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JÉSSICA ASSIS PEREIRA

AVALIAÇÃO DA DISBIOSE INTESTINAL CAUSADA PELA VANCOMICINA DURANTE A INFECÇÃO PELA BACTÉRIA *Pseudomonas aeruginosa*

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Dissertação apresentada como parte dos requisitos exigidos para obtenção do título de Mestre pelo Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Alfenas-MG. Área de concentração: Interação Patógeno-Hospedeiro.

Orientador: Prof. Dr. Leonardo Augusto de Almeida

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"AVALIAÇÃO DA DISBIOSE INTESTINAL CAUSADA PELA VANCOMICINA DURANTE A INFECÇÃO PELA BACTÉRIA *Pseudomonas aeruginosa*"

A Banca Examinadora, abaixo assinada, aprova a Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas.
Área de concentração: Interação Patógeno-Hospedeiro

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“O cientista não é o homem que fornece as verdadeiras respostas; é quem faz as verdadeiras perguntas”. (Claude Lévi-Strauss)

RESUMO

A *Pseudomonas aeruginosa* é uma das bactérias oportunistas mais comuns causadoras de infecções pulmonares em hospitais, e a vancomicina é amplamente utilizada em pacientes hospitalizados, influenciando sua microbiota intestinal. Dada a relevância da bactéria *P. aeruginosa* nos processos infecciosos e inflamatórios e estimulados pela necessidade de novas estratégias de tratamento, o objetivo deste trabalho foi analisar como o efeito da vancomicina sobre a microbiota intestinal murina pode favorecer ou não a infecção causada pela bactéria oportunista *P. aeruginosa* e como o uso do Transplante de Microbiota Fecal (TMF) pode atuar sobre essa resposta. Foi demonstrado neste trabalho que o conteúdo bacteriano fecal é alterado nos animais sob uso de vancomicina e que a disbiose intestinal acarretou na alteração do peso dos animais. Macrófagos derivados da medula óssea murina (BMDMs) estimulados com conteúdo cecal de camundongos em disbiose, apresentaram níveis mais altos de expressão de genes codificadores de citocinas pró-inflamatórias, tais como TNF-α, enquanto a expressão de IL-10 foi diminuída. Como foi confirmado que a disbiose bacteriana intestinal foi causada pela vancomicina e que essa alteração estimula diferencialmente macrófagos, foi avaliado se essa alteração da microbiota bacteriana intestinal altera a resposta do hospedeiro frente à infecção pulmonar pela bactéria oportunista *P. aeruginosa*. A quantidade de *P. aeruginosa* viável nos pulmões, baços e fígados de camundongos em disbiose mostraram níveis mais elevados, com mais danos no pulmão e ceco, além de apresentarem aumento da expressão de IL-10 e maior recrutamento de células CD11b+ no lavado bronco-alveolar. O fenótipo suscetível e dano tecidual foram revertidos quando camundongos em disbiose receberam o transplante de microbiota fecal. Em conjunto, os resultados demonstram que a vancomicina altera a microbiota intestinal, induzindo a disbiose intestinal murina, e o TMF demonstrou ser uma excelente estratégia para restaurar a comunidade microbiana intestinal e auxiliar no controle e prevenção de infecções bacterianas oportunistas de caráter nosocomial. Entretanto, embora nossos resultados possam esclarecer a relação entre os pulmões e os intestinos, é necessária uma investigação mais aprofundada para entender a resposta imunológica no interior dos pulmões, bem como a influência da disbiose intestinal neste contexto.

Palavras-Chave: *Pseudomonas aeruginosa*. Microbioma Gastrointestinal. Disbiose. Transplante de Microbiota Fecal.

ABSTRACT

P. aeruginosa is one of the most common opportunistic bacteria causing lung infections in hospitals, and vancomycin is widely used in hospitalized patients, influencing their intestinal microbiota. Given the relevance of P. aeruginosa bacteria in infectious and inflammatory processes and stimulated by the need for new treatment strategies, the objective of this study was to analyze how the effect of vancomycin on the murine intestinal microbiota may or may not favor the infection caused by opportunistic P bacteria. aeruginosa and how the use of TMF can affect this response. It was demonstrated in this work that the fecal bacterial content is altered in the animals using vancomycin and that the intestinal dysbiosis caused alteration in the animals weight. Murine bone marrow-derived macrophages (BMDMs) stimulated with cecal content of dysbiosis mice showed higher levels of expression of proinflammatory cytokine genes, such as TNF-α, while IL-10 expression was decreased. Since it was confirmed that intestinal bacterial dysbiosis was caused by vancomycin and that this change differentially stimulates macrophages, it was evaluated whether this change in the intestinal bacterial microbiota alters the host response to lung infection by the opportunistic bacterium P. aeruginosa. The amount of viable P. aeruginosa in the lungs, spleens, and livers of dysbiosis mice showed higher levels, more damage to the lung and cecum, and increased IL-10 expression and increased recruitment of CD11b + cells in bronchial lavage. alveolar. Susceptible phenotype and tissue damage were reversed when dysbiosis mice received fecal microbiota transplantation. Taken together, the results show that vancomycin alters the intestinal microbiota, inducing murine intestinal dysbiosis, and TMF has proved to be an excellent strategy for restoring the intestinal microbial community and assisting in the control and prevention of nosocomial opportunistic bacterial infections. However, although our results may shed light on the crosstalk between the lungs and intestines, further investigation is needed to understand the immune response within the lungs, as well as the influence of intestinal dysbiosis in this context.

Keywords: Pseudomonas aeruginosa. Gastrointestinal Microbiome. Dysbiosis. Fecal Microbiota Transplantation.

LISTA DE ABREVIATURAS E SIGLAS

BAL-		Líquido de lavado bronco-alveolar
BAL -		Bronchoalveolar Lavage
BMDM -		Macrófago derivados da medula óssea
BMDM -		Bone Marrow-Derived Macrophages
CDI	-	Infecção pelo *C. difficile*
CFU	-	Colony Forming Units
ICU	-	Intensive Care Unit
IL	-	Interleucina
OMS	-	Organização Mundial da Saúde
OTU	-	Operational Taxonomic Units
TNF	-	Fator de necrose tumoral
TLR	-	Receptor semelhante à Toll
TMF	-	Transplante de microbiota fecal
UFC	-	Unidades Formadoras de Colônias
UTO	-	Unidades Taxonômicas Operacionais

SUMÁRIO

1 INTRODUÇÃO

A *Pseudomonas aeruginosa* é um patógeno bacteriano oportunista que afeta pacientes imunocomprometidos. Devido a uma variedade de mecanismos de adaptação, sobrevivência e resistência a múltiplas classes de antibióticos, as infecções por cepas de *P. aeruginosa* resistentes a esses antibacterianos podem ser fatais, causar morbidade e são considerados como uma ameaça à saúde pública (MORADALI; GHODS; REHM, 2017). A *P. aeruginosa* é comumente associada às infecções do trato respiratório em diversos contextos clínicos, principalmente em infecções hospitalares, onde essa bactéria coloniza o trato respiratório gerando uma pneumonia aguda, sepse e morte devido ao estímulo do sistema imune em um processo inflamatório intenso. O processo inflamatório causador da pneumonia aguda pela *P. aeruginosa* é relacionada ao reconhecimento de componentes bacterianos como o lipopolissacarídeo e as proteínas flagelares pelos receptores do sistema imune inato como TLR4 e TLR5, respectivamente, acarretando na secreção de citocinas pró-inflamatórias como IL-1β, IL-6 e TNF-α (LIN; KAZMIERCZAK, 2017).

Segundo a Organização Mundial da Saúde (OMS) (2017) foi publicado uma lista de prioridades para patógenos com necessidade urgente de novas opções de tratamento onde a *P. aeruginosa* resistente a carbapenêmicos foi classificado na categoria mais alta. Diversos novos agentes foram desenvolvidos para evitar alguns dos mecanismos de resistência. A traquideavibactam, o ceftolozano-tazobactam, o cefiderocol e o imipenem cilastatina/relebactam podem fornecer atividade contra as cepas multirresistente de *Pseudomonas* sp.. Estudos publicados descrevem a eficácia clínica e microbiológica desses novos antimicrobianos. No entanto, resistências emergentes ilustram o desafio terapêutico contínuo no combate à *P. aruginosa* (NGUYEN et al., 2018).

Estratégias direcionadas para melhorar a defesa do hospedeiro e/ou limitar a inflamação excessiva podem ser importantes para melhorar o prognóstico de infecções pulmonares pela *P. aeruginosa* (SADIKOT et al, 2005). Nos últimos anos, especialmente a partir do desenvolvimento de sofisticados estudos metagenômicos, as pesquisas acerca da microbiota intestinal se intensificaram (ZHANG; FIGEYS, 2019). O advento de técnicas moleculares para o estudo de microrganismos forneceu muitos dados sobre composição, estrutura e montagem temporal da microbiota (KITISIOS et al., 2016), transformando de forma radical os nossos conhecimentos sobre o microbioma e sua relação com a manutenção

da saúde e o desenvolvimento de doenças no ser humano (PASSOS; MORAES-FILHO, 2017).

Evidências crescentes demonstram que uma alteração permanente ou não permanente da composição ou da função da microbiota, denominada disbiose, pode alterar as respostas imunológicas, o metabolismo, a permeabilidade intestinal e a motilidade digestiva, promovendo, dessa maneira, um estado pró- ou anti-inflamatório (ROSA et al 2018; GILBERT et al., 2018). Tais alterações favorecem o aparecimento de doenças metabólicas, digestivas, neurológicas, autoimunes e neoplásicas, além da maior ou menor suscetibilidade às infecções bacterianas (PASSOS; MORAES-FILHO, 2017).

Entre as causas de alteração da microbiota, o uso de antibacterianos é um dos principais, os antibióticos destroem a comunidade microbiana comensal e não patogênica e favorecem a colonização de bactérias oportunistas. Como modelo para disbiose foi escolhido o antibiótico vancomicina. É um antibiótico de amplo espectro utilizado há mais de 50 anos em infecções por bactérias Gram-positivas, especialmente para cepas resistentes a uma série de antibióticos, ocupa posição de destaque, nos pacientes em estado grave internados em hospitais. (LANES; BENDER; DELWING, 2016; WARN et al., 2016). Sendo assim a disbiose ocasionada por esse antibacteriano causaria mais susceptibilidade à infecções por *P. aeruginosa*, uma vez que esta bactéria é comum no ambiente hospitalar (ROYER et al.,2015). Além de não causar nenhuma influência nessa infecção já que a bactéria é um Gram-Negativo, e altera profundamente a microbiota intestinal que é constituída principalmente por bactéria Gram-positiva (CERF-BENSUSSAN; GABORIAU-ROTHIAU, 2010). Vários estudos têm demonstrado que o tratamento com vancomicina, que tem como alvo infecções bacterianas, culminam na disbiose intestinal, muitas vezes levando à recorrência das infecções (WARN et al., 2016).

Com intuito de restaurar a microbiota intestinal em processo de disbiose, tem sido usado o transplante de microbiota fecal (TMF) que restabelece a homeostase intestinal (RUPPÉ et al., 2018). Com o TMF, a microbiota intestinal de doadores saudáveis pode manter o microambiente dos receptores e eventualmente reconstruir o equilíbrio ecológico intestinal do receptor. Os mecanismos podem afetar os processos de doenças gastrintestinais e extra intestinais alterando a expressão gênica das células da mucosa, a função imune da mucosa intestinal, o ambiente ecológico intestinal e o metabolismo corporal, que regulam a resposta imune, a resposta inflamatória e o número e atividade de neurotransmissores (ZENG

et al., 2019). Devido a sua eficácia, o TMF é usado para tratamento de várias doenças, atualmente, o TMF tem sido aplicado para o tratamento da infecção por *Clostridium difficile* (CDI) (CAO et al., 2018). O mecanismo de ação do TMF no CDI ainda não está claro, mas seu sucesso é atribuído à restauração de uma comunidade microbiana intestinal saudável uma vez que a substancial mudança restaurativa no microbioma intestinal é uma característica fundamental do TMF (HOURIGAN et al., 2015; NADAI et al., 2017). E devido sua eficácia abriu novos caminhos terapêuticos para uma série de doenças mediadas por microbiomas, incluindo doença inflamatória intestinal, colonização por bactérias resistentes a antibióticos, distúrbios neuropsiquiátricos, síndrome metabólica e doenças autoimunes, havendo um aumento na progressão do uso de TMF ao longo dos anos (PANCHAL et al., 2018)

Dada a relevância da bactéria *P. aeruginosa* nos processos infecciosos e inflamatórios e estimulados pela necessidade de novas estratégias de tratamento, o objetivo deste trabalho foi analisar como efeito da vancomicina sobre a microbiota intestinal murina pode favorecer ou não a infecção causada pela bactéria oportunista *P. aeruginosa* e como o uso do TMF pode atuar sobre essa resposta.

CAPITULO 1:

ARTIGO: Vancomycin-induced gut dysbiosis during *Pseudomonas aeruginosa* pulmonary infection in a mice model

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BRIEF CONCLUSIVE REPORT

Vancomycin-induced gut dysbiosis during *Pseudomonas aeruginosa* pulmonary infection in a mice model

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Abstract

Pseudomonas aeruginosa is one of the most common opportunistic pathogens causing respiratory infections in hospitals. Vancomycin, the antimicrobial agent usually used to treat bacterial nosocomial infections, is associated with gut dysbiosis. As a lung-gut immunologic axis has been described, this study aimed to evaluate both the immunologic and histopathologic effects on the lungs and the large intestine resulting from vancomycin-induced gut dysbiosis in the *P. aeruginosa* pneumonia murine model. Metagenomic analysis demonstrated that vancomycin-induced gut dysbiosis resulted in higher *Proteobacteria* and lower *Bacteroidetes* populations in feces. Given that gut dysbiosis could augment the proinflammatory status of the intestines leading to a variety of acute inflammatory diseases, bone marrow-derived macrophages were stimulated with cecal content from dysbiotic mice showing a higher expression of proinflammatory cytokines and lower expression of *IL-10*. Dysbiotic mice showed higher levels of viable bacteria in the lungs and spleen when acutely infected with *P. aeruginosa*, with more lung and cecal damage and increased *IL-10* expression in bronchoalveolar lavage. The susceptible and tissue damage phenotype was reversed when dysbiotic mice received fecal microbiota transplantation. In spite of higher recruitment of CD11b+ cells in the lungs, there was no higher CD80+ expression, DC+ cell amounts or proinflammatory cytokine expression. Taken together, our results indicate that the bacterial community found in vancomycin-induced dysbiosis dysregulates the gut inflammatory status, influencing the lung-gut immunologic axis to favor increased opportunistic infections, for example, by *P. aeruginosa*.

KEYWORDS
Gut dysbiosis, Lung infection, *Pseudomonas aeruginosa*, Vancomycin

1 | INTRODUCTION

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium responsible for skin, ear, and pulmonary infections.[1] In particular, the latter occur as ventilator-related pneumonia in intensive care units (ICUs), representing one of the most common respiratory infections in these health settings.[1,2] This pathogen also causes disease in immunocompromised individuals and cystic fibrosis patients.[3] In order to treat *P. aeruginosa*-associated pneumonia, patients have to take broad-spectrum antibiotics,[2] which is followed by the development of dysbiosis, especially in the intestines,[4,5] with systemic repercussions.[6] The changes in commensal communities alter their interaction with intestinal mucosa, enhancing not only the production of certain T cell subsets, but also altering the cytokine expression in immune innate and adaptive cells.[7] These effects have also been recognized in the lungs, highlighting gut-lung crosstalk.[8] The available data suggest that changes in the operational taxonomic unit (OTU) load and/or diversity plays a significant role in predisposing patients to

Abbreviations: BAL, Bronchoalveolar lavage; BMDM, Bone marrow-derived macrophages; CFU, colony-forming unit; FMT, Fecal microbiota transplantation; ICU, Intensive care unit; OTU, Operational taxonomic units; VAN, Vancomycin.

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allergies,[9,10] asthma,[11,12] and respiratory infections.[1,13] The data also highlight the importance of macrophage and dendritic cells in directing T regulatory and T helper subsets in mouse models,[11,14] in addition to proinflammatory cytokine release in the gut,[8] in shaping lung immunologic-mediated outcomes. Recently, Dumas and colleagues demonstrated that antibiotic-induced dysbiotic mice had an increased early lung colonization and infection by *Mycobacterium tuberculosis*.[15] Respiratory capacity is mildly influenced by microbiota from other mucosal surfaces subject to a dysbiotic state.[16] Consequently, the present study aimed to evaluate the immunologic and histopathologic effects of vancomycin-induced gut dysbiosis in a *P. aeruginosa* pneumonia murine model on the lungs and the large intestines, in order to analyze lung-gut crosstalk.

2 | MATERIALS AND METHODS

2.1 | Mice, ethics statement, and bacterium

C57BL/6 mice were used according to the protocol approved by the Committee on the Ethics of Animal Experiments of the UNIFAL (CEUA 40/2017). The virulent strain PA14 of *P. aeruginosa* was grown in Luria Broth (LB) to the log phase.

2.2 | Antibiotic treatment, fecal microbiota transplantation (FMT), and *P. aeruginosa* intratracheal infection

Mice were randomly separated into 8 groups: PBS—received sterile PBS by gavage for 14 d; VA—received 10 mg/ml of vancomycin solution for 14 d; PA—received PBS and were intratracheally infected with PA14 strain; VA + PA—received both vancomycin and the aforementioned infection treatment; PBS + FZ—received the same treatment as the PBS group and FMT; VA + FZ—received the same antibiotic and FMT treatments; PBS + PA + FZ—received both infection and FMT regimens; and VA + PA + FZ—received antibiotic, infection, and FMT treatments. FMT occurred by gavage over 4 d with 1 mg fecal material, solubilized in 1 mL sterile PBS, from nontreated mice, followed by 14 d of microbiota recovery.

2.3 | Microbiome metagenomics from fecal samples and microbiome analysis from metagenomic sequences

Total DNA was extracted from fecal samples of the PBS or VA groups using a QIAamp DNA Stool Mini Kit (QIAGEN Inc., Valencia, CA, USA). Equal amounts of purified total DNA were used to analyze 16S rRNA sequences using the Illumina HiSeq platform (Illumina, San Diego, CA, USA). Metagenomic sequences were analyzed and OTUs were identified as contigs of sequences with ≥97% similarity. Proteobacteria and bacteroidetes were evaluated in fecal samples after FMT by real-time PCR (qPCR) using specific primers (Table S1). Data were presented as relative expression units compared to PBS group.

2.4 | Lung, liver, and spleen homogenates for residual *P. aeruginosa* CFU, histopathologic, and stereologic analysis

The lungs, livers, and spleens of animals from each group were homogenized in PBS and plated on LB agar to count CFUs. Histologic sections were captured, and the histopathologic changes were analyzed by Image Pro-Plus® 4.5 software (Media Cybernetics Inc., Silver Spring, MD, USA).

2.5 | RT-PCR for in vitro evaluation of bone marrow-derived macrophages (BMDMs) stimulated by cecal content

BMDMs were obtained as previously reported by our group[17] and stimulated with autoclaved cecum content from PBS or VA mice. The total RNA was obtained from cells and reverse transcription of 1 μg total RNA was performed using illustra™ Ready-To-Go RT-PCR Beads (GE Healthcare, Little Chalfont, United Kingdom). Real-time qPCR was performed with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) containing SYBR® Green PCR Master Mix (Applied Biosystems), with cDNA as the PCR template and primers to amplify specific fragments corresponding to specific gene targets (Table S1). All data were presented as relative expression units compared with nonstimulated cells after normalization to the β-*actin* gene.

2.6 | Bronchoalveolar lavage (BAL) and flow cytometry

Mice tracheas were cannulated after they were euthanized. The airways were perfused with sterile cold PBS providing the BAL fluid. Viable cells were evaluated by their differential expression of CD11b and CD80 molecules with a Guava® easyCyte Flow Cytometer (Millipore Sigma, Burlington, MA, USA), or their differential expression of pro- or anti-inflammatory genes by qPCR, as described above.

2.7 | Statistical analysis

Graphs and data analysis were performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA), by 1-way ANOVA or 2-way ANOVA (Bonferroni post hoc test).

3 | RESULTS AND DISCUSSION

3.1 | Vancomycin-induced gut dysbiosis in mice was characterized by an increase in proteobacteria and decrease in bacteroidetes

In order to evaluate if vancomycin could induce gut dysbiosis, mice underwent 14 d of antibiotic gavage and the microbiota from their feces were compared to untreated mice. Analysis of metabarcoding sequences showed that the microbiota from vancomycin-treated mice

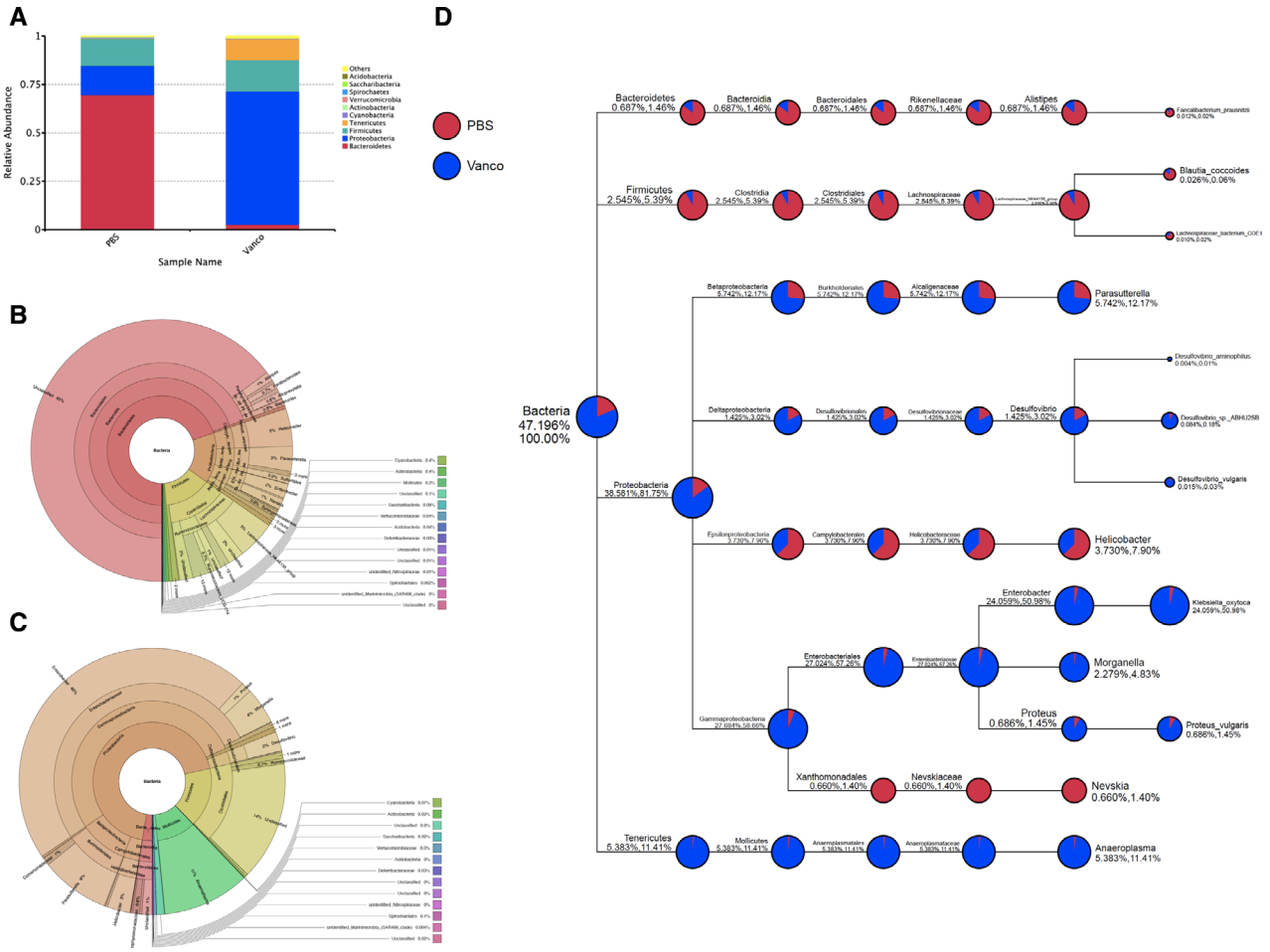

FIGURE 1 **Vancomycin induces gut dysbiosis in mice.** (**A**) Organizational taxonomic units (OTU) based on phyla found in feces from PBS mice and from vancomycin-induced gut dysbiosis. (**B**)–(**C**) Krona analysis from OTU showing the decrease of the *Bacteroidetes* population and increase of *Proteobacteria* in vancomycin-treated mice feces. (**D**) Taxonomic tree of specific species in PBS (red) or vancomycin- (blue) treated mice. The sizes of circles represent the relative abundance of species. The first number below the taxonomic name represents the percentage in the whole taxon, whereas the second number represents the percentage in the selected taxon

was quite different from that of nontreated mice, demonstrating gut dysbiosis caused by this antibiotic (Fig. 1A). High throughput metagenomic analysis showed that dysbiotic mice harbored a higher percentage of *Proteobacteria* (from 15% to 69%) and lower percentage of *Bacteroidetes* populations in feces (from 70% to 3%) when compared to the controls (Fig. 1B and C). Regarding specific taxa, *Enterobacter* sp. and *Proteus* sp. thrived among dysbiotic microbiota (Fig. 1D). Our results were consistent with the pharmacodynamics of vancomycin and previous reports.[18,19] Vancomycin-induced gut dysbiosis significantly disrupted OTUs in a study by Frölich et al., using principal coordinate analysis.[20] Another group showed that vancomycin-treated rats also displayed higher amounts of *Proteobacteria*, alongside increased *Verrumicrobiaceae* bacteria,[19] highlighting a similar microbiota disruption. Moreover, an *Akkermansia* population was also identified in a mouse model of dysbiosis.[21] Initial antibiotic treatment decreased the weight of mice compared to controls; however, after 5 d, no significant differences were observed. Conversely, when gut-dysbiotic mice received FMT, the animals gained weight and retained it until the end of the experiment (Fig. S1). In a previous study, vancomycin-treated rats weighted the same as controls.[19] To prove that the bacterial microbiota was recovered, we conducted qPCR for bacteroidetes and proteobacteria in fecal samples of mice under vancomycin or FMT treatment. The results showed that both bacterial phyla changed during antibiotic usage but their relative amounts were returned to nondysbiotic mice features (Fig. S2). Although FMT reverts the relative amount of the specific bacterial groups in feces, it is important to note that continuous vancomycin treatment can disturb the microbiota elsewhere in the mouse body. The recovery of gut microbiota by FMT does not necessary recover the disturbance that occurred in all mice tissues.

3.2 | BMDMs stimulated with cecal contents from dysbiotic mice demonstrated a high expression of proinflammatory genes

Because it was demonstrated that the fecal content is altered in mice under vancomycin treatment, BMDMs were obtained and we examined whether cecum contents could induce differential expression of pro- or anti-inflammatory genes. *TNF-α* was up-regulated (Fig. 2A)

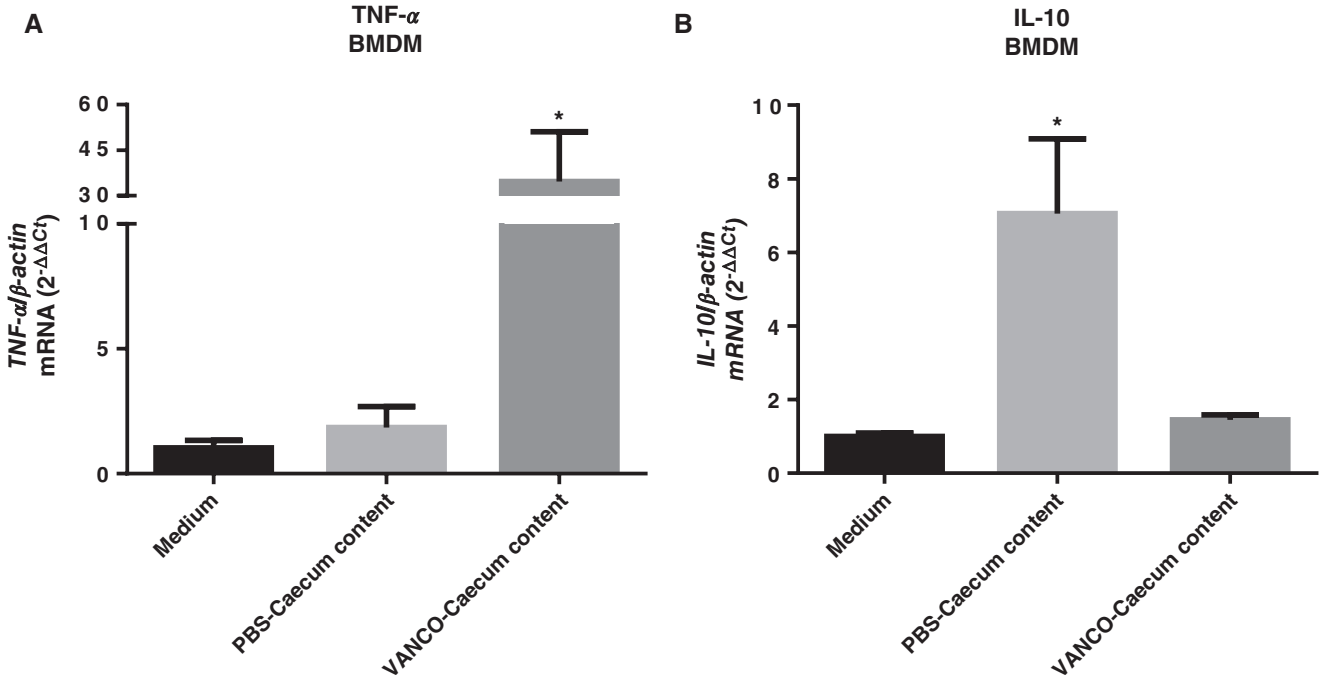

FIGURE 2 **BMDMs stimulated by cecal content from vancomycin dysbiotic mice induced high expression of proinflammatory genes.** Differential expression of (**A**) *TNF-α* or (**B**) *IL-10* of BMDM stimulated with medium, cecal content from nondysbiotic mice (PBS-cecum content) or cecal content from vancomycin-induced gut-dysbiotic mice (VANCO-cecum content). Transcript levels were measured by real-time RT-PCR. Error bars represent the mean ± SD of samples assayed in triplicate. A representative of at least 3 experiments is shown. $^{*}P < 0.05$ relative to the medium group

whereas *IL-10* (Fig. 2B) was down-regulated in BMDMs stimulated by cecal content from dysbiotic mice, when compared cecal content from nondysbiotic mice. This result was due to the differential content of bacterial groups found in dysbiotic and nondysbiotic mice (Fig. 1). It is well defined that dysbiosis can augment the proinflammatory status of the intestine, leading to a variety of diseases.[22,23] Notably, inflammatory bowel disease (IBD) carriers are more susceptible to *Clostridium difficile* infection, due to the intestinal proinflammatory microbiome found among these patients.[24] Possibly, the bacterial communities present in vancomycin-induced dysbiosis dysregulate the gut inflammatory status, increasing proinflammatory cytokine expression when compared to nondysbiotic mice. This hypothesis is well defined for IBDs.[25]

3.3 | Vancomycin-induced gut-dysbiotic mice displayed greater cecum measurements than control mice, but there were no differences regarding lung-infected animals with *P. aeruginosa*

An evaluation of intestinal patterns focusing on histologic changes of the cecum was made, due to an increased size of this structure during vancomycin treatment, and it was observed that all mice groups displayed statistically significant differences (Fig. 3). Mice under vancomycin treatment had a thicker cecal epithelium and mucosa compared to all other groups, in addition to a greater crypt width (Fig. 3B and D). There were significant changes when compared to control mice. However, there were no significant differences among these morphologic features between dysbiotic (Fig. 3B) and dysbiotic groups infected in the lung with the opportunistic bacterium *P. aeruginosa* (Fig. 3C), though the latter presented with lower average measures (Fig. 3H-J). Control mice had no overt changes compared with infected and FMT groups (Fig. 3H-J). Our analysis showed that the cecum showed changes specifically in antibiotic-treated groups, but nonsignificant differences among just infected mice. By contrast, Aguilera et al. found no statistical difference in crypt and mucous layer lengths, despite the tendency of these structures to be wider among antibiotic-treated mice.[26] Similarly, Cheng et al. found no difference in crypt width between vancomycin-treated and control mice.[27] Notably, a lower goblet cell density has been reported, as well as a significantly altered mucin content inside these vesicles.[26] In a streptomycin-induced gut-dysbiosis study, a cystic fibrosis mice model displayed no significant histopathologic changes.[28] This data highlights the specificity of the microbiota disruption in the cecum structure induced by vancomycin. Furthermore, mucin-2 and zonula occludens-1 were higher and lower, respectively, in the colon of antibiotic-treated mice.[27] These reports suggest that an altered gut microbiota has tissue and functional effects over immunologic and physiologic gastrointestinal tract homeostasis.

3.4 | Vancomycin-induced gut-dysbiotic mice were more susceptible to *P. aeruginosa* lung infection

The gut-lung crosstalk proposal indicates how changes in commensal intestinal microorganisms can influence lung responses.[8] In spite

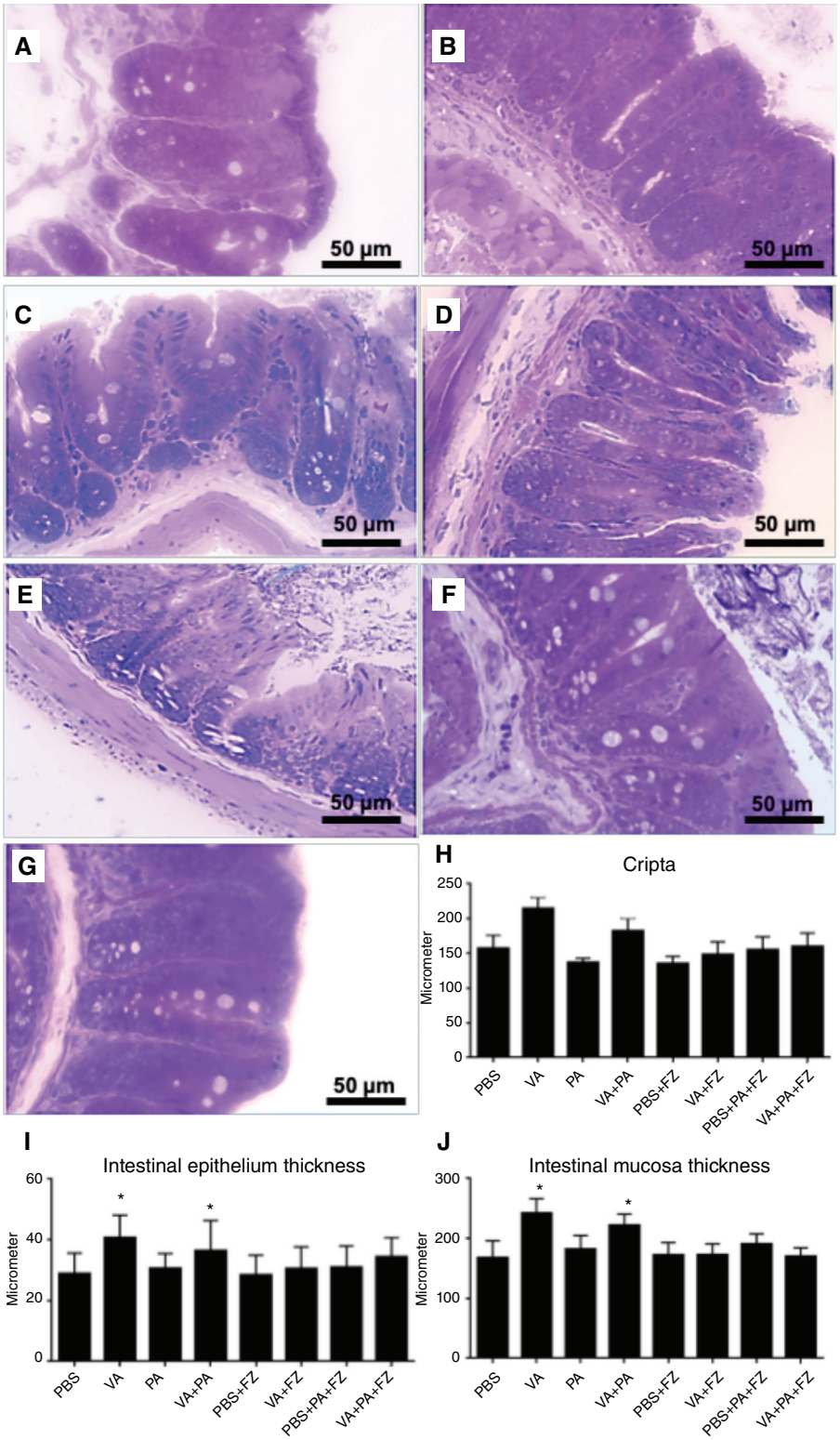

FIGURE 3 **Vancomycin-induced gut-dysbiotic mice showed increased damage to the cecum, independent of lung infection by *P. aeruginosa*.** Digital images from histopathologic analyses of: (**A**) PBS, noninfected C57BL/6 mice; (**B**) vancomycin, noninfected C57BL/6 mice; (**C**) PBS, infected C57BL/6 mice; (**D**) vancomycin, infected C57BL/6 mice; (**E**) PBS, FMT, and noninfected C57BL/6 mice; (**F**) PBS, FMT, and infected C57BL/6 mice; and (**G**) vancomycin, FMT, and infected C57BL/6 mice. Stereologic analyses of (**H**) cecum crypt size, (**I**) intestinal epithelium thickness, and (**J**) intestinal mucosa thickness. A representative of at least 2 experiments is shown. $^{*}P < 0.05$ related to PBS group; $\#P < 0.05$ related to PA group

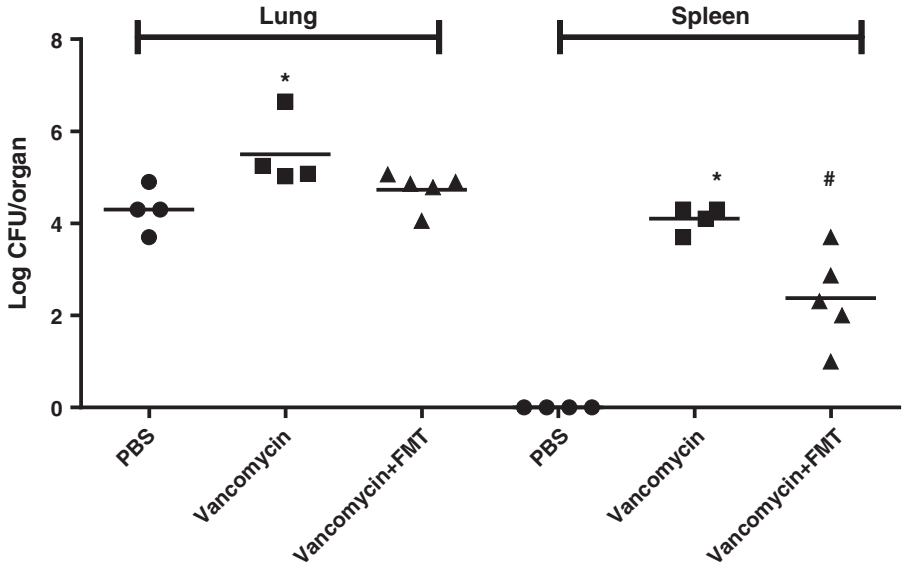

FIGURE 4 **Vancomycin-induced gut-dysbiotic mice were more susceptible to *P. aeruginosa* lung acute infection and recover the nondysbiotic phenotype after FMT.** Viable bacteria were recovered from lungs and spleen of nondysbiotic mice (PBS), dysbiotic mice (vancomycin) or dysbiotic mice that received FMT (vancomycin + FMT) 10 h post-infection. A representative of at least 3 experiments is shown. $^{*}P < 0.05$ related to the PBS group; $\#P < 0.05$ related to the vancomycin group

of the fact that no changes were observed in gut structure when nondysbiotic mice were lung-infected, we proposed that vancomycin treatment could favor the colonization of, and infection with, opportunistic lung bacteria, such as *P. aeruginosa*, one of the most common opportunistic bacteria causing nosocomial infections.[18] Moreover, vancomycin is widely used in hospitalized patients, influencing their gut microbiota.[18] Vancomycin-induced gut-dysbiotic mice and nondysbiotic mice were intratracheally infected and their residual bacteria were assessed. Viable bacteria were recovered from both groups of mice, with a greater number of bacteria in gut-dysbiotic mice (Fig. 4). Furthermore, *P. aeruginosa* was also recovered from the spleen of gut-dysbiotic mice, but not from control mice. This indicated that the dysbiosis caused by vancomycin in mice intestines could be related to an increased susceptibility to *P. aeruginosa* lung infection. To investigate further, dysbiotic mice received FMT from nondysbiotic mice and were challenged with *P. aeruginosa*. The recovered bacteria in these mice indicated a decrease in susceptibility when compared to dysbiotic mice, and no statistically significant difference was observed in CFUs when compared to nondysbiotic mice (Fig. 4). We showed that vancomycin-induced gut dysbiosis enhanced *P. aeruginosa* recovery in the lungs, and promoted lung infections to become systemic, as indicated by bacteria being found in the spleen and in the liver (Fig. S3). CFU amounts recovered from the lungs of control mice were similar to those from other reports.[29] In another study, *P. aeruginosa* CFU were recovered from the spleen of infected mice, but this has not previously been shown in a dysbiosis model.[30] Moreover, previous studies have not evaluated *P. aeruginosa* bacterial load in lungs, spleen, or liver during antibiotic-induced gut dysbiosis. Cheng et al. noted a lower spleen organ index in vancomycin-treated mice compared to controls, though T lymphocyte subsets did not diverge from controls.[27] Khailova et al. showed a higher proinflammatory cytokine expression in the spleen and colon of mice during pulmonary infection with *P. aeruginosa*, which was ameliorated by probiotic treatment, highlighting lung-gut crosstalk.[31]

3.5 | Vancomycin-induced gut-dysbiotic mice displayed greater lung lesions after *P. aeruginosa* infection

After CFU evaluation, histopathologic and stereologic analysis of the lungs of mice infected with *P. aeruginosa* were performed. An increase in inflammatory infiltrates was observed in lung sections from all groups after *P. aeruginosa* infection (Fig. 5C, D, F, and G). It was not possible to identify the cells by microscopy, though greater infiltrates of polymorphonuclear cells and monocytes were observed. Mice infected with bacteria displayed reduced alveolar spaces and a wider septum compared to the control group (Fig. 5H-J). Antibiotic-treated mice showed lower alveoli per unit area than controls, though there were more points in the septa (Fig. 5H). Vancomycin-induced dysbiosis showed no statistically significant differences as compared to controls with regard to septum width and alveoli per unit area (Fig. 5). Remarkably, among all groups, dysbiotic mice that were infected featured the greatest morphologic lung changes, compared to control and infected mice (Fig. 5D and E). The characteristics of the dysbiotic group resembled those seen in infected mice after FMT (Fig. 5C and G). The lungs of animals under the antibiotic regimen had substantial tissue damage, suggesting that gut microbiota disruption might have enhanced inflammatory responses against *P. aeruginosa*. Pulmonary septa were enlarged and the alveoli number per unit area was diminished. Other studies that have also assessed how intestinal dysbiosis affects lungs have found histopathologic changes in the respiratory system. The lung pathology score was

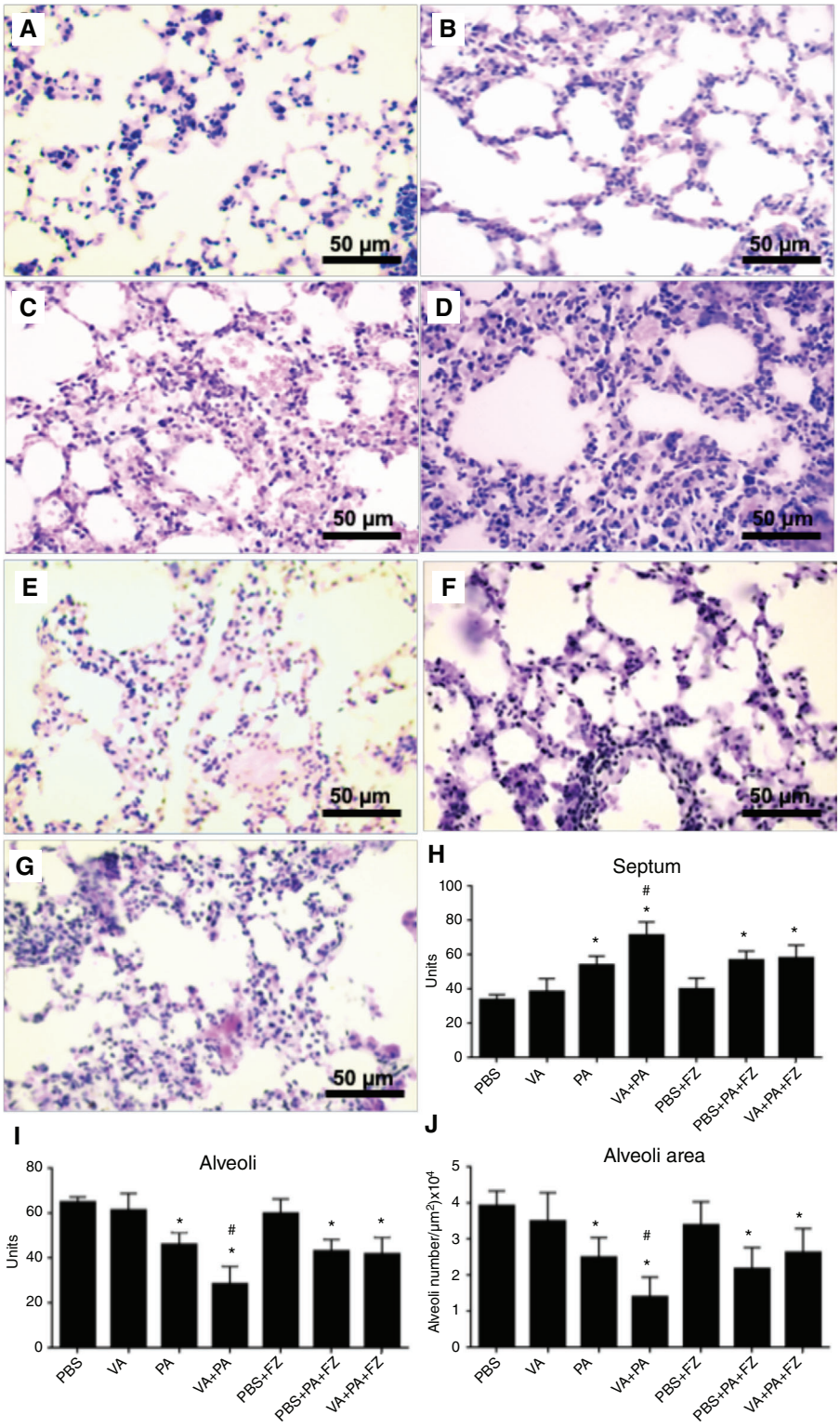

FIGURE 5 **Vancomycin-induced gut-dysbiotic mice displayed a greater lung lesion after *P. aeruginosa* acute infection.** Digital images from histopathologic analyses of: (**A**) PBS, noninfected C57BL/6 mice; (**B**) vancomycin, noninfected C57BL/6 mice; (**C**) PBS, infected C57BL/6 mice; (**D**) vancomycin, infected C57BL/6 mice; (**E**) PBS, FMT, and noninfected C57BL/6 mice; (**F**) PBS, FMT, and infected C57BL/6 mice; and (**G**) vancomycin, FMT, and infected C57BL/6 mice. Stereologic analyses of (**H**) lung septum thickness, (**I**) number of alveoli, and (**J**) alveoli area in the lungs. Mice lungs were evaluated 10 h post-infection. A representative of at least 2 experiments is shown. $^{*}P < 0.05$ related to PBS group; $\#P < 0.05$ related to the PA group

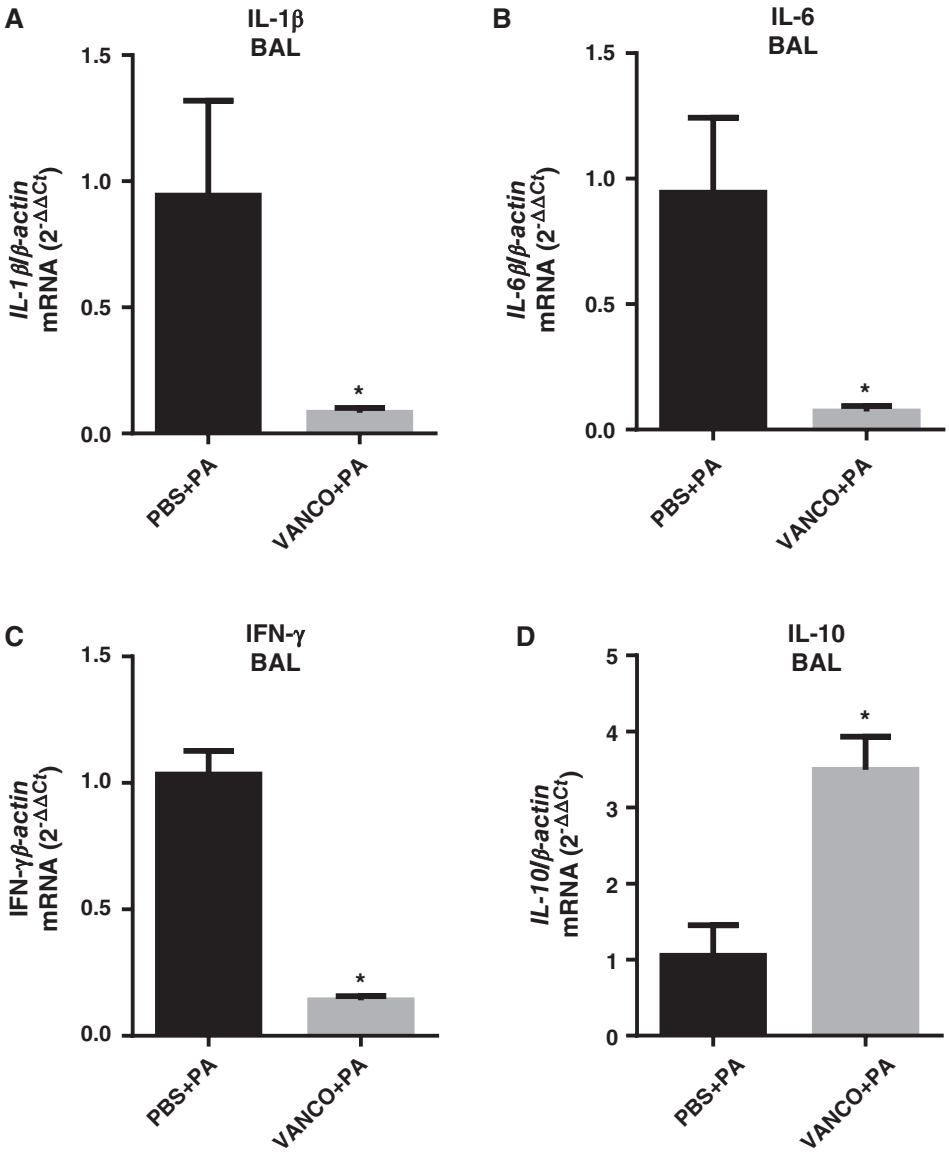

FIGURE 6 **BAL fluid showed increased *IL-10* expression in dysbiotic mice during P. aeruginosa infection in vancomycin-induced gut-dysbiotic mice.** Differential expression analysis of (**A**) *IL-1β*, (**B**) *IL-6*, (**C**) *IFN-γ*, and (**D**) *IL-10* from BAL fluid of nondysbiotic infected mice (PBS + PA) or vancomycin-induced gut-dysbiotic infected mice (VA + PA). BAL was evaluated ten hours post-infection. Transcript levels were measured by real-time RT-PCR. Error bars represent the mean ± SD of samples assayed in triplicate. A representative of at least 3 experiments is shown. $^{*}P < 0.05$ relative to the PBS + PA group

higher in an asthma mice model treated with vancomycin, enhancing airway responsiveness.[11,12]

3.6 | BAL fluid showed increased *IL-10* expression in dysbiotic mice independent of CD80 expression in CD11b+ or DC+ cells

BAL fluid from infected mice was collected and the percentages of CD11+ or DC+ cells were evaluated. In spite of an increased percentage of CD11+ cells in lungs from nondysbiotic infected mice, there was no difference in CD80+ expression when comparing dysbiotic to infected mice (Fig. S4). No differences were observed for DC+ cells, either in their influx or in their CD80+ expression. These results demonstrated that the influx of CD11+ cells to infected lungs expressing the co-stimulator molecule CD80+ was independent of gut dysbiosis. However, the expression of proinflammatory genes *IL-1β*, *IL-6*, or *IFN-γ* were down-regulated in cells coming from BAL fluid of dysbiotic infected mice when compared to nondysbiotic mice (Fig. 6A-C). By contrast, *IL-10* expression was up-regulated in dysbiotic BAL fluid (Fig. 6D). These results indicated that, even though the anti-inflammatory gene *IL-10* was up-regulated in BAL fluid from dysbiotic infected mice, the lung pathology demonstrated a proinflammatory profile. Vancomycin treatment has been associated with a decrease in T regulatory subsets in the colon, but not in the stroma of the lungs.[11] As our model of infection is based in an acute stimulation by *P. aeruginosa*, we hypothesized that innate immunity cells could

be differentially recruited to the mouse lung, showing differences in controlling this bacterium. Despite the greater influx of CD11b+ cells into the lungs of dysbiotic infected mice when compared to those of nondysbiotic infected mice, there were no observed differences in CD80 expression. By contrast, in dysbiotic infected mice, cytokine expression revealed decreased expression of *IL-1β*, *IL-6*, and *IFN-γ* in BAL fluid. Surprisingly *IL-10* expression increased, even though the inflammatory lung pattern was bigger in these mice when compared to nondysbiotic mice. This could be related to a greater number of viable bacteria being presented in the lungs from dysbiotic mice that still stimulated the influx of inflammatory cells but was independent of up-regulated of *IL-1β*, *IL-6*, and *IFN-γ* genes. Further studies would help to better understand why *IL-10* expression was up-regulated in BAL fluid from dysbiotic infected mice. IL-10 displayed higher concentrations in the lungs after probiotic treatment, which was due to higher T regulatory cell numbers in the lungs of mice.[31] During lung infection by *M. tuberculosis*, no significant differences were observed in the recruitment of macrophages, dendritic cells and neutrophils, or in the expression of IFN-γ, TNF-α, and IL-1.[15] However, mucosal-associated invariant T (MAIT) cells producing IL-17A were reduced in dysbiotic *M. tuberculosis*-infected mice.[15] Our results could open a new perspective in understanding if there are differences in MAIT cells involved in *P. aeruginosa* control, when compared to mice with normal microbiota. Notably, it has been demonstrated that IL-17A production mediated by monocyte and innate lymphoid cell interaction is necessary to clear *Klebsiella pneumoniae* lung infections.[32] NK cells, and their receptor NKG2D, have been implicated in *P. aeruginosa* clearance through *IFN-γ* expression.[30,33] This might indicate different cytokine and immune cell roles against specific pulmonary pathogens inside the lungs. Although our results shed light on lung-gut crosstalk, further investigation is needed to understand the immunologic response inside the lungs, as well as the influence of gut dysbiosis over it.

AUTHORSHIP

C.P.R., J.A.P., P.P.C., and L.A.A conceived and designed the experiments; C.P.R., J.A.P., N.C.M., G.A.B., E.N.S., R.D.N., and L.A.A. performed the experiments; C.P.R., J.A.P., N.C.M., C.A.T., R.D.N., E.N.S., P.P.C., and L.A.A. analyzed the data; and C.P.R., J.A.P., C.A.T., E.N.S., R.D.N., P.P.C., and L.A.A. wrote or contributed to the writing of the manuscript. All authors read and approved the final manuscript. C.P.R. and J.A.P. contributed equally to this work.

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DISCLOSURE

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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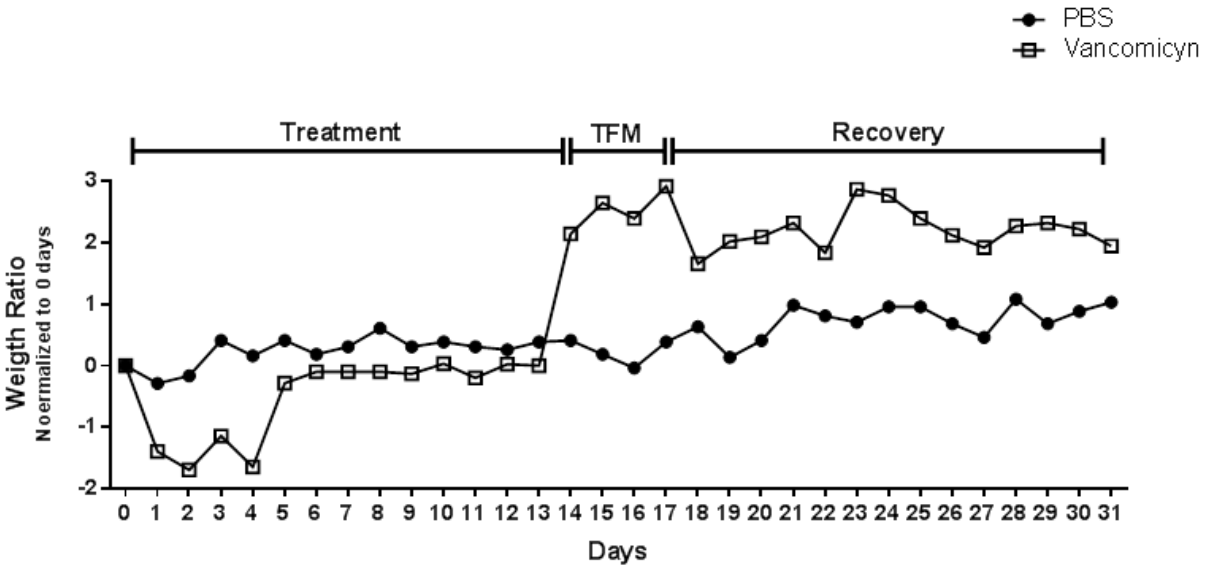
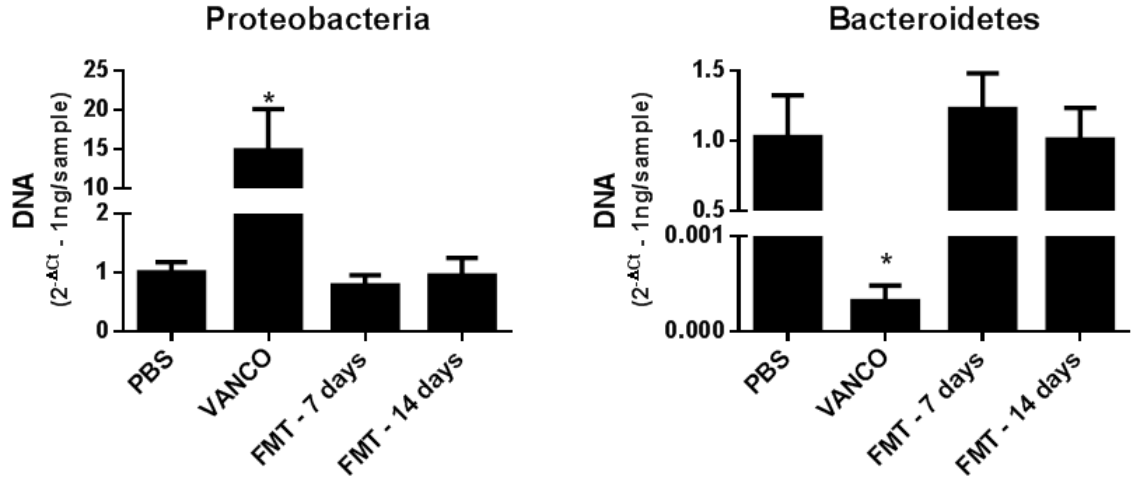
FIGURE S1. Animals receiving vancomycin lost weight in the first days of treatment but increased weight during FMT. Evaluation of the weight of C57BL/ 6 animals during treatment with the antibiotic vancomycin or PBS. The data are represented as mean weight ratios in relation to day 0.



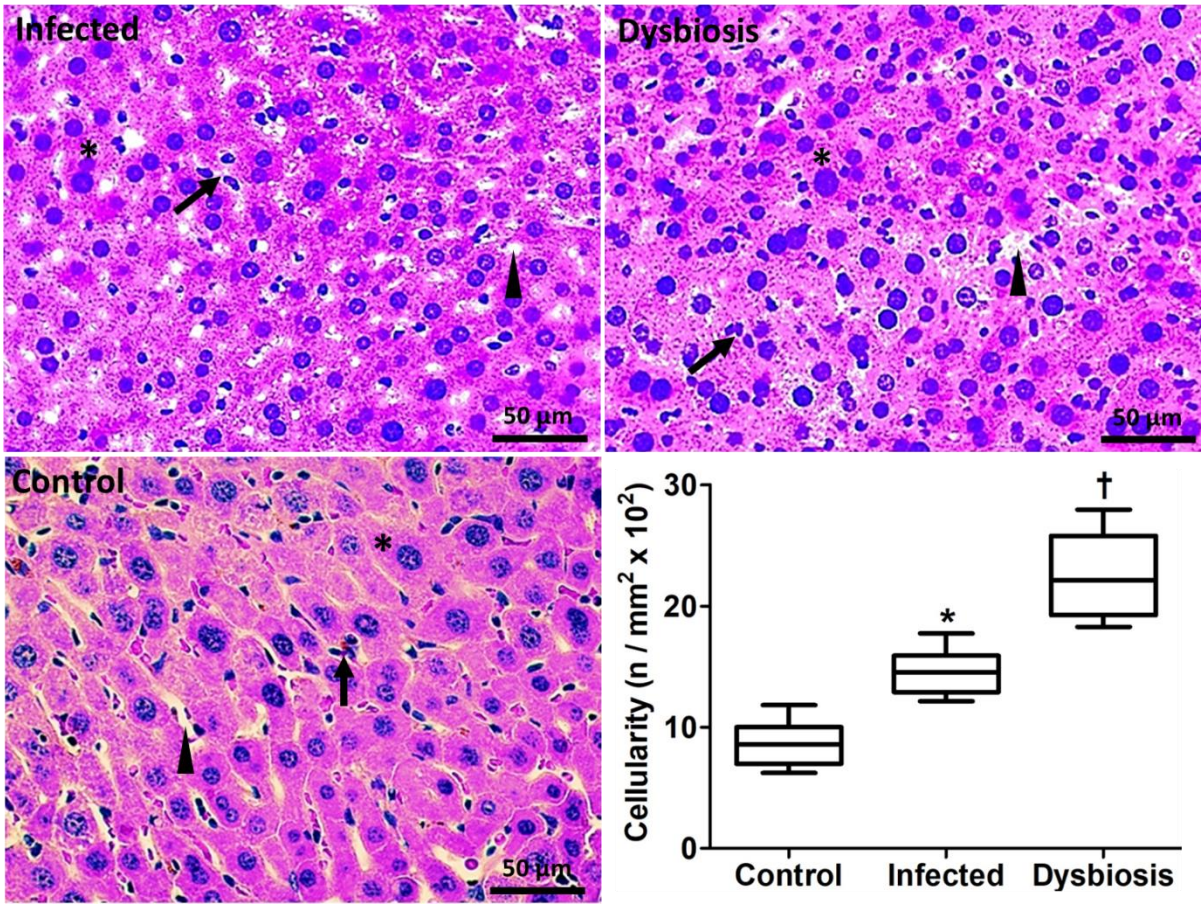
Fonte: (Rosa et al.,2019)

FIGURE S2. Fecal of microbiota transplantation recovery the relative abundance of Proteobacterias and Bacteroidetes in vancomycin-induced gut-dysbiotic mice. qPCR in fecal samples of PBS, vancomycin-treated mice or vancomycin-treated mice that received FMT by gavage for 4 d following 7 or 14 d of microbiota recovery. (A) Proteobacteria showed increase in dysbiotic mice but it decreases after FMT. On the other hand, Bacteroidetes population (B) showed decreased in dysbiotic mice but this bacterial population recovered in the feaces samples after FMT. *P < 0.05 related to PBS group. A representative of at least 2 experiments is shown.



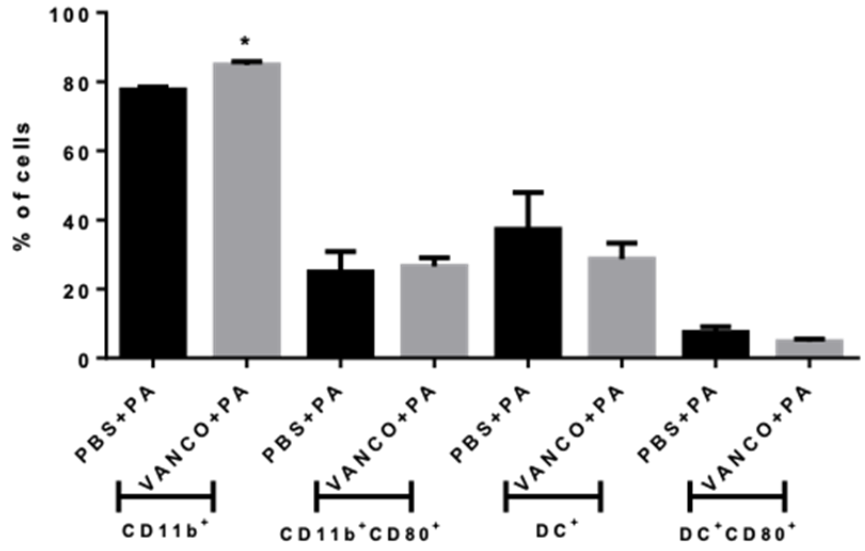
Fonte: (Rosa et al., 2019)

FIGURE S3. Vancomycin-induced gut-dysbiotic mice displayed a greater liver cellularity and susceptibility to P. aeruginosa acute infection. Digital images from histopathologic analyses of: (A) PBS, infected C57BL/6 mice; (B) vancomycin, infected C57BL/6 mice; (C) PBS, noninfected C57BL/6 mice. In all images: Asterisk, hepatocytes; Arrow, interstitial/inflammatory cells; Arrowhead, sinusoidal capillaries. (D) Cellularity analyses of hepatic tissues. Mice livers were evaluated ten hours post-infection. *P < 0.05 related to control group (PBS, noninfected C57BL/6 mice); †P < 0.05 related to infected group (PBS, infected C57BL/6 mice). (E) Viable bacteria were recovered from liver from PBS, infected C57BL/6 mice (PBS) or vancomycin, infected C57BL/6 mice (Vamcomycin). *P < 0.05 related to control group (PBS). A representative of at least 2 experiments is shown.



Fonte: (Rosa et al., 2019)

FIGURE S4. No differences of CD80+ expression was observed in BAL cells from dysbiotic and nondysbiotic infected mice in spite of increase of CD11b+ cells influx in lungs from vancomycin-induced gut-dysbiotic mice when infected with P. aeruginosa. Flow cytometry evaluation of CD80 marker in CD11b+ or DC+ cells from BAL fluid of dysbiotic or nondysbiotic infected mice. BAL was evaluated ten hours post-infection. Error bars represent the mean ± SD of samples assayed in triplicate. A representative of at least 3 experiments is shown. *P < 0.05 relative to PBS+PA group.



Fonte: (Rosa et al., 2019)

TABLE S1. Primers sequences used to evaluate the pro- or anti-inflammatory gene expression and also to evaluate the abundance of Bacteroidetes or Gammaproteobacteria in mice feaces targeting 16S rRNA gene.

Primer	Forward	Reverse
IL-12	5'-TGGTGTCTCCACTCATGG-3'	5'-AGCAGCAGATGTGAGTGG-3'
IL-1β	5'-TGACCTGGGCTGTCCAGATG-3'	5'-CTGTCCATTGAGGTGGAGAG-3'
TNF-α	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	5'-TGGGAGTAGACAAGGTACAACCC-3'
IL-6	5'-CCAGGTAGCTATGGTACTCCAGAA-3'	5'-GATGGATGCTACCAAACTGGA-3'
IL-10	5'-GGTTGCCAAGCCTTATCGGA-3'	5'-ACCTGCTCCACTGCCTTGCT-3'
IFN-γ	5'-TCTGGAGGAACTGGCAAAAG-3'	5'-TTCAAGACTTCAAAGAGTCTGAGG-3
β-actin	5'-AGGTGTGCACCTTTTATTGGTCTCAA-3'	5'-TGTATGAAGGTTTGGTCTCCCT-3
Bacteroidetes	5'-GTTTAATTCGATGATACGCGAG-3'	5'-TTAASCCGACACCTCACGG-3'
Gammaproteobacterias	5'-GCTAACGCATTAAGTRYCCCG-3'	5'-GCCATGCRGCACCTGTCT-3'

Nucleotide symbols: R = A or G; Y = C or T; S = C or G.

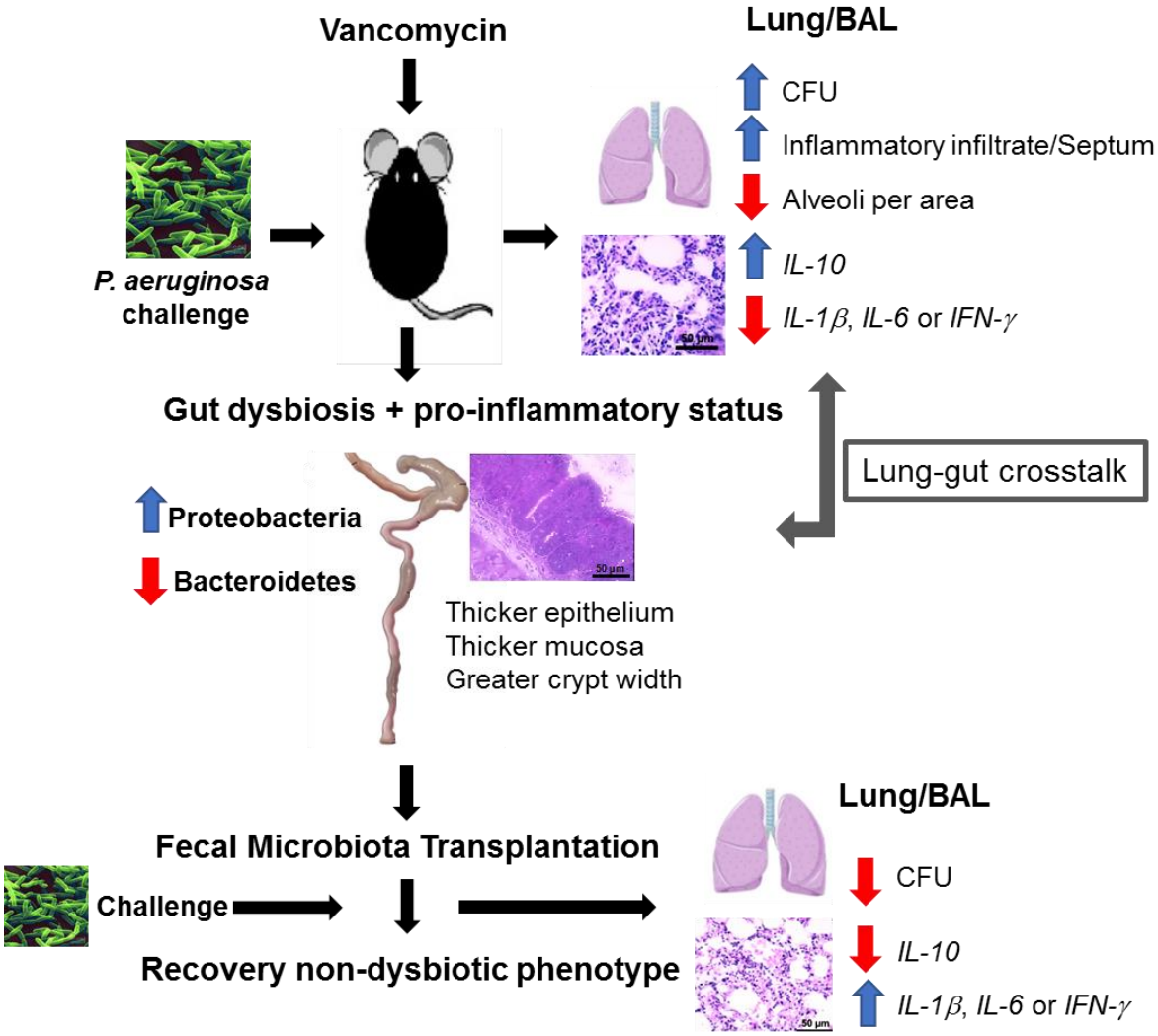
Fonte: (Rosa et al., 2019)

2 CONCLUSÕES

A vancomicina altera a microbiota intestinal, induzindo a disbiose intestinal murina com aumento relativo de Proteobactérias e diminuição de Bacteroidetes. Essa disbiose culminou com a exacerbação da infecção pulmonar causada pela *P. aeruginosa*. O TMF demonstrou ser uma excelente estratégia para restaurar a comunidade microbiana intestinal e auxilia no controle e prevenção da infecção bacteriana de caráter nosocomial. Houve maior dano tecidual nos pulmões dos animais disbióticos caracterizado por maior infiltrado inflamatório, aumento do septo e diminuição do espaço alveolar. Esses resultados da análise histopatológica e estereológicas dos pulmões, e os resultados de CFU demonstraram que, embora houvesse um padrão inflamatório maior nos camundongos em disbiose intestinal induzida por vancomicina, quando infectados por *P. aeruginosa*, o controle dessa bactéria não foi eficiente para eliminá-la, comparado a camundongos não disbióticos, mas após o TMF os camundongos voltaram ao padrão encontrado em camundongos infectados não-disbióticos.

Entretanto embora nossos resultados possam esclarecer a relação entre os pulmões e os intestinos, mediado pelas UTOs intestinais, é necessária uma investigação mais aprofundada para entender a resposta imunológica no interior dos pulmões, bem como a influência da disbiose intestinal sobre isso (Fig.1).

Figura 1- Graphical abstract do artigo "Vancomycin-induced gut dysbiosis during *Pseudomonas aeruginosa* pulmonary infection in a mice model". Fluxograma demonstrando a infecção com a *Pseudomonas aeruginosa* e os resultados obtidos da analise do pulmão de CFU, histopatologia, e da expressão de genes codificadores de citocinas. As alterações da disbiose no ceco, e analise do pulmão após os animais disbióticos receberem o TMF.



Fonte: (Rosa et al., 2019)

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