudomonas aeruginosa* lung infection

Ivermectin-induced bacterial gut dysbiosis does not increase susceptibility to *Pseudomonas aeruginosa* lung infection

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Abstract

Some studies have suggested the antiparasitic ivermectin use for Covid-19 treatment, even though it was later refuted by several studies and rejected by drug control agencies around the world. Excessive use of drugs, including antiparasitic drugs, can lead to bacterial gut dysbiosis, generating an imbalance in the intestinal microbiome, which in turn may increase or decrease susceptibility to infectious processes. To better understand the continuous ivermectin usage over bacterial gut community and susceptibility to *Pseudomonas aeruginosa* pulmonary infection, C57BL/6 isogenic mice were treated with ivermectin or phosphate buffer saline (PBS) by gavage. It was found that ivermectin-induced bacterial gut dysbiosis, is characterized by a decrease in *Bacteroidetes*, *Firmicutes*, *Proteobacteria,* and *Tenericutes* and an increase in

Verrucomicrobia. Furthermore, a pro-inflammatory immunostimulatory cecal content was observed, inducing an increase in CD86 expression and IL-6 secretion in bone marrow-derived macrophages (BMDM) and disruption in cecal tissue organization. In addition, liver tissue damage was observed in dysbiotic mice. However, the ivermectin-induced bacterial gut dysbiosis condition did not lead to acute susceptibility to intratracheal *Pseudomonas aeruginosa* infection, showing similarity between the gut-dysbiotic and non-dysbiotic groups infected in the recovery of viable bacteria in organs, histopathological analysis, and cytokine expression in the lung or secretion of pro- or anti-inflammatory cytokines from splenocytes. Therefore, an extension in liver damage and up-regulation in the pro and anti-inflammatory cytokines expression were observed in groups ivermectin-treated and infected with *P. aeruginosa*, evidencing that the ivermectin treatment generated liver damage in mice, which is exacerbated in infectious conditions.

Keywords: Gut dysbiosis, Ivermectin, Lung infection, *Pseudomonas aeruginosa*.

1.1 Introduction

Ivermectin is an endectocidal antiparasitic drug widely used in parasites treatment [1]. Discovered in 1970, through *Streptomyces avermitilis* bacterium studies, this drug acts on chlorine channels controlled by ligands, glutamate, in addition to having effects on γ-aminobutyric acid (GABA) receptors, paralyzing parasite muscle activities [2-4]. Ivermectin is a safe drug, since it does not cross the blood-brain barrier, not reaching the central nervous system, in which all GABA receptors are concentrated, aside from vertebrates do not have chloride channels controlled by glutamate, which is common in nematodes and insects [5-6].

The emergence and intensive spread of SARS-CoV-2, leading to a pandemic situation

from March 2020, led several researchers to suggest the ivermectin use as a possible treatment option for Covid-19. Wagstaff et al [7] checked that ivermectin can broadly inhibit importin α/β1-mediated nuclear import without interfering with other pathways of importation, and effectively combat RNA viruses, such as HIV-1 and dengue, which depend on these pathways for successful infection and viral production. In this way, Caly et al. [8] demonstrated in vitro that the ivermectin treatment in Vero-hSLAM cells reduced, in 24 hours, 93% of virion release and 99.8% of cell-associated SARS-CoV-2 viral RNA, reducing viral RNA by approximately 5,000-fold in samples ivermectin treated for 48 hours, without cellular toxicity.

Although it appeared to be a promising drug, it was proven that the ivermectin dose approved by the Food and Drug Administration (FDA) is not ideal for the Covid-19 treatment, since to obtain the result of 50% SARS-CoV-2 inhibition in vitro by Caly et al [8] the used dose was 35 times higher than the maximum approved plasma concentration after oral administration in fasting condition [9]. Even after demonstrating its ineffectiveness against SARS-CoV-2 in vivo and in clinical studies [10], it remained common sense in several countries, encouraged by government agencies, as a possible prevention and treatment of Covid-19, leading a large part of the population to use ivermectin, what may lead to gut microbiota disturbance. Little is known about the impact of ivermectin on the bacterial gut microbiota and the current literature lacks information about it as an inducer of gut dysbiosis and its impact on the maintenance of physiological, metabolic, immune balance and influencing the health-disease state, protecting the human organism from diseases related or accentuated by the imbalance of the microbiome [11-13].

Pseudomonas aeruginosa is a Gram-negative bacterium and the etiologic agent for several opportunistic pathologies, such as lung, urinary, and skin infections [14]. Due to the high antibiotic resistance, its ability to synthesize biofilms, and lead to severe therapeutic impasses to the infected, this is a microorganism of medical interest that has been widely studied

for treatment alternative forms [15-16]. Rosa et al. [17] evaluated that vancomycin-induced gut dysbiosis impacted the gut-lung crosstalk in a murine model for pneumonia infected with *Pseudomonas aeruginosa*, showing higher levels of viable bacteria in the lungs and spleen, a higher dosage of pro-inflammatory cytokines, and increased tissue damage, being this state reversed after fecal microbiota transplantation in the gut-dysbiotic mice.

Given the relevance of the *Pseudomonas aeruginosa* to public health, its impacts on the infectious and inflammatory process in the host, and the demonstration of accentuated pneumonic conditions in gut-dysbiotic murine models by Rosa et al. [17], the focus of this study was to evaluate how the ivermectin continuous usage affects the bacterial gut microbiota and what is its impact on the murine immune response against a pulmonary infection by the virulent strain of *Pseudomonas aeruginosa* PA14.

1.2 Material and Methods

1.2.1 Mice and ethics statement

This study was carried out in strict accordance with the Brazilian laws 6638 and 9605 in Animal Experimentation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Alfenas (CEUA 23/2021). The isogenic strain C57BL/6 mice aged 6-8 weeks were from the Central Vivarium of the Federal University of Alfenas. Mice were contained in isolated cages on a 12:12 light/dark cycle, fed *ad libitum* with a standard rodent diet, and with no water restrictions.

1.2.2 Ivermectin treatment and experimental groups

Ivermectin 1% Ivomec® was used and diluted in PBS, being administered by gavage at

5 mg/kg/day. C57BL/6 mice were randomly separated into 2 different groups. One group (PBS/C) was treated for 7 consecutive days with phosphate buffer solution - PBS and another group (IVM/C) was treated for 7 consecutive days with ivermectin (N=5 animals/group). Bodyweight, feed consumption, and fecal consistency were checked daily. Fecal consistency was based using the parameters described by Li et al. [18]: 0, normal; 1, slightly moist; 2, moderately moist; 3, undefined format; 4, watery stools.

1.2.3 Microbiome metagenomics from fecal samples

Total DNA was extracted from mice fecal samples from the PBS/C and IVM/C groups using PureLinkTM Microbiome DNA Purification Kit (Thermo Fischer Scientific, Van Allen Way Carlsbad, CA, USA) followed by quantification in Invitrogen Qubit Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA). Equal amounts of purified DNA were used to analyze the 16S ribosomal RNA (rRNA) sequences by the Illumina HiSeq platform (Illumina, San Diego, CA, USA). Distinct regions of the 16S rRNA / 18SrRNA / internal transcribed spacer (ITS), 16SV4 / 16SV3 / 16SV3-V4 / 16SV4 V5, 18S V4 / 18S V9, ITS1 / ITS2 and Arc V4 genes were amplified with specific primers (e.g. 16S V4: 515F-806R, 18S V4: 528F-706R, 18S V9: 126 1380F-1510R), with barcode. PCR reactions were performed with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The 400 – 450 bp segments were selected and mixed in equal amounts for further analysis. This mixture was then purified with a Qiagen Gel Extraction Kit (QIAGEN Inc, Valencia, CA, USA). Sequencing libraries were generated using a NEBNext® UltraTM Library Pre-Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's recommendations. The quality of the library was verified using an Invitrogen Qubit Fluorometer fluorimeter (Thermo Fischer Scientific, Waltham, MA, USA) and by the Agilent Bioanalyzer 2100 system (Agilent

Technologies, Santa Clara, CA, USA). The amplicons were sequenced on an Illumina paired-end platform, generating 250 bp paired-end reads (Raw PE), which were assembled and pre-treated. Chimeric sequences between clean tags were excluded to get effective tags.

1.2.4 Immunofluorescence analysis of BMDM surface CD11b and CD86 markers

Bone marrow cells were obtained from the femora and tibia of mice, and they have grown in bone marrow-derived macrophages (BMDMs) as previously described by our group [19]. BMDMs ($5x10^5$ cells per well) were plated on imaging slides (μ-Slide 12-well, glass-bottom, Ibidi GmbH, Munich, Germany), followed by cecal content stimulation from the PBS/C and IVM/C groups and heat-killed *Pseudomonas aeruginosa* (HKPa), as a positive control for 12 hours. The mice's cecal content was autoclaved, followed by protein quantification in Invitrogen Qubit Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA). Equal cecal content of 0,5 mcg protein amounts per well and HKPa at MOI 1:10 were used to stimulate. The cells were then washed three times with PBS and incubated with the anti-CD16/32 antibody (BD Biosciences, San Jose, CA) for 2 hours to block nonspecific bonds. The cells were then incubated with anti-CD86 and anti-CD11b, followed by staining with FITC-conjugated and PE-conjugated (BD Biosciences), respectively, overnight at 4°C. The slides were washed with PBS and the nuclei were stained with 150 ng/mL 40,6-diamino-2-phenylindole (DAPI; Thermo Scientific) for 1 hour. All images were captured using a Nikon Eclipse 80i fluorescence microscope (Melville, New York, U.S.A). Image J software was used to analyze the markings obtained for the nucleus (blue fluorescence), CD11b+ cells (green fluorescence), and CD86+ cells (red fluorescence).

1.2.5 Cultivation of the *Pseudomonas aeruginosa* PA14 strain, heat-killed PA14 (HKPa), and the mice intratracheal infection

The virulent strain of *Pseudomonas aeruginosa* PA14 was obtained from the bacterial culture collection of the Laboratory of Molecular Microbiology of Microorganisms of the Federal University of Alfenas. C57BL/6 mice have divided into two different groups at random (PBS/I and IVM/I) receiving PBS or ivermectin by gavage as described (N=5 animals/group). The mice were infected intratracheally on the seventh day with the virulent strain of *Pseudomonas aeruginosa* PA14, as described by Belo et al. [20]. Briefly, mice were ketamine/xylazine anesthetized (80/10 mg/kg) and the trachea was exposed by a small incision of the neck skin, and the infection was performed by instilling 10 µL of bacteria with 1×10^5 colony-forming units (CFUs). After installation, the neck skin was sutured. Mice were maintained in a vertical position for 5 min and transferred to a warming pad until full recovery from anesthesia. HKPa was obtained from exponential *P. aeruginosa* growth, following 15 minutes of incubation at 80°C. The bacterial killing was confirmed by plating the heat solution in Luria Bertani agar (LB).

1.2.6 Colony-forming unit count (CFUs)

P. aeruginosa PA14 loads in the lungs, liver, spleen and kidney organ fragments were macerated in 9 mL of sterile saline and serially diluted. The dilutions were plated in Luria Bertani agar (LB). Petri dishes were incubated at 37ºC for 24 hours and performed at CFU count. The results were expressed as CFU log/lung, liver, spleen, and kidney of each animal.

1.2.7 Real-Time RT-PCR for pro and anti-inflammatory cytokines expression

Lung and liver macerate of the experimental groups were homogenized with TRIzol

reagent (Invitrogen) to isolate total RNA. Reverse-transcription of 1 μg total RNA was performed using Illustra™ Ready-To-Go RT-PCR Beads (GE Healthcare, Buckinghamshire, UK). Real-time RT-PCR was conducted in a final volume of 10 μL containing the following: SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), with cDNA as the PCR template and primers to amplify specific fragments corresponding to specific gene targets: IL-6 F: 5'-CCAGGTAGCTATGGTACTCCAGAA-3', IL-6 R: 5'-GATGGATGCTACCAAACTGGA-3'; IL-10 F: 5'-GGTTGCCAAGCCTTATCGGA-3', IL-10 R: 5'-ACCTGCTCCACTGCCTTGCT-3', IL-1 β F: 5'-CATCTTCTCAAAATTCGAGTGACA-3', IL-1 β R: 5'-TGGGAGTAGACAAGGTACAACCC-3'. The PCR reaction was performed with ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using the following cycling parameters: 60°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min, and a dissociation stage of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec. All data are presented as relative expression units after normalization to the β-actin gene F: 5'-AGGTGTGCACTTTTTATTGGTCTCAA-3', R: 5'-TGTATGAAGGTTTGGTCTCCCT-3'. PCR measurements were conducted in triplicate. The differences in the relative expression were analyzed by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$ denotes statistical significance).

1.2.8 Splenocyte Culture

Splenocytes were cultured as previously described by our group [21] and stimulated with RPMI 1640 medium or HKPa (MOI 1:10), with supernatants collected at 48 hours.

1.2.9 Measurement of IL-6 and IL-10 from BMDM or splenocyte culture supernatants

BMDMs or splenocytes culture supernatants were used for quantitative analysis of IL-6 and IL-10 cytokine secretion using the Murine IL-6/IL-10 Mini ABTS ELISA Development kit (PeproTech, Cranbury, NJ, USA), following the manufacturer's instructions. Final cytokine concentrations were calculated using the standard curve for IL-6 or IL-10. The final reaction was measured using a microplate reader (Bio-Tropsch Tek Instruments, Winooski, Vt., USA) and read at 405 nm with wavelength correction set at 650 nm.

1.2.10 Histopathology and microscopic image processing

Fragments of the lung, liver, and cecum were fixed in a histological fixator (formaldehyde at 10% in 0.1M phosphate buffer, pH 7.2) for 48 hours, dehydrated in ethanol, diaphanized in xylene, and embedded in histological paraffin. 5μm thick sections were obtained using a rotary microtome (Leica Multicut 2045®, Reichert-Jung Products, Germany). At sections were stained with hematoxylin and eosin (H&E) for histopathological analysis and microstructural. To avoid histological analysis of the same area histological, 1/20 sections of tissue were made. The sections have been viewed and the images were captured using a bright-field photomicroscope (Axioscope A1, Carl Zeiss, Germany). For each animal and organ analyzed, ten microscopic fields were photographed randomly with a 40 × objective lens.

1.2.11 Statistical Analysis

Graphs were created and data analysis was performed using GraphPad Prism 8 software (San Diego, CA, USA), using one-way ANOVA or two-way ANOVA (Bonferroni post hoc test). P-values <0.05 were considered statistically significant.

1.3 RESULTS

1.3.1 Ivermectin treatment alters fecal consistency despite not influencing body weight or feed consumption by mice

C57BL/6 mice under ivermectin treatment conditions did not show weight loss or decrease in feed consumption when compared to the PBS group (Fig. 1A and B). However, the feces of the mice ivermectin treated (IVM/C) were mostly slightly moist in 80% and moderately moist in 20% of mice from the first day of treatment, maintaining it until the fifth day. From the fifth day onwards, the feces of the treated mice changed even more and remained between moderately moist and undefined consistency (Fig. 1C).

1.3.2 Ivermectin-induced bacterial gut dysbiosis in mice was characterized by a decrease in *Bacteroidetes*, *Firmicutes*, *Proteobacteria,* and *Tenericutes* and an increase in *Verrucomicrobia*

To verify if ivermectin induces bacterial gut dysbiosis, C57BL/6 mice were treated for seven consecutive days with ivermectin by gavage and the feces bacterial microbiota were evaluated and compared with untreated mice from the extraction of fecal total DNA and performed the metagenomic sequencing of 16S ribosomal RNA (rRNA). Metabaccording analysis showed that the bacterial gut microbiota of ivermectin-treated mice differed from the bacterial microbiota of untreated mice, in which the ivermectin-treated group presented a total of 60,895 16S rRNA sequences while the untreated presented 78,789 sequences. In the ivermectin-treated group, compared to untreated mice, the phyla that showed a decrease were

Bacteroidetes in 19.80%; *Firmicutes* in 56%, *Proteobacteria* in 65.6%, and *Tenericutes* in 71.1%, while the phylum *Verrucomicrobia* showed an increase in 82.3% (Fig. 2A). Related to specific taxa, *Akkermansia* unclassified species thrived among the bacterial gut dysbiotic microbiota while other bacterial species declined quantitatively (Fig. 2B).

1.3.3 BMDMs stimulated with cecal content from ivermectin-induced bacterial gut dysbiotic mice showed higher CD86 expression, IL6 secretion, and lower IL-10 secretion

Verified that ivermectin treatment induces bacterial gut dysbiosis in mice, the cecal content of these animals was used for BMDMs stimulation. The cecal content of bacterial gut-dysbiotic mice was shown to be more immunostimulatory by increasing CD86 expression on BMDMs (Fig. 3A). In addition, the quantification of the cytokines from stimulated BMDMs supernatant showed an increase in IL-6 secretion and a decrease in IL-10 secretion of cells with cecal content stimulated from bacterial gut dysbiotic mice compared to the non-treated mice, corroborating what was found in immunofluorescence staining (Fig. 3B and C).

1.3.4 Ivermectin-induced bacterial gut dysbiosis show changes in the mice's intestinal mucosa with cecal architecture derangement

After verifying bacterial gut dysbiosis, greater immunostimulation in BMDMs through cecal content stimulation and the reflex on cytokine secretion, histopathological sections of the cecum were performed and stained with hematoxylin and eosin (H&E). The cecum fragments from the untreated group showed typical morphological organization of the cecum wall, such as integrity in the lining epithelium, well-defined intestinal crypts, normal distribution, and organization of enterocytes and goblet cells (Fig. 4A). However, the cecum from the ivermectin-

treated group showed organizational changes in the cecum wall, atrophy in the submucosal layer, intestinal crypts, and enterocytes with irregular distribution and morphology (Fig. 4B).

1.3.5 Ivermectin-induced bacterial gut dysbiosis did not increase mice susceptible to *Pseudomonas aeruginosa* lung infection nor lung damage

Based on previous results by our group and others [17, 22-24] that there is a close relationship between the gut-lung axis when the change of the gut bacterial profile and lung susceptibility to opportunistic infection, the *Pseudomonas aeruginosa* PA14 intratracheal challenge was performed both in PBS-treated (PBS/I) or ivermectin-treated (IVM/I) mice. The IVM/I or PBS/I groups showed similar amounts of viable bacteria recovered from the lung, as well as in the spleen, the liver, and the kidney (Fig. 5).

Lung histological sections of mice treated or not with ivermectin without intratracheal infection with *P. aeruginosa* showed normal lung microstructural characteristics, low cellularity, well-defined alveolar septa, unobstructed alveolar lumen, and alveolar edges with an angular profile, with no differences between the two groups (Fig. 6A and B). Lung histopathological analyzes of the *P. aeruginosa* infected mice either previously treated with PBS or ivermectin showed the same histological patterns, emphasizing that the bacterial gut dysbiotic condition induced by ivermectin does not lead to a greater susceptibility to acute pneumonic conditions caused by the bacterium. The *P. aeruginosa* infection, independent of the previous treatment, showed increased cellularity, diffuse inflammatory infiltrate, alveolar septal thickening, alveolar lumen narrowing, and vascular congestion (Fig. 6C and D). Also, there were no observed differences in the pro-inflammatory cytokines IL-6 and IL-1β and the anti-inflammatory cytokine IL-10 encoding genes expression from *P. aeruginosa*-infected lung macerate mice (Fig. 6E-G).

In addition, ELISA measurements of IL-6 and IL-10 from re-stimulated splenocytes supernatant of IVM/I or PBS/I groups did not show differences (Fig. 7A and B), corroborating the CFU results and the non-increased susceptibility in bacterial gut dysbiotic mice.

1.3.6 Ivermectin induced liver damage in mice, that was accentuated with *P. aeruginosa* PA14 infection, but is not related to the bacterial gut dysbiotic condition

Liver histopathological analyzes of ivermectin-treated mice showed liver damage compared to PBS-treated mice, which was accentuated with *P. aeruginos*a infection. Untreated and non-infected mice had normal liver microstructure, evident hepatocytes and sinusoid capillaries, and low hydropic degeneration (Fig. 8A). Ivermectin-treated and uninfected mice had normal liver microstructure, but with an increase of hydropic degeneration in hepatocytes and inflammatory infiltrate (Fig. 8B). PBS-treated and infected mice showed normal liver microstructure with foci in sinusoid capillary congestion and microvesicular steatosis (Fig. 8C), while infected and ivermectin-treated mice showed an increase in tissue cellularity when compared to the other groups, with increased inflammatory infiltrate, foci of congestion in sinusoid capillaries and microvesicular steatosis (Fig. 8D). In addition, there was an increase in the differential expression of IL- 1β and IL-10 encoding genes in mice livers with a considerable increase in ivermectin-treated and infected mice (Fig 8E-J). Therefore, it was possible to verify that ivermectin induces liver damage, in addition to leading to bacterial gut dysbiosis, and that this damage is worsened in cases in which there is systemic bacterial dissemination after *P. aeruginosa* lung infection.

1.4 Discussion

Concerning the various evidence, the bacterial gut microbiota importance for the health-disease state maintenance of the host is well established, with the bacterial gut microbiota balance being a key point for pulmonary homeostasis through the lung-gut crosstalk. Changes in pulmonary immunity mediated by gut dysbiosis may favor the progression of lung diseases, such as in cases of acute pneumonia triggered by the opportunistic *Pseudomonas aeruginosa* bacterium [25]. Here we demonstrated that even causing mice bacterial gut dysbiosis, the ivermectin usage did not increase susceptibility to acute pneumonia caused by intratracheal *P. aeruginosa* infection. It was found that ivermectin treatment does not interfere with the mice's body weight curve, which is closely linked to the normal consumption of feed by animals, as previously demonstrated in mice and rats' studies [26-27]. However, the fecal mice consistency from ivermectin-treated mice showed moderately moist and undefined consistency. Fecal consistency is intimately related to the gut microbiota integrity composition and cecal tissue organization, suggesting that the stool score modification is reflected in the existence of a bacterial gut microbiota imbalance [28].

Metagenomic sequencing analysis demonstrated the onset of ivermectin-induced bacterial gut dysbiotic by consecutive ivermectin treatment. Current literature lacks information about the bacterial microbiological profile present in feces after treatment with ivermectin. In tiger, after treatment with fenbendazole and ivermectin pills was verified an alteration in the animal's bacterial gut microbiota and metabolic homeostasis, with an increase in *Firmicutes* and *Proteobacteria* and a decrease in *Actinobacteria* or an increase in *Bacteroidetes* after tribondimidine plus ivermectin administration in adolescents [29-30]. Our results indicated that ivermectin treatment proportionally reduced all the phyla studied in mice, except *Verrucomcirobia*, reflecting in a diversity decrease in the bacterial gut microbiota. Previous results from our group verified the increase of *Proteobacteria*, especially *Gammaproteobacteria* in the bacterial gut-dysbiotic fecal microbiota mice treated with

antibiotics [17,22], this fact was not observed in ivermectin-induced bacterial gut dysbiosis, where there was a decrease in *Proteobacteria*, being the *Betaproteobacteria* and *Epsilonproteobacteria* classes the most observed. Shin, Whon e Bae [31] showed that the *Proteobacteria* increase is an important sign of bacterial gut dysbiosis that makes it difficult to maintain a balanced microbiota and is an accentuated risk factor for several pathologies, such as type 2 diabetes mellitus, obesity, and inflammatory processes.

Macchione et al. [32] describe that *Akkermansia muciniphila*, from the *Verrucomicrobia* phylum, corresponds to 1 to 4% of the gut microbiota, corroborating what was found in our eubiotic group (PBS-treated mice), in which *Akkermansia* unclassified species corresponded to 1.58%, diverging from the ivermectin-induced gut dysbiotic group, which corresponded to 11.59%. Ganesh et al. [33] found that the presence of commensal *Akkermansia muciniphila* exacerbates intestinal inflammation in mice infected with *Salmonella typhimurium*, transforming it from a commensal organism to a pathobiont, and as *Akkermansia muciniphila* is capable of degrading mucin, it modifies the layer of mucus, thus evidencing the altered fecal consistency, a related fact to what was shown in this experiment.

The increased CD86 labeling in BMDMs with cecal content stimulation from ivermectin-treated mice and the reflex in the increase in IL-6 secretion and decrease in IL-10 secretion suggest a gut pro-inflammatory phenotype triggered by the imbalance in the bacterial microbiota. There is already described the existing relationship between the gut microbiota's impact on immunometabolism [34]. Although the lacking information regarding the pro-inflammatory state related to bacterial gut dysbiosis induced by antiparasitic drugs, upregulation in the expression of the IL6-coding gene in the colon of mice treated with ampicillin and vancomycin has been reported [35], likewise the up-regulation in BMDMs with cecal content stimulation in TNF-α encoding genes and down-regulation of IL-10 encoding genes from vancomycin-treated bacterial gut dysbiotic mice [17].

Therefore, our results demonstrated that ivermectin-induced bacterial gut dysbiosis directly reflects on the cecum tissue organization. Medonça et al. [36] found that the ivermectin administration impacted both the function and morphology of rat's gastrointestinal tract infected or not with *Strongyloides venezuelensis*. Similarly, Ganesh et al. [33] verified similar cecum histopathological sections to those demonstrated in the study of *Akkermania muciniphila* mice infected, increasing the symptoms of cecal inflammation in mice infected concomitantly with *Akkermania muciniphila* and *Salmonella typhimurium*, due to the *Akkermania muciniphil*a degradation of mucin have modified the mucus layer of these animals, increasing the exposure of the submucosa, which would explain the greater severity of salmonellosis.

The increase in the use of ivermectin by the population for the prevention and treatment of Covid-19 has been encouraged by many institutions worldwide, even without enough studies with in vivo combinations, clinical trials, randomized controlled trials, or dose-response studies performed [37-39], which led us to hypothesize whether this continuous use of ivermectin would generate bacterial gut-dysbiotic conditions capable of reflecting in a clinical worsening and impaired immune response in patients who contracted pneumonia caused by opportunistic microorganisms, mostly in the hospital environment. Dicks, Deane and Grobbelaar [40] questioned whether increased ivermectin use driven by the Covid-19 pandemic could lead to bacterial gut dysbiosis, and our study showed that it did in mice models. However, ivermectin-induced gut dysbiosis did not lead mice to a greater susceptibility to *Pseudomonas aeruginosa* pneumonia. Samuelson et al [41] evaluated that alcohol-induced gut dysbiotic mice increased susceptibility to *Streptococcus pneumoniae* pneumonia. Khailova et al [42] found that administration of *Lactobacillus rhamnosus* GG probiotic decreased the severity of acute *Pseudomonas aeruginosa* pneumonia in mice, with decreased CFU counts and pro-inflammatory markers. Similar results were achieved by our group, observing similar CFU counts between the bacterial eubiotic or dysbiotic groups that received the fecal microbiota

transplant [17]. Taken together, these results show the strong relationship between the bacterial gut microbiota and pulmonary homeostasis, even though this fact was not evidenced in our study.

The results obtained showed an established pneumonic condition, which is confirmed in lung histopathological sections, differential cytokines expression, and secretion of IL-6 and IL-10 in the splenocyte supernatant when stimulated by HKPa. Several factors may have influenced this state. Csóka et al. [43] checked that ivermectin is a P2X4 receptor allosteric activator in macrophages, acting to improve survival, decrease bacterial load, and organ damage in mice after sepsis and it can increase bacterial killing by macrophages. Zhang et al. [44] demonstrated that ivermectin treatment improves mice survival after a lethal dose of lipopolysaccharide (LPS), inhibits the production of pro-inflammatory cytokines, and it was also verified by the same group that ivermectin suppresses the nitric oxide and prostaglandin E2 production [45]. There is the possibility that ivermectin-induced gut dysbiosis may have interfered in the bidirectional lung-gut axis, however, ivermectin itself may have acted on immune system receptors and pathways that allow equality between bacterial eubiotic and dysbiotic groups. Further studies are needed to confirm this hypothesis

It was verified that ivermectin treatment generated an increase in liver tissue damage and differential expression of pro and anti-inflammatory cytokines before *P. aeruginosa* PA14 infection and that these damages worsened after infection, regardless of the established bacterial gut dysbiotic condition. Oularbi et al. [46] showed that rats emamectin-treated showed hepatotoxicity, with changes in histopathological patterns and increased liver markers. It is possible to cross these results since ivermectin has hepatic metabolism, and the continuous administration of this drug could lead to tissue damage, even ivermectin has a high degree of safety, which would directly imply the differential expression of pro and anti-inflammatory cytokines, which would logically accentuate these effects in the presence of a disseminated

bacterial infection [47-48].

1.5 Conclusion

Our results demonstrate that ivermectin can deregulate bacterial intestinal homeostasis, establishing a bacterial gut dysbiotic condition. Although our results do not demonstrate interference of this bacterial gut imbalance on the lung-gut crosstalk related to *P. aeruginosa* infection but worsened mice liver damage.

1.6 Conflict of Interest

The authors declare that research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.7 Funding Sources

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1.8 Author contributions

Thiago Caetano Andrade Belo: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing - original draft. **Natália Cristina de Melo:** Data curation; Formal analysis; Investigation; Methodology. **Bianca Silva Souto:**

Investigation; Methodology; Validation. **Ana de Souza Santos** Investigation; Methodology; Validation. **Caio Pupin Rosa:** Investigation; Methodology; Validation. **Karen Cristina Oliveira:** Investigation; Methodology; Validation. **Patrícia Paiva Corsetti:** Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Visualization. **Leonardo Augusto de Almeida:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Resources; Supervision; Validation; Visualization; Writing - original draft.

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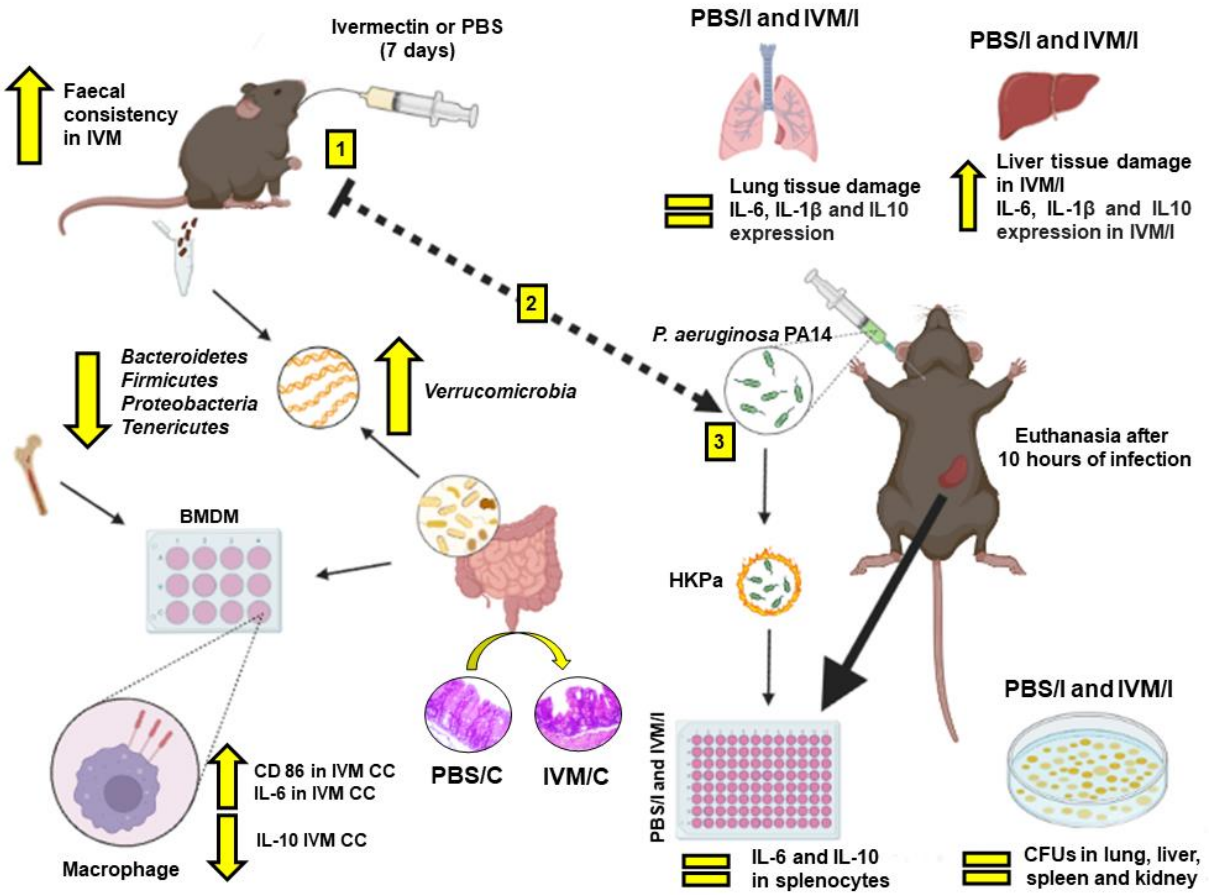
FIGURE LEGENDS:



Graphical abstract: The ivermectin treatment is able of inducing gut dysbiosis, reflected in fecal consistency changes, cecal tissue disorganization, and a pro-inflammatory intestinal profile. In addition, the consecutive treatment with ivermectin led to liver damage in mice. Ivermectin-induced gut dysbiosis in mice does not alter susceptibility to *Pseudomonas aeruginosa* infection, with amounts of viable bacteria recovered, lung damage, and pro- or anti-inflammatory cytokines secretion by splenocyte stimulated with heat-killed *P. aeruginosa* culture similar between gut-dysbiotic and non-dysbiotic groups. Created with BioRender.

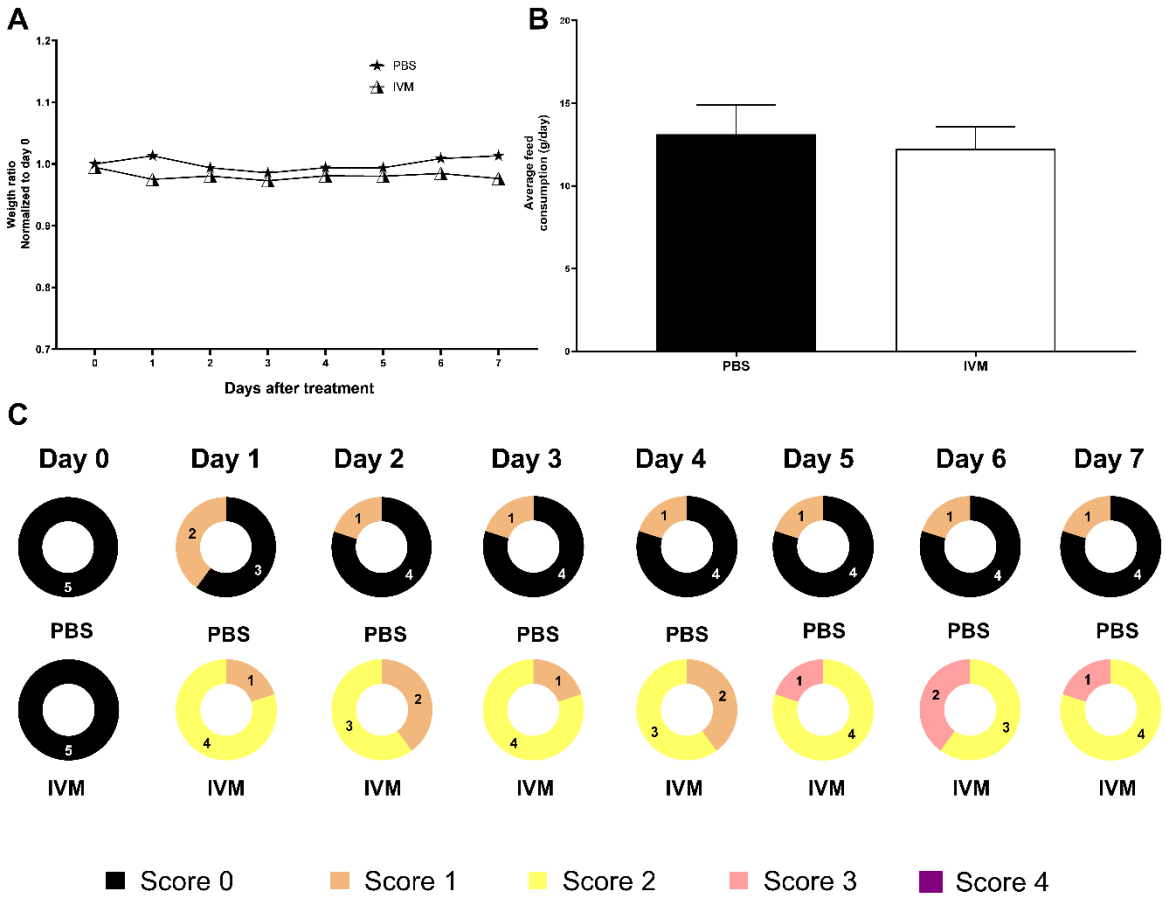
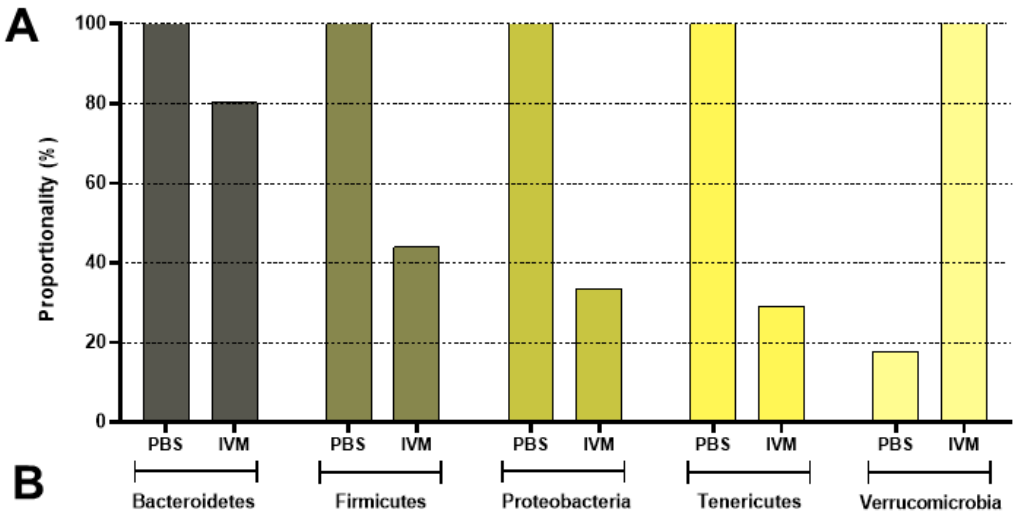


Figure 1: **Continuous ivermectin treatment alters C57BL/6 mice fecal consistency but no weight loss or difference in feed intake.** (A) Mice weights and (B) feed consumption, weighing performed daily under the same conditions of temperature and time. (C) Fecal consistency, considering the following parameters: 0, normal; 1, slightly moist; 2, moderately moist; 3, undefined format; 4, watery stools. (N=5 animals/group).


A
Proportionality (%)
100
80
60
40
20
0
PBS
IVM
PBS
IVM
PBS
IVM
PBS
IVM
PBS
IVM
B
Bacteroidetes
Firmicutes
Proteobacteria
Tenericutes
Verrucomicrobia

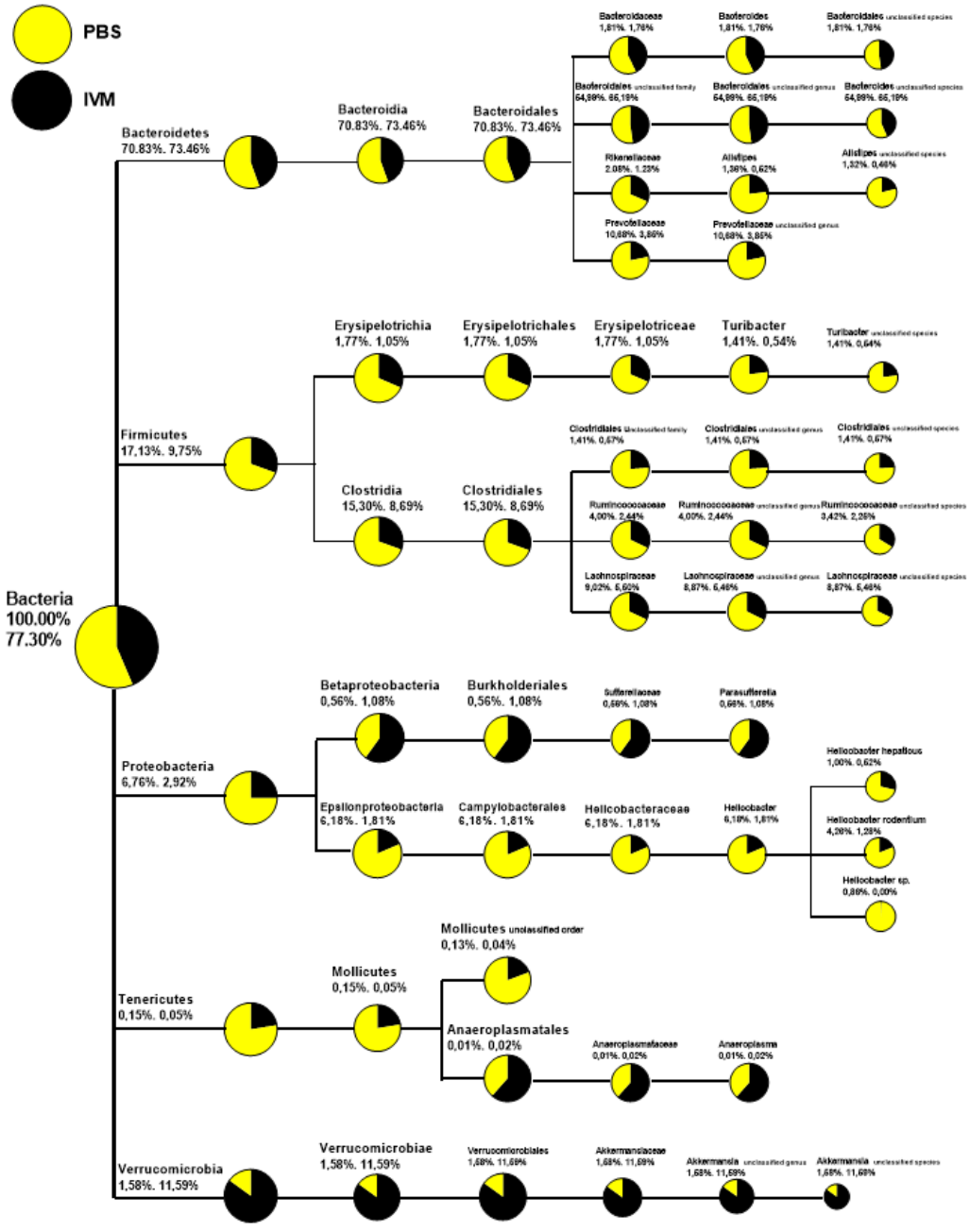

PBS
IVM
Bacteria
100.00%
77.30%
Bacteroidetes
70.83%. 73.46%
Bacteroidia
70.83%. 73.46%
Bacteroidales
70.83%. 73.46%
Bacteroidaceae
1,81%. 1,78%
Bacteroides
1,81%. 1,78%
Bacteroidales unclassified species
1,81%. 1,78%
Bacteroidales unclassified family
64,89%. 66,18%
Bacteroidales unclassified genus
64,89%. 66,18%
Bacteroides unclassified species
64,89%. 66,18%
Rikenellaceae
2,08%. 1,23%
Alistipes
1,38%. 0,62%
Alistipes unclassified species
1,32%. 0,46%
Prevotellaceae
10,68%. 3,86%
Prevotellaceae unclassified genus
10,68%. 3,86%
Firmicutes
17,13%. 9,75%
Erysipelotrichia
1,77%. 1,05%
Erysipelotrichales
1,77%. 1,05%
Erysipelotriceae
1,77%. 1,05%
Turibacter
1,41%. 0,54%
Turibacter unclassified species
1,41%. 0,64%
Clostridia
15,30%. 8,69%
Clostridiales
15,30%. 8,69%
Clostridiales unclassified family
1,41%. 0,67%
Clostridiales unclassified genus
1,41%. 0,67%
Clostridiales unclassified species
1,41%. 0,67%
Ruminococcaceae
4,00%. 2,44%
Ruminococcaceae unclassified genus
4,00%. 2,44%
Ruminococcaceae unclassified species
3,42%. 2,26%
Lachnospiraceae
9,02%. 5,60%
Lachnospiraceae unclassified genus
8,87%. 5,46%
Lachnospiraceae unclassified species
8,87%. 5,46%
Proteobacteria
6,76%. 2,92%
Betaproteobacteria
0,56%. 1,08%
Burkholderiales
0,56%. 1,08%
Sutterellaceae
0,56%. 1,08%
Parasutterella
0,56%. 1,08%
Epsilonproteobacteria
6,18%. 1,81%
Campylobacterales
6,18%. 1,81%
Helicobacteraceae
6,18%. 1,81%
Helicobacter
6,18%. 1,81%
Helicobacter hepaticus
1,00%. 0,62%
Helicobacter rodentium
4,26%. 1,28%
Helicobacter sp.
0,86%. 0,00%
Tenericutes
0,15%. 0,05%
Mollicutes
0,15%. 0,05%
Mollicutes unclassified order
0,13%. 0,04%
Anaeroplasmatales
0,01%. 0,02%
Anaeroplasmataceae
0,01%. 0,02%
Anaeroplasma
0,01%. 0,02%
Verrucomicrobia
1,58%. 11,59%
Verrucomicrobiae
1,58%. 11,59%
Verrucomicrobiales
1,58%. 11,59%
Akkermansiaceae
1,58%. 11,59%
Akkermansia unclassified genus
1,58%. 11,59%
Akkermansia unclassified species
1,58%. 11,59%

Figure 2**: Ivermectin induces gut dysbiosis in C57BL/6 mice**. (A) Proportionality of the phyla found in the fecal sample of mice treated or not with ivermectin. (B) Taxonomic tree of specific bacterial species in mice treated with PBS (yellow) or ivermectin (black). The sizes of the circles represent the relative abundance of the species. The first number below the taxonomic name represents the percentage compared to the entire taxon in the untreated group (PBS/C) and the second number represents the percentage compared to the entire taxon in the treated group (IVM/C).

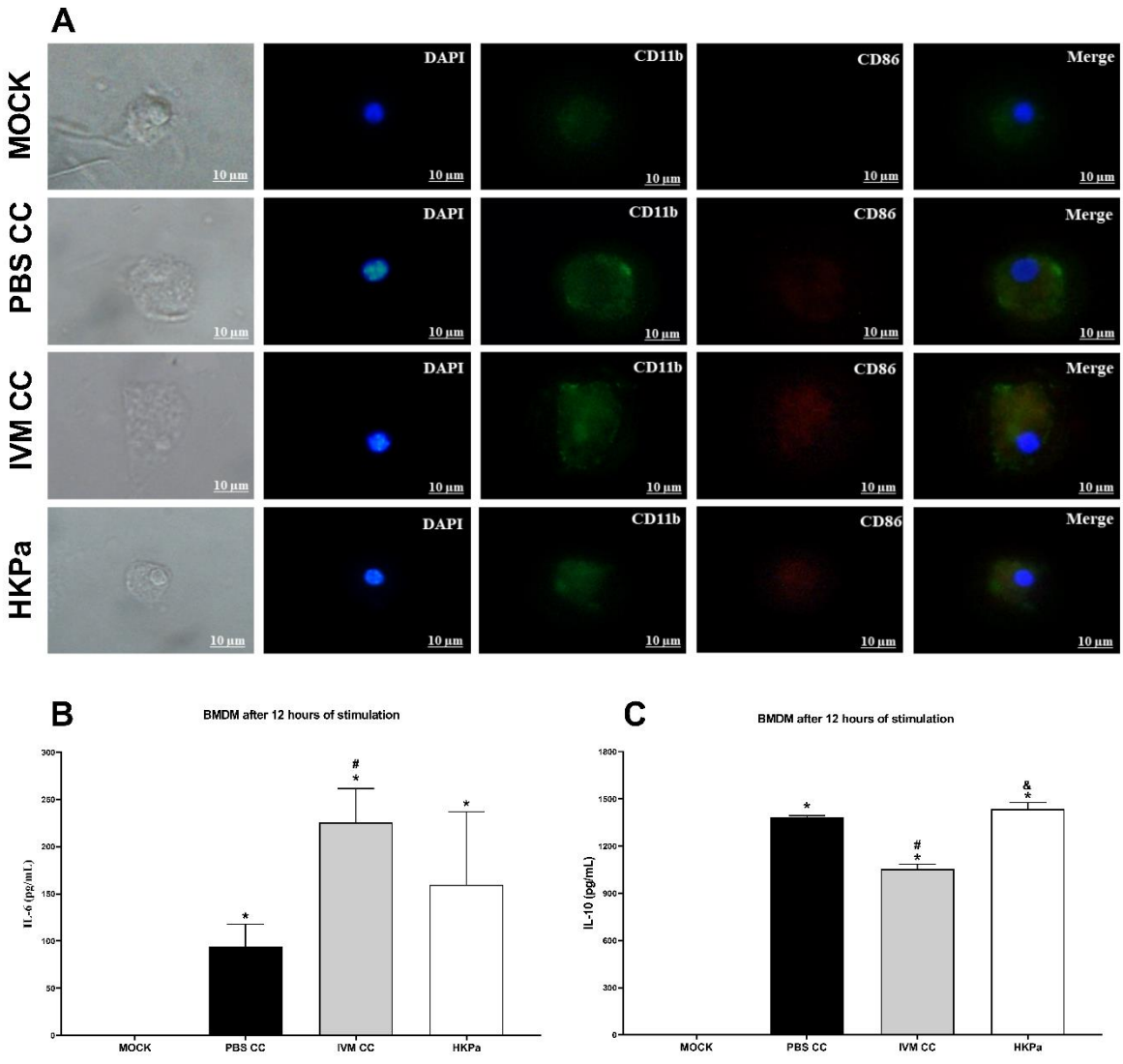


Figure 3: **BMDMs stimulation with cecal content from ivermectin-induced gut**

dysbiosis mice are more immunostimulatory, increasing CD86 labeling, IL-6 secretion, and decreasing IL-10 secretion. (A) Immunofluorescence after 12 hours of BMDMs stimulation with medium (mock), cecal content from untreated animals (PBS CC), cecal content from ivermectin-induced gut dysbiotic mice (IVM CC), or heat-killed *Pseudomonas aeruginosa* (HKPa). (B and C) Secretion of IL6 and IL10 by BMDMs stimulated with medium (mock), cecal content from untreated animals (PBS CC), cecal content from ivermectin-induced gut dysbiotic mice (IVM CC), or heat-killed *Pseudomonas aeruginosa* (HKPa). *$p<0.05$ compared to the mock, #$p<0.05$ compared to the PBS CC, and $^{\&}p<0.05$ compared to the IVM CC. The symbols used represent analyzes performed using the t-test $p<0.05$.

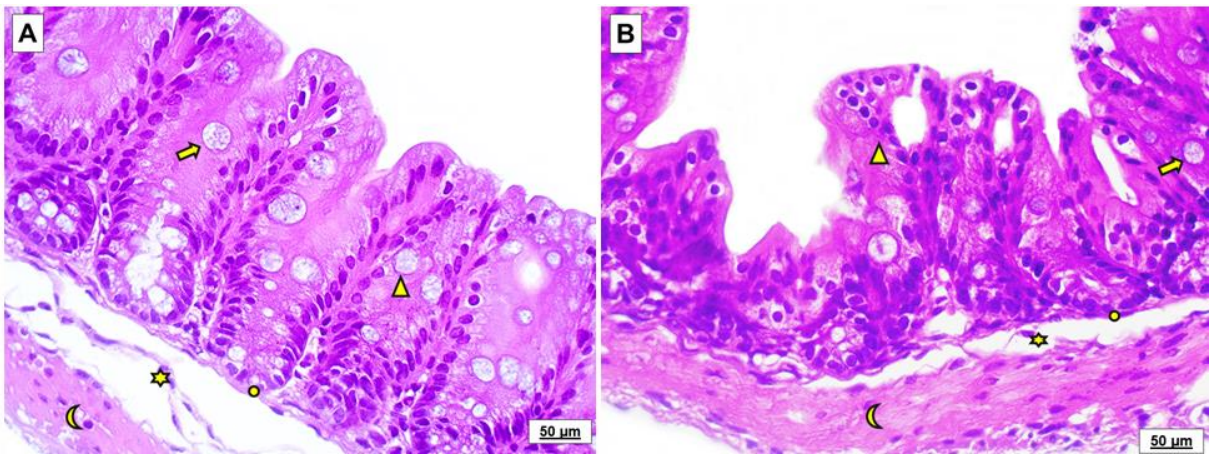


Figure 4: **Ivermectin-induced gut dysbiosis shows histopathological changes in the mice's intestinal mucosa**. A) Histopathological section of untreated C57BL/6 (PBS/C). (B) Histopathological section of C57BL/6 ivermectin-treated C57BL/6 (IVM/C). ☾ outer muscle layer; ☆ submucosal layer; ◯ muscular layer of the submucosa; ↗ goblet cell; △ enterocyte. Photomicrograph of a histological section of a representative animal cecum from each group (N=5 animals/group).

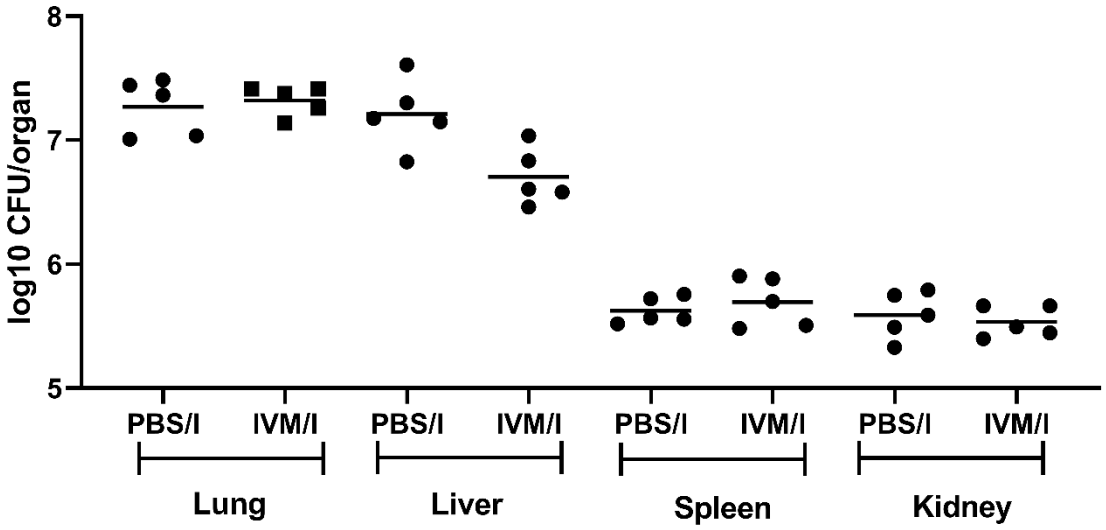


Figure 5: **Ivermectin-induced gut dysbiosis does not longer mice susceptibility to *Pseudomonas aeruginosa* pulmonary infection.** Viable bacteria were recovered from lung, liver, spleen, and kidney of mice treated (IVM/I) or not (PBS/I) with ivermectin 10 hours after infection with $1x10^5$ PA14. Data were transformed into CFU log values per organ (N=5 animals/group).

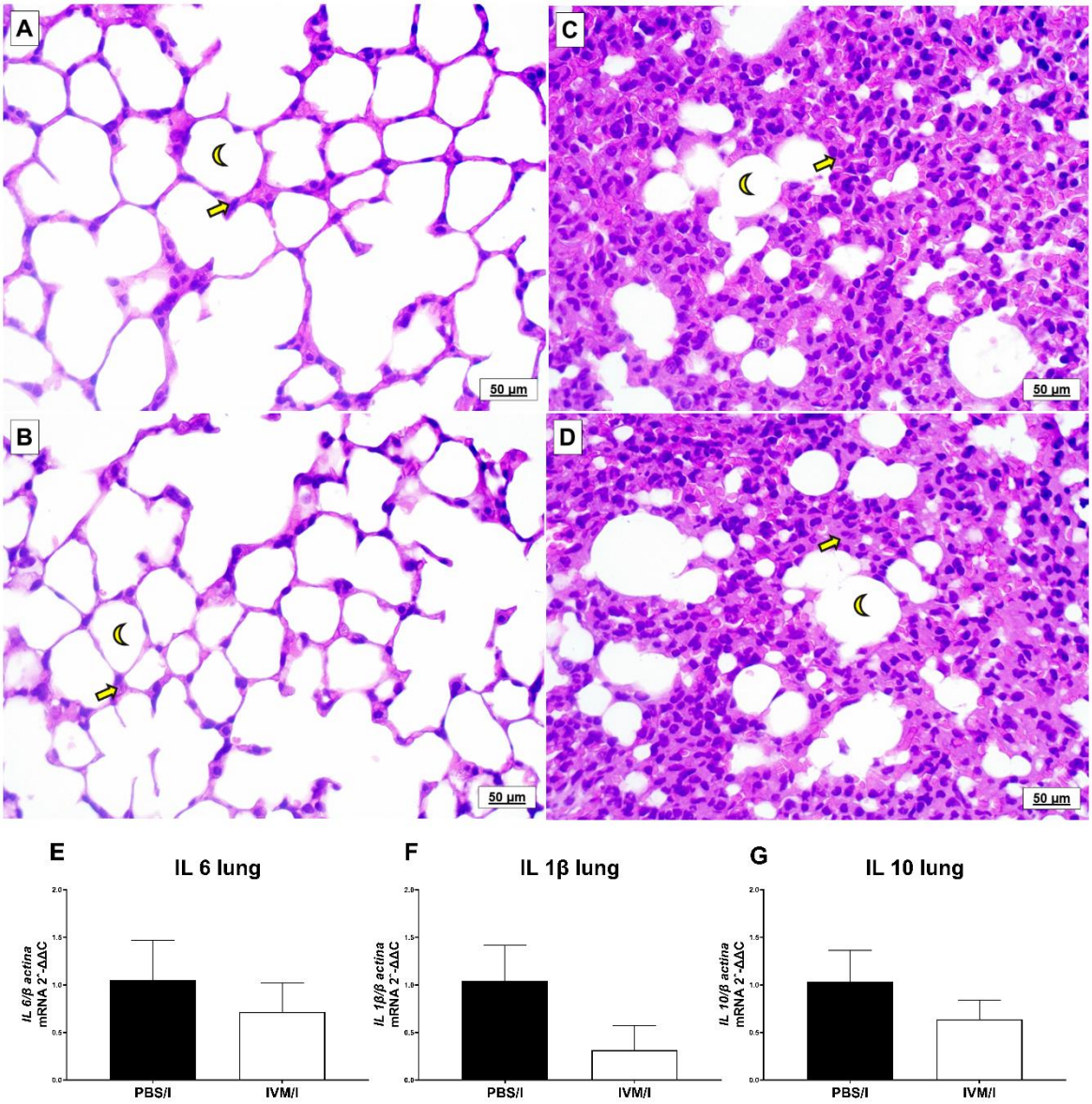


Figure 6: **Ivermectin-induced gut dysbiosis in mice did not show greater lung injury or differential expression of pro or anti-inflammatory cytokines compared to the untreated group after acute PA14 infection**. (A) Histopathological section of untreated and uninfected C57BL/6 (PBS/C). (B) Histopathological section of ivermectin-treated and uninfected C57BL/6 (IVM/C). (C) Histopathological section of untreated and infected C57BL/6 (PBS/I). (D) Histopathological section of C57L/6 ivermectin-treated and infected (IVM/I). Photomicrograph of a histological section of a representative animal lung from each group (N=5 animals/group). ☾ alveolus; ↗ alveolar septum. (EG) Analysis of differential

expression of (E) IL-6, (F) IL-1β, and (G) IL-10 in mice untreated or treated with ivermectin (PBS/I and IVM/I) and intratracheally infected with PA14 (N=5 animals/group).

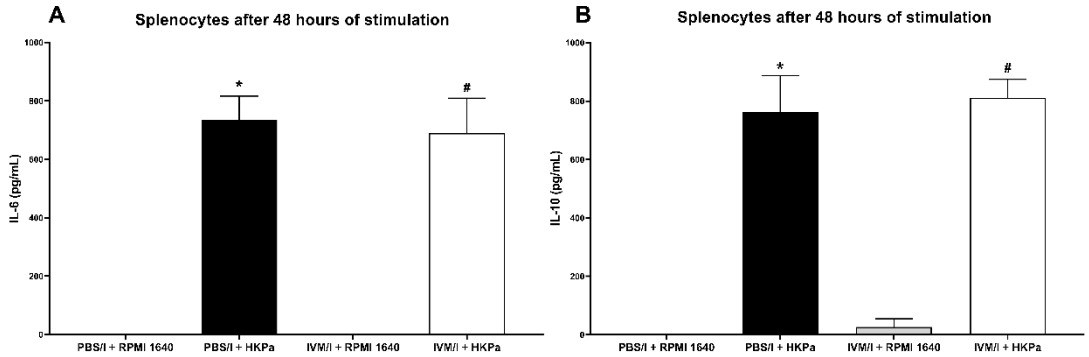


Figura 7: **HKPa-stimulated splenocytes from PA14-infected mice under ivermectin-induced gut dysbiosis show no difference in IL-6 and IL-10 secretion compared to untreated mice**. Secretion of IL-6 and IL-10 by splenocytes from untreated and infected (PBS/I) and treated and infected (IVM/I) mice stimulated with medium (RPMI 1640) or HKPa. *represents the medium stimulus compared to the HKPa of PBS/I and # represents the medium stimulus compared to the HKPa of IVM/I. The symbols used represent analyzes performed using the t-test $p<0.05$ about PBS/I + RPMI 1640 and IVM/I + RPMI 1640.

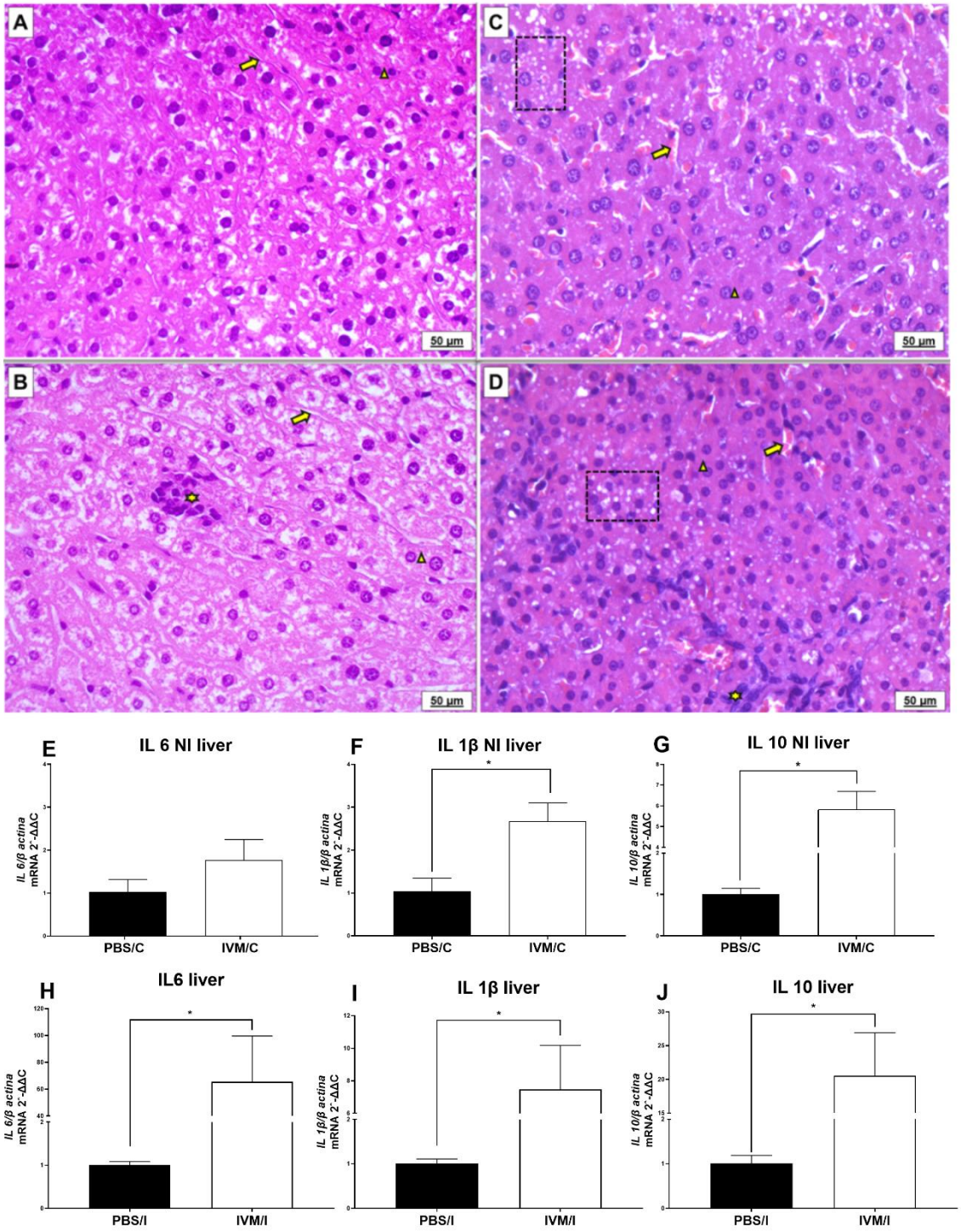


Figure 8: **Ivermectin treatment in mice generates liver damage that worsens with PA14 infection and increases the differential expression of IL-6, IL-1 β, and IL-10 regardless of the pre-established gut dysbiotic condition**. (A) Histopathological section of untreated and uninfected C57BL/6 (PBS/C). (B) Histopathological section of ivermectin-treated and uninfected C57BL/6 (IVM/C). (C) Histopathological section of untreated and

infected C57BL/6 (PBS/I). (D) Histopathological section of C57BL/6 ivermectin-treated and infected (IVM/I). Photomicrograph of a histological section of the liver of a representative animal from each group (N=5 animals/group). ☆ inflammatory infiltrate; ↗ sinusoid capillaries; △ hepatocytes; □ microvesicular steatosis. (EJ) Analysis of differential expression of (E) IL-6, (F) IL-1β, and (G) IL-10 in untreated or ivermectin treated and uninfected mice (PBS/C and IVM/C) and (H) IL-6, (I) IL-1β and (J) IL-10 in untreated or ivermectin-treated mice infected intratracheally with PA14. Analyzes were performed using the t-test *$p<0.05$ to PBS/C or PBS/I (N=5 animals/group).

2 CONCLUSÃO

O tratamento com ivermectina induziu disbiose intestinal murina caracterizada pela diminuição dos filos bacterianos *Bacteroidetes*, *Firmicutes*, *Proteobacterias* e *Tenericutes* e aumento de *Verrucomicrobia*. Este quadro disbiótico refletiu na alteração da consistência fecal e em uma maior imunoestimulação da molécula CD86 em BMDMs estimulados com conteúdo cecal de camundongos disbióticos tratados com ivermectina, impactando também no aumento da secreção de IL-6 e diminuição na secreção de IL-10 por estes macrófagos. Ademais, cortes histopatológicos verificaram um desarranjo tecidual no ceco de camundongos tratados com ivermectina. Contudo, essa disbiose intestinal não ocasionou uma maior susceptibilidade à pneumonia por *Pseudomonas aeruginosa* PA14, no qual a contagem de unidades formadoras de colônias (UFCs) no pulmão, fígado, rim e baço, cortes histopatológicos de pulmão e expressão diferencial de genes codificadores de IL-6, IL-1β e IL-10, secreção de IL-6 e IL-10 no sobrenadante de esplenócitos, reestimulados com *P. aeruginosa*, se mostraram semelhantes entre os grupos tratados com ivermectina ou PBS. Também foi verificado que o tratamento com ivermectina induziu danos hepáticos e maior expressão de genes codificadores de IL-6, IL-1β e IL-10, e que estes danos acentuaram-se após a infecção pulmonar aguda com *P. aeruginosa*. Portanto, é certo que a ivermectina induz disbiose intestinal, mas que este desequilíbrio do microbioma não impactou na homeostase pulmonar. Diversos fatores podem estar relacionados com o averiguado neste estudo, sendo necessário uma investigação mais aprofundada para compreender, por exemplo, a capacidade da ivermectina ter interagido com componentes do sistema imunológico.

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