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INSTITUTO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA

TRABALHO DE TESE

BIANCA SAMPAIO DOTTO FIUZA

PERFIL DA MICROBIOTA INTESTINAL EM INDIVÍDUOS ASMÁTICOS E SEU PAPEL IMUNOMODULADOR

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BIANCA SAMPAIO DOTTO FIUZA

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Tese apresentada ao Programa de Pós-graduação em Imunologia, da Universidade Federal da Bahia como requisito parcial para obtenção título de Doutora em Imunologia.

Orientadora: Profª. Drª Camila Alexandrina Viana de Figueiredo
Coorientador: Prof. Dr. Pedro Milet Meirelles

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A TESE:
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Elaborada por:

BIANCA SAMPAIO DOTTO FIUZA

Foi aprovada por todos os membros da banca examinadora e aceita pelo Programa de Pós-Graduação em Imunologia como requisito parcial à obtenção do título de **DOUTOR**

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Dedico este trabalho aos meus pais, pelo amor incondicional, apoio e sacrifícios que fizeram para que eu pudesse chegar até aqui.

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“If you put your mind to it, you can accomplish anything”
(De Volta para o Futuro)

RESUMO

O fenômeno da disbiose no microbioma intestinal pode influenciar o desenvolvimento imunológico do hospedeiro e a incidência de doenças alérgicas. Acredita-se que as interações microbioma-hospedeiro, ou seja, a simbiose, contribuam para o desenvolvimento adequado do sistema imunológico, enquanto a disbiose microbiana tem sido associada a uma variedade de distúrbios inflamatórios, incluindo a asma. O objetivo deste estudo é caracterizar o perfil taxonômico da microbiota intestinal de indivíduos asmáticos, participantes do *World Asthma Phenotypes* (WASP), e associá-lo a mecanismos imunomoduladores associados. A avaliação da microbiota intestinal foi realizada a partir do sequenciamento da região V4 do gene 16S rRNA do DNA bacteriano extraído de amostras de fezes, seguido de análise de bioinformática no *software* QIIME2. A associação com a resposta imune foi realizada utilizando marcadores como a positividade ao teste cutâneo, celularidade do escarro induzido e dosagem de citocinas no lavado nasal. Embora todos os filos predominantes, como *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes* e *Actinobacteria*, tenham sido consistentes entre asmáticos e não asmáticos, observaram-se diferenças nas abundâncias relativas. Não foram identificadas diferenças significativas na riqueza ou diversidade bacteriana entre asmáticos e não asmáticos, com base na diversidade alfa. No entanto, uma dissimilaridade estatisticamente significativa na diversidade beta foi observada. O gênero *Bacteroides* destacou-se como o mais abundante, contribuindo para a dissimilaridade entre o grupo asmático, enquanto *Prevotella* foi mais prevalente em não asmáticos. A presença de *Bacteroides* na microbiota dos asmáticos correlacionou-se com a produção de IL-4 no lavado nasal. Esses resultados reforçam a compreensão das diferenças na comunidade microbiana entre indivíduos asmáticos e não asmáticos.

Palavras-chave: asma, microbiota intestinal, sequenciamento, microbioma

ABSTRACT

The phenomenon of dysbiosis in the intestinal microbiome can influence the host's immune development and the incidence of allergic diseases. It is believed that microbiome-host interactions, i.e., symbiosis, contribute to the proper development of the immune system, while microbial dysbiosis has been associated with a variety of inflammatory disorders, including asthma. The aim of this study is to characterize the taxonomic profile of the intestinal microbiota in asthmatic individuals participating in the World Asthma Phenotypes (WASP) and associate it with related immunomodulatory mechanisms. The assessment of the intestinal microbiota was conducted through sequencing the V4 region of the 16S rRNA gene from bacterial DNA extracted from stool samples, followed by bioinformatic analysis using the QIIME2 software. Association with the immune response was investigated using markers such as skin prick test positivity, induced sputum cellularity, and cytokine levels in nasal lavage. Although all predominant phyla, such as *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, were consistent between asthmatics and non-asthmatics, differences in relative abundances were observed. No significant differences in bacterial richness or diversity were identified between asthmatics and non-asthmatics based on alpha diversity. However, a statistically significant dissimilarity in beta diversity was observed. The genus *Bacteroides* was the most abundant, contributing to the dissimilarity within the asthmatic group, while *Prevotella* was more prevalent in non-asthmatics. The presence of *Bacteroides* in the microbiota of asthmatics correlated with IL-4 production in nasal lavage. These results reinforce the understanding of differences in the microbial community between asthmatic and non-asthmatic individuals.

Keywords: asthma, gut microbiota, sequencing, microbiome

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LISTA DE ABREVIAÇÕES

AMPs: peptídeos antimicrobianos

APCs: células apresentadoras de antígenos

BALF: fluido do lavado broncoalveolar

BALT: tecido linfoide associado ao brônquio

CRTH2: receptor de quimiocinas expresso por células Th2

CXCL: quimiocina CXC Ligante

CXCR: receptor de quimiocina CXC

DC: células dendríticas

DNA: ácido Desoxirribonucleico

FeNO: fração de óxido nítrico exalado

FOXP3: forkhead box P3

GALT: tecido linfoide associado ao intestino

GI: gastrointestinal

GPR: receptores acoplados à proteína G

HDAC: histona desacetilase

IFN-γ: interferon

Ig: imunoglobulina

IL: interleucina

ILC: células linfoides inatas

iNOS: óxido nítrico-sintase induzida

LPS: lipopolissacarídeo

MALT: tecido linfático associado a mucosa

mLN: linfonodo mesentérico

NALT: tecido linfoide associado à nasofaringe

NF-kB: fator nuclear kappa B

NKT: células T *natural killer*

OTUs: unidades taxonômicas operacionais da comunidade

PCA: análise de componentes principais

PCoA: análise de coordenadas principais

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PPARγ: receptor ativado por proliferadores de peroxissoma gama

QIIME2: Quantitative Insight Into Microbial Ecology

RNA: ácido ribonucleico

SCFA: ácidos graxos de cadeia curta

SIM: sistema imunológico de mucosa

T2: inflamação tipo 2

TGF-β: Fator de transformação do crescimento beta

TGI: trato gastrointestinal

Th: T auxiliar, T *helper*

TLRs: receptor Toll-Like

TNF: fator de necrose tumoral

Tregs: células T reguladoras

TSLP: linfopoietina estromal tímica

SUMÁRIO

1. INTRODUÇÃO GERAL

O trato gastrointestinal (TGI) humano abriga um ecossistema microbiano complexo, estável, robusto, resiliente e diversificado, predominantemente composto por bactérias, mas também incluindo vírus, archaea, fungos e outros eucariotos (BARCIK et al., 2020c; CUNA et al., 2021).Esse sofisticado ecossistema, com aproximadamente 10^{14} células e de 100 a 1.000 espécies diferentes, é considerado a comunidade microbiana mais densa do planeta (CHEN et al., 2020; CUNA et al., 2021). Os dois filos principais, Bacteroidetes e Firmicutes, compõem 90% da microbiota intestinal, enquanto Proteobacteria, Actinobacteria, Fusobacteria e Verrucomicrobia são outros filos presentes no intestino humano (CUNA et al., 2021). A diversidade de espécies bacterianas pode ser mensurada dentro das amostras (alfa-diversidade) e entre amostras (beta-diversidade) (HUFNAGL et al., 2020).

O microbioma intestinal desempenha um papel importante na manutenção da saúde humana, contribuindo para uma ampla gama de atividades bioquímicas e metabólicas. Está envolvido em processos fisiológicos e imunológicos, como equilíbrio energético e metabolismo, síntese de vitaminas e nutrientes, sinalização endócrina, prevenção da colonização de enteropatógenos, modulação da função imunológica e metabolismo químico xenobiótico (BARKO et al., 2018; CHIU et al., 2019). Essas descobertas sustentam a concepção de que os mamíferos são holobiontes, dependendo tanto do hospedeiro quanto do genoma microbiano para operar adequadamente (ROOKS; GARRETT, 2016).

Evidências indicam que uma colonização bacteriana diversificada na mucosa intestinal ao longo da infância é essencial para a formação, manutenção e modulação do sistema imunológico (HUFNAGL et al., 2020). Mudanças na composição ou diversidade microbiana podem influenciar não apenas o órgão colonizado, mas também órgãos e sistemas distantes (Chunxi et al., 2020). Assim, as interações microbioma-hospedeiro são consideradas fundamentais para a homeostase, enquanto a disbiose microbiana pode resultar em um desenvolvimento imunológico alterado, associado a diversos distúrbios inflamatórios, incluindo a asma (BARCIK et al., 2016a; CHRISTIANSEN; ZURAW, 2019; HUFNAGL et al., 2020).

A asma é uma condição inflamatória crônica das vias aéreas que se manifesta através de tosse, chiado, falta de ar e aperto no peito (HAMMAD; LAMBRECHT,

2021). Esses sintomas resultam da inflamação das vias aéreas, desencadeando processos como produção de muco, remodelamento da parede das vias aéreas e hiper-responsividade brônquica (HAMMAD; LAMBRECHT, 2021b). Acometendo aproximadamente 350 milhões de pessoas globalmente, a asma deve atingir 400 milhões até 2025 (BARCIK et al., 2020b; KOMLÓSI et al., 2022). Todos os anos, cerca de 250.000 mortes anuais são relacionadas à asma, muitas das quais evitáveis (BARCIK et al., 2020b).

O mecanismo convencional da fisiopatologia asmática é a inflamação eosinofílica das vias aéreas mediada por IgE, e a maioria das pesquisas recentes sobre a asma se concentram nas respostas imunes adaptativas relacionadas ao T helper (Th) 2 (OZTURK et al., 2017). Estudos recentes sobre a fisiopatologia de várias doenças, incluindo a asma, têm se concentrado na imunidade inata e na microbiota, o ecossistema com todos os microrganismos presentes nos tecidos e fluidos do organismo.

A asma é uma doença complexa com vários endotipos e manifestações clínicas. Pesquisas em adultos revelaram a inflamação tipo 2 (T2) como uma resposta imunológica crítica na patobiologia da asma, levando às amplas categorias de asma com T2-alta e T2-baixa (MAISON et al., 2022).

Na asma T2-alta, alérgenos, microrganismos e poluentes inalados interagem com o epitélio das vias aéreas, ativando mediadores como linfopoietina estromal tímica (TSLP), IL-25 e IL-33. Isso leva à ativação de IL-4, IL-5 e IL-13, que podem atrair e ativar basófilos, eosinófilos e mastócitos; havendo também a produção de IgE pelas células B; e ativação de células inatas como o epitélio das vias aéreas e o músculo liso, resultando em broncoconstrição, hiper-responsividade das vias aéreas, produção de muco e remodelamento das vias aéreas (RIJAVEC et al., 2021). A asma T2-baixa, associada à obesidade, tabagismo, poluição e idade avançada, é caracterizada por inflamação neutrofílica ou paucigranulocítica, com células Th1 e/ou Th17 como efetores predominantes(BARCIK et al., 2020a; KYRIAKOPOULOS et al., 2021).

A asma é influenciada por variáveis genéticas e ambientais, incluindo exposição a vírus e perfil dietético (CHRISTIANSEN; ZURAW, 2019). Há evidências de interação entre o trato respiratório e o TGI, ou mais precisamente, entre a microbiota intestinal

e os pulmões, e essa relação é conhecida como eixo intestino-pulmão (DE OLIVEIRA et al., 2021).

O presente trabalho buscou compreender a relação entre a microbiota intestinal, o sistema imunológico e a asma. Ao caracterizar o perfil taxonômico da microbiota em indivíduos asmáticos e não asmáticos, exploramos a hipótese de que suas diferenças podem influenciar a produção de citocinas, desempenhando um papel crucial na homeostase do sistema imunológico, impactando a suscetibilidade à doença. Os objetivos incluem uma revisão abrangente da literatura sobre a influência da microbiota e exposições ambientais na asma e alergias, a caracterização do perfil taxonômico bacteriano, a avaliação da diversidade microbiana, a comparação dos níveis de citocinas e a investigação da associação entre a microbiota gastrointestinal e sua função imunomoduladora. Esta abordagem visa lançar luz sobre os mecanismos que conectam a microbiota intestinal à saúde respiratória, contribuindo assim para uma compreensão mais profunda dos fatores subjacentes à asma. Neste documento serão apresentados os dois capítulos da Tese no formato de artigo científico, sendo o capítulo I já publicado (revisão de literatura) e o capítulo II submetido ao periódico *Journal of Allergy and Clinical Immunology: Global* (JACIG-D-23-00054_R1).

2. REVISÃO DE LITERATURA

2.1. Histórico, Fisiopatologia e Epidemiologia da Asma

Hipócrates inicialmente identificou a asma como um distúrbio de falta de ar induzido por estresse mental (KOZIK; HUANG, 2019). As obras de Sir John Floyer do século XVII mencionam a constrição brônquica, bem como as noções de ataques e gatilhos (KOZIK; HUANG, 2019). A asma foi classificada como doença inflamatória no século XX, sendo uma condição crônica que afeta mais de 350 milhões de pessoas em todo o mundo e deve chegar a 400 milhões até 2025 (BARCIK et al., 2020b; BRUSSELLE; KOPPELMAN, 2022; KOMLÓSI et al., 2022; KOZIK; HUANG, 2019).

Apesar das inúmeras melhorias científicas, a asma continua a ser um grave problema de saúde pública global (KOZIK; HUANG, 2019). As exacerbações da asma causam perda de dias escolares e de trabalho, hospitalizações, atendimentos de emergência e cerca de 250.000 mortes relacionadas à asma são documentadas todos os anos, muitas das quais poderiam ser evitadas (BARCIK et al., 2020b; GANS; GAVRILOVA, 2020; ZOU et al., 2021).

Persiste em todo o mundo uma preocupante taxa de mortalidade relacionada à asma, refletindo um sério problema de saúde pública, especialmente nos países de baixa e média renda (PITREZ et al., 2021) . No Brasil, embora tenhamos observado uma ligeira redução de 10% no número total de mortes entre 2008 e 2013, a média de cinco óbitos diários devido à asma destaca a urgência de abordagens mais eficazes no controle dessa doença tratável (CARDOSO et al., 2017). Um exemplo elucidativo provém de Salvador, no estado da Bahia, onde a implementação do Programa para o Controle da Asma na Bahia (ProAR) demonstrou resultados notáveis. Quando os pacientes com asma grave alcançaram estabilidade no controle da doença, ocorreu uma expressiva diminuição nos custos diretos associados à asma para as famílias, registrando uma queda significativa de 89%, ao passo que a renda familiar global aumentou (FRANCO et al., 2009). Essas iniciativas locais não apenas sublinham a necessidade premente de implementação de programas de controle eficazes nos sistemas de saúde pública, mas também evidenciam os benefícios tangíveis que tais intervenções podem trazer para a qualidade de vida das famílias afetadas (CARDOSO et al., 2017).

Diversos fatores contribuem para a manifestação simultânea ou individual de alterações patológicas específicas nas vias aéreas (SANTOS et al., 2023), como observado na Figura 1.

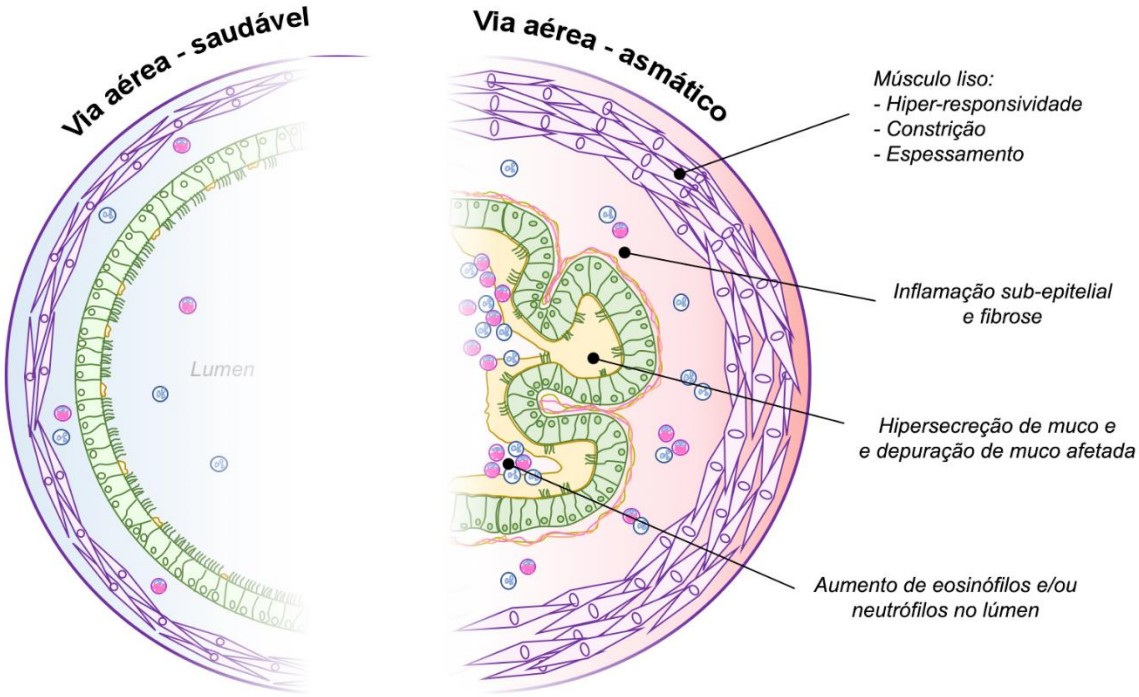

Figura 1. Comparação visual de um brônquio: à esquerda, a via aérea normal e à direita, alterações patológicas associadas à asma, incluindo contração da musculatura lisa brônquica, produção de muco, espessamento devido a edema e inflamação. Adicionalmente, destacam-se o aumento de eosinófilos e/ou neutrófilos. **Adaptado de**: Severe Asthma Toolkit, 2022

Tais alterações se manifestarão com sintomas variados de falta de ar, tosse e aperto no peito e está associada à inflamação crônica e edema das vias aéreas, hipersecreção das glândulas mucosas brônquicas e células caliciformes, resultando na restrição reversível do fluxo aéreo expiratório e hiper-responsividade das vias aéreas (BRUSSELLE; KOPPELMAN, 2022; SANTOS et al., 2023).

A imunofisiopatologia da asma inclui a ativação do sistema imunológico inato e adaptativo para causar inflamação persistente das vias aéreas. A fisiopatologia de como esses estímulos conhecidos geram mudanças estruturais persistentes nos diferentes endotipos da asma envolve uma mistura de respostas T helper (Th) 1, 2 e 17, ilustrados na Figura 2, bem como, uma suscetibilidade genética subjacente (GANS; GAVRILOVA, 2020).

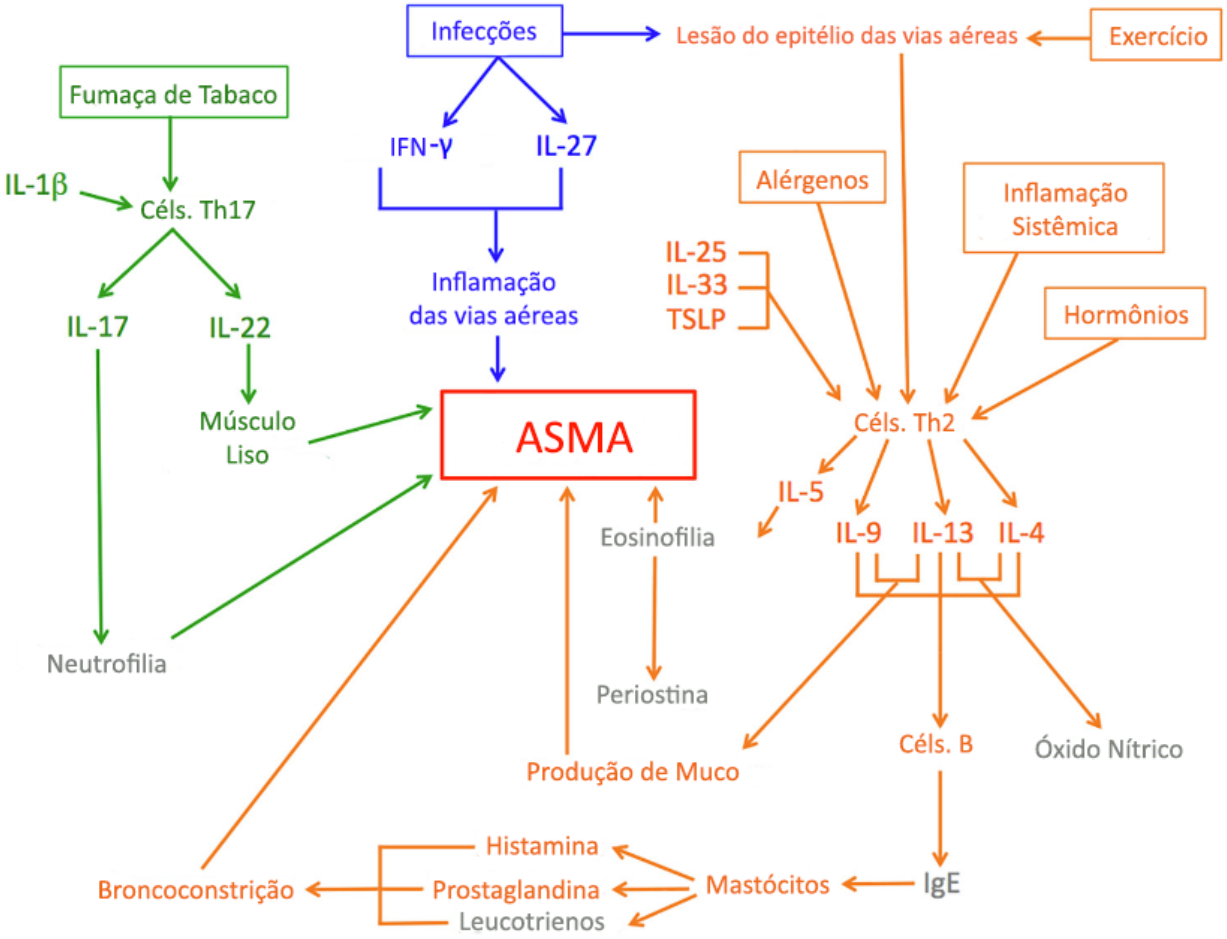

Figura 2. Representação da fisiopatologia da asma, destacando como os fatores conhecidos induzem mudanças nos diferentes endótipos da doença. Essas alterações são resultado de uma combinação de respostas dos linfócitos T auxiliares (Th) 1, 2 e 17, além de uma predisposição genética subjacente. As respostas Th17 estão em verde, Th2 em laranja e Th1 em azul, com potenciais biomarcadores indicados em cinza. **Adaptado de**: Gans et al. 2020

A resposta Th1 é normalmente ativada em infecções, especialmente as virais (HOWARD et al., 2022). Os vírus estimulam a produção de interferon-γ e interleucina (IL)-27, que auxiliam na eliminação do patógeno, mas também desempenham um papel na inflamação das vias aéreas (AMSDEN et al., 2022; GANS; GAVRILOVA, 2020).

As células Th17 desempenham um papel significativo na fisiopatologia da asma ao produzirem tanto a IL-17 quanto a IL-22, que, por sua vez, induzem a remodelação das vias aéreas (MARGELIDON-COZZOLINO et al., 2022). A IL-17 é associada à promoção da infiltração neutrofílica nas vias aéreas, desencadeando um processo inflamatório caracterizado pela presença de neutrófilos (HYNES; HINKS, 2020). Por outro lado, a IL-22 está envolvida no aumento da massa muscular lisa,

desempenhando um papel essencial na hiper-responsividade brônquica (HIROSE; ITO; NAKAJIMA, 2018).

As células dendríticas presentes nas vias aéreas desempenham um papel crucial ao apresentar alérgenos às células T virgens, desencadeando a ativação e produção das células Th2 (GANS; GAVRILOVA, 2020). As células Th2, por sua vez, liberam citocinas Th2, incluindo IL-4, IL-5, IL-9 e IL-13. As citocinas IL-4, IL-9 e IL-13 estimulam as células B a liberarem imunoglobulina E (IgE) (SADEGHALVAD; MOHAMMADI-MOTLAGH; REZAEI, 2022). A IgE, por sua vez, desencadeia a degranulação das células de mastócitos e a liberação de mediadores, como histamina e leucotrienos, resultando em broncoconstrição (GALLI; TSAI, 2012; MÉNDEZ-ENRÍQUEZ; HALLGREN, 2019). Esses mecanismos são perpetuados por citocinas como IL-25, IL-33 e linfopoietina estromal tímica (TSLP) (GANS; GAVRILOVA, 2020). A IL-25 induz a expressão de IL-4, IL-5, IL-9 e IL-13 (SAHINER; AKDIS; AKDIS, 2022), enquanto a IL-33 ativa as células dendríticas para produzirem IL-5 e IL-13 (PROENCE et al., 2020). A IL-5 é essencial para a manutenção de eosinófilos, enquanto a IL-9 e a IL-13 contribuem para a produção de muco.

A asma não é uma doença única, mas sim um espectro de manifestações clínicas (fenótipos) e diversas causas fisiopatológicas subjacentes (endotipos) (ANDREA et al., 2021a). Estudos em adultos identificaram a inflamação do tipo 2 (T2) como uma resposta imunológica fundamental na patobiologia da asma, levando à ampla classificação da condição em dois grupos distintos: asma com predomínio de resposta T2 (T2-alta) e asma com baixa influência da resposta T2 (T2-baixa) (MAISON et al., 2022). Essa categorização baseada na inflamação tipo 2 tem sido crucial para uma compreensão mais refinada dos fenótipos asmáticos, oferecendo percepções valiosas para o desenvolvimento de abordagens terapêuticas mais específicas e personalizadas (GONZALEZ-URIBE; ROMERO-TAPIA; CASTRO-RODRIGUEZ, 2023; KURUVILLA; LEE; LEE, 2019).

A asma T2-alta é distinguida pela inflamação eosinofílica das vias aéreas, que está associada a contagens elevadas de eosinófilos no sangue ou fração de óxido nítrico exalado (FeNO), enquanto a asma T2-baixa inclui asma neutrofílica e asma

paucigranulocítica; a asma granulocítica é distinguida pela coexistência de inflamação eosinofílica e neutrofílica das vias aéreas (BRUSSELLE; KOPPELMAN, 2022),

As células Th2 podem produzir IL-4, IL-5 e IL-13, que orquestram a fisiopatologia da asma T2 (ANDREA et al., 2021a; HAMMAD; LAMBRECHT, 2021). Recentemente, há um grande interesse na função das células imunes inatas na geração dessas citocinas. Foi encontrado um papel crescente para as células linfoides inatas do tipo 2 (ILC2s) capazes de produzir citocinas típicas do tipo 2 (ANDREA et al., 2021b). As células Th2 e ILC2s compartilham várias características, incluindo a expressão de receptores de citocinas, o fator de transcrição GATA3 e o gene CRTH2 (receptor de quimiocinas expresso por células Th2), ilustrados na figura 3 (HAMMAD; LAMBRECHT, 2021). A IL-4 estimula a expressão de CD23 (FcεRII), o receptor de IgE de baixa afinidade, em linfócitos B e macrófagos, e promove o desenvolvimento de células T CD4+ virgens para um fenótipo Th2 (COVERSTONE; SEIBOLD; PETERS, 2020).

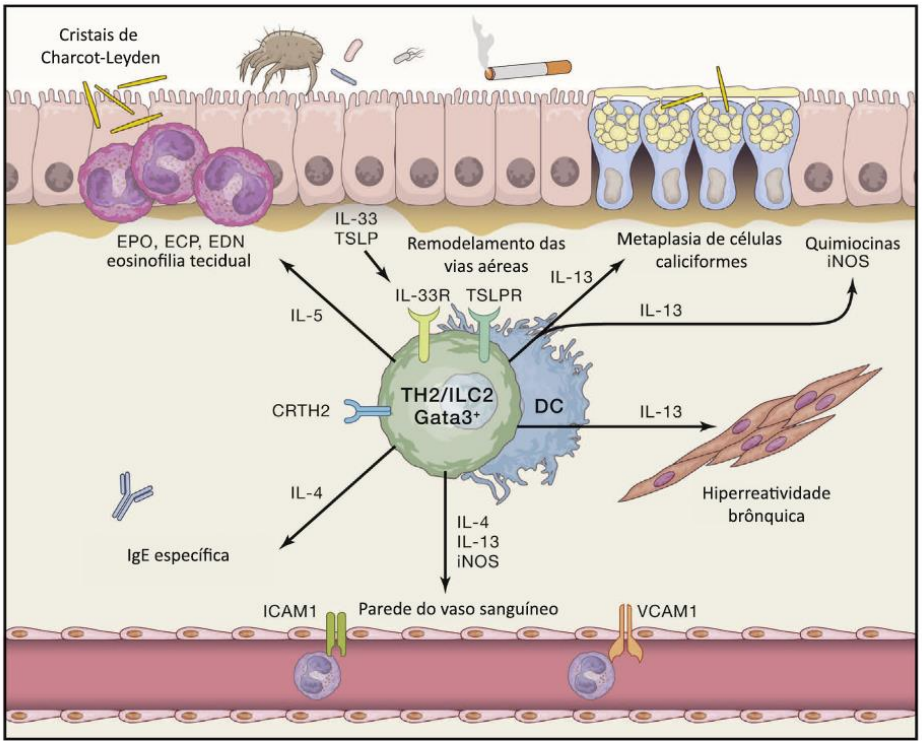

Figura 3. Papel central dos linfócitos Th2 e inatos no controle das principais características da asma T2-alta. A asma T2-alta é caracterizada pela presença de eosinófilos e biomarcadores como iNOS nas vias aéreas. Linfócitos Th2 reestimulados por células dendríticas produzem citocinas, como IL-4, IL-5 e IL-13, associadas a asmáticos alérgicos. Além disso, alguns pacientes apresentam ativação direta de linfócitos inatos tipo 2 (ILC2). IL-5 impulsiona eosinófilos, gerando substâncias prejudiciais. IL-13 está associada a metaplasia e hiper-reatividade brônquica. IL-4 promove síntese de IgE e prepara vasos para extravasamento de eosinófilos. iNOS: Óxido nítrico sintase induzida. **Adaptado de**: Hammad & Lambrecht, 2021.

A IL-5 aumenta a proliferação, diferenciação, ativação e sobrevivência de eosinófilos (BRUSSELLE; KOPPELMAN, 2022). A inflamação eosinofílica contribui significativamente para as alterações fisiológicas e remodelamento observadas em asmáticos. Os eosinófilos são encontrados na mucosa brônquica e no lúmen das vias aéreas de indivíduos asmáticos, e seus números aumentam quando a asma está descontrolada ou grave, e diminuem quando a asma está sob controle (SMITH et al., 2016).

A IL-13 estimula a síntese de óxido nítrico-sintase induzida (iNOS) nas células epiteliais das vias aéreas, bem como a metaplasia das células caliciformes e a hiper-reatividade brônquica (HAMMAD; LAMBRECHT, 2021). IL-13 faz com que as células musculares lisas nas vias aéreas se contraiam e ative a sintase de óxido nítrico induzível nas células epiteliais brônquicas, resultando em um aumento de FeNO (BRUSSELLE; KOPPELMAN, 2022). A eosinofilia é uma característica celular predominante das vias aéreas na asma tipo 2, dada a importância de IL-5 e IL-13 na inflamação de T2 e seu impacto nos eosinófilos (COVERSTONE; SEIBOLD; PETERS, 2020).

Tais citocinas fazem com que as células epiteliais das vias aéreas sejam ativadas, assim como, o recrutamento de outras células efetoras, como mastócitos, basófilos e eosinófilos, bem como a remodelamento epitelial e subepitelial, resultando em hiper-responsividade das vias aéreas (RIJAVEC et al., 2021).

A asma T2-baixa é um fenótipo muito diversificado e é caracterizado pela ativação de células Th1 e Th17, infiltrados neutrofílicos e paucigranulocíticos nas vias aéreas que são induzidos por IL-6, IL-8, IL-17, IL-22, TNF-α, IFN-γ e citocinas derivadas de células epiteliais (RICCIARDOLO et al., 2021; RIJAVEC et al., 2021) (Figura 4).

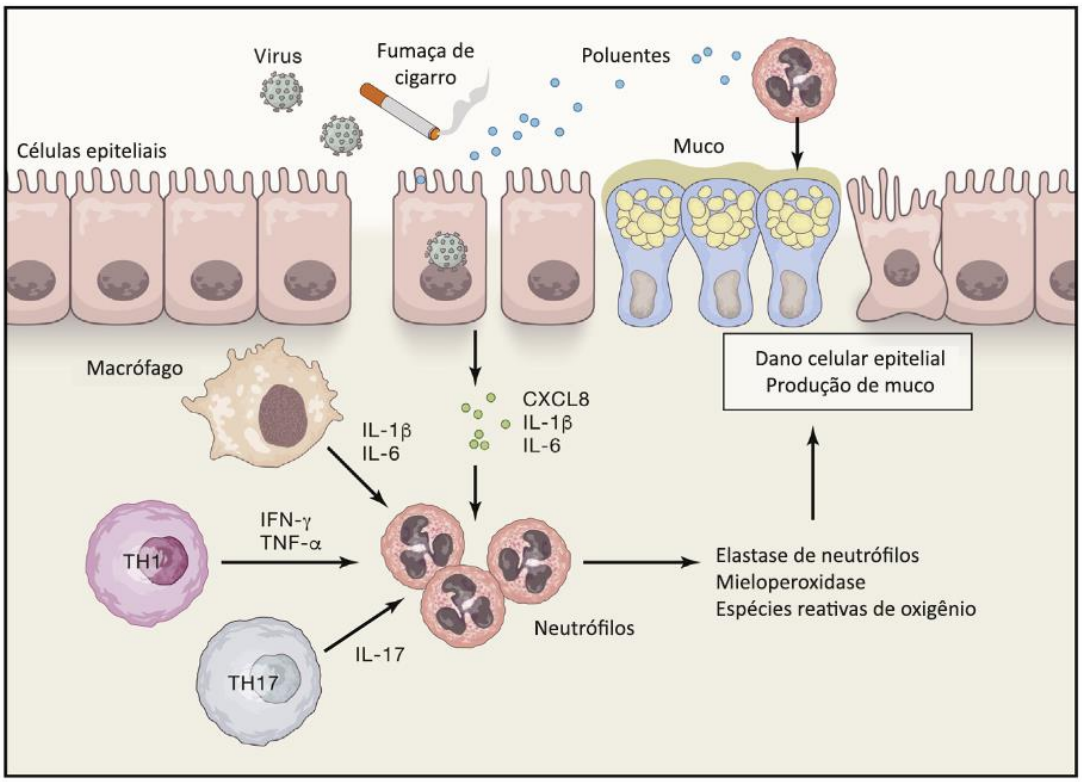

Figura 4. Papel proposto dos neutrófilos na asma T2-baixa. Ativação do epitélio das vias aéreas e macrófagos alveolares em resposta a gatilhos ambientais, como micro-organismos ou poluentes. Essas células respondem gerando citocinas pró-inflamatórias (IL-1β, IL-6), enquanto o epitélio também produz CXCL8 (IL-8), atraindo neutrófilos. Sob influência dessas citocinas, células Th1 e Th17 são induzidas, contribuindo para o recrutamento e ativação de neutrófilos. Fatores liberados por neutrófilos ativados, como elastase, mieloperoxidase e ROS, causam danos ao epitélio e aumentam a produção de muco. ROS**:** espécies reativas de oxigênio. **Adaptado de**: Hammad & Lambrecht, 2021

A IL-8 (também conhecida como CXCL8) e outras quimioatraentes de neutrófilos, incluindo CXCL1 e CXCL5, modificam seu efeito através dos receptores de quimiocina CXC CXCR1 e CXCR2 e a expressão de ambos os receptores foi significativamente elevada no escarro de asma neutrofílica (SAMITAS; ZERVAS; GAGA, 2017).

As células Th17 liberam citocinas IL-17, particularmente IL-17A, que aumentam o recrutamento de neutrófilos nas vias aéreas estimulando liberação de quimiocinas, desta forma, os níveis de IL-17 correlacionam-se com a infiltração de neutrófilos nas vias aéreas e são mais elevados em indivíduos com asma grave e propensa a exacerbações do que naqueles com doença mais leve (HUDEY; LEDFORD; CARDET, 2020).

O fator de necrose tumoral (TNF), uma citocina pró-inflamatória implicada em muitos aspectos da asma refratária grave, tem sido associado ao acúmulo e ativação

de neutrófilos, com níveis de elevados TNF-α no soro e no esputo na asma grave (SAMITAS; ZERVAS; GAGA, 2017).

No entanto, atualmente não existem biomarcadores clinicamente úteis ou tratamentos direcionados disponíveis na asma T2-baixa, portanto, a terapia nesse grupo é baseada no tratamento tradicional com fármacos de controle disponíveis comercialmente (MCGREGOR et al., 2019).

A asma possui uma etiologia variada, com inúmeras manifestações divergentes (ZOU et al., 2021). Anteriormente, pensava-se que a asma era um diagnóstico único com tratamentos uniformes para todos os pacientes; no entanto, o conhecimento atual da asma compreende uma condição diversa e complexa com uma grande quantidade de variáveis genéticas, epigenéticas, exposições ambientais (poluição, contato com biodiversidade) e internas (alérgenos domésticos, tabaco) e fatores atrelados ao indivíduo (gênero, envelhecimento, alimentação) (GANS; GAVRILOVA, 2020; MIMS, 2015; SANTOS et al., 2023).

A interação entre fatores ambientais e a microbiota intestinal desempenha um papel vital na determinação do estado imunológico das vias aéreas. De acordo com Marathe et al. (2022), uma dieta equilibrada e a qualidade do ar contribuem para a manutenção de um microbioma intestinal saudável, cuja liberação de metabólitos e ácidos graxos de cadeia curta (SCFA) exerce influência benéfica sobre o sistema imunológico pulmonar. Este ambiente promove a não inflamação, atuando como uma defesa contra o desenvolvimento da asma e suas exacerbações. Por outro lado, uma dieta inadequada, exposição à poluição do ar e certos medicamentos podem desencadear disbiose nas comunidades microbianas do intestino e pulmão, associando-se a condições de comorbidade como a obesidade e uma ativação imunológica inadequada (MARATHE et al., 2022). Além disso, fatores como o modo de parto, padrões de alimentação e o uso de antibióticos durante a gravidez e infância desempenham um papel crucial na homeostase microbiana das vias aéreas, influenciando o início e a progressão da asma (MARATHE et al., 2022; YAO et al., 2021).

Diversos estudos conectaram a disbiose da microbiota intestinal no início da vida a um risco aumentado de desenvolvimento de asma mais tarde na vida, uma vez que há fatores importantes para a formação da microbiota no pulmão e no intestino incluem

uma janela inicial para o estabelecimento do microbioma, variedade e riqueza bacteriana e impactos bacterianos no sistema imunológico (HUFNAGL et al., 2020).

2.2. Microbiota intestinal

O trato gastrointestinal (TGI) começa na cavidade oral, faringe, seguido do esôfago, estômago, intestino delgado, cólon e reto (OGOBUIRO et al., 2023; TOBIAS; SADIQ, 2023). Sua superfície maciça de 150-200 m^2 (do esôfago ao reto) oferece bactérias com opções de colonização ou habitação transitória (HILLMAN et al., 2017). Dependendo da área, o intestino humano tem entre 100.000 e 100 bilhões de bactérias por mL de fluido luminal, tornando-o o órgão mais densamente povoado (BARCIK et al., 2020b).

O TGI humano contém aproximadamente 10^{14} bactérias de até 1.000 espécies distintas, e os avanços na tecnologia de sequenciamento tornaram a microbiota intestinal, o microbioma do corpo humano mais pesquisado (CHUNXI et al., 2020). O termo "microbiota" refere-se a comunidades ecológicas de microrganismos comensais, simbióticos e patogênicos (TRIVEDI; BARVE, 2020), enquanto o “microbioma” é descrito como todos os microrganismos, bactérias, archaea, vírus e fungos, bem como seus genomas e metabólitos (SHUKLA et al., 2019), A hierarquia da taxonomia da microbiota está descrita na figura 5, do mais geral ao mais específico.

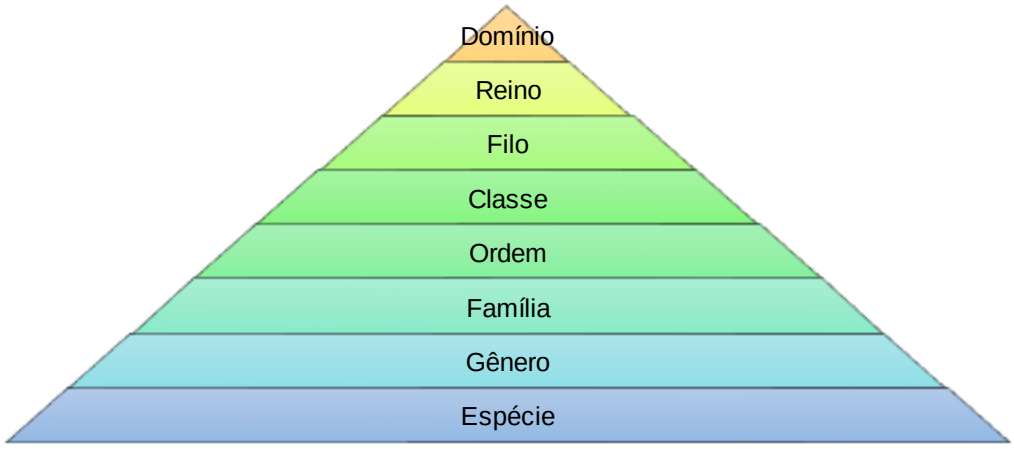

Figura 5. Hierarquia taxonômica. **Fonte:** elaboração própria

A microbiota interage com a fisiologia do hospedeiro (BORBET et al., 2019). Equilíbrio energético, metabolismo, saúde epitelial intestinal, atividade imunológica e neurodesenvolvimento, todos se beneficiam do microbioma (BARKO et al., 2018). O

microbioma é dinâmico e propenso a mudanças significativas durante a vida do hospedeiro como resultado de uma série de variáveis, como nutrição, ambiente, tratamentos médicos e estados de saúde/doença (BARKO et al., 2018).

Uma microbiota intestinal saudável realiza uma variedade de tarefas vitais para a saúde humana. Essas tarefas incluem digestão, estimulação imunológica da mucosa, exclusão e/ou controle de patógenos e a síntese de metabólitos úteis (HOU et al., 2022). Da mesma forma, a falha das comunidades microbianas intestinais em desempenhar essas funções tem sido relacionada a doenças inflamatórias intestinais, obesidade, desnutrição e diarreia associada a antibióticos (VER HEUL; PLANER; KAU, 2019).

As bactérias no intestino fornecem uma variedade de atividades para o hospedeiro, incluindo geração de vitaminas, absorção de íons, defesa de patógenos, desenvolvimento histológico, aumento das capacidades imunológicas e fermentação de alimentos (BARCIK et al., 2020b). A microbiota é taxonomicamente variada entre os indivíduos, resultando em uma “impressão digital” microbiana única no intestino (BORBET et al., 2019; TRIVEDI; BARVE, 2020). Os indivíduos diferem significativamente na composição da microbiota em níveis taxonômicos mais baixos, e cada indivíduo pode ter seu próprio padrão particular de perfil microbiano (CUNA et al., 2021).

A tabela 1 descreve os filos e gêneros mais abundantes na microbiota intestinal humana saudável. No geral, a composição da microbiota intestinal muda progressivamente à medida que as pessoas envelhecem, apesar da variabilidade interindividual e do efeito de variáveis externas, como nutrição, medicamentos, tipo de exercício ou mobilidade e a localização geográfica do hospedeiro (RAGONNAUD; BIRAGYN, 2021).

Avaliações de diversidade alfa e diversidade beta são usadas para estimar a diversidade de espécies em populações bacterianas em ambientes específicos, como o intestino (CHEN et al., 2020; WALTERS; MARTINY, 2020). A riqueza de cada amostra é avaliada usando a diversidade alfa, a partir de quatro parâmetros: o índice Chao1, o índice de Shannon, o índice de Simpson e as *espécies observadas* (CHEN et al., 2020). O índice *espécies observadas* representa os números de unidades taxonômicas operacionais da comunidade (OTUs) e os índices Chao1, Shannon e

Simpson, representa a riqueza e equidade da comunidade (CHEN et al., 2020) A diferença de composição entre amostras distintas é avaliada usando beta-diversidade. Para visualizar os efeitos da diversidade beta, a análise de componentes principais (PCA) e a análise de coordenadas principais (PCoA) são comumente utilizadas (FINOTELLO; MASTRORILLI; DI CAMILLO, 2018).

Tabela 1. Filos e gêneros mais abundantes na microbiota intestinal humana. **Adaptado de**: Trivedi et al. (2020); Barcik et al. (2020)

Filo	Gênero
Firmicutes	*Faecalibacterium*
	Rumminococcus
	Roseburia
	Eubacterium
Bacteroidetes	*Provetella*
	Bacteroides
Actinobacteria	*Bifidobacterium*
Proteobacteria	

Existem diversas variáveis que podem alterar a composição, função e o estabelecimento da microbiota intestinal (GOMAA, 2020). Grande parte da microbiota neonatal transmitida após o nascimento varia dependendo da via de parto (KUMBHARE et al., 2019). Ou seja, a microbiota das crianças nascidas por via vaginal é semelhante a microbiota da vagina e do intestino da mãe (CARR; ALKATIB; KRAFT, 2019). A microbiota de recém-nascidos por cesariana, por outro lado, imita a microbiota da pele. Aliás, a amamentação também modifica a seleção de quais microrganismos podem colonizar (OZTURK et al., 2017). Além disso, exposição na primeira infância a animais de fazenda ou domésticos, principalmente cães, também influenciam na formação da microbiota (CARR; ALKATIB; KRAFT, 2019).

Carboidratos não digeríveis como celulose, hemiceluloses, amido resistente, pectina, oligossacarídeos e lignina podem ser convertidos em ácidos graxos de cadeia curta (SCFA, *short chain fatty acids*) como ácidos acético, propiônico e butírico pelas bactérias intestinais (GOMAA, 2020). Esses produtos de fermentação fornecem energia substancial às células epiteliais intestinais, fortalecendo assim a barreira da

mucosa e possuem efeitos anti-inflamatórios e quimiopreventivos (GOMAA, 2020; MCKENZIE et al., 2017). Tais metabólitos podem ativar receptores acoplados à proteína G (GPCRs) sensíveis a metabólitos ou influenciar processos epigenéticos e de transcrição gênica, ambos com consequências imunológicas (MCKENZIE et al., 2017).

Esses sistemas podem ser interrompidos como resultado da alteração da composição microbiana, uma condição conhecida como disbiose (THURSBY; JUGE, 2017). Uma mudança ou desequilíbrio nos tipos e quantidades de microrganismos no intestino altera a microbiota e a tolerância imunológica da mucosa (TRIVEDI; BARVE, 2020). Muitas doenças clínicas estão ligadas à disbiose intestinal, incluindo doença inflamatória intestinal, obesidade, diabetes, doenças cardiovasculares e doenças neurodegenerativas (KIM; JAZWINSKI, 2018).

De acordo com Takiishi et al. (2017), a barreira epitelial no intestino não representa uma fronteira física estática, mas sim interage de maneira robusta com o microbioma intestinal e as células do sistema imunológico. A tolerância e as atividades imunes efetoras são equilibradas pela estreita interação entre as células epiteliais, as células do sistema imunológico e o microbioma, formando respostas imunes distintas aos antígenos (TAKIISHI; FENERO; CÂMARA, 2017). Dessa forma, a disbiose pode favorecer perturbações da barreira intestinal e estar associada a uma maior suscetibilidade a certas doenças (TALAPKO et al., 2022).

2.3. Microbiota e o Sistema Imune

O intestino é o único órgão onde um grande número de microrganismos coexiste em harmonia com o sistema imunológico. Esta função do TGI só pode ser alcançada através de um mecanismo de defesa bem projetado, incorporando nutrientes, água, eletrólitos para facilitar a digestão, mantendo o equilíbrio imunológico (SOLÉ et al., 2018),

A figura 6 retrata o epitélio intestinal composto por uma única camada de enterócitos ou colonócitos, e é função do sistema imunológico manter essa barreira intacta (BROWN; SADARANGANI; FINLAY, 2013). Os processos de imunidade inata incluem a utilização de uma camada de muco, peptídeos antimicrobianos (AMPs) e

células linfoides inatas (ILCs) que trabalham em conjunto para manter a maioria da população no lúmen intestinal (BROWN; SADARANGANI; FINLAY, 2013).

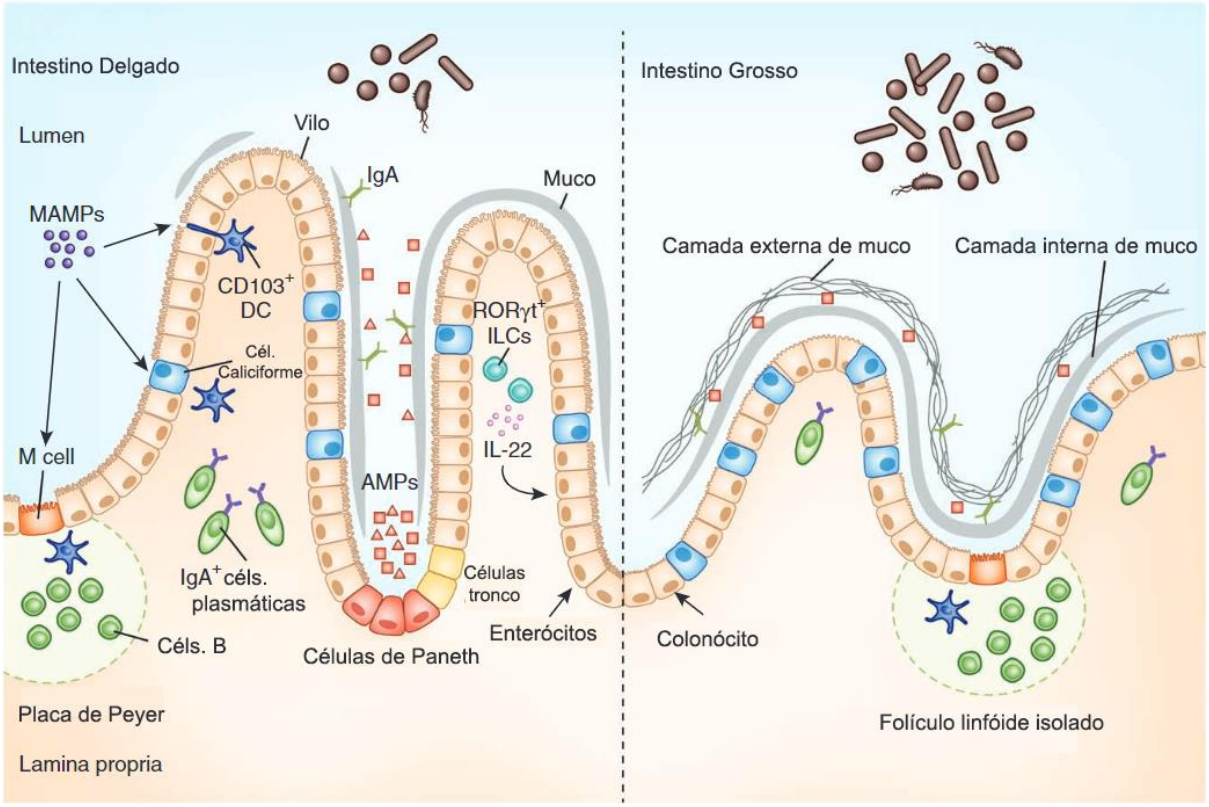

Figura 6. Abrangência anatômica da microbiota ao longo do intestino. O epitélio intestinal, composto por uma única camada de enterócitos ou colonócitos, depende do sistema imunológico para preservar sua integridade. No intestino delgado, as demandas absorventes resultam em uma camada de muco descontínua, com células de Paneth nas criptas secretando AMPs que interagem com o muco. Esta barreira permite a amostragem de MAMPs, mediada pela captação de antígenos por células M e caliciformes para células dendríticas, além da amostragem luminal transepitelial por DCs. ILCs RORγt detectam sinais microbianos, produzindo IL-22 para reforçar a função de barreira. No intestino grosso, uma camada de muco espessa e contínua isola a microbiota, com IgA e AMPs desempenhando um papel secundário. AMPs: peptídeos antimicrobianos; MAMPs: padrões moleculares associados a microrganismos; ILCs: células linfoides inatas. **Adaptado de:** Brown et al. 2013

A microbiota habita quase todos os nichos do corpo humano e desempenha um papel significativo na maturação e função do sistema imunológico (SOZAŃSKA, 2019). Evidências de pesquisas ambientais e da microbiota intestinal sugerem que a redução da composição da comunidade bacteriana do trato gastrointestinal pode alterar o desenvolvimento da função imunológica, com consequências negativas para

a saúde do sistema imunológico, incluindo o risco de alergia e asma (BONAMICHI-SANTOS et al., 2015; HUANG; BOUSHEY, 2015).

Ao modular a resposta imune do sistema TGI e dos órgãos distais, as bactérias comensais intestinais influenciam e mantêm a homeostase do corpo (CHUNXI et al., 2020). O sistema imunológico de mucosa (SIM) pode desencadear respostas imunológicas em diferentes órgãos, composto por sítios indutivos e efetores que se distinguem por suas características funcionais e arquitetura (ANAND; MANDE, 2018; TRIVEDI; BARVE, 2020). As células imunes migram através do sistema linfático desde os locais indutores da mucosa até os locais efetores, influenciando a resposta imunológica em vários órgãos (ANAND; MANDE, 2018).

O sistema imunológico de mucosa contribui para a formação do tecido linfático associado a mucosa (MALT), que é inclui: tecido linfoide associado ao intestino (GALT – do inglês *gut-associated lymphoid tissue*), tecido linfoide associado à nasofaringe (NALT – do inglês *nasal-associated lymphoid tissue*) e tecido linfoide associado ao brônquio (BALT – do inglês *bronchus-associated lymphoid tissue*) (TRIVEDI; BARVE, 2020).

O MALT é dividido em quatro seções: 1) tecido linfoide mucoso organizado composto por folículos linfoides reativos que produzem placas de Peyer quando concentrados no íleo terminal; 2) lâmina própria; 3) linfócitos intraepiteliais; e 4) linfonodos mesentéricos (BORIE et al., 2016).

O GALT é uma rede de estruturas imunológicas altamente estruturadas estrategicamente localizadas em todo o trato gastrointestinal, compreendendo microambientes especializados onde os antígenos derivados do intestino são apresentados aos linfócitos por células apresentadoras de antígenos (APCs) (BRUCKLACHER-WALDERT et al., 2014). O GALT é formado por tecidos linfoides que são estruturados (linfonodos mesentéricos e placas de Peyer) e atuam como sítios indutores (ANAND; MANDE, 2018).

O GALT tem três funções (Figura 7): fornece amostras antigênicas de todo o trato GI, maximiza a oportunidade para linfócitos virgens encontrarem o antígeno e, por último, promove a ativação de linfócitos e sua diferenciação precoce (BRUCKLACHER-WALDERT et al., 2014). Além disso, iniciam a síntese de IgA e outros mediadores imunológicos, que posteriormente circulam para os sítios efetores

de GALT através da circulação sistêmica, i.e., podem então ser transferidos para locais extra intestinais, como o epitélio brônquico, onde induzem uma resposta imunológica (TRIVEDI; BARVE, 2020).

As células T reguladoras (Tregs) são mais prevalentes na mucosa colônica e desempenham um papel importante na manutenção da homeostase imunológica (CHEN et al., 2020). Para induzir Tregs colônicas, são necessárias bactérias comensais com capacidades específicas em situações inflamatórias e não inflamatórias no intestino.

O polissacarídeo A de *Bacteroides fragilis* melhora os déficits sistêmicos de células T, a organogênese linfoide e a diferenciação de células T CD4+ em células T reguladoras (Tregs), promovendo a imunomodulação da mucosa e a síntese de TGF-β (MEZOUAR et al., 2018). As células T auxiliares (Th) 17 são induzidas a amadurecer para coordenar o equilíbrio com as Tregs e manter uma inflamação saudável de baixo grau. Os benefícios protetores alcançados pelas células T reguladoras (Treg) regulam negativamente as citocinas pró-inflamatórias (IL-8, IL-12, IL-23) e regulam positivamente as citocinas anti-inflamatórias, como a IL-10 (BARKO et al., 2018).

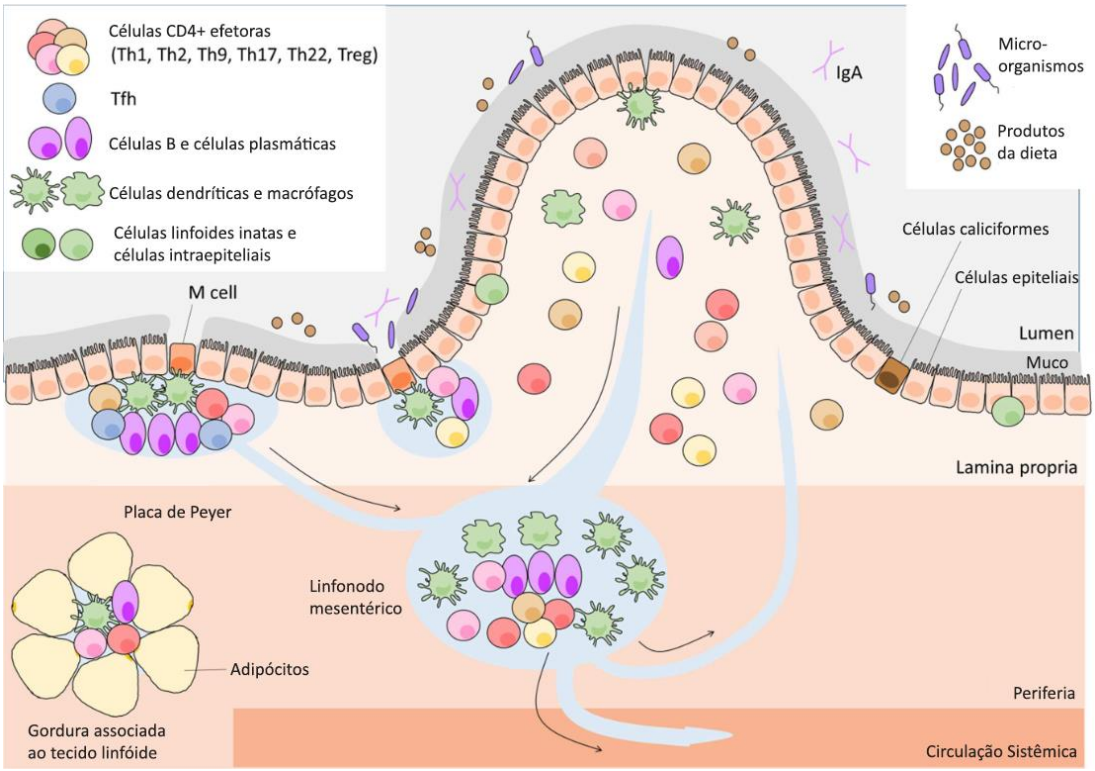

Figura 7. Arquitetura do tecido linfoide associado ao intestino. O GALT é uma rede altamente organizada de estruturas linfoides, incluindo linfonodos mesentéricos, placas de Peyer, folículos linfoides isolados e tecidos linfoides associados à gordura. Além disso, envolve o tecido conjuntivo frouxo da lâmina própria. **Adaptado de**: Brucklacher-Waldert et al. 2016

Estudos demonstraram que o aumento da proporção de *Firmicutes* intestinais para *Bacteroidetes*, induziu níveis elevados de ácidos graxos de cadeia curta circulantes e protegeu contra a inflamação alérgica no pulmão através da alteração da capacidade das células dendríticas de estimular a atividade efetora das células Th2 (HUANG; BOUSHEY, 2015).

Além da presença das bactérias em si, com seus padrões moleculares associados a patógenos (PAMPs) e padrões moleculares associados a danos (DAMPs), metabólitos microbianos também afetam as respostas imunes das mucosas, além de contribuir para as condições metabólicas sistêmicas (GRAHAM; XAVIER, 2023). Células imunológicas e citocinas desencadeadas pela microbiota intestinal e seus metabólitos, podem entrar na circulação sistêmica via sangue e sistema linfático e modular as respostas imunes e inflamatórias no pulmão, influenciando a saúde e a doença respiratória (CHUNXI et al., 2020).

2.4. Eixo intestino-pulmão

Tanto em pesquisas em humanos quanto em camundongos, uma ligação entre os pulmões e o intestino tem sido frequentemente comprovada (BARCIK et al., 2020a). A diferença na composição da microbiota intestinal mostra que as bactérias podem ter um papel de regulação das respostas imunológicas para a microbiota que vive em outros locais, como o pulmão (FRATI et al., 2019). Isso deu origem à noção do "eixo intestino-pulmão", no qual a disbiose da microbiota intestinal está conectada à doença das vias aéreas por meio da modulação da resposta imune (ZOU et al., 2021).

Apesar das variações na composição, os epitélios do trato TGI e respiratório surgem de uma única estrutura embrionária – endoderma -, as características e funções anatômicas das duas localizações da mucosa são comparáveis, e a colonização microbiana precoce do intestino e do pulmão é semelhante (CHUNXI et al., 2020).

Interrupções nessa interação bidirecional têm sido relacionadas a um aumento de distúrbios das vias aéreas, como a asma (SULLIVAN et al., 2016),

Em nível da estrutura do filo, a microbiota intestinal e pulmonar humana são semelhantes (Tabela 2), com as principais diferenças em nível de gênero.

Tabela 2. Comparação taxonômica da microbiota intestinal e pulmonar. **Adaptado de**: Trivedi 2020

	Intestino	**Pulmão**
Filo	Actinobacteria Firmicutes Bacteroidetes	Proteobacteria Bacteroidetes
Gênero	*Faecalibacterium* *Ruminococcus* *Bifidobacterium* *Bacteroides*	*Veillonella* *Prevotella* *Porphyromonas*

O mecanismo subjacente proposto é que células epiteliais, outras células estruturais e células imunes absorvem sinais endoteliais para formar um microambiente local de citocinas, o que leva a alterações nas respostas imunes em locais distais (BARCIK et al., 2020a). As células imunes *naive* que são ativadas no intestino vão para o pulmão, através do sistema mesentério para a circulação sanguínea, onde desempenham funções efetoras (MA et al., 2021).

A figura 8 ilustra o papel da microbiota intestinal nas doenças respiratórias e na homeostase. A disbiose da microbiota intestinal contribui para doenças respiratórias enquanto uma microbiota intestinal saudável desempenha um papel protetor no pulmão (ANAND; MANDE, 2018). A microbiota intestinal é influenciada por vários fatores, incluindo antibióticos, probióticos, fumaça de cigarro, dietas e transplante de microbiota fecal, e está associada à saúde e doença pulmonar, regulando a imunidade respiratória e a inflamação através do sangue e do sistema linfático (CHUNXI et al., 2020).

As células dendríticas (DC) apresentam os microrganismos intestinais diretamente do lúmen ou depois de terem passado pelas células M e alcançado o GALT (COOMBES; POWRIE, 2008; SAMUELSON; WELSH; SHELLITO, 2015). Essas células estimulam a produção de várias citocinas, como IL-10, TGF-β, INF- γ e IL-6, bem como a ativação de diferentes subconjuntos de células T dentro do linfonodo mesentérico (mLN) (SAMUELSON; WELSH; SHELLITO, 2015). As células ativadas do GALT e mLN vão para a mucosa respiratória onde suportam respostas protetoras e anti-inflamatórias.

Além disso, as células epiteliais intestinais e os macrófagos expressam receptores Toll-Like (TLRs) podendo ser alvos de subprodutos bacterianos como LPS,

causando a produção de diferentes citocinas e quimiocinas (BARCIK et al., 2020a). A expressão de NF-kB em macrófagos também é um componente da ativação de TLR.

Inúmeras ideias foram propostas sobre a translocação da microbiota intestinal para os pulmões, que é a causa de muitas respostas imunológicas e, portanto, de doenças respiratórias (TRIVEDI; BARVE, 2020).

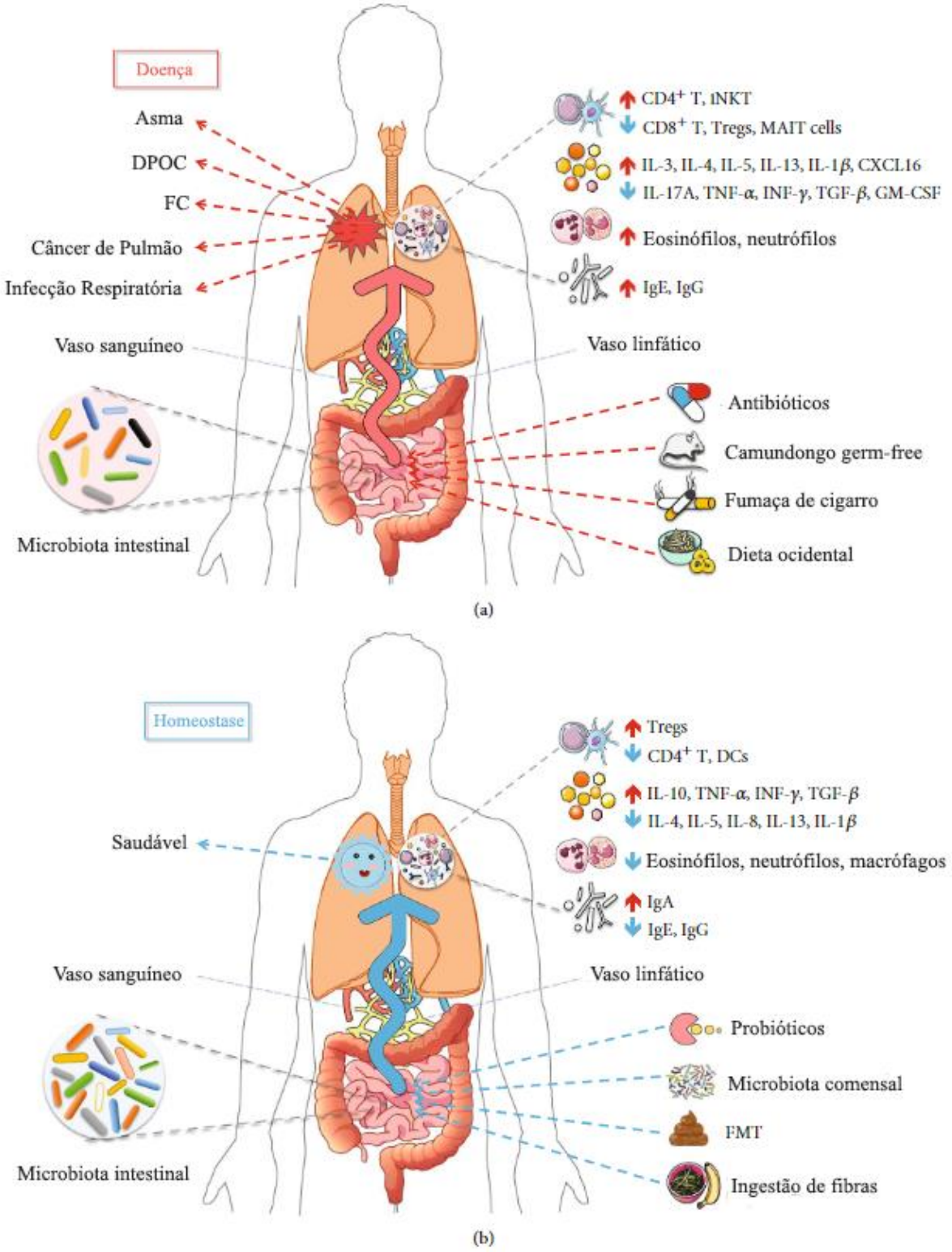

Figura 8. Papel da microbiota intestinal nas doenças respiratórias e na homeostase. A disbiose intestinal contribui para doenças (**a**), enquanto uma microbiota saudável exerce um papel protetor nos pulmões (**b**). Vários fatores, como antibióticos, probióticos, fumaça de cigarro, dietas e transplante fecal de microbiota (FMT), influenciam a microbiota intestinal. Essa interação está associada à saúde pulmonar e doenças, regulando a imunidade respiratória e a inflamação através do sangue e do sistema linfático. ↑: aumento; ↓: diminuição. **Adaptado de:** Chunxi et al. 2020

2.5. Microbiota intestinal e asma

O recente avanço das ferramentas de sequenciamento de DNA/RNA para identificação de bactérias permitiu um estudo mais aprofundado do microbioma em vários contextos diferentes (SATAM et al., 2023; SHI; GRODNER; DE VLAMINCK, 2021). Vários estudos conectaram a disbiose inicial da microbiota intestinal a um risco aumentado de desenvolvimento de asma mais tarde na vida (ZOU et al., 2021). A "hipótese da higiene" sustenta que a exposição precoce a diferentes bactérias e outras microbiotas na infância pode reduzir a incidência de alergia e asma mais tarde na vida (CARR; ALKATIB; KRAFT, 2019).

A diversidade microbiana, estimulação da resposta imune inata, exposição a animais de fazenda e domésticos, bem como tipo de parto, amamentação e uso de antimicrobianos, influenciam na colonização do pulmão e do intestino. Todos esses impactos levam efeitos na imunidade da mucosa das vias áreas, como ao aumento da hiper-responsividade brônquica, resistência a corticoides e aumento da infiltração de linfócitos Th17, Th2 ou eosinófilos e efeitos na imunidade da mucosa do intestino com a indução de células Treg pelos SCFAs, resposta de IgA aos micro-organismos intestinais e modulação de células T *natural killer* (NKT). Essas são inúmeras rotas que contribuem para o impacto potencial da microbiota no desenvolvimento da asma (VER HEUL; PLANER; KAU, 2019) (Figura 9).

Adultos com asma alérgica têm mais bactérias secretoras de histamina no intestino do que indivíduos saudáveis, indicando uma fonte microbiana de histamina que influencia as apresentações alérgicas da asma (BARCIK et al., 2016b; WAN et al., 2023; ZOU et al., 2021). Alterações na microbiota intestinal também foram associadas à sensibilidade a vários aeroalérgenos e diferenças na função pulmonar em indivíduos asmáticos (ZOU et al., 2021),

A baixa variedade intestinal durante o primeiro mês de nascimento está ligada a um risco aumentado de doenças alérgicas mais tarde na vida (ABRAHAMSSON et al., 2014). A disbiose da microbiota intestinal precoce em crianças está ligada ao desenvolvimento da asma e à diminuição de quatro gêneros bacterianos específicos: *Faecalibacterium*, *Lachnospira*, *Veillonella* e *Rothia* (KOZIK; HUANG, 2019). A adição dessas quatro espécies bacterianas a camundongos *germ-free* reduziu a prevalência de asma em seus descendentes adultos, diminuindo o recrutamento de neutrófilos

nos pulmões e a resposta imune Th1/Th17 associada à asma humana grave (RIVAS; CROTHER; ARDITI, 2016).

Como a microbiota intestinal influencia na patogênese da asma pode estar relacionado com a presença de metabólitos microbianos, tais como os ácidos graxos de cadeia curta (SCFAs) cujos níveis têm sido relacionados à disbiose intestinal em crianças com risco de asma-, metabólitos de histamina e triptofano, têm sido implicados nos processos de asma (KOZIK; HUANG, 2019).

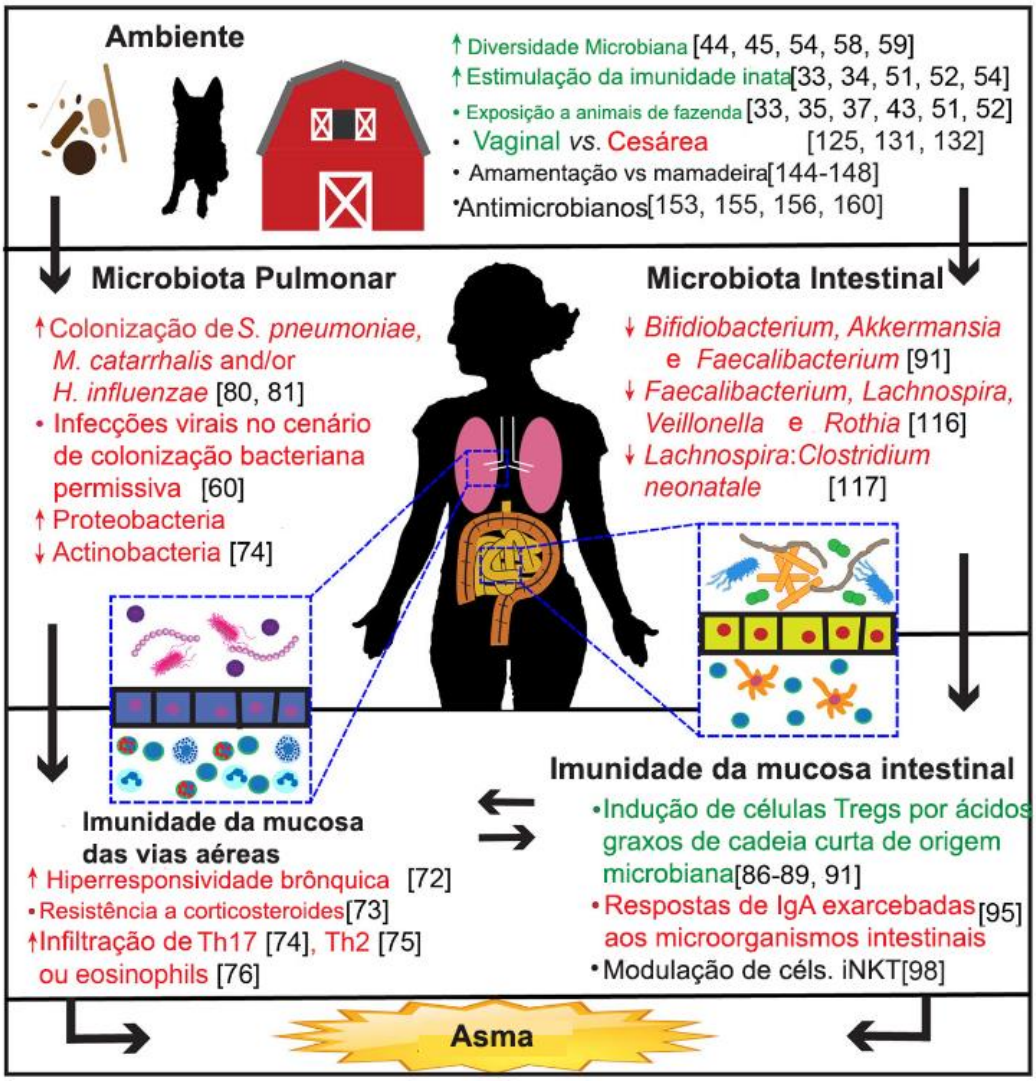

Figura 9. Visão geral dos supostos impactos da microbiota na asma. A colonização do pulmão e do intestino é influenciada pela diversidade microbiana, estimulação da resposta imune inata, exposição a animais de fazenda e domésticos, tipo de parto, amamentação e uso de antimicrobianos. Esses fatores têm impactos na imunidade da mucosa das vias aéreas, resultando em aumento da hiper-responsividade brônquica, resistência a corticoides e aumento da infiltração de linfócitos Th17, Th2 ou eosinófilos. Além disso, na imunidade da mucosa intestinal, há a indução de células Treg pelos SCFAs, resposta de IgA aos micro-organismos intestinais e modulação de células T natural killer (NKT). ↑: aumento; ↓: diminuição**. Adaptado de**: Ver Heul 2019

Outros processos potenciais pelos quais a microbiota intestinal afeta a asma estão relacionados com o uso de antibióticos, probióticos e estudos com camundongos *germ-free* levam a mecanismos efetores em doenças respiratórias (CHUNXI et al., 2020).

Estudos demonstraram que antibióticos afetam o microbioma intestinal em asmáticos levando ao aumento da infiltração de células inflamatórias e a geração de citocinas inflamatórias para exacerbar as respostas Th2 (IL-4 e IL-13) (CAIT et al., 2018; YANG et al., 2019), reduz o número de Tregs nos pulmões (ADAMI et al., 2018) e leva a respostas imunes adaptativas Th1/Th17 no pulmão exageradas (RUSSELL et al., 2015).

Camundongos *germ-free* com inflamação alérgica nas vias aéreas apresentam altos níveis de eosinófilos, quantidade de células T CD4+ e citocinas Th2 nas vias aéreas, bem como a quantidade e o fenótipo das DCs convencionais (HERBST et al., 2011); aumento da expressão de CXCL16 e o acúmulo de células iNKT no intestino e nos pulmões (OLSZAK et al., 2012) e reversão do desequilíbrio Th1/Th2 aumentando a citocina anti-inflamatória IL-10 enquanto diminui as citocinas pró-inflamatórias, incluindo IL-4, IL-5 e IL-13 (CHEN et al., 2018), particularmente após exposição a alérgenos, consistente com uma transição da imunidade do tipo Th1 para Th2 após depleção da microbiota (KENNEDY; KING; BALDRIDGE, 2018).

O uso de probióticos demonstrou aumento na expressão de receptor ativado por proliferadores de peroxissoma gama (PPARγ) de células dendríticas no pulmão (HSIEH et al., 2018), aumento no número de células T CD4+ e CD4+Foxp3+Tregs enquanto diminui o número de CD11b+DCs ativados(RAFTIS et al., 2018) e redução da expressão de MMP9 no fluido do lavado broncoalveolar (BALF) e soro, bem como a infiltração de células inflamatórias no pulmão (WU et al., 2016).

A composição microbiana do trato respiratório varia entre asmáticos e pessoas saudáveis. Proteobacterias foram as bactérias mais comuns identificadas em pessoas com asma, enquanto *Bacteroides* e *Firmicutes* apresentaram abundância relativa mais baixa (SOZAŃSKA, 2019). O *cluster* com a maior abundância de *Prevotella* foi relacionado com a maioria dos desequilíbrios bacterianos no intestino, aumento da hiper-responsividade das vias aéreas e baixa diversidade da comunidade (TRIVEDI; BARVE, 2020). Outras pesquisas com indivíduos mais jovens relacionaram a conexão

entre pulmão-intestino com a síntese de IgA, que está implicada nas respostas imunológicas, bem como no aumento dos níveis de *Firmicutes* e *Proteobacteria* (TRIVEDI; BARVE, 2020).

Também se levanta a questão que os asmáticos apresentam disbiose, embora seja difícil definir a função e os mecanismos de interação entre a composição bacteriana e o fenótipo da asma (SMITS et al., 2016).

Diante disso, percebe-se uma notável interação entre a microbiota intestinal, o sistema imunológico e a asma. Para obter uma compreensão mais abrangente da relação entre a microbiota gastrointestinal e a suscetibilidade à asma, realizou-se o estudo do perfil taxonômico bacteriano em asmáticos e não asmáticos, para delinear as possíveis implicações dessas diferenças na produção de citocinas e, consequentemente, na modulação do sistema imunológico.

3. HIPÓTESE E OBJETIVOS

HIPÓTESE

Indivíduos asmáticos possuem um perfil de microbiota intestinal diferente dos não asmáticos que interfere na produção das citocinas IL-4, IL-5, IL-8, IL-10, IL-13 e IL-17A, contribuindo para a mudança na homeostase do sistema imune e na ocorrência da doença.

OBJETIVO GERAL

Caracterizar o perfil taxonômico da microbiota intestinal de indivíduos asmáticos e não asmáticos e explorar os mecanismos e imunológicos associados.

OBJETIVOS ESPECIFÍCOS

- Realizar uma revisão de literatura descrevendo o papel da microbiota e demais exposições ambientais sobre asma e alergias;
- Caracterizar o perfil taxonômico de bactérias da microbiota nas amostras de fezes de indivíduos asmáticos;
- Avaliar a diversidade e diferença microbiana entre asmáticos e não asmáticos;
- Correlacionar os níveis de citocinas IL-4, IL-5, IL-8, IL-10, IL-13 e IL-17A entre asmáticos e não asmáticos com o perfil taxonômico encontrado;
- Prever, *in silico*, as vias metabólicas e biossintéticas associadas com o microbioma intestinal em asmáticos e não asmáticos

4. CAPÍTULO I: ARTIGO CIENTÍFICO

Understanding asthma and allergies by the lens of biodiversity and epigenetic changes

Artigo científico publicado no periódico *Frontiers in Immunology*

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REVIEW
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Understanding Asthma and Allergies by the Lens of Biodiversity and Epigenetic Changes

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Exposure to different organisms (bacteria, mold, virus, protozoan, helminths, among others) can induce epigenetic changes affecting the modulation of immune responses and consequently increasing the susceptibility to inflammatory diseases. Epigenomic regulatory features are highly affected during embryonic development and are responsible for the expression or repression of different genes associated with cell development and targeting/conducting immune responses. The well-known, "window of opportunity" that includes maternal and post-natal environmental exposures, which include maternal infections, microbiota, diet, drugs, and pollutant exposures are of fundamental importance to immune modulation and these events are almost always accompanied by epigenetic changes. Recently, it has been shown that these alterations could be involved in both risk and protection of allergic diseases through mechanisms, such as DNA methylation and histone modifications, which can enhance Th2 responses and maintain memory Th2 cells or decrease Treg cells differentiation. In addition, epigenetic changes may differ according to the microbial agent involved and may even influence different asthma or allergy phenotypes. In this review, we discuss how exposure to different organisms, including bacteria, viruses, and helminths can lead to epigenetic modulations and how this correlates with allergic diseases considering different genetic backgrounds of several ancestral populations.

Keywords: asthma, allergies, holobiont, microbiome, epigenetics

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INTRODUCTION

Asthma and allergy are the most common chronic inflammatory diseases, especially in children (1). The prevalence of asthma is elevated in economically developed countries in Western and Eastern Europe and higher in the United States compared to other countries (2, 3). A progressive increase in the prevalence of asthma in low-income countries has also been observed (4, 5), which makes asthma prevalent worldwide. According to the World Health Organization over 80% of asthma-related deaths occur in low- and low-middle-income countries, and difficulties in accessing treatment and management are also related to that (6). On the other hand, the prevalence of eczema, allergic rhinitis, and food allergies in childhood is distributed differently

between tropical countries and temperate zones (7–14). Geographic differences in the prevalence of allergies between and within populations may reflect both exposure to common environmental factors and a host genetic background, which can either increase or decrease risk (15). In terms of genetics, large genome-wide association studies (GWAS) initiatives were unable to completely explain such high and still increasing prevalence of allergic disorders as well as their phenotypic heterogeneity (1). Among the top pathways linked to asthma in such initiatives, include those related to epithelial barrier dysfunction and reduction of immune tolerance (16). In addition, studies have found not only shared but also distinct genetic components between asthma subtypes, indicating that heterogeneity is related to individual genotype (17, 18) but still do not completely explain everything.

Thus, the knowledge about the interactions between the genetic pool and the environment is increasing with several lines of evidence explaining those trends (19–21). In this context, some hypotheses explain the links between environmental changes that occurred in recent decades with the prevalence of allergies across the globe, such as urbanization, housing condition, diet, and fewer exposures to organisms such as bacteria, virus and helminths (21, 22). In fact, there is a link between the higher incidence of allergic diseases and reduced infections/exposure to organisms in Western countries and across the globe and this has been studied for several years now and appears to reflect the economy and sanitation in each territory. Additionally, the degree of industrialization and consequent changes in the habits and lifestyle of the population imply that limited exposure to several environmental factors for reducing biodiversity may contribute to an increased risk of developing or exacerbating asthma and allergies (23). David Strachan observed in 1989 that infections transmitted in early childhood, through contact between older siblings, could restrict the development of allergies (22, 24). Urbanization and improvements in hygiene, better housing conditions, and reduced chances of cross-infection in younger members of the family are the basis for what we know as the "hygiene hypothesis" (24). The initial mechanistic explanation of the hygiene hypothesis emphasized the role of Th1 cells in regulating Th2 responses. Later, the role of regulatory T cells was emphasized in the regulation of both Th1 and Th2-induced inflammatory responses through mechanisms that include the production of regulatory cytokines (25). The mechanistic pathways of the hygiene hypothesis were described extensively in the literature, other theories amplified the initial concept such as the "old friends" hypothesis (26) and, afterwards, the biodiversity hypothesis, proposed by The Karelia Allergy Study from 1998 (27). Both theories attempt to explain the impact of modifications in human living conditions and habits on the prevalence of immune-mediated diseases (28, 29).

Studies show that early exposure to antibiotics during childhood increases the risk of developing allergic diseases (30) and also regular anthelmintic use (31). Numerous epidemiologic studies reinforce that the increase in allergic diseases, eczema, and food allergies is inversely related to parasitic infections (32–36). Soil biodiversity and climatic characteristics of a country are also determinants in the types of environmental exposures and consequent development of infectious diseases and allergic sensitization. The climate and biodiversity of the tropics (fauna and flora) favor intestinal helminth infections and the dissemination of human infectious diseases transmitted by vectors like insects (37–42). According to (6), Soil-transmitted helminth infections are distributed in tropical and subtropical areas, with the highest incidence in sub-Saharan Africa, the Americas, China, and East Asia.

The tropics are also marked by sharp economic and social inequalities that reflect health and sanitary conditions and an increased risk of spreading fecal-oral transmission diseases (toxoplasmosis, giardiasis, hepatitis A, worms). In addition, the relationship between helminths and allergies is complex and is influenced by the parasite burden, chronicity, first infection or reinfection, coinfections, and parasite species present in the environment (33). In contrast, allergic sensitization to house dust mite species such as *Dermatophagoides pteronyssinus*, *D. farina,* and *Blomia tropicalis* is prevalent in the tropics, markedly in individuals living in better sanitary conditions and urban areas (43–45).

The importance of environmental exposures does not underestimate the fundamental participation of the family history of atopy and/or asthma and genetic background. Thus, we still have an enormous challenge to explain the occurrence of allergies and asthma. Increasing attention has been given to epigenetic modifications, i.e., modifications in DNA without sequence changes, triggered by individual exposure to environmental factors, for instance, by products of combustion, drugs, diet, and infections. Epigenetic mechanisms, such as DNA methylation and histone modifications, can modulate gene expression upon exposure to a specific environmental agent (46). Such biochemical alterations can alter different targets within the body, leading to the risk or protection of several conditions. In this review, we present the concept of holobiont and discuss how exposure to different organisms, including bacteria, viruses, and helminths, can lead to epigenetic modulations and how this modulation correlates with allergic diseases, taking into account different genetic backgrounds of several ancestral populations.

THE CONTEXT OF MICROBIAL EXPOSURE, THE CONCEPT OF HOLOBIONT, AND THE MECHANISMS INVOLVED IN IMMUNE MODULATION

Holobiont Concept

Microbes are the most ancient, abundant and arguably the greatest successful form of life on Earth, contributing to the evolution and function of all more complex multicellular organisms (47). Since the early days of life, microbes interacted

Abbreviations: AAI, allergic airway inflammation; BCG, Bacillus Calmette–Guérin; CpG, cytosine-phosphate-guanine; ERK, Extracellular signal-regulated kinases; ES, excretory/secretory antigens; EWAS, epigenome-wide association studies; GWAS, genome-wide association studies; HAT, histone acetyltransferase; HAV, Hepatitis A virus; HDAC, histone deacetylase; ICS, inhaled corticosteroids; MAPK, mitogen-activated protein kinase; miRNAs, MicroRNA; SOCS, suppressor of cytokine signaling; STH, soil-transmitted helminths; VNN1, Vanin-1.

and established intrinsic symbiotic relationships which could evolve as a unit. The term holobiont was first coined by Lynn Margulis (48) and consisted of a simple and elegant way to explain how a host and its symbiont would evolve (49). This concept has been expanded, and it is well-accepted that a holobiont consists of a set comprised of the host and its associated microbial communities, i.e., the microbiota composed of the three domains of life, and viruses (50). According to this concept, the host (i.e., plant or animal) is subject to ecological and evolutionary pressures, so the entire community would evolve according to natural selection (51–53). This concept has been widely adopted, especially in the coral and human microbiome literature (54, 55), and it is relevant to understand its implications on human health. Understanding the relationships and interactions between microorganisms and parasites, such as helminths and protozoans, with host cells and tissues within a holistic approach is of paramount importance (49) and may provide practical solutions for challenging problems such as antibiotic resistance, allergies and asthma (56). This concept is tightly linked with the One Health framework, which is a multidisciplinary collaborative effort to achieve most appropriate health for people, animals and environment (50, 57).

The advances in DNA sequencing technologies and computational tools enabled us to explore in great detail the microbial communities and their ecological relationships on several times and space scales (58). This is a flourishing time for microbiome studies and a robust body of literature has already elucidated how environmental drivers shape free-living and host-associated microbial communities (58). Several lines of evidence show that human health is tightly linked with the equilibrium of the commensal microbial community, ultimately holobiont homeostasis. The microbial biodiversity and the relationships and interactions among microbes lead to functional outcomes. Reducing diversity, usually by a dominant microorganism, promotes a more variable and less resilient microbiota, a phenomenon known as dysbiosis, which can alter the ecosystem services provided by the microbiota, leading to a disease state.

More specifically, for the scope of the present review, the mammalian gastrointestinal tract harbors a wide diversity of microorganisms. It is estimated that *Homo sapiens* DNA makes up only a small percentage of the overall DNA on and within the human body—far greater genetic contributions are derived from bacteria, fungi, viruses, archaea, and other microorganisms as part of a vast (and individually distinct) residential community collectively known as the human microbiome (48). Additionally, more than 100 trillion microorganisms, colonize the oral–gastrointestinal tract (59). The microbiota interacts and stimulates the host immune system by activating bacterial metabolism through biochemical pathways (60), mediated by diet, host and microbiota metabolites, and antimicrobial compounds (60). The commensal microbiota is essential not only for the use of nutrients through good digestion and resistance to infections by pathogens but also supports the regulation of the host immune system, influencing innate, and adaptive immune responses (61). Dysbiosis can lead to a disruption on immune homeostasis and, consequently, to diseases such as allergy, asthma, neurodegenerative disorders, autoimmune, cardiovascular, and metabolic diseases (60, 62).

Host-Bacterial Interactions

The presence of organisms/microbes in the human body is important to induce a proper immune response, including a regulatory mechanism that could even have a bystander effect of inflammatory conditions (63). The immune system is regulated by immune organs and cells, soluble cytokines, and cell receptors (64). The gut-associated lymphoid tissue is composed of three different lymphoid structures of the mucosa: immune cells present in the compartments of the intestinal epithelium, lamina propria, and Peyer's patches of the small intestine (61, 64). Commensal human host bacteria modulate the immune system through a bridge between epithelial cells and lymphoid structures (65). It has been previously described that microbiota can induce both Th17 and T regulatory (Treg) immune responses (66). The interaction with epithelial cells induces Th17 cell polarization and a positive regulation of antimicrobial proteins. Th17 cells are vital for protective host immunity and have been implicated in autoimmune disease development by producing the pro-inflammatory cytokines IL-17A, IL-17F, and IL-22 (59, 66).

Clostridia, segmented filamentous bacteria, *Bacteroides fragilis,* and other microorganisms can induce the development and/or activation of Treg cells by stimulating intestinal epithelial cells, lamina propria dendritic cells (DCs) and macrophages (59). However, it is unclear which molecular mechanisms commensal microbiota induce Treg cells in the gut (67, 68). Treg cells control autoimmune reactivity, suppress inflammatory responses, and maintain homeostasis of the microbiota (69). According to Kamada et al. (59), the reduction of Treg cells can increase the expansion of CD4+ Th cells expressing commensal bacteria-specific T cell receptors (TCRs), leading to intestinal inflammation.

In fact, the mechanisms whereby commensal microbiota can modulate immune response is an area of increasing interest. In this context, the immune cells in the Peyer's patches are responsible for the surveillance of the intestinal lumen (70). Peyer's patches contribute to the generation of B cells, which, once activated, produce intestinal secretory IgA (sIgA) (64). IgA is the most abundant class of immunoglobulin produced in mucosal tissues, mostly the gut (59, 71). sIgA is essential for the neutralization of toxins and response to pathogens. It promotes intestinal barrier function and supports maintaining host–commensal mutualism. In addition, IgA is involved in determining the diversity and regulating the composition and function of the gut microbiota (59, 70). Innate lymphoid cells (ILCs), categorized into three subsets (groups 1, 2, and 3), help also with the homeostasis, control the composition of the microbiota, contribute to the resistance to pathogens and heal the gut (59, 64). ILC1s promote homeostasis through the production of IFN-γ, while ILC2s are activated by IL-25 (induced by commensal microbiota) to release amphiregulin (Areg), which is responsible for tissue repair, and IL-5/6, which has a role in the production of IgA by B cells (72). IL-22 induces the production of ILC3s, leading to mucus production, the release of the antimicrobial peptide, fucosylation (a type of glycosylation)

of the proteins from the lumina and lipids that offer energy for the microbiota (72).

Some commensal bacteria, such as *Clostridia* strains, have been shown to suppress the immune response by promoting the differentiation of Tregs and IL-10 production in the gut (65, 73). The induction of colonic Tregs can depend on *Clostridium* cluster IV and XIV and the production of metabolites, such as short-chain fatty acids (SCFAs), which have immune and metabolic functions involved in the regulation of cellular processes (74, 75). SCFAs are metabolites synthesized by bacterial fermentation of indigestible carbohydrates, in the colon, and decomposition of dietary fibers (61, 76). Propionate, butyrate, and acetate are the most predominant SCFAs in the gut and enable Treg production (73, 75). Butyrate is involved in Treg differentiation by binding G-protein-coupled receptor 43 (GPR43), a receptor of SCFAs present in colonic T cells (76). Butyrate has also been shown to induce Treg cell differentiation via dendritic cells dependent on GPR109a (77). This metabolite also can regulate central steps of the eosinophil lifecycle and function (78), inhibit ILC2 proliferation and cytokine production likely through inhibition of GATA3 expression (79), inhibit nuclear factor- κB (NF-κB) signaling via protein acetylation by a HDAC inhibitor (80) and limit the production of TNF by lipopolysaccharide (LPS)-stimulated neutrophils (81) and peripheral blood mononuclear cells (66) (**Table 1**).

Escherichia coli trytophanase produces indole from tryptophan (94). This metabolite activate aryl hydrocarbon receptor, a transcription factor that induces expression of genes such as CYP4501A1, which cleans chemicals and toxins (95). Indole has an immunomodulatory function by maintaining the integrity of the enteral mucosa and promoting the epithelial barrier defense against pathogens by stimulating the production of anti-microbial peptides, mucins, and proliferation of intestinal goblet cells (62) (**Table 1**).

Polysaccharide produced by *Bacteroides fragilis,* a species of gut microbiota, was described to conduct systemic immunological maturity and could restore the balance between Th1 and Th2 cells and CD4+ T cell deficiency in germ-free mice (65, 66, 74). *B. fragilis* triggers toll-like receptors to create a symbiosis between the host and microbiota and affects the differentiation and development of T cells (74). *Lactobacillus reuteri* is a Gram-positive facultative anaerobic bacterium that also resides in the gut microbiota. This microorganism has many benefits as a probiotic, such as reducing infection, influencing the integrity of gut mucosa, and modulating the host's immune responses (96). *L. reuteri* has a role in protecting lung infections, stimulating the production of gut granulocyte-macrophage colony-stimulating factor, which promotes clearance of pathogens by alveolar macrophages (74, 96).

Host-Fungus and Viruses Interactions

Although bacteria are a main component of the human microbiota, there are other organisms also composing the holobiont such as fungi, viruses, and multicellular parasites that are also important for a good balance, with potential effects on human health. The most-reported fungi in the intestines of mice and humans include *Saccharomycetes* (*Candida* and *Saccharomyces* spp.), *Eurotiomycetes* (*Aspergillus* and *Penicillium* spp.), *Tremellomycetes* (*Cryptococcus* and *Trichosporon* spp.) along with *Cladosporium*, *Wallemia*, and *Malassezia* spp. (97).

Candida albicans interacts with intestinal epithelial cells through some events, including adhesion, invasion, damage, and apoptosis (98). This interaction can lead to superficial overgrowth and epithelial invasion, followed by disease and immune activation (82). The Candidalysin, a cytolytic peptide toxin released by *C. albicans*, induces proinflammatory cytokines, chemokines and antimicrobial peptides of epithelial cells that are necessary for the recruitment of immune cells, via MAPK signaling, specifically the p38 pathway, resulting in the activation of the AP-1 transcription factor c-Fos, and the ERK1/2 pathway, leading to the activation of MKP1 (MAPK phosphatase 1), which regulates the immune response (82).

Aspergillus fumigatus produces a variety of precursors of toxins such as gliotoxin, which represses IFN-γ responses and induces neutrophil apoptosis through inhibition of NF-κB, a transcriptional regulator of the host proinflammatory response (99); and fumigaclavine C that down-regulates Th1 cytokines, by binding to IFN-ɤ receptor 1 (IFN-ɤ R1) (100) and induces host cell apoptosis via caspases-3,−8, and−9 (83, 101).

In addition to bacteria and fungi, the intestinal virome is composed of DNA and RNA viruses and includes eukaryotic viruses, endogenous retroviruses and bacterial viruses (102). According to (84), eukaryotic viruses and bacteriophages can stimulate changes in the immune response. Eukaryotic virus by altering the hematopoiesis or immune activation, improving a secondary infection. Bacteriophages by stimulating the production of inflammatory cytokines and type I interferon. These changes in immune responses can contribute to inflammatory diseases. In this review, we will focus in unicellular and multicellular organisms leading to immune modulation.

Host-Helminths Interactions

Moreover, the different life cycle stages of helminths and protozoa challenge host immune responses to recognize and respond to different antigens. Distinct pattern recognition receptors members participate in the recognition of these parasites and are responsible for driving the TCD4 + cells polarization. Many molecules secreted by adult intestinal worms known as "excretory/secretory antigens" (ES) can stimulate different effects on the host's immune cells. The helminth ES products activate basophils, eosinophils, mast cells, innate lymphocyte T cells 2 (ILC2) and TCD4 + cells and drive the production of innate and adaptive cytokines. Different classes of lipids extracted from schistosome eggs and adult worms have been able to stimulate the production of several inflammatory cytokines (IL-6, IL-8, IL-10, IL-12, TNF-α). Schistosomal lysophosphatidylserine through TLR2 stimulates activation of dendritic cells with subsequent development of IL-10 producing Treg cells (85) and *Ascaris lumbricoides* derived phosphatidylserine containing preparations in the presence of interaction between TLR4 and LPS induced TLR2 with activation of TH2 response (91).

Schistosomal-Derived Lysophosphatidylcholine *in vivo* was able to induce cytokine production and eosinophil

TABLE 1 | Summary of the main products (molecules) from holobionts with immunomodulatory potential and biological activities in the host.

Microbial	Molecules	Biological activities	References
Clostridium	SCFAs	• Anti-inflammatory activities • Regulate Treg production • Inhibit nuclear factor- κB (NF-κB) signaling • Limit the production of TNF in neutrophils and peripheral blood mononuclear cells	(60, 77, 80, 81)
Escherichia coli	Indoles	• Immunomodulatory function • Integrity of the enteral mucosa • Promotes epithelial cell barrier function	(62)
Bacteroides fragilis	Polysaccharide A	• Influences T cells fate through its • Interaction with the toll-like receptor 2.	(66)
Candida albicans	Candidalysin	• Induces proinflammatory cytokines, chemokines, and antimicrobial peptides	(82)
Aspergillus fumigatus	Gliotoxin and Fumigaclavine C	• Suppresses interferon (IFN)-γ • Downregulates Th1 cytokines • apoptosis	(83)
Eukaryotic Virus		Alteration in hematopoiese or immune activation	(84)
Bacteriophages		Production of inflammatory cytokines and type I interferon	(84)
Schistosoma mansoni	Schistosomal-Derived Lysophosphatidylcholine; The soluble extract of eggs (SEA) and lacto-N-fucopentose III; Schistosomula tegument (Smteg) Sm22·6, PIII, and Sm29 antigens Schistosomula tegument (Smteg) Sm22·6, PIII, and Sm29 antigens	TLR2 activation • IL-10 producing Treg cells • Eosinophil recruitment • DC2 maturation • Polarization of the Th2 response. • Phosphorylation of ERK • Up-regulation of CD40 and CD86 expression • IL-12 and TNF-α production • Reduction of eosinophils in the BAL • Reduction of specific IgE • Increase in IL-10 (Sm22·6) • Reduction in IL-4 and IL-5 levels in the BAL. (PIII and Sm29)	(85–90)
Ascaris lumbricoides	Phosphatidylserine containing preparations (PS)	• TLR2 activation • Polarization of the Th2 response.	(91)
Schistosoma ssp. A. lumbricoides	Glutathione transferases	• Stimulate specific IgE antibodies	(43, 92)
Leishmania spp., *Toxoplasma gondii*	The glycosylphosphatidylinositol (GPI) anchors	• TLR2 and TLR4 activation induce of TNF-α	(93)

recruitment potentially through TLR2 recognition (86). Lysophosphatidylcholine participates in the recruitment of eosinophils (85) IL-5 and IL-3 stimulate eosinophilia, and recruitment is mediated mainly by chemoattractant CCL11 and CCL26 (eotaxins). Activation of eosinophils results in degranulation of chemical mediators such as Matrix metalloproteinases, cysteinyl leukotrienes, major basic protein and others (103). It has been shown that patients with *Schistosoma* infection exhibit a higher concentration of CCL3, CCL5, and CCL11 in plasma compared to uninfected individuals. These chemokines favor granulocyte recruitment, granulomatous response against egg antigens (104, 105).

Antigens from *Schistosoma mansoni*, Sm22·6 (soluble protein from the tegument of *S. mansoni*), PIII (multivalent antigen from the *S. mansoni* adult worm) and Sm29 (a membrane-bound glycoprotein from the adult worm tegument) were tested in a murine model of induced airway inflammation and showed immunomodulatory ability. These antigens induced a reduction in the number of eosinophils in bronchoalveolar lavage (BAL) and lower levels of specific IgE. In addition, Sm22·6 was associated with an increase in IL-10 while PIII and Sm29 showed a reduction in IL-4 and IL-5 levels in the BAL (90).

The soluble extract of *Schistosoma mansoni* eggs and lacto-N-fucopentose III (carbohydrates group in *S. mansoni*) has been associated with DC2 maturation and induction of the Th2 response dependent on recognition by TLR4, as well as induces phosphorylation of ERK (87, 88). In addition, schistosomula tegument (Smteg) can induce up-regulation of CD40 and CD86 expression and production of proinflammatory cytokines, such as IL-12 and TNF-α, and such activation is TLR4-dependent (89).

The glutathione transferases from helminths (*Schistosoma ssp.* and *A. lumbricoides*) stimulate specific IgE antibodies (92, 106) The glycosylphosphatidylinositol anchors from protozoan (*Leishmania* spp., *Toxoplasma gondii*) is involved in the activation of cells of lymphoid and myeloid lineage, such molecules are recognized by TLR2 and TLR4 with activation of NF-κB and subsequent induction of TNF-α in murine macrophage cells (93).

The secretion of ES products from hookworms induces activation of ILC2s and tolerogenic dendritic cells, followed by increased expression of molecules associated with tolerance and

reduced expression of co-stimulatory molecules with expansion of Treg cell numbers in the gut and suppresses Th17 cell, this implies a decrease in inflammation and proliferative capacity of the parasite (107, 108). Interestingly, the Howkworms' tolerance ability was demonstrated in experimental hookworm infection in patients with celiac disease, *Necator americanus* infection suppressed gluten-induced IFNγ, IL-17, and IL-23 expression and increased the expression of IL-10, TGFβ, and IL-22 in the gut (107, 109).

It has been shown that infection with geohelminths (*A. lumbricoides, Trichuris trichiura,* hookworm) induces IL-10 and a higher mRNA expression of the Foxp3, PD-1, and regulatory molecules suppressor of cytokine signaling (SOCS) (−3) (110), reinforcing the immunomodulatory capacity of geohelminths. In addition, many of the ES components have pleiotropic immunomodulatory properties.

Taken together, it is possible to see that a balanced holobiont is necessary to maintain homeostasis. Any alteration in this environment can lead to dysregulation of the immune system and metabolism. Further studies are needed to exactly describe how holobionts changes regulate the host immune system, and which changes in its composition is associated with specific diseases.

THE RELATIONSHIP BETWEEN THE SHIFTS IN HOLOBIONT COMMUNITY'S COMPOSITION WITH ASTHMA AND ALLERGIES

Exposures during the peri- and post-natal periods are critical for the host's immune homeostasis, reflecting immune maturation, the development of immune tolerance mechanisms, and susceptibility to disease, also known as the first "window of opportunity" (111). This exposure includes fetal environment conditioned to the individual to the mother's lifestyle, type of delivery, diet, use of antibiotics, exposure to other children and animals, and contact with parasites and environmental microbes (112). Studies have reported that exposure to specific immunostimulatory molecules (from helminths and bacteria mainly) in childhood could reduce or block allergic disease development or progression (113). In embryonic development the immunological regulation of pregnancy is complex and an increased production of Th2 cytokines is observed, along with decreased production of Th1 cytokines. In addition, TGF-β1 appears to be involved in the differentiation of the trophoblast being an important inducer of regulatory T cells (CD4 + CD25 +) and Th17 cells, this seems to be essential for avoiding fetal allorejection (114, 115). Microbial exposures in childhood determine factors in modulation and gradual replacement for T cells and cytokines other than Th2 (116).

Moreover, universal initiatives seeking to improve the population's health conditions, such as immunization in children, improved hygiene and sanitation, access to clean water, indiscriminate use of antibiotics and anti-parasitic drugs, have been implied in reducing opportunities of microorganism's exposure/infections in early childhood with decreased Th1 responses and or decreasing Treg activation and polarizing the immune response to the Th2 profile, breaking homeostasis. Changes in the exposure of antigen patterns, including proteins released from environmental particles or infections in childhood, can impact the diversity of commensal microorganisms that make up the microbiota (117).

The use of antibiotics by mothers during pregnancy is associated with a child's asthma risk, promoting an imbalance between commensal, and pathogenic bacteria (118). Changes in the colonization of the lung microbiota of neonatal mice have (119, 120) been associated with decreased aeroallergen responsiveness induced by Helios– regulatory T cells (Helios–Treg cells) activated depending on interaction with programmed death-ligand 1 early in life, widely known as a regulator of allergic responses. Imbalance in the formation of these cells implies increased susceptibility to atopy in adulthood (119). Likewise, the altered composition of the airway microbiota is often found in asthmatic patients (120, 121). This could be explained partially by differences in environment.

Rural vs. Urban

For instance, the prevalence and severity of asthma differ between urban and rural areas. An agricultural environment has been associated as a protective factor against the development of asthma, hay fever, and atopic sensitization in children (12, 122). An explanation would be associated with concentrations of endotoxin significantly higher in rural homes than in urban centers (123). Exposure to higher levels of endotoxin and other bacterial components in early childhood can play a protective role against allergies and asthma (123). The endotoxin constitutes the membrane of gram-negative bacteria, inducing the Th1 response by stimulating cytokines such as IL-12 and IFN-ɤ (12). In addition to that, helminth infections caused by *Ascaris lumbricoides*, *Trichuris trichiura*, are more prevalent among children living in areas of the rural tropics in poverty and poor access to clean water and sanitation (124).

Helminths vs. Asthma/Allergies

Helminths and allergic asthma induce similar immune responses, including elevated serum IgE, systemic eosinophilia, and cytokines such as IL-4, IL-5, IL-9, and IL-13, the hallmark of an immune Th2 response (125). Additionally, basophils, mast cells, neutrophils and innate lymphoid cells are involved (126). Interestingly, infections by parasite species such as *A. lumbricoides*, *Schistosoma mansoni*, *Strongyloides stercoralis*, and *T. trichiura,* have been associated with a reduction in airway allergic inflammation (34) with decreased Th1 responses (**Table 2**). The immunomodulatory ability of geohelminths to reduce susceptibility to allergies in humans has been recognized, and it is related to the immune-regulatory network, including helminth-derived products. Recombinant proteins of *S. mansoni* were associated with an increase in IL-10 and TGF-β, an increased frequency of regulatory T and B cells, and a reduction in the frequency of activated T lymphocytes that produce IL-4 and IL-13 in individuals with severe asthma and animal models (142, 143). In addition, *T. trichiura* infection appears to modulate the immune response among asthmatics, with some studies

TABLE 2 | Summary of the immunomodulatory effects of some halobiont's organisms on asthma and allergy.

Holobiont	Immunomodulatory effects	Consequences
Schistosoma mansoni	↑TNF-a and IFN-g in acute phase	Prevent against the development of allergies and asthma (32, 34)
	↑ IL-10 in chronic phase	Down-modulate the inflammatory response in murine model of ovalbumin (OVA)-induced airway inflammation (90)
Ascaris lumbricoides,	↑IL-4, IL-5, and IL-10	*Ascaris lumbricoides* eggs was associated with an increased prevalence of asthma (124) Reduced risk of wheeze (127) Anti-A lumbricoides IgE antibodies were associated with risk of wheezing in atopic children and atopia (36)
Trichuris trichiuria	Modulation of pro and anti-inflammatory cytokine (35) ↓TNF-α and IL-6 levels among asthmatics infected ↑IL-10	↓allergen skin test reactivity (33) Positively associated with wheezing (36)
Helicobacter pylori	Th1 polarization ↓Th2 response ↑ (IFN)-γ, IL-12, IL-18, IL-23 (128)	Negative association between H. pylori infection and asthma, eczema, and rhinitis (128)
Hookworm (*Ancylostoma duodenale* and *Necator americanus*)	Induction of IL-25 and ILC2s (129) ↑IgG1, IgG4, and IgE Expansion of Treg cell numbers in the gut (107, 108)	Protect against wheezing, asthma, and allergic diseases. Reduction in risk of wheeze (130)
Toxoplasma gondii	Induces IL-10 production, IL-27, and activity of lipoxins (131, 132).	Protective effect against atopy (133) Suspend the development of airway inflammation and atopy in mice (133) Decrease in specific IgE for *Dermatophagoides pteronyssinus* (134)
Toxocara spp.	↑ levels of total IgE Cross reactivity with aeroallergens	Positive skin tests to allergens, and asthma prevalence and morbidity (135, 136)
Bifidobacterium	Stimulating IL-10 or IL-12 synthesis	Protective factor for high risk of allergic asthma and atopic dermatitis in children from Turkey (137)
Bacteroides fragilis	Stimulate Th2 cytokines by biding TLR2	Risk factor in children with a positive API (138)
Penicillium	High counts in patients with atopy	Risk factor for atopic asthma (139, 140)
Aspergillus fumigatus	Decrease the expression of GCR	Aggravate airway hyper-responsiveness and increase the level of TLR2 (141)

↑*increase;* ↓*decrease.*

reporting a risk association for asthma among infected people and positively associated with wheezing (33, 35, 36).

Maternal soil-transmitted helminths (STH) infections can sensitize the individual still in the fetal phase. Cooper et al. (144) reported a strong association between Maternal STH infections during pregnancy (mainly moderate to chronic *A. lumbricoides* infection) and childhood STH infections. During pregnancy, infected mothers have an increased number of CD4 + T cells and production of IL-10 in cord blood from newborns demonstrating immunomodulation mediated by parasite antigens (145, 146). In the same study, poor hygiene conditions, with the prevalence of STH infections, were not associated with reduced eczema-asthma-rhinitis symptoms (144). Co-exposure to mites and *Ascaris lumbricoides* in the context of low worm burdens promotes allergic sensitization and asthmatic symptoms by increasing parasite-specific IgE production, mite-specific and mite–parasite cross-reacting IgE antibodies, observed mainly in urban areas, once in rural areas the exposure to helminths tends to be chronic (40). *A. lumbricoides* extract was associated with inhibition of pulmonary eosinophilia in mice sensitized with ovalbumin (OVA) and a decrease in allergic inflammation independent of IL-10 (147) (**Table 2**). In contrast, Anti-*A. lumbricoides* IgE (but not active infection), were associated with risk of wheezing in atopy in atopic children (36).

Viruses and Protozoans vs. Asthma/Allergies

Some viral and protozoan infections have been associated with decreased reactivity to skin prick tests for aeroallergens (148, 149) and asthma (150). The host's defenses against viruses are marked by a predominance of the Th1 response and interaction with different Toll-like receptors with probable biological and immunomodulatory effects on Th2 responses. *Toxoplasma gondii* infection has been reported to suspend the development of airway inflammation and atopy in mice (133) and induces IL-10 production, IL-27 and activity of lipoxins (131, 132). In addition, a negative association was reported between *T gondii* seropositivity and specific IgE to *Dermatophagoides pteronyssinus* (134). Hepatitis A virus (HAV) exposure has been inversely associated with allergies (151). In the United States, positive serology for HAV was associated with a lower chance of developing hay fever and asthma and skin reactivity to airborne allergens (152). In Turkey, the prevalence of atopy was lower among individuals with positive serology for HAV and hepatitis

B virus (anti-HAV IgG, HBsAg, anti-HBc IgG) (153). The accumulated infection burden, considering HAV, herpes simplex virus, Epstein–Barr virus, Cytomegalovirus, *Helicobacter pylori*, and *Toxoplasma gondii* (> 3 microbes), was associated with a protective effect against atopy (149, 153). According to Amedei et al. (128) *H. pylori* infection was negatively associated with asthma, eczema and rhinitis and induces Th1 polarization (**Table 2**). Moreover, BCG vaccination at an earlier age was associated with a decreased risk of atopy in children without a family history of asthma and atopy (154). However, there are controversies regarding some types of vaccines (155, 156).

Bacteria vs. Asthma/Allergies

A study (157) from the Copenhagen Prospective Study on Asthma in Childhood has shown that the lack of development of the gut microbiome in the first year of life is the determinant to the occurrence of childhood asthma, increasing asthma risk. The lower number of *Lachnospiraceae* and *Ruminococcaceae* genera was observed in asthmatic children and was associated with allergic wheezy phenotype (157). The production of SCFAs was suggested to be associated with asthma development in a study of high vegetable fiber intake by children from Manitoba Prospective Cohort Study of Allergy, Genes and the Environment, acting as a protective factor against to airway hyperresponsiveness (158, 159). *Bifidobacterium longum* has been described influencing the prevalence of allergic disease being a protective factor for allergic asthma and atopic dermatitis in children from Turkey (**Table 2**) (137). In contrast, *Bacteroides fragilis* count was significantly higher in children with a positive Asthma Predictive Index as compared with those negative (138). It seems that *Bacteroides* species maybe stimulate Th2 cytokines and some studies have found an association between this genera and higher IgG in children with allergies (138) (**Table 2**).

Fungus vs. Asthma/Allergies

Skin-test for fungal allergens is usually characterized with the presence of immediate cutaneous hyperreactivity or positive results for specific IgE antibodies to fungal antigens and has been related to be especially common in patients with life-threatening asthma (139, 160). *Aspergillus*, *Alternaria*, *Penicillium*, *Cladosporium,* and *Trichophyton*, have been described to be associated with exacerbation and severity of asthma (139). *Penicillium* species was higher in patients with atopy compared with healthy control subjects, suggesting to be a risk factor for atopic asthma since this genera is one of the most common fungi related to allergic asthma exacerbations among adults (**Table 2**) (140, 161). A study using rats with asthma shows that *Aspergillus fumigatus* may decrease the expression of glucocorticoid receptor aggravating airway hyper-responsiveness and increase the level of TLR2, involved in airway inflammation (141) (**Table 2**).

The immune response in the context of asthma and atopy as well as its development, differentiation of cell subtypes and expression of receptors and cytokines are influenced by exposures to holobionts. This immunological modulation is often accompanied by epigenetic changes. In part, such modifications that allow such plasticity of immune responses, also promote homeostasis through the balance of adaptive immune responses in certain conditions and are responsible for the maintenance and intensification of Th2 responses, increasing the risk for allergic diseases and other inflammatory diseases.

EPIGENETIC MECHANISMS: BASIC CONCEPTS

Currently, epigenetics can be defined as changes above the DNA without changing the nucleotide sequence (162). Different mechanisms of epigenetic regulation have been described, such as DNA methylation, histone modifications and non-coding RNAs. Since the first Waddington epigenetics works (163, 164), many studies have been conducted to determine the influences of epigenetics in several conditions. The epigenetic mechanisms are widespread in the different cell types of the human body, including cells that participate in an immune response pathway directly involved in the etiopathogenesis of asthma and other allergic diseases. Understanding the impact of epigenetic changes on the normal and abnormal functioning of these cells, therefore, is an important piece to compose the complex puzzle that allergic diseases represent. Bellow, are described the main mechanisms of epigenetics-induced changes in gene expression.

DNA Methylation

DNA methylation is the addition of a methyl group (CH3) to a cytosine by DNA methyltransferases, generating 5-methyl-cytosine (165). Promoter regions of genes have a large amount of CpG (cytosine-phosphate-guanine), known as CpG islands, that when methylated prevents the binding of transcription factors and represses gene expression (166).

Several factors can contribute to DNA methylation changes, such as aging, environmental exposure, cell type, and age. These modifications can be passed through cell division through either mitosis or meiosis (167). Recently, many epigenome-wide association studies (EWAS) have described the association between DNA methylation and asthma, and several genes were identified, including EPX, IL4, IL5RA, PRG2, SIGLEC8, CLU, AP2A2, and KCNH2 (168–170).

Histone Modifications

Histone is a protein involved in the organization of chromatin and regulation of gene expression. They are grouped into 8 subunits, two of each H2A, H2B, H3, and H4 forming an octameric nucleosome where the DNA coils. Histone H1 is associated with this complex and stabilizes the chromatin structure. Some modifications can occur in the N-terminal tails of histones, including acetylation, methylation, ubiquitylation, and phosphorylation (171).

Histone acetylation occurs when acetyltransferases add lysine residues to histone tails. Histone acetylation increases DNA access and facilitates the process of transcription, increasing gene expression. Previous studies reported that H3K4me3 and H3K27me3 were associated with T helper cell differentiation and IL-5 expression (172), and higher histone 3 acetylation levels at the IL13 locus were associated with higher protein levels of IL13 (173).

Methylation in histones is performed by methyltransferases and usually occurs at lysine (K) or arginine (A) residues and can increase or decrease gene expression depending on the modified residue. For instance, inactivation can occur by methylation on H3K9, H3K27, and H4K20, while activation occurs by methylation on H3K4 and H3K36 (174).

Non-coding RNAs

Non-coding RNAs are a group of RNAs that do not encode proteins but can play an important role in the regulation of gene expression acting at the post-transcriptional level (175). Regarding size, RNAs with regulatory functions are divided into short non-coding RNAs (siRNAs, miRNAs and piRNAs) and long non-coding RNAs (lncRNAs) (176). They can silence genes through the RNA interference pathway and modulate several biological processes, including immunological functions (177).

SHAPING IMMUNE RESPONSES THROUGH EPIGENETICS MECHANISMS: REGULATION OF CYTOKINE GENE EXPRESSION, TRANSCRIPTION FACTORS, AND REGULATION OF IMMUNE RESPONSES IN ASTHMA AND ALLERGY

The epigenetic mechanisms previously described are present in the different contexts and cell types of the human body, including driven immune cell pathways directly involved in the etiopathogenesis of asthma and other allergic diseases. Understanding the impact of epigenetic changes on the normal and abnormal functioning of these cells, therefore, is an important piece to compose the complex puzzle that allergic diseases represent. Some advances in this direction have recently been achieved. Thus, epigenetic modifications play a role in regulating the expression of cytokines related to T cell differentiation and transcription factors (178). The development of cell types and, consequently, the specificity of immunological responses occur through internal stimuli or driven by stimulatory molecules of microorganisms. They act on surface receptors such as TLR signaling, signal transduction proteins, and lineage-specifying transcription factors, promoting intracellular events. Even the development of T lymphocytes and maturation for helper (CD4+) and cytotoxic (CD8+) cells are influenced by epigenetic control. This promotes CD4+ silencing in CD8+ thymocytes and the development of T helper cell subsets (Th1, Th2, and Th17) accompanied by epigenetic changes (179, 180). Epigenetic changes have also been linked to the activation and polarization of macrophages (M1/M2 phenotypes) (181).

Allergic diseases, e.g., asthma, result from a strong interaction of genetic and environmental components with remarkable phenotypic heterogeneity. This heterogeneity of asthma can be partially explained by dysregulated epigenetic mechanisms correlated with environmental exposures, pharmacological treatments, and airway inflammation and function (182). There is evidence that the induction of Th2 cells, maintenance, and the resurgence of memory Th2 cells are controlled by epigenetic regulation since this induction is mediated by signal transducer and activator of transcription 6 and the consequent production of the Th2 cytokine profile (183). Hypermethylation in GATA3 CpG loci was associated with a decreased risk of asthma at birth (184), and hypomethylation of IL-13 and interleukin 5 receptor subunit alpha (IL5RA) was associated with an increased risk of asthma in teenagers (169).

Epigenetic mechanisms are essential in controlling gene expression or silencing and the consequent balance of Th1/Th2 responses. Corroborating the principle that Th1/Th2 imbalance is involved in the pathogenesis of asthma and atopy, experimental studies in mice showed hypermethylation in the IFN-ɤ gene promoter in TCD4+ cells, leading to the silencing of the *IFNG* gene (Th1 pattern) (185). During initiation of a Th2 immune response, an increase in histone acetylation was observed at the Th2 cytokine *loci*. It has been demonstrated that the *IL4* and *IL13* genes are hypomethylated in asthmatic patients, critical genes in amplifying the Th2 response (186).

A balance of histone deacetylase (HDAC) and histone acetyltransferase (HAT) activity has been considered to regulate gene expression. Reduced HDAC expression was observed among adults with severe asthma compared to mild asthma (187). In atopic asthmatic children, a relationship was found between HDAC/HAT activity and increased histone acetylation, and the degree of acetylation was associated with an increase in bronchial hyperresponsiveness (188).

miRNAs have also shown an essential role in the inflammatory response of asthma. Studies have shown that miR-155 and miR-221 are associated with modulation of the Th2 response (189) and hyperproliferation of airway smooth muscle in asthmatic patients, respectively (190). In the asthma context, non-coding RNAs have been observed as markers of disease diagnosis, phenotypes, and response to treatments. For example, a negative correlation between the levels of miR-323-3p and IL22 and IL17 was observed in PBMCs from patients with asthma, suggesting that non-coding RNA acts as negative feedback in the production of these cytokines influencing the immune response of these individuals (191). Moreover, elevated levels of miRNA-21 in the peripheral blood of children with asthma were identified, suggesting that this non-coding RNA may be a biomarker in the diagnosis of asthma (192). Furthermore, high expression of microRNA-155 and decreased expression of Let-7a were observed in the plasma of asthmatic patients and were associated with the degree of asthma severity, suggesting that these markers can be used both in diagnosis and in the prediction of the severity of disease (193).

In the context of regulatory T cells (Tregs), cells that play a substantial role in immune homeostasis through mechanisms of tolerance and immune de-activation during a regular immune response and suppression of a self-destructive immune response, the repressive phenotype of Tregs is conferred, in part, by the expression of Forkhead box protein 3 (FOXP3) (194). Hypermethylation of CpG islands in the promoter region in the *FOXP3* locus impacts transcriptional silencing and consequent reduction in Treg cell function. Air pollutants have been recognized as acting on epigenetic changes. Increased exposure to polycyclic aromatic hydrocarbons has been associated with an increase in DNA methylation at the *FOXP3* locus in peripheral

blood mononuclear cells and elevated total IgE with significant effects on asthmatics (195). Furthermore, an increase in *FOXP3* DNA methylation has been associated with an increased risk of asthma and persistent wheezing (196).

Figure 1 shows different factors shaping asthma and allergy, such as environmental factors, epigenetic changes, and exposure to holobionts components which can be modulated by disturbances in the homeostasis.

EPIGENETIC CHANGES ASSOCIATED WITH HOLOBIONT INTERACTIONS

Since immune dysregulation is linked to allergies and asthma through the lack of certain environmental exposure, one could think that potential epigenetic mechanisms may play a role in this phenomenon. A question raised by these new findings is at "what point in the development of the human being the epigenetic mechanisms could act to drive the maturation of the immune system in early life?." In this sense, in recent years, the hygiene hypothesis has been expanded to encompass the potential effect of prenatal exposure to microbial agents on modulating the individual risk of asthma and other allergic diseases (115). Although some evidence in this regard was already available through epidemiological studies that assessed the impact of maternal microbial exposure on the risk of developing allergic conditions in the offspring (116), the elucidation of the molecular mechanisms underlying these processes has been a relatively new and fascinating field of investigation.

Alterations in the gut microbiota, called dysbiosis, is related to infections and inflammatory diseases and comes with irregular immune responses, e.g., particular inflammatory cytokines (66, 75). Changed gut microbiota can also increase the production of NK-κ-B and TNF-α and the overexpression and activation of Th1 and Th17 cells (197). Studies have shown that changes in the gut bacterial composition and the production of its metabolites can influence epigenetic levels, such as reducing methylation and inhibiting histone deacetylases (197). Specifically, the metabolites influencing epigenetic enzyme activity are a substrate needed for epigenetic changes (197). For example, Butyrate, a metabolite from microbiota, can also inhibit HDAC, increasing the expression of FOXP3 through the acetylation of histone H3 in the promoter and enhancing Treg generation (164).

One of the first mechanistic studies on the allergoprotective effects of maternal exposure to microbes used a mouse model with the farm bacterium *Acinetobacter lwoffii* (198). In this study, the protective effect for allergic airway inflammation (AAI) in the offspring was dependent on maternal TLR signaling, since this protection was abolished when mothers were knocked out for multiple TLR genes. The authors also demonstrated that the immune dampening observed in the progeny of pregnant mice was not due to microbial components able to pass the fetus-maternal interface and directly activate the developing fetal immune system. This last observation suggests the possible involvement of epigenetic factors operating in the fetuses of mothers exposed to *Acinetobacter lwoffii*. Indeed, another study by the same group reported epigenetic changes in Th1/Th2 cytokine genes in offspring from pregnant mice exposed to *A. lwoffii* (199). While the IFNγ promoter on CD4+ T cells exhibited significant protection against the loss of histone 4 (H4) acetylation, with the consequent increase in IFN-ɤ expression in OVA-induced AAI, the IL4 promoter showed a significant decrease in H4 acetylation and diminished gene expression. A protective effect against induced AAI has also been shown in the progeny of pregnant mice exposed to *Helicobacter pylori* extracts (200). An epigenetic consequence observed in the offspring was the enhanced demethylation of the regulatory T cell-specific demethylated region in Foxp3+ Treg cells. Intriguingly, this protective effect extended to the second generation (F2) of mice exposed to *H. pylori* antigens during pregnancy, with both sexes exhibiting similar levels of protection. This indicates that the epigenetic changes in the offspring induced by transmaternal exposure to *H. pylori* may extend to chromosomal *loci* other than just the TSDR linked to the X chromosome. The transfer of allergoprotective effects during the prenatal phase through maternal infection with the helminth *Schistosoma mansoni* has also been previously investigated in experimental models of AAI in mice (201). Interestingly, this protective effect was dependent on the stage of the immune responses to *S. mansoni* in the females at the time of mating. While the offspring of the mothers mated during the Th1 and regulatory phases showed protection against OVA-induced AAI, those born to mothers mated during the Th2 phase showed an exacerbation of the allergic inflammatory response compared to the controls. The authors also demonstrated that the protective effect of transmaternal exposure to *S mansoni* was mediated by maternally produced IFN-ɤ and not by the transfer of helminth antigens to the fetus. Potential epigenetic changes in the offspring associated with the protective immune phenotype, however, were not further investigated and remain to be clarified (202).

In humans, data on epigenetic changes induced by pre- or post-natal exposure to microbial agents and their relationship to asthma and other allergic conditions are still scarce. A pilot study evaluated the effect of maternal exposure to the farm environment on offspring epigenetic changes for genes known to be associated with asthma and allergies (203). Significant differences between non-asthmatic children born to mothers exposed to the farm environment and asthmatic children born to unexposed mothers were observed for the methylation pattern of the *ORMDL3* and *STAT6* genes in cord blood. In a recent study, Lund et al. (204) reported that changes in the methylation pattern in chromosomal previously linked to asthma, such as the SMAD3 promoter at 15q22.33 and intronic regions of the DDO/METTL24 genes at 6q21, were associated with atopic asthma in children with early rhinovirus-induced wheezing. In turn, DNA methylation changes linked to the prostaglandin D2 synthase gene were associated with non-atopic asthma in children with rhinovirus etiology at the first severe wheezing episode (204). This suggests that the epigenetic changes triggered by the same microbial agent may differ according to the specific phenotype of asthma or other allergic diseases, which needs to be further investigated in the future.

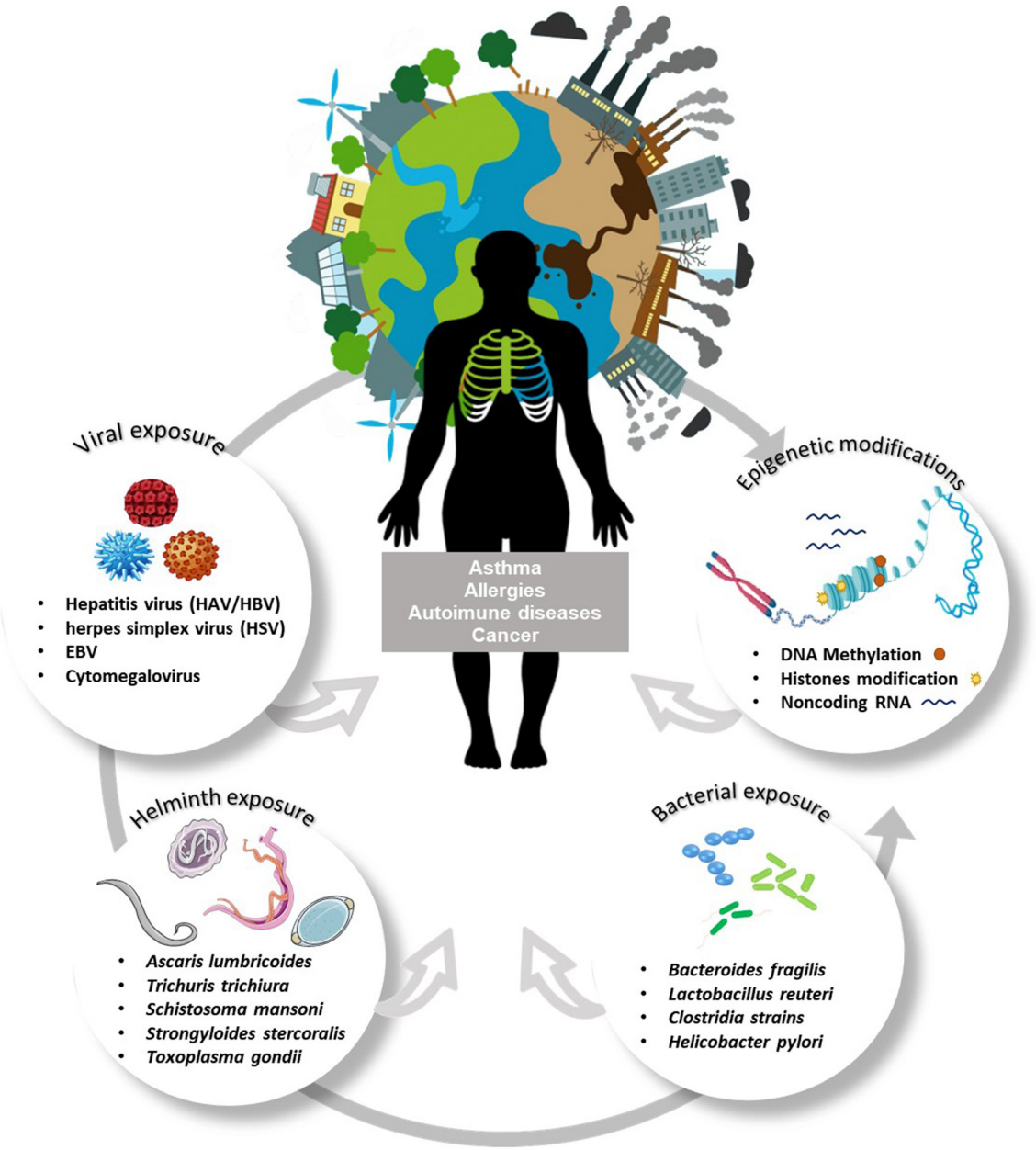

FIGURE 1 | Interaction between multiple environmental exposures and epigenetic changes: impact on immune-mediated diseases. Polluting agents, environmental exposure, diet, age, drugs, and especially exposure to organisms (species of bacteria, fungi, protozoan, and helminths) act as inducers of epigenetic changes. Among the epigenetic modifications are DNA methylation and histone modifications. Histone acetylation increases DNA access and facilitates the process of transcription, increasing gene expression. The addition of a methyl group in CpG islands prevents the binding of transcription factors and represses gene expression. The interaction between environmental exposures and epigenetic variations begins in the embryonic period and continues throughout life, being strongly dependent on the environmental experiences/challenges of everyone. Such alterations can be highly modifiable by instantaneous adaptation to the environment or generate inheritable epigenetic patterns and consequences to offspring. Epigenetic mechanisms influence cell differentiation and polarization of immune responses, these events modulate biological responses, and can interfere with the development of different immune-mediated diseases such as cancer, asthma, allergies, autoimmune diseases.

Taken together, although several studies related to epigenetics of asthma and allergies have been published so far, very few initiatives explores the role of the environmental changes, in special, exposure to organisms such as bacteria, fungi, protozoan and helminths as important modulators of those biochemical changes in human DNA. Further studies are needed to better understand such associations.

FUTURE THERAPIES AS POTENTIAL MODULATORS OF EPIGENETICS CHANGES IN ASTHMA AND ALLERGIES: OBSERVATIONS AND FUTURE PERSPECTIVES

The usual immunotherapy and pharmacological therapy in the treatment of asthma and allergies act in the modulation of immune responses, with a focus on reducing inflammation and increasing immunological tolerance. This immunological modulation is almost always accompanied by epigenetic changes. It is even possible to distinguish different epigenetic signatures between untreated individuals and individuals under treatment (205).

The use of inhaled corticosteroids (ICS) in the management of moderate to severe asthma is recommended by asthma management guidelines (206) and several studies have shown that corticosteroids are potent epigenetic modifiers (207–209). Children with better response to corticosteroids have been shown to have hypermethylation in Vanin-1 (VNN1) promoter compared to the group with poor response, in addition VNN1 mRNA expression was higher among good responders. VNN1 appears to have an important role in corticosteroid responsiveness among asthmatics, and can be used as a biomarker for treatment response (209, 210). Acetylation of histones by HATs activity was reported to be reduced in asthmatics treated with inhaled steroids (211). Variations in serum IgE concentrations can be influenced by DNA methylation patterns. An association between total serum IgE concentration and low methylation at 36 *loci* has been demonstrated, this observation may be useful in optimizing therapies with anti-IgE antibodies such as omalizumab (212).

Studies evaluating the effectiveness of peanut oral immunotherapy found a great suppressive function of Treg cells and higher levels of FOXP3 hypomethylation among treated individuals (213). In addition, a study involving cow's milk allergy children and dietary intervention using probiotic *Lactobacillus rhamnosus* (abundant in butyrate-producer bacteria strains) demonstrated that oral tolerance in children with IgE-mediated CMA involves epigenetic regulation of the FOXP3 gene. Difference in the methylation status of FOXP3 was found among children who developed oral tolerance after probiotic therapy (205). Prenatal administration of *Acinetobacter lwoffii* F78 in murine demonstrated a modulation in Th1/Th2 balance genes, with protection for asthma in the progeny, accompanied by changes in DNA acetylation (199).

Although some studies using probiotic supplementation in animal models have indicated a protective effect of probiotics on asthma and allergic Rhinitis (214, 215), studies in humans are still limited due to couple limitations such as the duration of supplementation.

Many efforts have been focused on understanding and developing microbial therapies using technological approaches involving parasitology, genomics, transcriptomics, and proteomics methods. Currently with the help of bioinformatics and helminth genome sequencing initiatives it is possible through *in silico* analyzes to identify molecules with potential immunomodulatory properties. These databases are available on WormBase Parasite, HelmDB, and Heminth.net (216–218).

The identification of genomic sequences of helminth parasites known to down-modulate the immune system of mammalian hosts such as *Ascaris suum*, *Necator americanus*, *Schistosoma mansoni*, *Strongyloides* spp. as mentioned in previous topics in this review, have motivated the development of recombinant helminth proteins with therapeutic potential for immune-mediated diseases such as protease inhibitors, cytokine homologs and lectins (219). High immunogenicity has been observed for these therapeutic recombinant proteins (220) which may be able to mimic the immunomodulation observed in helminth infections. However, standardized studies in humans as well as adequacy of doses and treatment duration are still necessary.

Epigenetic mechanisms play an important role in the regulation of immune response and are strongly influenced by microbial exposures and drug use, advancing the knowledge about such interactions may be used to both development of future target therapeutic strategies for asthma and allergies but also to discover new biological properties in current drugs in use.

The genetic susceptibility to allergic disorders is known be polygenic and recent studies have established that the presence of the gut microbiota is essential for normal gene expression (221, 222). The presence of certain bacterial species in the gut, such as *Helicobacter pylori* increases the CpG methylation in the promoter region of O6-methylguanine DNA methyltransferase, which ends up decreasing the expression of this DNA methyltransferase in gastric mucosa cells (222).

Lactobacilli and *Bifidobacteria* are the major source of butyrate and the absence of these species is important. By inhibiting HDACs, butyrate suppresses nuclear NF-κB activation, upregulates PPARɤ expression, and decreases IFNɤ production in the residing gut immune cells, promoting an anti-inflammatory gut environment (222). In a study with patients with allergic rhinitis, blocking the HDAC activity restored the integrity of the nasal epithelium and restored mucosal function and prevented the development of airway inflammation and hyperresponsiveness in experimental models (223).

Studies in dietary manipulation have demonstrated that diets high in methyl-donating nutrients are associated with hypermethylation of the epigenome, impacting the gene expression, especially during early development when the epigenome is first established, and can have long-term effects in adult life (224, 225). According to Bae et al. (225), in humans, methyl donors for DNA methylation are mostly derived from dietary methyl groups nutrients such as folate, vitamin B12, and choline. Methyl donors affect DNA methylation and immune responses such as Th17, Th1/Th2 balance, and Treg generation (225).

Additional studies are needed to better characterize the mechanisms underlying the different asthma phenotypes and their correlation with clinical characteristics, and those that contemplate the complex interaction of different epigenetic

mechanisms and those that focus on a single-cell type or investigations at the single cell level (221, 226). In this sense, EWAS can be useful to identify patterns of epigenetic signatures among asthma and allergy phenotypes and clinical characteristics, which reinforces the potential of epigenetic changes as future biomarkers for diagnosis and target personalized therapies.

AUTHOR CONTRIBUTIONS

BF, HF, PM, CM, TS, and CF have contributed for the first draft. HF designed the figure. CF designed the work. All authors listed co-authored and proofread the manuscript and approved the final manuscript.

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5. CAPÍTULO II: ARTIGO CIENTÍFICO

Gut microbiome signature and nasal lavage inflammatory markers in young asthmatics

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Gut microbiome signature and nasal lavage inflammatory markers in young asthmatics

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Abstract:	Background Asthma is a complex disease and a severe global public health problem resulting from interactions between genetic background and environmental exposures. It has been suggested that the gut microbiota may be related to asthma development, however, these relationships between needs further investigation. Objective This study aimed to characterize the gut microbiota as well as the nasal lavage cytokine profile of asthmatic and non-asthmatic individuals.Methods Stool and nasal lavage samples were collected from 29 children and adolescents with Type 2-asthma (defined as asthma with >2.5% eosinophils in sputum, and a positive skin prick test) and 28 without asthma in Brazil. Amplicon sequencing of the stool bacterial V4 region of the 16S rRNA gene was performed using Illumina MiSeq. Microbiota analysis was performed using QIIME2 and PICRUST. Type 2-asthma phenotype was characterized by high sputum eosinophil counts and positive skin prick tests for house dust mites, cockroaches, cat or dog dander. The nasal immune marker

	profile was assessed using a customized multiplex panel. Results The stool microbiota differed significantly between asthmatic and non-asthmatic participants (p=0.001). Bacteroides was more abundant in participants with asthma (p<0.05), while Prevotella was more abundant in non-asthmatics (p<0.05). In asthmatics, the relative abundance of Bacteroides correlated with IL-4 concentration in nasal lavage. Inference of microbiota functional capacity identified differential fatty acid biosynthesis in asthmatics compared to non-asthmatics. Conclusion The stool microbiota differed between asthmatics and non-asthmatics in young people in Brazil. Asthma was associated with higher Bacteroides which correlated with nasal IL-4 concentration.

Gut microbiome signature and nasal lavage inflammatory markers in young asthmatics

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Abstract (250 words)

Background: Asthma is a complex disease and a severe global public health problem resulting from interactions between genetic background and environmental exposures. It has been suggested that the gut microbiota may be related to asthma development, however, such relationships needs further investigation.

Objective: This study aimed to characterize the gut microbiota as well as the nasal lavage cytokine profile of asthmatic and non-asthmatic individuals.

Methods: Stool and nasal lavage samples were collected from 29 children and adolescents with Type 2-asthma (defined as asthma with $\geq$2.5% eosinophils in sputum, and a positive skin prick test) and 28 children without asthma in Brazil. Amplicon sequencing of the stool bacterial V4 region of the 16S rRNA gene was performed using Illumina MiSeq. Microbiota analysis was performed using QIIME2 and PICRUST. Type 2-asthma phenotype was characterized by high sputum eosinophil counts and positive skin prick tests for house dust mites, cockroaches, cat or dog dander. The nasal immune marker profile was assessed using a customized multiplex panel.

Results: The stool microbiota differed significantly between asthmatic and non-asthmatic participants ($p=0.001$). *Bacteroides* was more abundant in participants with asthma ($p<0.05$), while *Prevotella* was more abundant in non-asthmatics ($p<0.05$). In asthmatics, the relative abundance of *Bacteroides* correlated with IL-4 concentration in nasal lavage. Inference of microbiota functional capacity identified differential fatty acid biosynthesis in asthmatics compared to non-asthmatics.

Conclusion: The stool microbiota differed between asthmatics and non-asthmatics in young people in Brazil. Asthma was associated with higher *Bacteroides* which correlated with nasal IL-4 concentration.

Capsule summary

Bacteroides is associated with T2-type asthma.

Keywords: asthma, microbiome, stool, gut microbiota

Abbreviations

ANOSIM: analysis of similarities

ANOVA: one-way analysis of variance

ASV: amplicon sequence variant

BMI: Body Mass Index

CD4: cluster of differentiation 4

COPD: chronic obstructive pulmonary disease

Ig: immunoglobulin

IL: interleukin

ISAAC: International Study of Asthma and Allergies in Childhood

KEGG: Kyoto Encyclopedia of Genes and Genomes

nMDS: non-metric multi-dimensional scaling

OUT: operational taxonomic unit

PCoA: principal coordinate analysis

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PSA: polysaccharide A

QIIME: Quantitative Insights into Microbial Ecology

SCFAs: short-chain fatty acids

SD: standard deviation

SIMPER: similarity percentage analysis

STAMP: Statistical Analysis of Metagenomic Profiles

Tregs: regulatory T cells

Th: T helper cell

TSLP: thymic stromal lymphopoietin

Introduction

Asthma is a serious global public health problem which affects 339 million people [1]. Its prevalence, severity, and mortality vary across the world[2]. . However, the disease has multiple clinical phenotypes and pathophysiological characteristics [3] and is a result of complex interactions between genetic, immunological status, and environmental exposures[4].

The type 2 (T2) is the most studied asthma endotype, characterized by inflammation with eosinophils, including the expression of cytokines such as IL-4, IL-5, and IL-13[5]. These cytokines may be secreted by T-helper cell type 2 (Th2)- CD4 lymphocytes or type 2 innate lymphoid cells (ILC2s)[5–7]. T2 high asthma also involves increased activation of dendritic cells (DCs) and B cells, mediated by an IgE-dependent mechanism[8]. These biomarkers are hypothesized to contribute to the establishment and persistence of T2 asthma.

Evidence suggests that early-life environmental exposures can shape the composition of the gut microbiome, which can modulate the development of immune function, play a role in disease causation, as well as provide protection through mechanisms such as attenuation of allergic sensitization[9]. The immune system of the gut mucosa represents the major immune component in vertebrates, working in close collaboration with the intestinal microbiome, with which it interacts to achieve intestinal homeostasis[10]. The epithelium controls the local immunological activities of IgA, defensins, and lysozymes, which are also regulated by the production of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which, in turn, stimulate a T2-type inflammation, which is classically known to support the development of asthma[11].

Microbial dysbiosis has been associated with lung disorders and respiratory infections[12] and can be caused by many factors related to lifestyle and environmental exposures[13]. Studies have suggested the role of gut microbiota on asthma development is related to an imbalance in microbiota composition[12] and others have shown that the composition of gut microbes differed considerably between asthmatic and non- asthmatics[14] and also that severity of asthma is linked to changing gut microbiome composition[15].

The gut-lung relationship has been studied predominately in European and North American asthmatics. The characteristics of the gut microbiota and its role in T2 asthma in low and middle-income country settings is poorly understood. The ability to understand

pathophysiological mechanisms related to T2 asthma, and geographical variation, is crucial for the development and appropriate evaluation of novel therapies.

In this study, we aimed to characterize the gut microbiota from T2 asthmatic and non-asthmatic subjects from Brazil and investigate the association with asthma status and cytokine production in nasal lavage.

Methods

Study Population

Fifty-seven Brazilian adolescents and young adults were selected for the present study based on asthma diagnosis, presence of eosinophils in respiratory secretions and skin prick test for common aeroallergens. *a) twenty-nine T2-asthma cases* (asthma group) defined as subjects with an asthma diagnosis, presence of eosinophils in sputum greater than ≥2.5% eosinophils, and positive skin prick test for at least one allergen; *b) twenty-eight controls* (non-asthma group) without asthma diagnosis. The inclusion and exclusion criteria are described in Table 1.

Table S1. Inclusion and Exclusion Criteria

CASES	CONTROLS
Inclusion:	**Inclusion:**
• Asthma symptoms and/or use of asthma medications in the last 12 months	• No past or current history of asthma
• Must not have used cromolyn, fast-acting beta-agonists, or ipratropium bromide 6 hours before the test	**Non-inclusion:**
• Must not have used theophylline 12 hours before the test	• Having used antihistamines 48 hours before the test
• Must not have used long-acting beta-agonists 24 hours before the test	
• Not having used antihistamines 48 hours before the test	
Exclusion:	**Exclusion:**
• Acute exacerbation of asthma	• Any infection detected in the last 4 weeks
• FEV1 < 75% (of the reference value)	

• Any infection detected in the last 4 weeks • Other chronic diseases (in addition to asthma) • Pregnancy	• Any chronic illness (including asthma) • Pregnancy

Recruitment and sample collection of the selected individuals was carried out at the reference outpatient clinic for severe asthma in the city of Salvador, Brazil, the ProAR (Programa para o Controle da Asma na Bahia) [16]. The recruitment was done by applying questionnaires based on the ISAAC (International Study of Asthma and Allergies in Childhood) phase II (asthma management) and phase III (environmental risk factors) modules, with additional validated questions about the clinical severity of asthma[17]. Written informed consent was obtained from each participant or child's legal guardian.

Sputum induction

To characterize the eosinophilic asthma phenotype sputum induction was conducted according to a protocol previously used by our team[18,19]. Individuals were pre-treated with 400 mg of inhaled salbutamol by inhalation and, on average, 100 µL of induced sputum was collected. A differential count of 200 non-squamous cells was performed using an optical microscope. An eosinophilic sputum inflammatory phenotype was defined as having an eosinophil count greater than ≥2.5% eosinophils[19].

Skin Prick Test

To define atopic asthma all cases and controls underwent skin prick tests against house dust mites (*Dermatophagoides pteronyssinus* and *Blomia tropicallis*), cockroaches (*Blatela germanica* and *Periplaneta americana*), cat and dog dander. Histamine and saline were used as positive and negative controls, respectively. The diameter of the wheals was measured after 15 minutes and considered positive if the mean of the largest perpendicular diameters was at least 3 mm larger than the negative control.

Nasal lavage and cytokines measurements

Nasal lavage was performed using nasal atomizers (MAD Nasal ™) by instilling 5 mL of sterile saline solution (0.9% NaCl) into each nostril, with the individual's head tilted 30° backward[20]. After 10 seconds, a sample of at least 7 mL (considering two nostrils) was

collected and stored in a sterile conical tube and frozen at -80 °C until use. Cytokines were measured in the supernatants of the nasal lavage samples, as follows.

For the analysis of inflammatory and anti-inflammatory cytokines, a customized multiplex panel (PROCARTAPLEX CUSTOM ASSAY KIT, 9 PPX-l246 INVITROGEN PLEX) for the Luminex® MAGPIX (Life Technologies, USA) instrument was used. The panel allowed us to measure the levels of various cytokines, including inflammatory IL-4 (11.47/47000 pg/ml), IL-5 (5.37/22000 pg/ml), IL-8 (2.18/8950 pg/ml), IL-13 (2.46/10100 pg/ml), and IL-17A (1.86/7650 pg/ml)), as well as the anti-inflammatory cytokine IL-10 (1.50/6150 pg/ml), in the supernatants of nasal lavage. *Stool sample collection*

To perform sequencing of the gut microbiome, stool samples were collected during the participant's visits using a commercial sterile collector. Stool DNA was extracted and purified according to the protocol of the Human Microbiome Project[21], which uses the PowerSoil kit protocol (Qiagen).

16S rRNA amplicon sequencing

The 16S rRNA gene amplicon library preparation and sequencing were conducted following the Earth Microbiome Project protocol[22]. Briefly, PCR amplification of the extracted DNA was performed for 30 cycles targeting the V4 hypervariable 16S region using the universal primers: 515F and 806R (515F: GTGCCAGCMGCCGCGGTAA; 806R: GGACTACHVGGGTWTCTAAT), with a Golay barcode on the forward primer. The sequencing was performed using the Illumina MiSeq Reagent Kit v2 (500-cycles) on an Illumina MiSeq platform in the Genomics and Microarray Core of the University of Colorado Anschutz Medical Campus, USA. 57 paired end read samples were generated by the end of the sequencing runs. Raw files and associated metadata are available at Sequence Read Archive (SRA) data at the PRJNA950484 accession number (https://www.ncbi.nlm.nih.gov/sra/PRJNA950484).

Bioinformatics analysis

Bioinformatic analyses and sample quality checks were performed using QIIME2 2021.4 software[23]. Using the q2-demux plugin, raw sequence data were demultiplexed and quality filtered before being denoised with DADA2, through q2-dada2[24]. To construct phylogenetic trees, a fragment insertion tree was created using the q2-fragment-insertion

plugin[25]. The q2-feature-classifier[26] classify□sklearn naïve Bayes taxonomy classifier was used to classify ASVs against the Greengenes 13_8_99% OTUs reference sequences set trimmed to 250 bp of the V4 hypervariable region (corresponding to the 515F-806R primers)[27].

To test if the microbial community structure and diversity were different between the groups, the alpha (i.e., observed features and Shannon index[28]) and beta diversity (i.e., Principal Coordinate Analysis (PCoA) using Bray-Curtis distances) were calculated using R Statistical Software (v4.1.0; Core Team 2023) via phyloseq R package, after samples were rarefied to 26300 sequences per sample. To test if the potential functional of the microbiota was different between groups, we used the PICRUSt2[29] to predict pathway abundances of the Kyoto Encyclopedia of Genes and Genomes (KEGG) features.

Statistical analysis

Statistical analysis was conducted using R Statistical Software and STAMP (version 2.1.3)[30]. Demographic data and clinical characteristics are expressed as a mean ± standard deviation (SD) for numeric variables and the differences between groups were evaluated by one-way analysis of variance (ANOVA), and the chi-square test was used for categorical variables. The Wilcoxon rank-sum test was used to analyze the alpha diversity and the analysis of similarities (ANOSIM) was evaluated for the beta diversity between-group comparisons. Adjustments for multiple comparisons were made using the False Discovery Rate (FDR) method. Only results meeting these criteria were deemed statistically significant. P-values <0.05 were considered statistically significant. To identify which features contributed to the differences between groups, the similarity percentage analysis (SIMPER) was used. The correlation analysis between microbial composition and cytokines was performed by the Spearman correlation test.

Results

The study population characteristics

Clinical parameters and demographic characteristics are shown in Table 2. There were 18 female and 11 male participants with asthma, mean±SD age of 17.21±2.66 years. By comparison, the non-asthmatics individuals were comprised of 19 females and 9 males,

mean±SD age of 19.36±1.70 years. All subjects had a normal body mass index (18.5-24.9) and most individuals had self-declared black skin color.

Table 2. Characteristics of the study population according to asthma status.

	Asthma Status	
	Asthma	Non-asthma
	(N = 29)	(N = 28)
Gender		
Female	18 (62,1%)	19 (67,8%)
Male	11 (37,9%)	9 (32,2%)
Ethnicity		
White	2 (6,9%)	3 (11,1%)
Black	26 (8962%)	24 (85,7%)
Asian	1 (3,4%)	0 (0%)
Age	17.21±2.66	19.36±1.70
BMI (kg/m^2)	22.62±4.19	22.91±6.72
Height (m)	1.65±0.08	1.62±0.33
Weight (kg)	62.80±15.82	65.81±21.64
SPT positive for at least one allergen tested	29 (100%)	18 (69,2%)

Notes: Data were presented by mean ± standard deviation (continuous).

Abbreviations: BMI, Body Mass Index.

Stool microbiota differs between asthmatics and non-asthmatics

Sequencing depth ranged from 26,318 to 108,737 reads per sample (mean = 50,931) (Supplementary Table. S2), and the number of unique ASVs was 2,436 after denoising with DADA2. After rarefying to 26,300 sequences per sample (Supplementary Fig. S1), overall composition, taxonomic richness (number of unique ASVs) and diversity (Shannon-Wiener Index) were calculated. There was a divergence in the gut microbiota

between asthmatics and non-asthmatics (p = 0.01, R^2 = 0.038, PERMANOVA) (Figure 1).

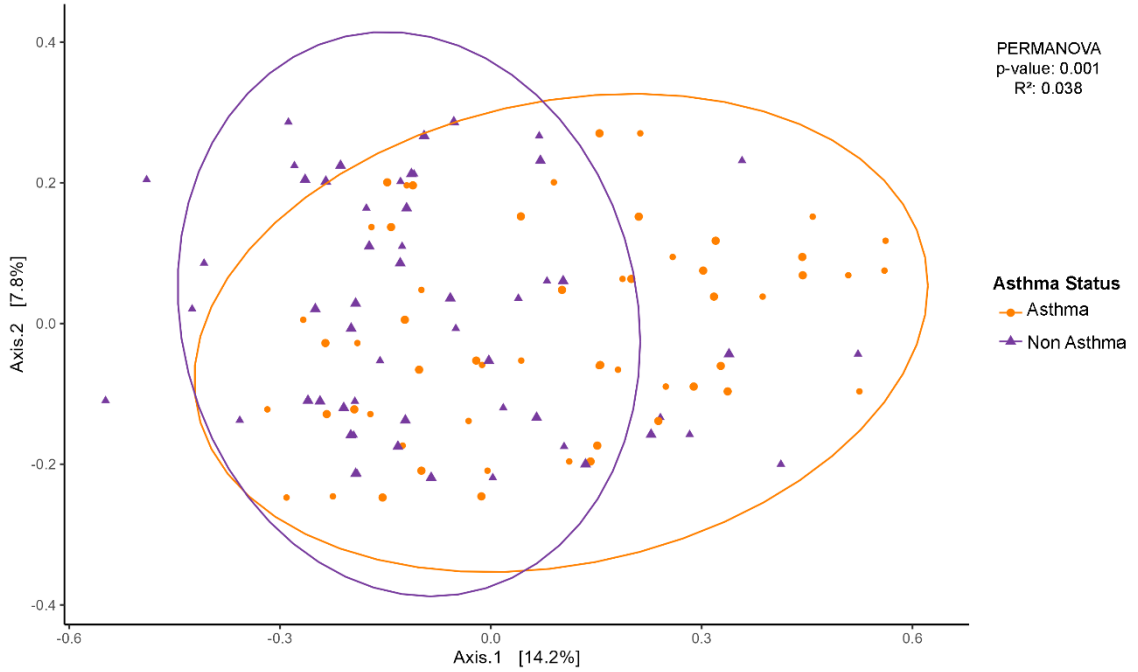

Figure 1. Differences between asthmatics and non-asthmatics groups using Bray–Curtis dissimilarity indices using principal coordinate analysis. Each dot represents a sample, and the corresponding group can be found in the legend. There was a significant difference in β-diversity between both groups. Statistics were calculated using pairwise PERMANOVA.

Although not statistically significant, those with asthma had a lower richness (p=0.144, Supplementary Table. S3) and diversity (p=0.145, Supplementary Table. S3) compared to non-asthmatic subjects (Figure 2).

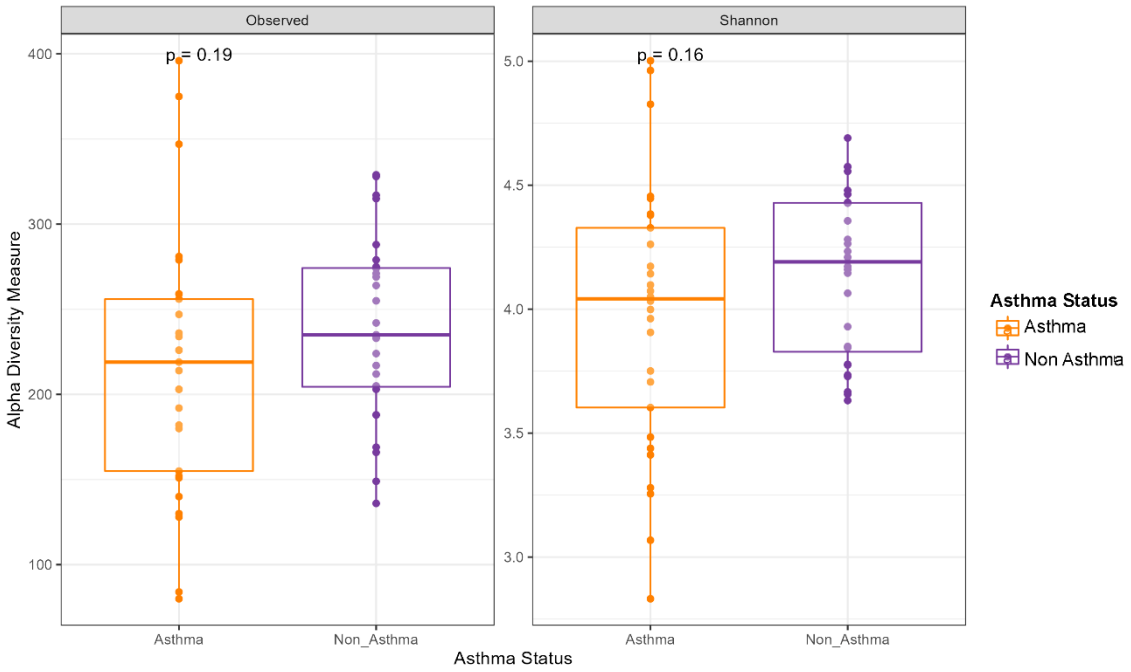

Figure 2. The comparison of gut microbiota alpha diversity between groups, including **A.** species richness (represented by observed species) and **B.** evenness (represented by Shannon). The asthmatic group had less bacterial diversity, richness, and evenness, although non-significant, in comparison to non-asthmatics (Student's t-Tests p-value>0,05, Table S3).

A total of 15 phyla, 25 classes, 39 orders, 75 families, 165 genera, and 235 species were detected across all samples. The predominant phyla were largely consistent in both groups, but different relative abundances were observed (Supplementary Fig. S2). When comparing the relative abundance of the top 10 (> 2%) most common genera between asthmatics and non-asthmatics, some differences were observed, with a higher abundance of *Bacteroides* in the asthma group and a higher abundance of *Prevotella* in the non-asthma group (Figure 3).

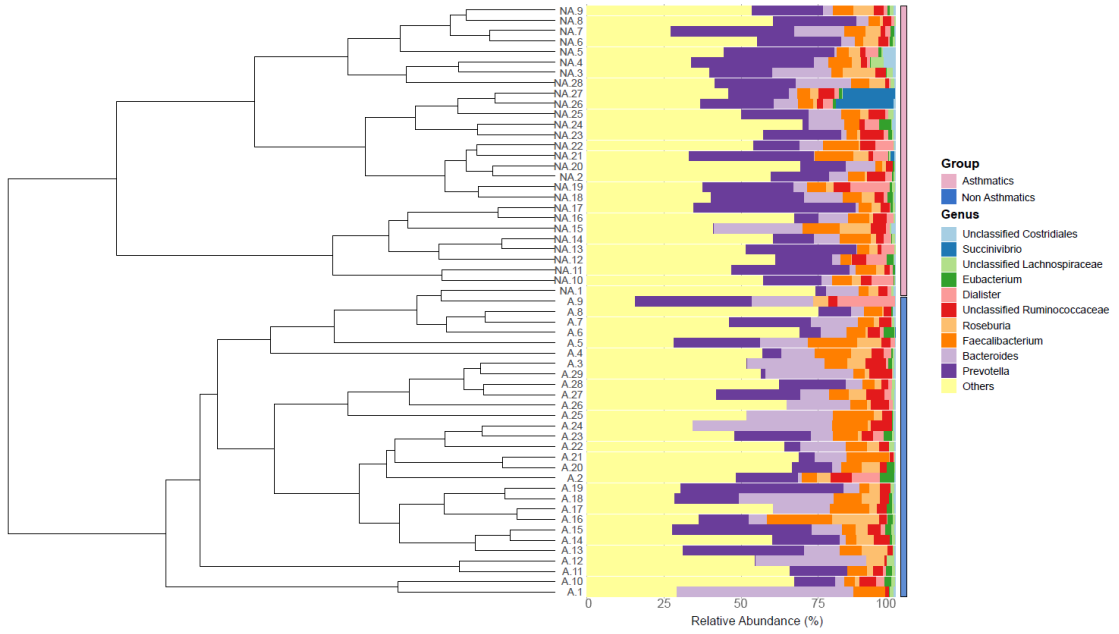

Figure 3. The relative abundance of the ten most abundant genera in asthmatics and non-asthmatics. The genera are shown in different colors and the height of bars represents relative abundance.

We found that the asthma group had an increased abundance of the genera *Bacteroides* (p-value: 0.007), *Parabacteroides* (p-value: 0.01) and *Phascolarctobacterium* compared to the non-asthma group. On the other hand, *Prevotella* had an increased abundance in the non-asthma group compared to the asthma group (Figure 4).

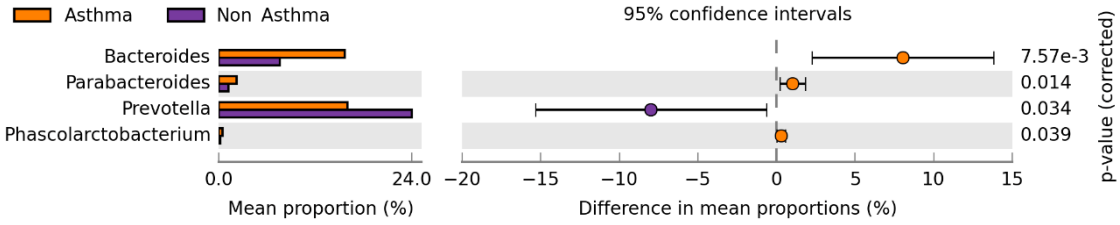

Figure 4. Extended bar plot for the significantly different genera between groups showing the mean proportion and the difference in mean proportion. The asthmatic individuals are shown in orange, and the non-asthmatic individuals are shown in purple. Error bars indicate standard deviation and corrected p-values are indicated to the right.

Asthmatics had a decreased IL-10 production in nasal lavage

A total of 24 asthmatic patients and 20 non-asthmatics had detectable levels of pro-inflammatory (IL-5, IL-8 and IL-17A) and anti-inflammatory (IL-10, IL-4 and IL-13) cytokines in nasal lavage fluids. Among the cytokines evaluated, the levels of IL-10 in nasal lavage were decreased compared to non-asthmatics subjects ($p < 0.04$) (Figure 5A). No significant differences were found for IL-13, IL-17A, IL-4, IL-5 and IL-8 (Figure 5B-F, respectively).

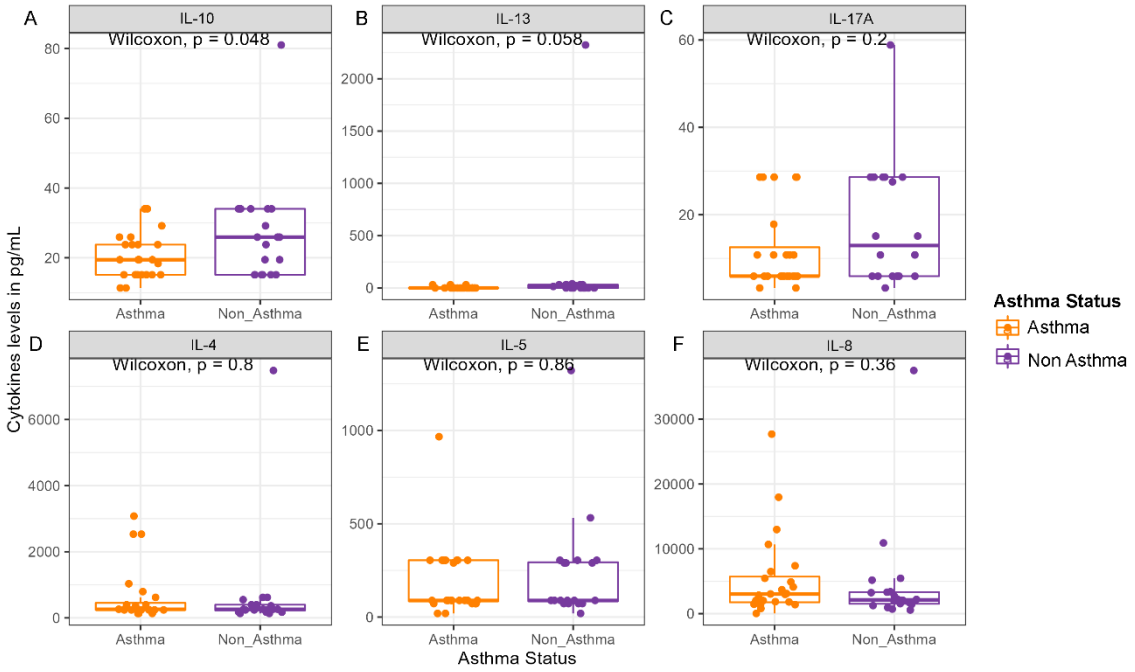

Figure 5. Cytokines IL-10 **(A)**, IL-13 **(B)**. IL-17A **(C)**, IL-4 **(D)**, IL-5 **(E)** and IL-8 **(F)** levels of asthmatic patients quantified by Luminex in the nasal lavage of 24 asthmatic patients and 20 non-asthmatics. Statistics were calculated using Mann-Whitney-Wilcoxon test.

Bacteroides abundance is correlated with nasal IL-4 levels in asthmatics

Spearman's correlation coefficients were estimated between the relative abundance of the significantly different genera (i.e., *Bacteroides, Parabacteroides, Prevotella, Phascolarctobacterium and Dialister*) and asthma and non-asthma groups with cytokine levels (Figure 6). There was a positive correlation between the presence of *Bacteroides* and IL-4 production (p-value: 0.04, r=0.12) in asthmatics.

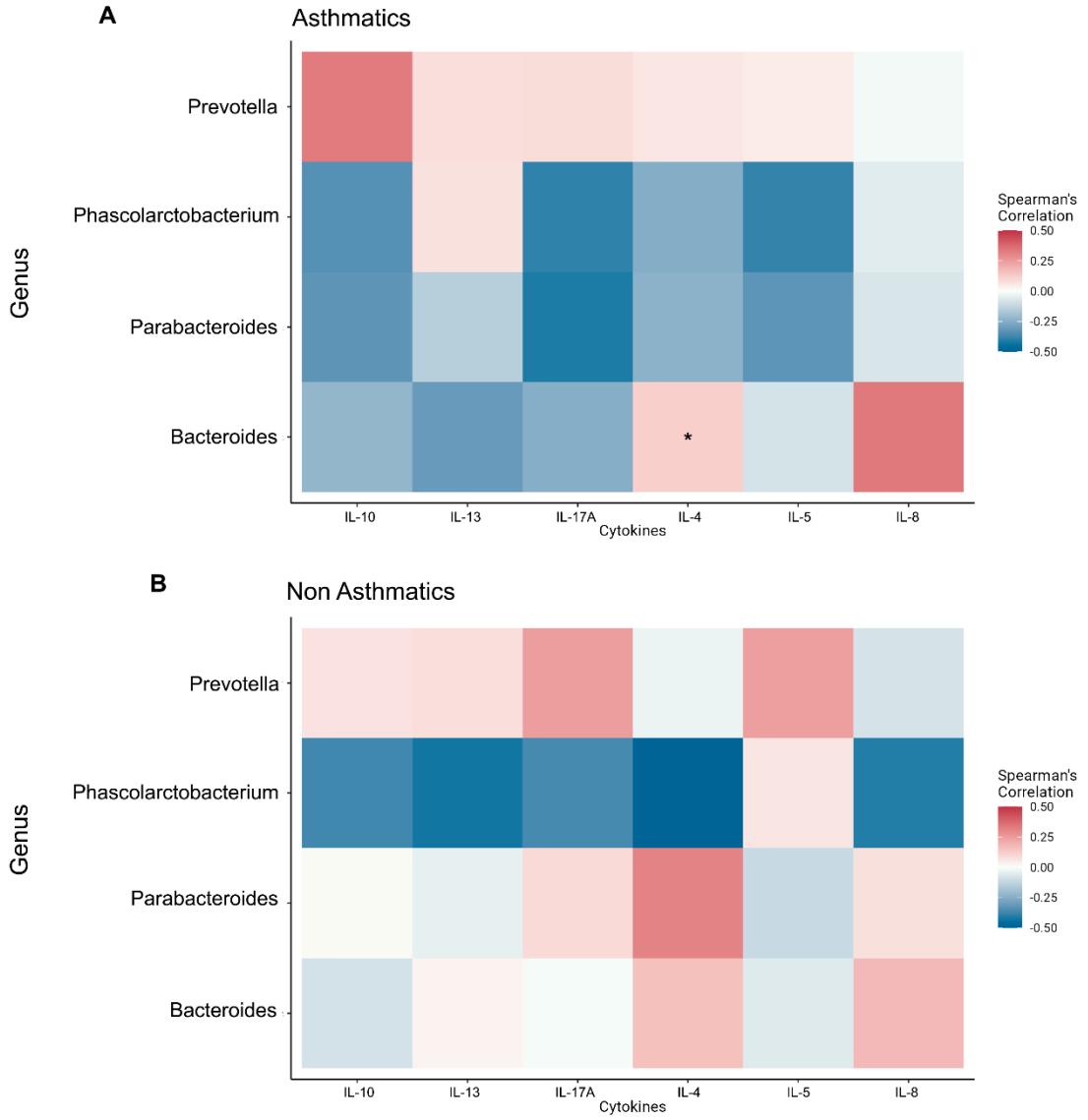

Figure 6. Correlations between asthma (n: 24) and non-asthma (n: 20) gut microbiome relative abundance of genera and nasal lavage concentrations of inflammatory cytokines. The color is according to the Spearman coefficient distribution: red represents a positive correlation and blue represents a negative correlation. IL: interleukin. *p-value < 0.05

IL-10 is correlated with the other cytokines in the population

IL-10 exhibits correlations with the other cytokines within the study population. Figure 7 illustrates the correlations among the cytokines in both non-asthmatic individuals and

those with asthma. There was a positive correlation between IL-10 and IL-13, IL-17A, IL-4, and IL-5.

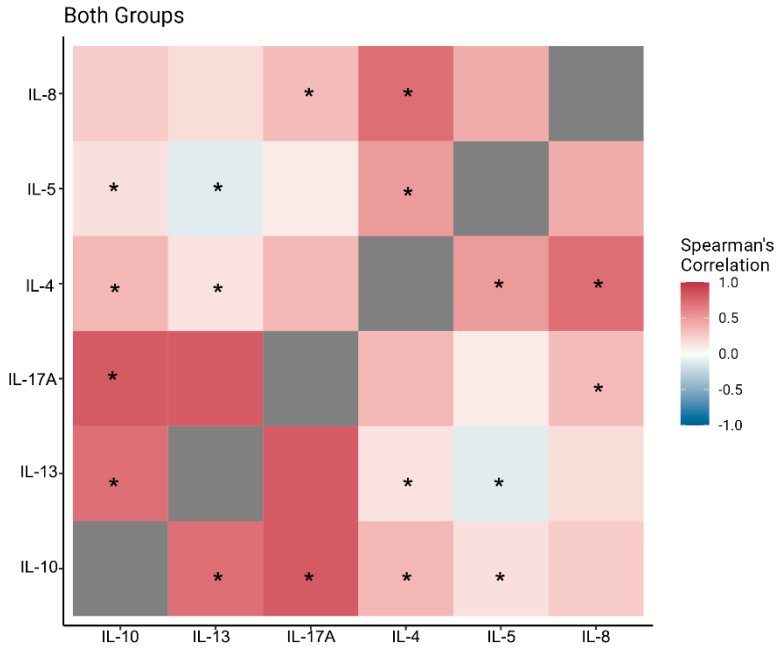

Figure 7. Correlations of the nasal lavage cytokines levels with each other. The color is according to the Spearman coefficient distribution: red represents a positive correlation and blue represents a negative correlation. IL: interleukin. *p-value < 0.05

Predicted metabolic and biosynthetic pathways are different between asthmatic and non-asthmatic subjects

KEGG Pathway database is a collection of human produced pathway maps that represent the understanding of molecular interactions. Inference of KEGG Pathways from the taxonomic composition identified 13 metabolic pathways that differed between asthmatic and non-asthmatic subjects (Figure 8). Compared to the non-asthmatic group, fatty acid, and monobactam biosynthesis, riboflavin, and purine metabolism were less abundant in the asthma group. Secondary bile acid, penicillin, and cephalosporin, O-antigen nucleotide sugar, and steroid hormone biosynthesis were enriched in the asthma group. Lipoic acid, galactose, sphingolipid, glyoxylate, and dicarboxylate, and amino sugar and nucleotide sugar metabolism were also less abundant in asthmatics. Supplementary Table S3 summarizes the findings of the significant metabolic pathways and their association with asthma and allergy in other studies.

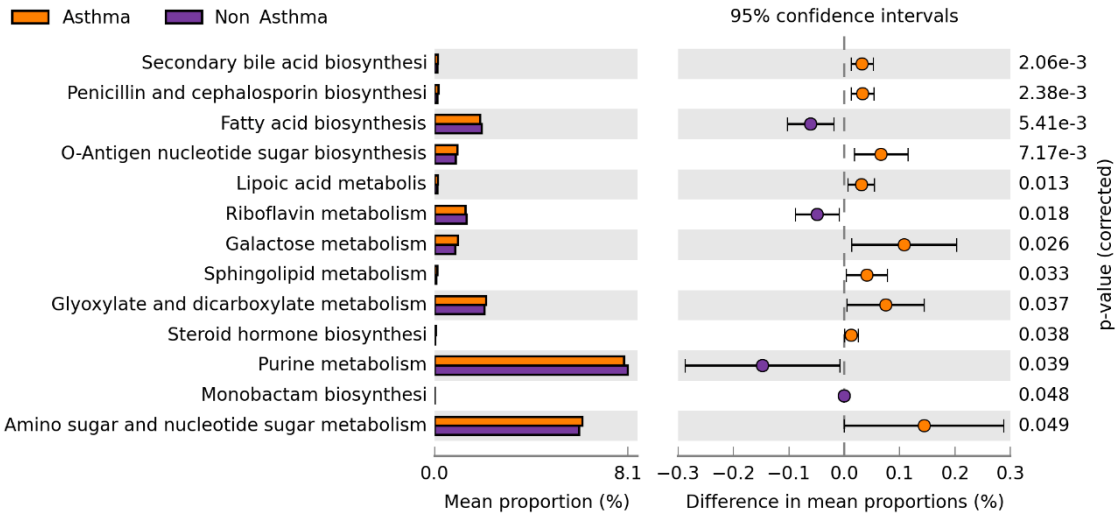

Figure 8. Prediction of differential pathways presented in asthmatic and non-asthmatics groups with PICRUSt2 analysis. Extended error bar plot for each pathway indicating differences in mean proportions for each pair of groups. Dot plots on the right show the differences in mean proportions between the two indicated groups (asthma in orange and non-asthma in purple) using p-values.

Discussion

The current study used the 16S rRNA amplicon sequencing technique to assess the stool bacteria compositions in children and young asthmatic patients from Brazil. Our findings revealed that there were differences in the gut microbiota between groups. Beta diversity indices indicated an overall significant difference between groups. This difference may be related to the distinct microbial contacts to which individuals are exposed, such as contact to greenery[31], mode of delivery[32], and diet[12].

A rich diet in fermented foods increases microbial diversity and lowers several inflammation-related indicators[33]. The modification of intestinal metabolites and microorganisms is more likely to causes the impact[34]. The diet of the participants was greatly influenced by socioeconomic factors and regional dietary habits, with both the case and control groups coming from the same geographic area and sharing similar dietary patterns. To provide a proxy for diet in our study population, we analyzed the weekly consumption of prevalent foods among the participants, which included beans, milk, goodies, and vegetables (Supplementary Figure S3).

Environmental variables evaluated, i.e., humidity, exposure to domestic animals, tobacco, in our population did not show significant differences (data not shown) in terms of microbiota profiling.

It is well known that gut microbiota plays several important roles in the development, regulation, and maintenance of healthy immune responses. In our study, we examined the microbiome profiles in T2 asthma endotype and found a link between gut microbiota and asthma-related immune responses. The results can help us gain a complete and comprehensive description of the bacterial community that can be associated with asthma in our population. In Brazil, a previous study has focused on the relationship between obesity and microbiome development in childhood in connection to delivery mode and socioeconomic class[34]. In addition, Melli et al (2020) showed the relationship between gut microbiota and atopic dermatitis in school-age children from Brazil and, in accordance with, our study, *Bacteroidetes* and *Firmicutes* were also found in all the samples examined[35].

Bacteroides was the most abundant genus among asthmatics. This genus has been implicated in the development of asthma through the production of short-chain fatty acids (SCFA), which regulate several leukocyte functions including the production of cytokines (TNF-α, IL-2, IL-6, and IL-10)[36,37]. Higher prevalence of *Bacteroides* was observed in the early microbiota of children who later developed allergies[38], in asthmatics[39] and in patients with atopic dermatitis[40]. Type 2 (T2)-asthma is characterized by upregulation of Th2 cytokine profile and eosinophilic airway inflammation[8], which was also observed in the present work, where *Bacteroides* has a positive correlation with IL-4 production, increasing the T2-asthma risk through the activation of Th2 cells and their effectors mechanisms. The present study was the first to observe a higher abundance of *Parabacteroides and Phascolarctobacterium* in T2 asthma.

In contrast, *Prevotella* was the most abundant genus among non-asthmatics. The abundance of the Prevotella genus was reduced in a study that evaluated the lung microbiota of patients with asthma and chronic obstructive pulmonary disease (COPD) compared to healthy controls[41,42]. Larsen (2017) reported that *Prevotella* has a capability of driving Th17 immune responses *in vitro* and increased abundances in this genus have been associated with enhanced Th17 response mediated by mucosal inflammation. Although the correlation between the relative abundance of *Prevotella* and the levels of

IL-17A was not significant (Figure 5), it was possible to observe a higher production of this cytokine in non-asthmatics.

Our results have shown that asthmatics had decreased IL-10 production in nasal lavage. The powerful inflammatory response regulator interleukin-10 (IL-10) is essential for regulating allergic airway inflammation. According to previous reports, people with asthma had lower amounts of IL-10 in their bronchoalveolar lavage fluid and less IL-10 being secreted by their alveolar macrophages [43]. Previously, a lower production of IL-10 has been associated with increased T2-asthma risk[44]. We observed that the production of pro-inflammatory cytokines is positively correlated with the production of IL-10 in non-asthmatic individuals, and this interaction demonstrates the importance of the production of these cytokines in modulating the immune system, ensuring an appropriate regulatory response.

IL-10 interacts directly with CD4+ T cells, and suppresses neutrophils, eosinophils, and mast cells in lungs, inhibits the production of IL-4 and IL-5 by Th2 cells[45,46]. As a result, IL-10 plays a crucial role in asthma and lung inflammation[46], since it has been shown that microbiota metabolites, such as short-chain fatty acids, can promote IL-10 production to maintain the intestinal homeostasis.

Additionally, we have previous observed the link between childhood exposure to high levels of microbes and the development of regulatory mechanisms, leading to increased IL-10 production [47]. This information provides further context for the potential role of the gut microbiome in modulating immune responses in individuals with asthma.Moreover, using predicted pathway analysis we found that fatty acids biosynthesis related pathways were more common in asthmatics compared to non-asthmatics. Despite the caution required when interpreting 16S data to infer the metabolic composition of microbial communities, several studies (Supplementary Table S3) show that fatty acids signaling plays an important role in the pathogenesis of asthma[48]. For instance, SCFAs have been recognized for their important role in regulating the immune system, by regulating host immune homeostasis. Specifically, they are crucial for promoting the development of regulatory T cells (Tregs) in the colon, which help to prevent an excessive Th2 response that could potentially contribute to the development of allergic asthma. Previous studies[49,50] have demonstrated that *Bacteriodes* and *Prevotella* genera are linked to the production of SCFAs. Thus, additional studies should be conducted to further investigate this.

This study had a couple limitations. First, this was a cross-sectional study so we cannot determine whether changes in the microbiota occurred before or after asthma onset. As a result, it is challenging to conclude a cause-and-effect relationship between microbiota changes and asthma development. Secondly, the small sample size was limited. With a limited number of participants, the findings may not adequately represent the entire population of interest. And, thirdly, we focused on T2-type asthma and so we cannot generalize our findings to other phenotypes of the disease, leading to incomplete insights into the overall disease complexity. The traditional understanding of asthma has evolved over time, and recent research has highlighted the heterogeneity of the disease. Asthma is now recognized as a complex syndrome with diverse pathophysiological mechanisms[51].

Nevertheless, to our knowledge, our study is the first study evaluating the profile of intestinal microbiota, nasal lavage cytokines levels and its relationship with T2-asthma phenotype in Brazilian children and young adults. Our data strongly suggest that microbial composition that colonize the gut can significantly influence chronic lung disorders such as asthma.

In conclusion, in young individuals from Brazil, there were observable differences in the stool microbiota between asthmatics and non-asthmatics. Specifically, those who had asthma had also higher levels of *Bacteroides* present in their stool. Interestingly, this abundance of in *Bacteroides* was positively correlated with nasal IL-4 concentration, indicating a potential association between the gut microbiota and the immune system's response in individuals with asthma**.**

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Authors' contributions: BSDF, PMM, and CAF conceived and designed the study. CMA collected the stool samples and did the DNA extraction. MJS, CMA, and BSDF performed multiplex assays. LP worked on the sputum characterization. BSDF and JSS performed the data and statistical analyses. BSDF wrote the manuscript. BSDF, JSS, PMM, and CAF contributed to the interpretation of the data. All authors revised and approved the final version before submission.

Ethics committee approval: The project was approved by the Brazilian National Research Ethics Council (CONEP –CAAE: 47840415.3.0000.5030).

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Supplementary Materials

Table S1. General features of the sequencing

Sample Name	Asthma Status	Sequence count	Feature count	Base pairs	Average GC Content (%)
A.1	Asthma	45455	29358	23343	36.26
A.2	Asthma	90870	58808	47690	36.26
A.3	Asthma	87462	56505	98894	36.26
A.4	Asthma	87748	59068	4267	35.16
A.5	Asthma	64428	42873	169174	36.26
A.6	Asthma	78416	57546	13554	36.26
A.7	Asthma	76961	49243	22339	35.16
A.8	Asthma	148429	108737	7530	34.06
A.9	Asthma	45584	33204	14809	34.06
A.10	Asthma	102504	80030	19327	35.16
A.11	Asthma	74613	51693	10040	34.06
A.12	Asthma	67172	42032	75551	36.26
A.13	Asthma	127293	96896	32881	36.26
A.14	Asthma	100086	64099	26355	35.16
A.15	Asthma	46052	31131	16315	36.26
A.16	Asthma	85508	57581	211593	36.26
A.17	Asthma	89401	62130	297435	35.16

A.18	Asthma	66793	45043	626245	36.26
A.19	Asthma	47123	33082	31375	35.16
A.20	Asthma	55040	35644	21837	36.26
A.21	Asthma	73703	51740	34387	35.16
A.22	Asthma	63137	40877	29618	36.26
A.23	Asthma	71829	47513	31877	34.06
A.24	Asthma	58316	39363	19829	34.06
A.25	Asthma	93760	58617	260538	31.86
A.26	Asthma	75670	47676	99145	36.26
A.27	Asthma	78603	54266	182728	31.86
A.28	Asthma	84154	63343	41917	34.06
A.29	Asthma	64791	42202	11876065	36.26
NA.1	No_ Asthma	101953	70714	25473739	36.26
NA.2	No_ Asthma	93168	66806		
NA.3	No_ Asthma	80355	50756	107930	36.26
NA.4	No_ Asthma	45278	34512	17433707	35.16
NA.5	No_ Asthma	37877	29571	153612	36.26
NA.6	No_ Asthma	78019	53156	47690	35.16
NA.7	No_ Asthma	65412	41105	12048	35.16
NA.8	No_ Asthma	84710	59705	19327	36.26
NA.9	No_ Asthma	54997	40516	30120	35.16

NA.10	No_ Asthma	65047	41977	47188	36.26
NA.11	No_ Asthma	66032	43790	13052	35.16
NA.12	No_ Asthma	90779	63913	40662	35.16
NA.13	No_ Asthma	50937	35680	129516	35.16
NA.14	No_ Asthma	76963	50861	19076	36.26
NA.15	No_ Asthma	33014	26318	5020	36.26
NA.16	No_ Asthma	54929	37598	28363	34.06
NA.17	No_ Asthma	44082	31500	16566	35.71
NA.18	No_ Asthma	81430	55668	83081	31.86
NA.19	No_ Asthma	51003	38811	123492	36.26
NA.20	No_ Asthma	85398	57945	40913	31.86
NA.21	No_ Asthma	41644	29918	9789	35.16
NA.22	No_ Asthma	139105	97242	57479	35.16
NA.23	No_ Asthma	82084	54553	337595	35.16
NA.24	No_ Asthma	85448	60945	663644	36.26
NA.25	No_ Asthma	72911	53627	260036	35.16
NA.26	No_ Asthma	51271	37446	13554	36.26
NA.27	No_ Asthma	87133	58200	13303	35.16
NA.28	No_ Asthma	58217	39985	69025	36.26

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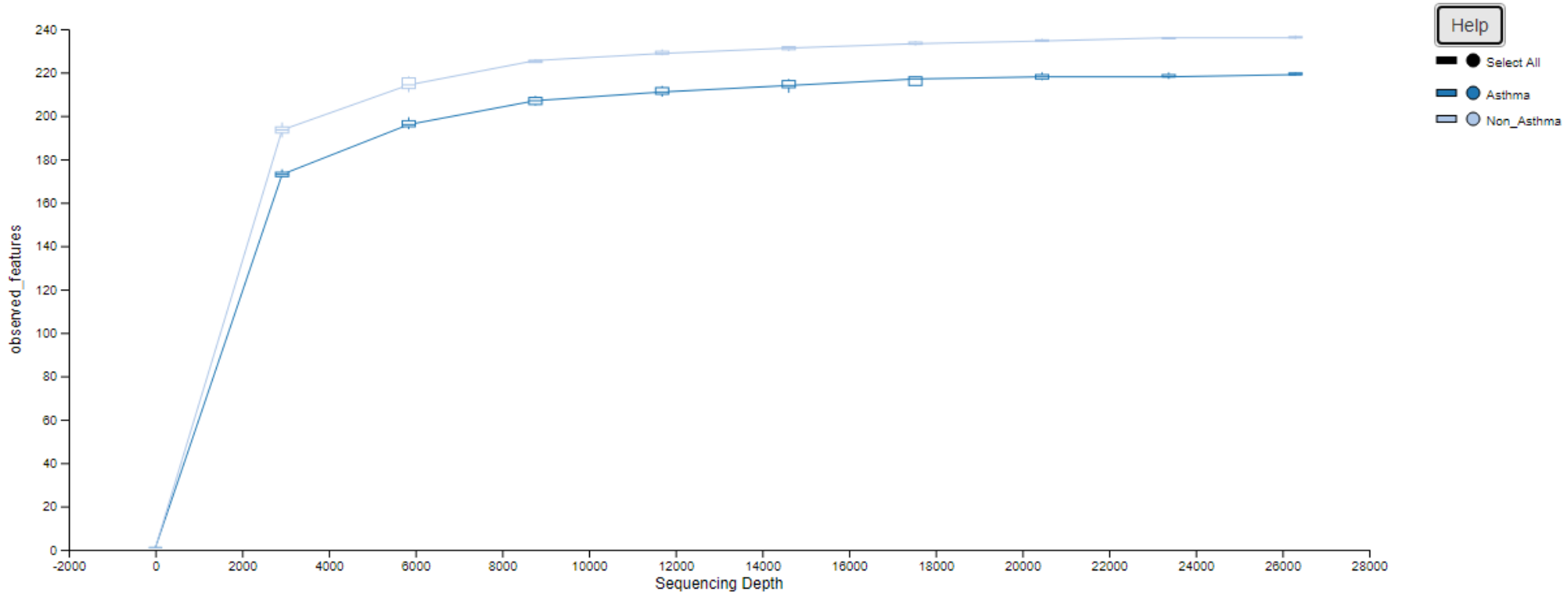


Figure S1. Alpha rarefaction curves for features

Table S2. P values based on Wilcoxon signed-rank test for Observed and Shannon index differences among asthmatics and non-asthmatics

Observed		
	Non_Asthma	Asthma
Non_Asthma	NA	0.144
Asthma	0.144	NA
Shannon		
	Non_Asthma	Asthma
Non_Asthma	NA	0.145
Asthma	0.145	NA

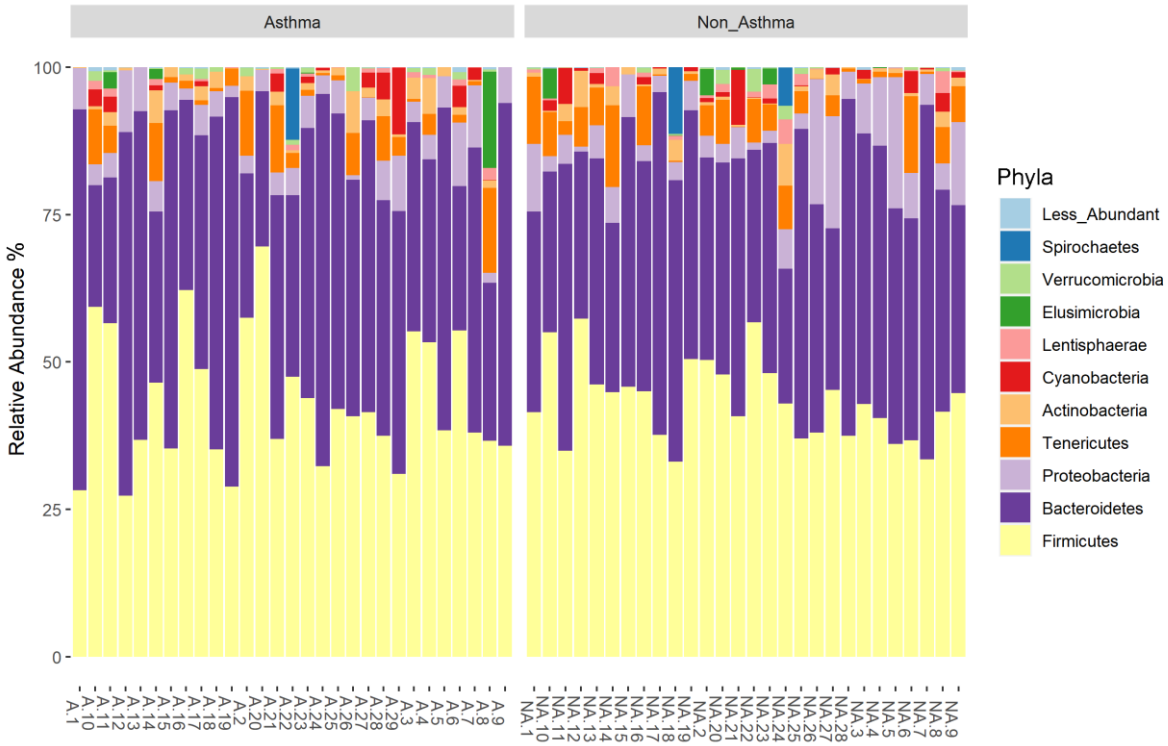
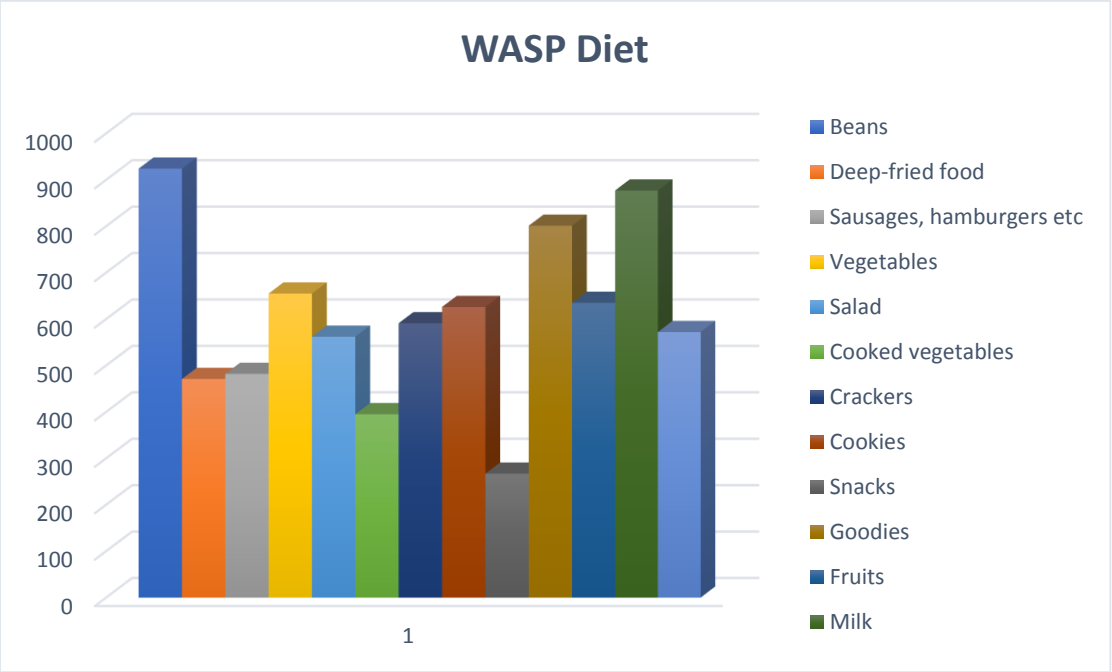


Figure S2. Relative abundance of predominant bacteria.



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686 **Figure S3. Diet profile based on weekly consumption.**

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689 **Table S3.** Predicted pathways and relationship with asthma and allergies.

Reference	Pathway	Major Findings
Joyce and Gahan, 2017	Secondary bile acid biosynthesis	"Extraintestinal diseases and syndromes such as asthma and obesity may be linked to aberrant bile acid profiles in the host"
Zhao et al., 2001	Penicillin and cephalosporin biosynthesis	"IgE antibodies in the sera of subjects allergic to beta-lactam antibiotics detect a spectrum of specificities ranging from side-chain groups to an entire penicillin or cephalosporin molecule"
Rodriguez-Perez et al., 2017	Fatty acids biosynthesis	"Fatty acids and lipid mediator signaling play an important role in the pathogenesis of asthma, yet this area remains largely underexplored"
Cho et al., 2004	Lipoic acid metabolism	"alpha-Lipoic acid inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma"
Revyakina et al., 2019	Riboflavin metabolism	"The results indicate a decrease in the concentration of magnesium and normal levels of vitamin B2 in serum in patients with bronchial asthma and obesity"
Ho et al., 2012	Galactose metabolism	"These metabolite changes suggest alterations of energy metabolism in asthmatic lungs, with (…) reductions in carbohydrates, such as (…) galactose"
Kowal et al., 2019	Sphingolipid metabolism	"Altered sphingolipid metabolism is associated with asthma phenotype in house dust mite-allergic patients"
Liu et al., 2018	Glyoxylate and dicarboxylate metabolism	"Pathway topology enrichment analysis revealed that (…) glyoxylate and dicarboxylate metabolism (…) pathway in serum are suggested to be significant pathways related to obese asthma"
Payne and Freishtat, 2012	Steroid hormones biosynthesis	"Steroid hormones (eg, glucocorticoids) are ubiquitous in the short-term and long-term management of all types of asthma"
Yu et al., 2016	Purine metabolism	"Purine metabolism was the most prominently influenced in OVA-induced asthma mice according to the metabolic pathway analysis (MetPA), suggesting that significant changes in inflammatory responses in the pathophysiologic process of asthma"

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6. CONCLUSÃO GERAL

As interações entre o hospedeiro e a microbiota são bidirecionais, com procedimentos específicos de espécies e cepas moldados pelo *background* genético e microambiente em que existem. Os fatores microbianos estão evolutivamente conectados ao circuito molecular que conduz os processos de tomada de decisão das células imunes.

Este estudo enfocou a assinatura microbiana da asma considerando o diagnóstico de asma, presença de eosinófilos e teste cutâneo positivo para alérgenos comuns, e análise baseada na diversidade bacteriana e estrutura da comunidade de amostras de fezes de asmáticos e não asmáticos.

O presente estudo detalha a lista de gêneros bacterianos associados à asma associada à produção de citocinas. Houve diferenças significativas na composição microbiana da microbiota intestinal em asmáticos em comparação com indivíduos não asmáticos, e foi demonstrado quais gêneros e espécies influenciaram a dissimilaridade da estrutura bacteriana.

Os nossos dados confirmam que as diferenças taxonômicas da microbiota intestinal influenciam o risco de asma e sugere que as comunidades de bactérias selecionadas estão associadas à imunopatologia da asma. Assim, mais estudos mecanísticos, avaliando fatores ambientais, produtos microbianos e variantes genéticas são necessários para explorar a influência da composição microbiana na patogênese da asma, para posteriormente esclarecer como o perfil da microbiota intestinal pode influenciar os distúrbios pulmonares.

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