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# Efeito dos probióticos no tratamento da esteatohepatite não alcoólica: um ensaio clínico randomizado

**Porto Alegre**
**2018**

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Co-orientadora: Dra. Cristiane Valle Tovo

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Aos pacientes, razão deste trabalho.

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*“Les microbes auront le dernier mot.”*

“Os micróbios terão a última palavra.”

Louis Pasteur

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## LISTA DE ABREVIATURAS E SIGLAS

ALT - Alanina aminotrasferase

APRI - *AST to Platelet Ratio Index*

AST - Aspartato aminotransferase

DAMPs - Padrões Moleculares Associados ao Dano

DHGNA - Doença hepática gordurosa não alcoólica

DNL - Lipogênese *de novo*

EHNA - Esteatohepatite não alcoólica

FNT-α - Fator de necrose tumoral alfa

HDL - Lipoproteína de alta densidade

HOMA-IR - *Homeostasis model assessment*

IL-6 - Interleucina 6

IMC - Índice de massa corporal

LDL - Lipoproteína de baixa densidade

LPS - Lipopolissacarídeo

MCP-1 - Proteína quimiotática de monócitos-1

NAFLD - *Nonalcoholic fatty liver disease*

NASH - *Nonalcoholic steatohepatitis*

PAMPs - Padrões Moleculares Associados a Patógenos

TLR - Receptores do tipo Toll

VLDL-C - Lipoproteína de muito baixa densidade

# RESUMO

Introdução: O eixo fígado-intestino apresenta papel importante no desenvolvimento e progressão da doença hepática gordurosa não alcoólica (DHGNA) e esteatohepatite não alcoólica (EHNA). Estudos com intervenção utilizando probióticos apresentaram resultados positivos na melhoria da DHGNA e EHNA, no entanto ainda existe a necessidade de estudos que avaliem diferentes doses, cepas e tempo de tratamento. O objetivo deste trabalho foi avaliar o efeito da suplementação de probióticos nas enzimas hepáticas, perfil inflamatório sistêmico e metabólico em pacientes com EHNA.

Métodos: Ensaio clínico randomizado duplo cego, controlado por placebo. Pacientes com EHNA comprovada por histologia (n=43) foram randomizados para receber probióticos ($1x10^9$ UFC *Lactobacillus acidophilus* e $1x10^9$ UFC *Bifidobacterium lactis*) (Grupo Probióticos) ou placebo (Grupo Placebo) por 6 meses. Os pacientes foram avaliados aos 0, 3 e 6 meses, incluindo índice de massa corporal (IMC), testes laboratoriais (AST, ALT, colesterol total, colesterol HDL, colesterol LDL, triglicerídeos, glicose, insulina, proteína C-reativa, ferritina, interleucina-6, fator de necrose tumoral, proteína quimiotática de monócitos-1 e leptina), *AST to Platelet Ratio Index* (APRI) e escore NAFLD. A biópsia hepática foi realizada na inclusão e repetida após 6 meses de tratamento, além da avaliação não invasiva da fibrose por meio do teste Fibromax$^{TM}$ que avalia fibrose, inflamação e esteatose hepática.

Resultados: Após 6 meses de tratamento, o Grupo Probióticos apresentou redução significativa no nível de AST (37.0 vs. 34.0 $p = 0.012$), escore APRI (0.41 vs. 0.35 $p = 0.042$, proteína C reativa (6.6 vs. 4.5 $p = 0.026$) e ferritina (169.0 vs. 138.5 $p = 0.008$) comparado com os valores encontrados na inclusão. Os níveis de HDL (47.5 vs. 52.0 $p = 0.020$) e colesterol total (186.0 vs. 205.0 $p <0.001$) aumentaram significativamente no Grupo Probióticos comparado com os valores pré tratamento. Não houve diferenças entre os grupos após o término do estudo.

Conclusão: Este estudo demonstrou que, em pacientes com EHNA, o grupo que recebeu suplementação de probióticos apresentou melhora em parâmetros hepáticos, inflamatórios e metabólicos.



Palavras-chave: Fígado; Esteatohepatite; Estatose hepática; Probióticos; Suplementação. Microbiota intestinal; Eixo fígado-intestino

**ABSTRACT**

Introduction: The gut liver axis plays an important role in the development and progression of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NAFLD). Some promising results in the improvement of NAFLD and NASH have been identified following the treatment with probiotics, However, further studies on the subject are needed that evaluate different doses, strains and treatment time. The aim of the present study was to evaluate the effect of probiotic supplementation on liver function, inflammatory and metabolic profile in NASH patients.

Methods: Double-blind, placebo-controlled clinical trial. A total of 43 patients with histologically proven NASH were randomized to receive probiotics (*Lactobacillus acidophil*us $1x10^9$ CFU and *Bifidobacterium lactis* $1x10^9$ CFU – Probiotic Group) or placebo (Placebo Group) for 6 months. Patients were evaluated at 0, 3 and 6 months, including BMI and laboratory tests (AST, ALT, HDL, LDL and total cholesterol, triglycerides, glucose, insulin, C-reactive protein, ferritin, interleukin-6, tumor necrosis factor-α, monocyte chemoattractant protein-1 and leptin). Liver biopsy and/or Fibromax™, a noninvasive test to evaluate liver fibrosis, were performed at baseline and repeated after 6 months of treatment.

Results: After 6 months of treatment, the Probiotic Group presented a significant reduction in AST level (37.0 vs. 34.0 p = 0.012), APRI score (0.41 vs. 0.35 p = 0.042), C-reactive protein (6.6 vs. 4.5 p = 0.026) and ferritin (169.0 vs. 138.5 p = 0.008) compared to baseline. Compared to baseline HDL (47.5 vs. 52.0 p = 0.020) and total cholesterol (186.0 vs. 205.0 p <0.001) increased significantly in the Probiotic Group compared to baseline. There were no significant differences between groups after treatment.

Conclusion: This study identified that NASH patients treated with probiotic supplementation presented reduced AST levels and APRI score and had some inflammatory and metabolic parameters improved.

Key words: Liver; Steatohepatitis; Liver Steatosis; Probiotic; Dietary Supplementation

## SUMÁRIO

# REFERENCIAL TEÓRICO

## Doença Hepática Gordurosa Não Alcoólica e Esteatohepatite - Definição

A doença hepática gordurosa não alcoólica (DHGNA) é uma doença hepática crônica que está aumentando em prevalência com a epidemia mundial de obesidade. (1). É caracterizada pelo acúmulo excessivo de triglicerídeos (esteatose) no fígado (concentração superior a 5% dos hepatócitos) (2). É critério de exclusão para o diagnóstico de DHGNA o consumo de álcool (>21 doses por semana para homens e 14 doses por semana para mulheres) e outras causas específicas de doença hepática (3).

O termo DHGNA descreve um espectro de patologia do fígado, desde esteatose simples até esteatose com inflamação, esteatohepatite não alcoólica e cirrose (3,4).

Tabela 1 – Definições relacionadas a DHGNA.

| | |
|---|---|
| DHGNA | Compreende todo o espectro de doença hepática gordurosa em indivíduos sem consumo significativo de álcool, que vão desde fígado gorduroso até esteatohepatite e cirrose. |
| Fígado gorduroso não alcoólico | Presença de esteatose hepática sem evidência de lesão hepatocelular na forma de balonização dos hepatócitos ou sem evidência de fibrose. O risco de progressão para cirrose e insuficiência hepática é mínimo. |
| EHNA | Presença de esteatose hepática e inflamação com lesão de hepatócitos (balonização) com ou sem fibrose. Isso pode progredir para cirrose, insuficiência hepática e raramente carcinoma hepatocelular. |
| Cirrose | Presença de cirrose com evidência histológica atual ou anterior de esteatose ou esteatohepatite. |

Adaptado de: Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. AASLD PRACTICE GUIDELINE The Diagnosis and Management of Non-Alcoholic Fatty Liver Disease : Practice Guideline by the American Association for the Study of Liver Diseases , American College of Gastroenterology , and the American Gastroenterological Assoc. 2012;2005–23. (3)

## Epidemiologia

A prevalência global de DHGNA é 25%, com maior prevalência na América do Sul e Oriente Médio e menor na África. Nos últimos 20 anos esta prevalência duplicou, tornando DHGNA e EHNA as principais causas de doença hepática nos países ocidentais. (2,5) A DHGNA está associada a fatores de risco metabólicos como obesidade (51%), diabetes mellitus tipo 2 (22%), dislipidemia (69%) e síndrome metabólica (42%). A taxa reportada de progressão de DHGNA para EHNA é de 25% (Figura 1) (6).

Estima-se que 5 a 6% dos indivíduos adultos são portadores de EHNA (6). No Brasil, a esteatose hepática está estimada em 18%. Em análise de 226 biópsias hepáticas de obesos mórbidos, a frequência de esteatose, EHNA, fibrose e cirrose foram, respectivamente, de 90,4%, 70,4%, 43,2% e 1,6% (7).

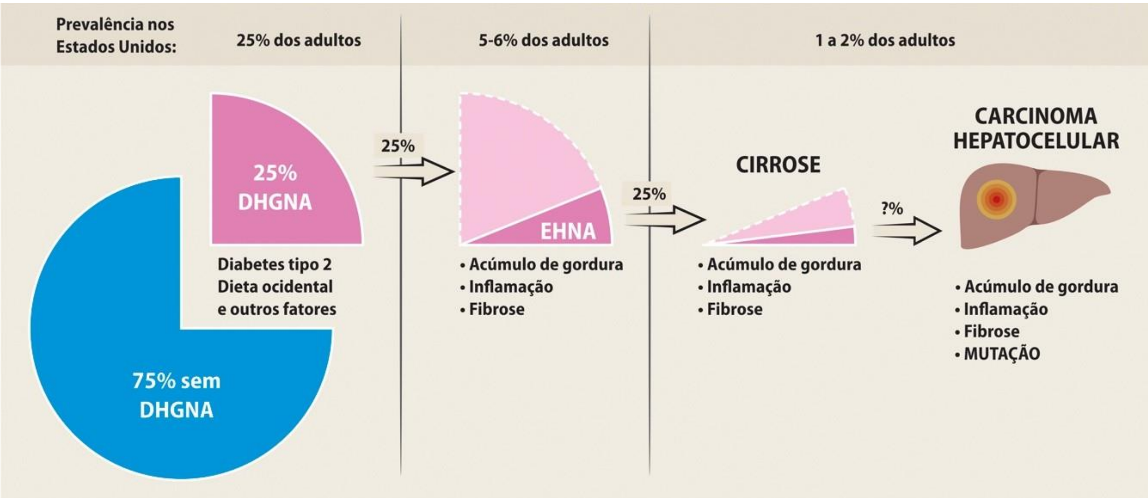


Figura 1 – Prevalência e progressão da DHGNA.

Distribuição relativa de DHGNA, EHNA, cirrose e carcinoma hepatocelular na população adulta dos EUA. A EHNA é uma manifestação potencialmente progressiva de DHGNA.

Adaptado de: Anna M. Diehl, M.D., and Christopher Day, M.D. 2017. Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis (6)

## Diagnóstico

Pacientes portadores de DHGNA e EHNA geralmente são assintomáticos e apresentam alterações nos níveis de transaminases (8). Estas enzimas costumam ter pouca sensibilidade e especificidade para diagnosticar EHNA, podendo se encontrar em níveis normais em pacientes com EHNA avançada e cirrose hepática (3).

A biópsia hepática ainda é considerada o padrão ouro para a o diagnóstico de EHNA (9) e é indicada nos *Guidelines* da Associação Americana para o Estudo de Doenças do Fígado - AASLD (3) para pacientes com DHGNA que apresentam riscos de EHNA e fibrose, como portadores de Síndrome Metabólica (SM) e pacientes que apresentem o NAFLD escore com indicação de risco de fibrose, sinalizado como risco significativo ou indeterminado. Porém, a biópsia é um procedimento invasivo que apresenta riscos de complicações e morte e os resultados da biópsia dependem da experiência do patologista (10,11).

Os métodos não invasivos para diagnóstico e classificação da DHGNA e EHNA utilizados atualmente incluem marcadores séricos e escores calculados a partir deles como NAFLD escore que utiliza idade, índice de massa corporal (IMC), aspartato aminotransferase (AST), alanina aminotransferase (ALT), albumina, plaquetas e a presença de diabetes ou alteração glicêmica, e o APRI escore que utiliza AST e plaquetas.

Exames de imagem convencionais como ecografia abdominal, tomografia computadorizada e ressonância magnética podem fornecer informação sobre o grau de esteatose, mas não informam grau de fibrose e atividade necroinflamatória. Recentemente novas tecnologias em exames de imagem como a Elastrografia vêm sendo utilizados (11).

A avaliação não invasiva pode ser realizada também por meio do teste Fibromax$^{TM}$ que avalia fibrose, inflamação e esteatose hepática. Este teste combina dez biomarcadores padrão: gama-GT, bilirrubina total, alfa-2-macroglobulina, apolipoproteína a1, haptoglobina, ALT, AST, triglicerídeos, colesterol e glicemia em jejum, estes marcadores são ponderados em função da idade do paciente, sexo, peso e altura (9,11,12).

**Patogênese e fatores de risco**

A esteatose hepática foi reconhecida como uma das principais causas de morbidade e mortalidade relacionadas ao fígado (1,2,13). Pode evoluir para EHNA, fibrose hepática e entre 10 e 30% dos pacientes com DHGNA têm EHNA que podem progredir para a cirrose (14). Carcinoma hepatocelular (CHC) ocorre em 70 a 90% de todos os pacientes com doença hepática crônica (4,6,15). A combinação de fatores genéticos, eventos externos e intracelulares somados ou não às agressões hepáticas, resultam em diferentes caminhos que levam à esteatose ou EHNA. Podendo a EHNA ser consequência da DHGNA e/ou a lesão inicial do fígado (16).

Os mecanismos envolvidos na progressão da DHGNA ainda não estão bem esclarecidos (17). A hipótese de *"múltiplos hits"* engloba algumas alterações que juntamente à predisposição genética ao desenvolvimento de DHGNA, explicam de certa forma a patogênese da doença, incluindo resistência à insulina, fatores inflamatórios secretados pelo tecido adiposo, fatores nutricionais, fatores genéticos e epigenéticos e a microbiota intestinal (6,18), foco deste trabalho.

**Hipótese dos Múltiplos *"hits"***

Fatores dietéticos e ambientais, juntamente com a obesidade, levam a níveis elevados de ácidos graxos e colesterol, desenvolvimento de resistência à insulina, proliferação de adipócitos e alterações na microbiota intestinal (18).

A resistência à insulina é um dos fatores-chave para o desenvolvimento de DHGNA e EHNA, resulta em aumento da lipogênese *de novo* (DNL) no fígado, promove o agravamento da disfunção de adipócitos no tecido adiposo, induz lipólise, com consequente aumento do fluxo de ácidos graxos para o fígado (19), alteração na produção e secreção de adipocinas e citocinas pró-inflamatórias como fator de necrose tumoral alfa (FNT-$\alpha$) e interleucina 6 (IL-6) (20), que também contribuem para manter o estado de resistência à insulina (18).

A leptina é um hormônio anorexígeno produzido pelo tecido adiposo, com ação pro-inflamatória que previne o acúmulo de lipídios em tecidos não adiposos. Contudo, a leptina aumenta em sujeitos obesos como uma consequência da resistência a leptina e exerce efeito profibrogênico (21,22). A ferritina pode ser considerada um marcador de inflamação porque apresenta associação direta com numerosas doenças inflamatórias e degenerativas (23).

As quimiocinas e seus receptores desempenham um papel crucial no processo inflamatório, recrutando macrófagos para tecidos metabolicamente comprometidos. A MCP1 é uma quimiocina pro-inflamatória, altamente envolvida no desenvolvimento da obesidade e esteatose hepática, induzida por uma desregulação do metabolismo de lipídios e energia (24).

O aumento do fluxo de ácidos graxos livres hepáticos, tem como consequência síntese e acúmulo de triglicerídeos (TG), e a produção de níveis "tóxicos" de ácidos graxos, colesterol livre e outros metabólitos lipídicos que causam disfunção mitocondrial com estresse oxidativo e produção de espécies reativas de oxigênio e ativação de mecanismos de estresse do retículo endoplasmático levando a inflamação hepática (18,25).

Além disso, a alteração da composição da microbiota intestinal e o aumento da permeabilidade no intestino delgado, levam ao aumento da produção e absorção de ácidos graxos, moléculas e endotoxinas como lipopolissacarídeos (LPS) que contribuem para a ativação das vias inflamatórias e a liberação de citocinas proinflamatórias como IL-6 e FNT-α (26).

Em sujeitos com predisposição genética ou modificações epigenéticas, todos esses fatores afetam o acúmulo de gordura nos hepatócitos, processos enzimáticos e perfil inflamatório do fígado, levando assim ao estado de inflamação hepática crônica por meio de diversas vias. Estas influenciam o risco de progressão para inflamação e fibrose (EHNA) ou persistência em estágio estável da doença (DHGNA), tanto pela toxicidade direta quanto pela ativação dos mecanismos de apoptose celular e ativação de células estreladas (18).

**Microbiota Intestinal e Eixo fígado-intestino**

A microbiota intestinal humana é composta por $10^{13}$ - $10^{14}$ micro-organismos cujo genoma contém pelo menos 100 vezes mais genes do que o genoma humano, que representa 500 a 1000 espécies no total (27,28).

É crescente o reconhecimento sobre a influência da microbiota intestinal na patogênese da DHGNA. Esta relação é explicada pelos mecanismos envolvidos no eixo fígado-intestino (18,29,30). Sendo o fígado irrigado por sangue proveniente da veia porta, este órgão acaba sendo o mais exposto às toxinas derivadas do intestino e representa a primeira linha de defesa contra dos produtos derivados das bactérias intestinais. Esta troca é de vital importância para a homeostase do sistema imune adaptativo (31).

A associação de esteatose hepática com microbiota intestinal em humanos é relacionada com aumento da permeabilidade intestinal e super crescimento bacteriano no intestino delgado de pacientes (32). O fígado é constantemente exposto a frações bacterianas derivadas de intestino ou metabólitos, e é um local importante para fagocitose bacteriana e depuração, pois hospeda mais de 80% dos macrófagos do corpo. As células de Kupffer, os macrófagos residentes do fígado, limitam a quantidade de endotoxina e fagocitam bactérias transportadas através da veia porta, desempenhando assim um papel central na depuração de infecções bacterianas sistêmicas (33). Os *toll like receptors* (TLR) reconhecem padrões moleculares associados a patógenos (PAMPs) para detectar a presença de agentes patogênicos. Mesmo pequenas quantidades de PAMPs, tais como lipopolisacarídeos, lipopeptídos, DNA não metilado e RNA de cadeia dupla, provocam reações inflamatórias intensas (34).

Considerando que o intestino hospeda mais de 99% do volume bacteriano no organismo, a microbiota intestinal é a principal fonte de PAMPs derivadas de bactérias tanto na saúde quanto na doença. Além do seu papel na imunidade inata, os ativadores de TLRs também desempenham um papel importante na regulação da inflamação, se ligam a moléculas endógenas, denominadas padrões moleculares associados ao dano (DAMPs) ou alarminas, que atuam como um sinal da presença de necrose e posterior gatilho da inflamação (35–37).

O fígado saudável contém baixos níveis de RNAm de TLRs (TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10) e moléculas de sinalização (ou seja, CD14, MD-2 e MyD88) em comparação com outros órgãos, sugerindo que a baixa expressão de moléculas de sinalização TLR pode contribuir para a alta tolerância do fígado aos ativadores de TLR provenientes da microbiota intestinal (38,39).

Determinados perfis de microbiota exercem efeito negativo na mucosa intestinal causando hiperpermeabilidade e “lipopolissacaridemia”. Pacientes com DHGNA e EHNA possuem permeabilidade aumentada significativamente correlacionada com a prevalência aumentada de supercrescimento bacteriano no intestino delgado (32,40). Este desequilíbrio entre micro-organismos benéficos e patogênicos é chamado disbiose intestinal.

Em doenças hepáticas crônicas, mudanças estruturais da mucosa intestinal como aumento da permeabilidade devido ao afrouxamento das *tight junctions*, ampliação de espaços intercelulares, congestionamento vascular e defeitos no sistema imunológico da mucosa, promovem a perda da função de barreira e permitem a translocação de bactérias e bactérias PAMPs (39). Muitos efeitos pró-inflamatórios dos PAMPs são uma consequência da secreção induzida por TLR de mediadores inflamatórios, como FNT-$\alpha$ e interleucina-1$\beta$ (41).

Os lipopolissacarídeos são um dos principais produtos bacterianos tóxicos e podem agir, ligados ao co-receptor CD-14, como um ligante de TLR com consequente ativação da cascata inflamatória, incluindo proteínas quinase ativadas pelo estresse, fator regulatório do interferon e NF-κβ, gatilhos que possuem relação com a resistência a insulina, obesidade, acúmulo de lipídios no fígado e desenvolvimento e progressão de esteatohepatite (31,42).

A disbiose intestinal também induz a produção de enzimas que catalisam a conversão da colina proveniente da dieta em compostos tóxicos (particularmente metilaminas). Estas aminas podem ser retomadas pelo fígado, transformando-se em N-oxido de trimetilamina (TMAO) induzindo inflamação e dano ao tecido hepático (43). Em recente estudo foi demonstrado que a disbiose intestinal pode promover EHNA pela redução dos níveis de colina e/ou pelo aumento dos níveis de metilaminas. Outro mecanismo que liga a microbiota intestinal ao surgimento de DHGNA é a alteração no metabolismo dos sais biliares que influencia a sinalização do receptor de sais biliares *farnesoid* X e, portanto, processos hepáticos: DNL e exportação de VLDL (44).

A composição da microbiota intestinal tem impacto no balanço energético pela fermentação de amido resistente e polissacarídeos não amiláceos para a produção de ácidos graxos de cadeia curta, principalmente acetato, butirato e propionato tornando-os absorvíveis pelo epitélio intestinal que exercem ação anti-inflamatória (43). Além disso, as bactérias entéricas suprimem a síntese de fator de tecido adiposo induzido pelo jejum resultando no aumento da atividade da lipoproteína lipase e aumento do acúmulo de triglicerídeos (45).

A fermentação realizada pelas bactérias da microbiota intestinal é também a maior fonte de álcool endógeno e alterações associadas a obesidade (46). Estudo realizado com crianças obesas portadoras de EHNA demonstrou abundância de álcool produzido pelas bactérias na microbiota com subsequente aumento da concentração de etanol sanguíneo quando comparado com crianças saudáveis (47). Esta hipótese pode explicar a similaridade entre as características histológicas e genéticas nas doenças hepáticas alcoólicas e não alcoólicas.

Já foram identificados diferentes perfis de microbiota relacionados com diferentes fenótipos (30). Estudos experimentais comprovaram que o transplante de microbiota do doador com EHNA transmite o fenótipo desta doença (48). Atualmente as pesquisas voltam-se para o transplante de microbiota de doadores saudáveis com o objetivo de reverter a doença.

O eixo do intestino-fígado é indicativo de uma forte ligação entre a saúde do trato intestinal e a do fígado. Com base em que uma mudança no perfil de microbiota intestinal, devido ao crescimento excessivo bacteriano, pode contribuir para a patogênese de DHGNA, foram propostos tratamentos capazes de manipular flora entérica, como a suplementação de probióticos ou prebióticos (34).

As mudanças nas terapias de dieta e estilo de vida são discutidas com frequência no tratamento dos pacientes portadores de DHGNA e EHNA (1). Inúmeros estudos com tratamentos farmacológicos para EHNA foram desenvolvidos, com sensibilizadores de insulina (49,50), fármacos hipolipemiantes (51), antioxidantes (52) e agentes do fator de necrose antitumoral (53). Apesar da abundância de ensaios clínicos, estas terapias ainda não podem ser utilizadas na prática clínica (1,13).

Nos últimos anos tem sido proposta uma nova estratégia de tratamento com probióticos (1,13) com o objetivo de modular a microbiota intestinal e reverter a inflamação e danos hepáticos.

**Tratamento com Prebióticos, Probióticos e Simbióticos**

Prebióticos são substâncias dietéticas não digeríveis, principalmente constituídas por polissacarídeos e oligossacarídeos não amiláceos (oligofructose, inulina, galacto-oligossacarídeos, lactulose, oligosacarídeos de leite materno, lactulose) (54,55). Probióticos são micro-organismos vivos que, quando administrados em quantidades adequadas, conferem um benefício para a saúde ao hospedeiro. Simbióticos são a combinação de probióticos e prebióticos, que conferem benefícios para a saúde, exercendo tanto um efeito prebiótico como probiótico (54).

A presença de prebióticos no cólon resulta em mudanças específicas na composição e/ou atividade da microbiota gastrointestinal, aumentando o número de bactérias anaeróbicas benéficas e diminuindo a população de micro-organismos potencialmente patogênicos, conferindo benefícios sobre a saúde do hospedeiro, (34,54).

As funções de probióticos e prebióticos são envolvidas com os micro-organismos que colonizam humanos. Os prebióticos servem como fonte de alimento para membros benéficos da comunidade microbiológica comensal, promovendo a saúde. A interação entre probióticos e células hospedeiras, ou probióticos e micróbios residentes, fornecem um meio chave para influenciar a saúde do hospedeiro (34).

Lactobacilos e bifidobactérias são considerados probióticos. Produzem compostos orgânicos, decorrentes da atividade fermentativa, como ácido láctico, peróxido de hidrogênio e ácido acético que aumentam acidez intestinal e produzem bacteriocinas, proteínas metabolicamente ativas, que auxiliam na destruição de micro-organismos indesejáveis inibindo proliferação bacteriana e dano ao epitélio intestinal (56).

Os probióticos afetam o ecossistema intestinal impactando os mecanismos imunes das mucosas, interagindo com patógenos, gerando produtos metabólicos finais, como ácidos graxos de cadeia curta e comunicando com células hospedeiras através de sinalização química. Esses mecanismos podem levar ao antagonismo de possíveis agentes patogênicos, melhora do ambiente intestinal, reforçando a barreira intestinal, regulando as respostas imunológicas e inflamatórias como DHGNA e EHNA (34,57).

Os probióticos podem contribuir para a redução da DHGNA, inflamação e melhora da função hepática modulando a microbiota intestinal por meio de alguns mecanismos: exclusão ou inibição de agentes patogênicos bacterianos, redução do crescimento bacteriano no intestino delgado, restauração da função de barreira epitelial gastrointestinal, modulação do sistema imune (58,59), produção de fatores antimicrobianos. As substâncias antimicrobianas incluem ácidos graxos de cadeia curta (produzidos na fermentação de prebióticos) (60), bacteriocinas, substâncias inibidoras semelhantes a bacteriocina e bacteriófagos (Figura 2) (34,61,62).

No metabolismo lipídico, os mecanismos pelos quais os probióticos reduzem as concentrações de colesterol permanecem em estudo (63). As hipóteses que têm sido propostas incluem: assimilação do colesterol pelas bactérias, algumas cepas bacterianas são capazes de assimilar e/ou incorporar o colesterol à parede celular, tornando indisponível a sua absorção do intestino para a corrente sanguínea (64); desconjugação enzimática dos sais biliares, tornando-os menos eficientemente reabsorvidos resultando em aumento da excreção de ácidos biliares nas fezes (65), a redução das concentrações plasmáticas de colesterol ocorre à medida que aumenta a demanda de colesterol para síntese de ácidos biliares que precisam ser repostos e alteração do metabolismo lipídico pela atuação dos ácidos graxos de cadeia curta (66).

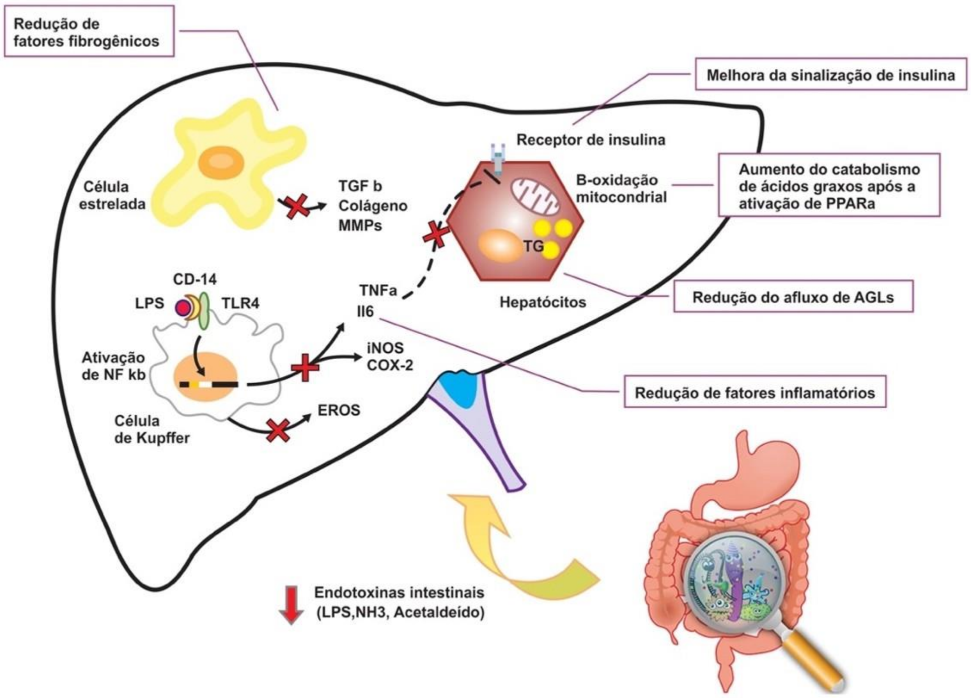


Figura 2 - Mecanismos celulares dos probióticos no fígado.

Os probióticos induzem a redução das endotoxinas derivadas do intestino conduzindo a uma diminuição da estimulação das células de Kupffer, do receptor de TLR4 e da transcrição de genes relacionados com NF-κB, reduzindo inflamação e fatores profibróticos. LPS: lipopolissacarídeo; NF-κB: fator nuclear-κB; TLR: Toll like receptor; ROS: espécies reativas de oxigênio; iNOS: sintase de óxido nítrico induzível; COX: ciclooxigenase; IL: interleucina; FNT-α: fator de necrose tumoral; TGF: factor de crescimento; MMPs: metaloproteinases; TG: triglicerídeos; PPAR: receptor ativado por proliferador de peroxissoma; AGL: ácidos graxos livres

Adaptado de: Iacono A, Raso GM, Canani RB, Calignano A, Meli R. 2011. Probiotics as an emerging therapeutic strategy to treat NAFLD: Focus on molecular and biochemical mechanisms.

Alguns ensaios clínicos testaram o uso de probióticos no tratamento de DHGNA e EHNA, os resultados foram a redução nos níveis séricos de transaminases, *Homeostasis model assessment* (HOMA-IR), FNT-α, proteína C-reativa e redução de índices de esteatose e atividade da esteatohepatite não alcoólica (67–76), mas as cepas de probióticos, doses, duração do tratamento, tratamentos paralelos e resultados não foram semelhantes nesses estudos. Além disso, pouco estudos realizaram avaliação histopatológica antes e após a intervenção.

## JUSTIFICATIVA

Considerando a alta prevalência de DHGNA, o aumento de evidências da influência do eixo fígado-intestino e poucos estudos sobre o tema, é crescente a necessidade de tratamentos não medicamentosos e mais estudos sobre o tema para a comparação de diferentes doses, cepas e tempo de tratamento.

# OBJETIVOS

## Objetivo Geral

Avaliar o efeito da suplementação de probióticos em parâmetros hepáticos (enzimáticos e histológicos) em pacientes com esteatohepatite não alcoólica.

## Objetivos Específicos

- Avaliar efeito da suplementação de probióticos nos parâmetros inflamatórios (proteína C-reativa, ferritina, interleucina-6, fator de necrose tumoral, proteína quimiotática de monócitos-1 e leptina) em pacientes com EHNA.
- Avaliar efeito da suplementação de probióticos nos parâmetros metabólicos (colesterol total, colesterol HDL, colesterol LDL, triglicerídeos, glicose, insulina) em pacientes com EHNA.

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## CONCLUSÕES GERAIS

A análise dos resultados dessa pesquisa permite concluir que os pacientes portadores de EHNA que receberam suplementação de probióticos em por 6 meses apresentaram:

- redução no parâmetro enzimático AST e o escore de fibrose APRI;
- nenhuma alteração significativa nos parâmetros histológicos:
- diminuição nos parâmetros inflamatórios proteína C reativa e ferritina;
- melhora no parâmetro metabólico colesterol HDL (com consequente aumento de colesterol total).

Não houve diferença estatisticamente significante entre os grupos após 6 meses de tratamento com probióticos.

Percebe-se potencial promissor para o desenvolvimento de terapias alternativas ou adjuntos ao tratamento da Esteatohepatite Não Alcoólica utilizando-se probióticos.

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# Probiotic Supplementation, Hepatic Fibrosis, and the Microbiota Profile in Patients with Nonalcoholic Steatohepatitis: A Randomized Controlled Trial

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A B S T R A C T

Background: Promising results in improvement of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (NASH) have been identified following probiotic (PRO) treatment.

Objectives: To evaluate PRO supplementation on hepatic fibrosis, inflammatory and metabolic markers, and gut microbiota in NASH patients.

Methods: In a double-blind, placebo-controlled clinical trial, 48 patients with NASH with a median age of 58 y and median BMI of 32.7 kg/m$^2$ were randomly assigned to receive PROs (*Lactobacillus acidophilus* $1 \times 10^9$ colony forming units and *Bifidobacterium lactis* $1 \times 10^9$ colony forming units) or a placebo daily for 6 mo. Serum aminotransferases, total cholesterol and fractions, C-reactive protein, ferritin, interleukin-6, tumor necrosis factor-$\alpha$, monocyte chemoattractant protein-1, and leptin were assessed. To evaluate liver fibrosis, Fibromax was used. In addition, 16S rRNA gene-based analysis was performed to evaluate gut microbiota composition. All assessments were performed at baseline and after 6 mo. For the assessment of outcomes after treatment, mixed generalized linear models were used to evaluate the main effects of the group-moment interaction. For multiple comparisons, Bonferroni correction was applied ($\alpha = 0.05/4 = 0.0125$). Results for the outcomes are presented as mean and SE.

Results: The AST to Platelet Ratio Index (APRI) score was the primary outcome that decreased over time in the PRO group. Aspartate aminotransferase presented a statistically significant result in the group-moment interaction analyses, but no statistical significance was found after the Bonferroni correction. Liver fibrosis, steatosis, and inflammatory activity presented no statistically significant differences between the groups. No major shifts in gut microbiota composition were identified between groups after PRO treatment.

Conclusions: Patients with NASH who received PRO supplementation for 6 mo presented improvement in the APRI score after treatment. These results draw attention to clinical practice and suggest that supplementation with PROs alone is not sufficient to improve enzymatic liver markers, inflammatory parameters, and gut microbiota in patients with NASH.

This trial was registered at clinicaltrials.gov as NCT02764047.

*Keywords*: NAFLD, liver steatosis, microbiota, inflammation, probiotics, treatment

*Abbreviations used:* MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PLA, placebo; PRO, probiotic; UFCSPA, Universidade Federal de Ci^encias da Saúde de Porto Alegre.

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## Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide [1]. The term NAFLD describes a spectrum of liver pathologies ranging from simple steatosis to steatosis with inflammation, nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [2,3]. Although the mechanisms involved in the progression of the disease have not been fully elucidated, the multiple-hit hypothesis [3] suggests possible aggravating factors, including alterations in fat metabolism, lipotoxicity, insulin resistance, adipose tissue dysfunction, genetic and epigenetic factors, dietary factors, and the influence of microbiota on the gut-liver axis

Recently, a new role for gut microbiota has emerged as an important player in NAFLD. Indeed, gut microbiota dysbiosis affects the gut-liver axis by increasing gut leakiness and endotoxemia absorption [4–6]. The relationship between the microbiota and NAFLD has been explained based on the gut-liver axis and 4 factors: energy balance and weight gain, increased inflammatory parameters, FA homeostasis dysregulation, and bacterial translocation. Therefore, the gut microbiota has drawn attention in nutrition research and clinical practice. The gut microbiota has been considered an effective target for therapeutic interventions in managing NAFLD [4–6], and there is now substantial evidence about the effects of these interventions in patients with NAFLD [7–9]. In patients with NASH, however, who present with a more advanced stage of liver disease and limited treatment options, the evidence focusing on gut microbiota and hepatic, inflammatory, and metabolic marker alterations is still incipient. Pilot trials performed exclusively with patients with NASH have tested the effect of prebiotics [10] and the effects of a combination of prebiotics and probiotics (PROs) [11].

PROs have been shown to inhibit the proliferation of pathogenic bacteria, produce antibacterial substances, reduce bacterial growth in the small intestine, restore gastrointestinal barrier function, and modulate the immune system, thereby contributing to the reduction of NAFLD and improvement of liver function [12,13]. A healthy microbiota profile improves intestinal permeability and reduces circulating LPS, endotoxins, and harmful metabolites [14,15]. A recent systematic review has shown that PROs, prebiotics, and symbiotics exert positive effects on inflammation and metabolic markers as well as liver injury in patients with NAFLD and NASH [8]. However, there are studies in children and studies with multiple co-interventions. The assessment of the effects of PROs alone in adult patients with NASH has yet to be investigated.

Considering the need for novel studies on the subject, especially in advanced stages of nonalcoholic fatty liver disease such as NASH, this study aimed to evaluate the effect of PRO supplementation on hepatic fibrosis, liver aminotransferases, systemic inflammatory markers, metabolic profile, and microbiota composition in patients with NASH. The hypothesis prior to the study was that PRO supplementation would decrease liver aminotransferase concentrations and improve hepatic fibrosis and systemic inflammation. It was also hypothesized that PROs would promote the modification of the intestinal microbiota.

## Methods

### Trial design

This was a single-center, randomly assigned clinical trial and a double-blind, placebo (PLA)-controlled parallel-group study. There was a baseline assessment with a follow-up assessment at 6 mo. The trial was registered, and the protocol can be accessed at www.cinicaltrials.gov, PROs in NAFLD (NCT02764047). The manuscript was elaborated according to the Consolidated Standards of Reporting Trials guidelines [16]. The protocol was approved by the Universidade Federal de Ci^encias da Saúde de Porto Alegre (UFCSPA) (approval number 852.771) and by the Hospital Santa Casa de Porto Alegre, Brazil (approval number 932.452) ethics in research committees. The study was conducted following the Declaration of Helsinki and with all applicable laws and regulations of the study locations. All participants provided written informed consent.

Subjects, investigators, and all site personnel involved in dispensing study supplementation, conducting study procedures, evaluating subjects, entering study data, and/or analyzing study data remained blinded to individual treatment assignments until all subjects completed the treatment and the data analyses were completed.

### Intervention

Patients were randomly assigned to receive either PRO supplement (PRO group) containing $1 \times 10^9$ colony forming units = 1 billion *Lactobacillus acidophilus* ATCC SD5221 + $1 \times 10^9$ colony forming units = 1 billion *Bifidobacterium lactis* HN019 (Beneflora Caps; Biolab Sanus Farmaceutica Ltda.) or PLA (PLA group) containing maltodextrin for 6 mo. This choice of PRO supplementation was based on safety, functionality, and usability [17], and it was also based on previous trials that tested the effects of *L. acidophilus* and *B. lactis* in patients with NAFLD [18] and children with NASH [19]. Because the preliminary evidence commonly presented a treatment length of 12–16 wk, we aimed to test a longer intervention (6 mo of treatment). Randomization was performed using www.randomization.com. The allocation sequence was concealed throughout the study period.

The first 90 capsules were dispensed at inclusion, and the remaining 90 capsules at the 3-mo follow-up visit. At this visit and at the final visit after treatment, patients were instructed to bring empty packages for compliance checking. The PRO and PLA capsules were visually indistinguishable, and the packaging was identical except for a unique bottle identification number. The patients were instructed to take 1 capsule/d with 1 glass of water before bedtime. Additional orientation was given to the patients to keep medications in use without any change until the end of the protocol. The patients were also instructed to communicate about antibiotic use. All patients received a standard dietary orientation regarding their healthy habits.

### Participants and study setting

Adult patients (>18 y old) with risk factors for NAFLD attending the gastroenterology outpatient clinic at the Hospital Santa Casa de Porto Alegre, Brazil, were recruited. Patients who met the inclusion criteria were invited to participate in the study.

### Eligibility criteria

Inclusion criteria comprised histology-proven NASH confirmed by a liver biopsy performed ≤6 mo prior to inclusion. In addition, the exclusion criteria were hepatitis B virus and/or hepatitis C virus and/or HIV infection; significant alcohol consumption (>14 drinks/wk for women and >21 drinks/wk for men) [2]; decompensated liver disease (ascites, esophageal varices, and encephalopathy); hepatocellular carcinoma; use of steroids, methotrexate, amiodarone, tamoxifen, valproate, vitamin E, or other medications potentially causing steatosis; previous surgery (bariatric, gastric, and intestinal resection); total parenteral nutrition in 6 mo preceding inclusion; pregnancy or breastfeeding; history of hypothyroidism; Cushing syndrome; and type 1 diabetes. Other causes of secondary steatosis were also evaluated and excluded accordingly.

## Data collection

### Demographic and clinical profile, anthropometric assessment

Data were collected on sex, age, hypertension, diabetes, dyslipidemia, metabolic syndrome, use of medications, and lifestyle habits (physical activity, smoking, and alcohol consumption). Hypertension, diabetes, and metabolic syndrome were defined according to the guidelines [15,20,21].

Height was measured at baseline to confirm the BMI. Weight was measured at baseline and after 6 mo. Anthropometric measurements were performed using a calibrated mechanical scale and ruler (Filizola). Patients wore light clothing and no shoes.

### Hepatic, inflammatory, and metabolic tests

Improvement in liver parameters was defined by the reduction of fibrosis, steatosis, and inflammation in the Fibromax test [22] (Biopredictive), AST to Platelet Ratio Index (APRI), and NAFLD fibrosis scores. Fibromax tests were performed at baseline and at 6 mo.

Fibromax is a noninvasive test that evaluates liver steatosis, fibrosis, and inflammation grade. This test combines 10 standard biomarkers: GGT, total bilirubin, α-2-macroglobulin, apoA1, haptoglobin, ALT, AST, TG, cholesterol, and fasting glycemia, combined and weighed according to patient age, sex, weight, and height [22]. Evaluation of posttreatment results in relation to baseline was performed based on alterations (improvement or worsening) in the stage of fibrosis graded F0–F4, steatosis graded S0–S3, and inflammatory activity graded A0–A2 according to the Fibromax reference values [22]. The cutoff points used were those standardized by the test. In cases of intermediate results, the most advanced concentration was chosen for data analysis. Thus, Fibrotest scores 0.00–0.21 indicated F0 (without fibrosis); 0.22–0.31 indicated F1 (portal fibrosis); 0.32–0.58 indicated F2 (portal fibrosis with rare septa); 0.58–0.72 indicated F3 (portal fibrosis with many septa) and 0.73–1.00 indicated F4 (cirrhosis). For Steatotest, values of 0.00–0.3 defined S0 (without steatosis), 0.31–0.48 defined S1 (minimal steatosis), 0.49–0.67 defined S2 (significant steatosis), and 0.68–1.00 indicated S3 (severe steatosis). In the inflammatory activity evaluation, the values of 0.25, 0.50, and 0.75 were defined as A0 (without NASH), A1 (probable NASH), and A2 (presence of NASH), respectively.

APRI score was calculated using AST and platelets [23]. For the evaluation of significant fibrosis, the APRI score was categorized as the absence (APRI ≤ 0.5) or presence (APRI ≥ 1.5) of significant fibrosis. The NAFLD fibrosis score includes age, BMI, insulin resistance or diabetes mellitus type 2, aminotransferases, platelets, and albumin values [24]. A NAFLD fibrosis score cut-of-value under —1.455 suggests no significant fibrosis. A NAFLD fibrosis score cutoff value of >0.675 was considered to suggest the presence of advanced fibrosis.

Blood samples were collected after overnight fasting at baseline and 6 mo. Blood samples were processed for biochemical analyses at the Santa Casa Hospital Central Laboratory or Immunology Laboratory of UFCSPA. Blood samples (8 mL) were collected from the antecubital vein in tubes without anticoagulants. Samples were centrifuged (1000 g, 10 min), separated into aliquots in microtubes, and stored at —80°C until analysis. Biochemical analyses of serum GGT, total bilirubin, α-2-macroglobulin, apoA1, haptoglobin, ALT, AST, TG, total cholesterol, HDL cholesterol, and fasting insulin and glycemia were performed in an automated machine at Santa Casa Hospital Central Laboratory using appropriate controls and calibrators. In addition, the LDL cholesterol [25] and HOMA-IR [26] were calculated. The systemic amount of IL-6, monocyte chemoattractant protein-1 (MCP-1), TNF-α, and leptin (all from Peprotech Inc.) were analyzed by ELISA following the manufacturer's recommendations using a microplate reader (SpectraMax M2e; Molecular Devices). The intra-assay coefficient of variation was <7.5%. The detection limits of each cytokine were as follows: TNF-α, 2–500 pg/mL; IL-6, 2–400 pg/mL; MCP-1, 50–1500 pg/mL; leptin, 6–800 ng/mL.

### 16S rRNA gene-based analysis of gut microbiota

Fecal samples were collected by patients and refrigerated until they arrived at the Immunology Laboratory at UFCSPA, 4 h after collection. Samples were divided into 4 aliquots and stored at —20°C or —80°C. DNA was extracted using a MoBio PowerFecal DNA Isolation Kit (Qiagen, The Netherlands) according to the manufacturer's protocol [27]. The DNA samples were sent to the University Institute Research Center of Cardiology and Pneumology at the University of Laval, Quebec. The total extracted DNA was quantified using ND-1000 Nanodrop (Nanodrop Technologies). Samples that met quality requirements with values of 260/280 and 260/230 ratios of >1.8 and 2.0, respectively, were selected for sequencing. Amplicons of the 16S rRNA V3-V4 region were generated using degenerate primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') adapted to incorporate the transposon-based Illumina Nextera adapters (Illumina) and a sample barcode sequence allowing multiplexed paired-end sequencing. Constructed 16S metagenomic libraries were purified using 35 μL of magnetic beads (AxyPrep Mag PCR Clean-up Kit; Axygen Biosciences) per 50 μL of PCR reaction. Library quality control was performed using Bioanalyzer 2100 using DNA 7500 chips (Agilent Technologies). An equimolar pool was obtained and checked for quality prior to further processing. The pool was quantified using PicoGreen (Life Technologies) and loaded on a MiSeq platform using 2 × 300-bp paired-end sequencing (Illumina). High-throughput sequencing was performed at Institut de Biologie Int´egrative et des Syst`emes -

Universit´e Laval. A total of 5,729,902 sequences (ranging from 46,677 to 129,170) were generated from libraries.

Demultiplexed reads were analyzed using the QIIME software package (QIIME is an open-source project, developed primarily in the Knight and Caporaso labs, version 1.9.1) and customs scripts. The Paired-End reAd mergeR, version 0.9.5 [28], was used to pair the forward and reverse reads of sequences in each sample with ≥50-bp overlap. Sequences were discarded if they had an ambiguous base (N), a Phred score of ≤25, or were <450 bp in length. Chimeras were filtered with a reference-based approach using UCHIME, version 4.2, drive5.com [29], and a representative set of chimera-checked sequences Greengenes version 13.8 [30]. The resulting sequences comprised a total of 5,185,953 reads (representing a range from 24,846 to 69,759 and a mean of 47,071 reads per sample) and were clustered into operational taxonomic units (OTUs) at 97% identity threshold using an open-reference methodology performed with USEARCH 61, version 6.1.544, drive5.com [29]. The taxonomy of OTUs was assigned to a representative set of 16S rRNA sequences in the Greengenes database [30] using the RDP classifier [31]. All OTUs that were observed <3 times in the entire dataset or with a number of sequences <0.01% of the total number of reads were discarded. A total of 657 OTUs were kept after the filtering step. In order to normalize the sampling effort, samples were rarefied to an even sampling depth of 24,800 sequences.

## Study outcomes

The primary outcomes were enzymatic liver markers, hepatic fibrosis, inflammatory parameters, and gut microbiota alterations evaluated at baseline and at the end of treatment (6 mo). These outcomes were evaluated based on the results of Fibromax, NAFLD fibrosis score, AST to platelet ratio index (APRI score), ALT, AST, CRP, ferritin, TNF-α, IL-6, MCP-1, leptin, and 16S rRNA gene-based analysis of gut microbiota. In addition, all downstream analyses were performed on the rarefied abundance of taxa. The secondary outcomes were anthropometric and metabolic parameters. These outcomes were evaluated based on BMI, total cholesterol, HDL cholesterol, LDL cholesterol, TG, glucose, insulin, and HOMA-IR.

## Sample size

For sample size calculation, we used the measure of effect size (Cohen "d") based on the study by Aller et al. [14] regarding improvement in posttreatment AST in the PRO group. The study presented an effect size (d) of 0.81. Because it is considered a high effect size, we used d = 0.8 at a significance level of 0.05 and a power of 80%. The calculation estimated 15 individuals/group. In addition to possible losses to follow-up (30%), 20 patients were required for each group. As an outcome, a change of 5 units in AST was considered the minimum change for clinical relevance, as presented in the literature [14].

## Statistical analysis

Statistical analysis was performed using SPSS software, version 20.0 (SPSS Inc.). Continuous variables are described as median and minimum–maximum. Categorical variables are described as absolute and relative frequencies. The Shapiro-Wilk test was used to determine whether continuous variables were normally distributed. For baseline assessment, continuous variables were compared between the intervention and control groups using the Student's t-test (normal distribution) or Mann-Whitney U test (asymmetrical distribution). Categorical variables were described by frequencies and percentages and were associated with the $\chi^2$ test or Fisher's exact test.

For the assessment of outcomes after treatment, mixed GLM was used, with an unstructured correlation matrix, to evaluate the main effects of the group and evaluated moment and of the group*moment interaction. For multiple comparisons, Bonferroni correction was applied ($\alpha = 0.05/4 = 0.0125$). Results are presented as mean and SE.

The statistical significance of differentially abundant bacteria between 2 distinct biological conditions was measured using Linear discriminant analysis Effect Size (LEfSe) [32]. A linear discriminant analysis score of ≥2.5 was considered statistically significant. For all tests, the significance level was set at 0.05.

# Results

A total of 48 patients were included and randomly assigned to 2 groups receiving PROs or PLA (Table 1). Five patients did not complete 6 mo of treatment, but following the intention-to-treat analysis principle, they were included in the analysis (Figure 1). None of the patients reported any discomfort, side effects, or symptoms associated with treatment. In addition, patients had high compliance with treatment as they all brought empty packages of the treatment received.

TABLE 1
Baseline characteristics of 48 patients with nonalcoholic steatohepatitis treated with probiotics or placebo for 6 mo

| Parameter[1] | PRO $n = 23$ | PLA $n = 25$ | $P$ value[2] |
|---|---|---|---|
| Female, $n$ (%) | 20 (87.0%) | 18 (72.0%) | 0.292 |
| Age, y | 58.0 (28.0–71.0) | 57.0 (33.0–71.0) | 0.860 |
| BMI, kg/m² | 31.6 (26.5–66.4) | 31.6 (22.7–46.0) | 0.865 |
| Comorbidities | | | |
| Hypertension, $n$ (%)[3] | 17 (73.9%) | 19 (79.2%) | 0.671 |
| Diabetes, $n$ (%)[3] | 17 (73.9%) | 18 (75.0%) | 0.932 |
| Dyslipidemia, $n$ (%)[3] | 13 (56.5%) | 15 (62.5%) | 0.433 |
| Metabolic syndrome, $n$ (%)[3] | 18 (78.3%) | 17 (94.4%) | 0.559 |
| Medications | | | |
| Metformin use, $n$ (%) | 14 (60.9%) | 17 (68.0%) | 0.471 |
| Statin use, $n$ (%)[3] | 11 (47.8%) | 08 (32.0%) | 0.855 |
| Antibiotic use, $n$ (%) | 05 (21.7%) | 04 (16.0%) | 0.708 |
| Fibrosis stage, $n$ (%)[3] | | | 0.441 |
| F0 | 07 (38.9%) | 13 (59.1%) | |
| F1–F2 | 07 (38.9%) | 06 (27.3%) | |
| F3–F4 | 04 (22.2%) | 03 (13.6%) | |

Abbreviations: F, fibrosis; PLA, placebo; PRO, probiotic.

[1] Data presented as absolute and relative frequencies ($n$, %) for categorical variables and as medians (minimum–maximum) for continuous variables.

[2] Continuous variables were compared between the intervention and control groups using Student's t-test. Categorical variables were compared with the $\chi^2$ test or Fisher's exact test.

[3] Percentage calculated based on valid cases. Fibrosis stage based on Fibromax test.

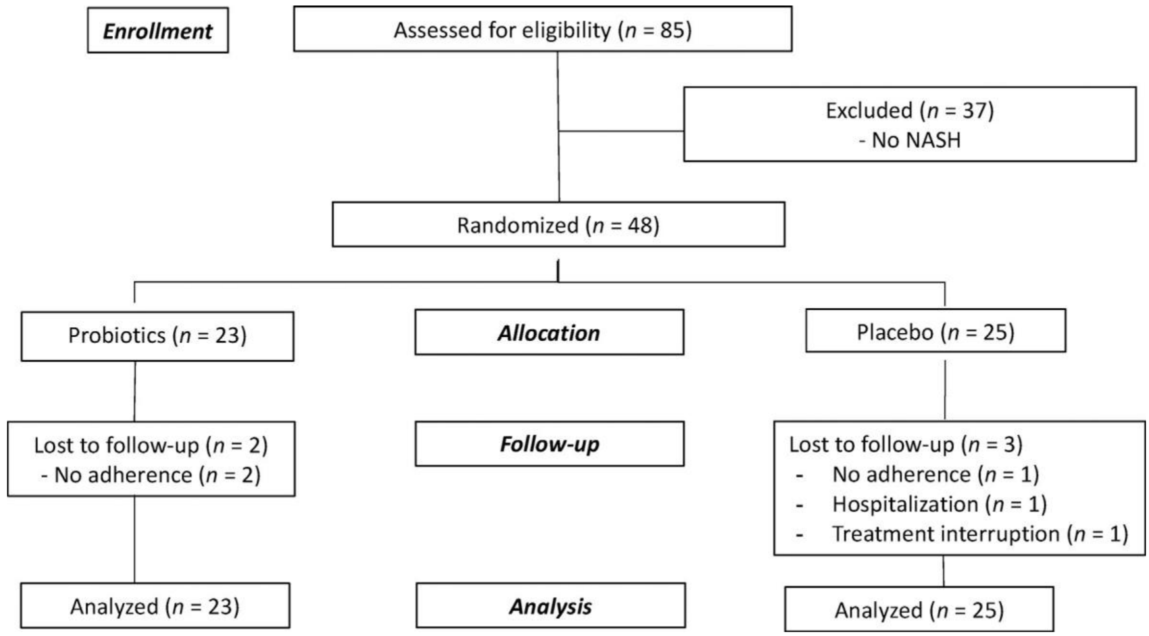


FIGURE 1. CONSORT flow diagram of patients with nonalcoholic steatohepatitis treated with probiotics or a placebo for 6 mo. NASH, nonalcoholic steatohepatitis.

## Primary outcomes

The APRI score was the primary outcome that presented a significant decrease in the PRO group between baseline and 6 mo (Table 2). AST presented a significant group*moment interaction, but the Bonferroni correction failed to identify where the differences were. Other enzymatic liver markers and the inflammatory parameters presented no significant differences between groups after treatment.

There were no statistically significant differences in liver fibrosis scores, steatosis, and inflammatory activity assessed by the Fibromax test after treatment (Table 2). Maintenance of baseline stage was identified in 88.9% compared with 72.7% of patients; $P = 0.417$ for fibrosis, 77.8% compared with 54.5%; $P = 0.294$ for steatosis and 77.8% compared with 72.7%; $P = 0.690$ for inflammatory activity between the PRO and PLA groups, respectively.

Eighteen DNA samples that met quality control criteria, 10 from the PRO group and 8 from the PLA group, were used for 16S rRNA gene analysis (Figure 2). According to the LEfSe analysis, Mitsuokella was the only bacterial genus overrepresented in the PRO group compared with that in the PLA group after treatment (Figure 3B). However, this is likely because these bacteria were more abundant in the group treated with the PROs than in the PLA group at baseline (Figure 3A).

## Secondary outcomes

Regarding the metabolic profile, metabolic markers (total cholesterol, HDL cholesterol, LDL cholesterol, TG, glucose, insulin, and HOMA-IR) showed no statistically significant differences between groups at the end of the study (Table 2).

# Discussion

This study evaluated the effect of 6 mo of PRO supplementation on hepatic fibrosis, liver aminotransferases, systemic inflammatory markers, metabolic profile, and microbiota composition in patients with NASH. The strengths of this study were the length of treatment (6 mo), which was designed to assess effects over a longer period than most trials performed to date [33], and the assessment of gut microbiota alterations in patients with NASH. Mixed-linear model analyses revealed that the group treated with PROs showed a reduction in APRI scores after treatment when compared to baseline. However, as for other hepatic, inflammatory, and metabolic markers, no statistically significant differences were detected between the groups after treatment. In addition, no major shifts in the gut microbiota composition were identified between the groups after PRO treatment.

The APRI score was significantly decreased in the PRO group after treatment. As a marker of fibrosis, this score may reflect a reduction in AST concentrations. To date, no previous published trials testing PROs have assessed this score, which is widely applied in clinical practice [34,35]. In view of our results, however, we believe there is little clinical significance in this finding, as the reductions were very slight.

AST concentrations showed a statistically significant group*moment interaction in our study but with no identification of what group or moment was different from others. Nevertheless, other studies have reported that some PROs may improve liver enzyme concentrations. Indeed, Shavakhi et al. [36] performed a clinical trial with patients with NASH using PROs (*L. acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Streptococcus thermophilus*) plus metformin or metformin alone for 6 mo. In within-group comparisons, a decrease in AST and ALT concentrations was observed in the PRO plus metformin group, and a significant reduction in AST concentrations was observed in the metformin group. Wong et al. [37] analyzed 20 patients with NASH who received PROs (*Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *L. acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacterium bifidum*) plus lifestyle advice or lifestyle advice alone for 6 mo. The treated group presented a significant reduction in

TABLE 2

Comparison of anthropometric, hepatic, inflammatory, biochemical, and lipid serum parameters between the treatment groups of 48 nonalcoholic steatohepatitis patients treated with probiotics or placebo for 6 mo

| Parameter[1] | Treatment | Basal | 6 mo | Group-moment interaction (*P* value)[2] |
|---|---|---|---|---|
| BMI, kg/m² | PRO | 33.0  1.1 | 32.7  1.1 | 0.848 |
| | PLA | 32.7  1.1 | 32.4  1.1 | |
| ALT, U/L | PRO | 52.3  10.1 | 49.3  10.3 | 0.716 |
| | PLA | 45.6  10.3 | 44.6  9.9 | |
| AST, U/L | PRO | 46.2  5.0 | 39.0  5.2 | 0.028 |
| | PLA | 30.6  4.7 | 33.2  5.0 | |
| APRI score | PRO | 0.6  0.1 | 0.4  0.1 | 0.046 |
| | PLA | 0.5  0.1 | 0.5  0.1 | |
| NAFLD fibrosis score | PRO | —0.6  0.3 | —0.7  0.3 | 0.137 |
| | PLA | —0.8  0.3 | —0.6  0.3 | |
| Fibrotest score | PRO | 1.3  0.3 | 1.3  0.3 | 0.630 |
| | PLA | 1.0  0.3 | 1.2  0.3 | |
| ActiTest score | PRO | 0.8  0.2 | 0.6  0.2 | 0.392 |
| | PLA | 0.5  0.2 | 0.5  0.2 | |
| Steatotest score | PRO | 2.4  0.2 | 2.5  0.2 | 0.495 |
| | PLA | 2.3  0.2 | 2.3  0.2 | |
| C-reactive protein, mg/dL | PRO | 8.4  1.4 | 6.4  1.0 | 0.055 |
| | PLA | 5.5  1.4 | 6.6  1.0 | |
| Ferritin, mg/dL | PRO | 307.9  78.0 | 235.2  59.0 | 0.102 |
| | PLA | 210.2  76.3 | 204.6  57.8 | |
| MCP-1, pg/mL | PRO | 400.1  35.1 | 398.9  33.2 | 0.644 |
| | PLA | 420.2  33.7 | 435.1  31.9 | |
| Leptin, ng/mL | PRO | 46.5  4.7 | 34.7  4.1 | 0.296 |
| | PLA | 40.6  4.0 | 37.7  3.5 | |
| Tumor necrosis factor, pg/mL | PRO | 16.5  1.2 | 15.4  1.2 | 0.755 |
| | PLA | 17.7  1.2 | 16.0  1.2 | |
| Total cholesterol, mg/dL | PRO | 186.4  8.4 | 208.1  8.8 | 0.110 |
| | PLA | 195.9  8.1 | 201.8  8.4 | |
| HDL-C, mg/dL | PRO | 98.6  7.3 | 113.2  8.5 | 0.529 |
| | PLA | 110.1  7.3 | 119.1  8.5 | |
| LDL-C, mg/dL | PRO | 47.4  2.0 | 51.7  2.1 | 0.237 |
| | PLA | 49.8  2.0 | 52.0  2.1 | |
| Triglycerides, mg/dL | PRO | 206.8  27.5 | 216.8  25.6 | 0.124 |
| | PLA | 192.9  26.5 | 171.6  24.5 | |
| Glucose, mg/dL | PRO | 137.2  15.2 | 130.2  10.8 | 0.731 |
| | PLA | 162.9  14.4 | 150.6  10.4 | |
| Insulin, mg/dL | PRO | 28.5  4.8 | 27.0  4.7 | 0.935 |
| | PLA | 30.2  4.7 | 28.4  4.6 | |
| HOMA-IR | PRO | 9.1  2.2 | 8.8  2.1 | 0.451 |
| | PLA | 13.0  2.1 | 11.2  2.1 | |

Abbreviations: ALT, alanine aminotransferase; APRI, AST to Platelet Ratio Index; AST, aspartate aminotransferase; HDL-C, HDL cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; LDL-C, LDL cholesterol; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; PLA, placebo group; PRO, probiotics group.

[1] Data presented as mean  SE.

[2] Bolded *P* values are significant at $P < 0.05$.

AST concentrations relative to the baseline in the intragroup analysis [37]. This evidence considered PROs to be a promising treatment for AST reduction [36,37]; however, there are multiple interventions combined.

The present study found no alterations in fibrosis assessed by the Fibromax test results between groups after treatment with PROs. Mofidi et al. [38] conducted a randomly assigned clinical trial with 50 patients with NAFLD who received symbiotics or a PLA for 28 wk. Hepatic steatosis and fibrosis were reduced in both groups; however, the mean reduction was significantly greater in the symbiotic group than in the PLA group. Malaguarnera et al. [39] evaluated 66 patients with NASH. One group received symbiotic (*Bifidobacterium longum* with fructo-oligosaccharides) plus lifestyle modifications, and the other group received lifestyle modifications alone. This study showed significant differences between groups in steatosis and NASH activity index in histological analyses. Another study [40] analyzed 50 biopsy-proven patients with NASH; 1 group received *Lactobacillus reuteri* with guar gum and inulin plus healthy balanced nutritional counseling, and the other group received nutritional counseling alone for 3 mo. The symbiotic group showed significant reduction in steatosis. It is worth noting that studies do not clearly indicate the number of patients according to liver fibrosis grading and the number of patients with cirrhosis. Compared to our findings, these conflicting results could be attributed to differences in the treatment administered (dose, strains, duration, combination of PROs and prebiotics, and the application of multiple

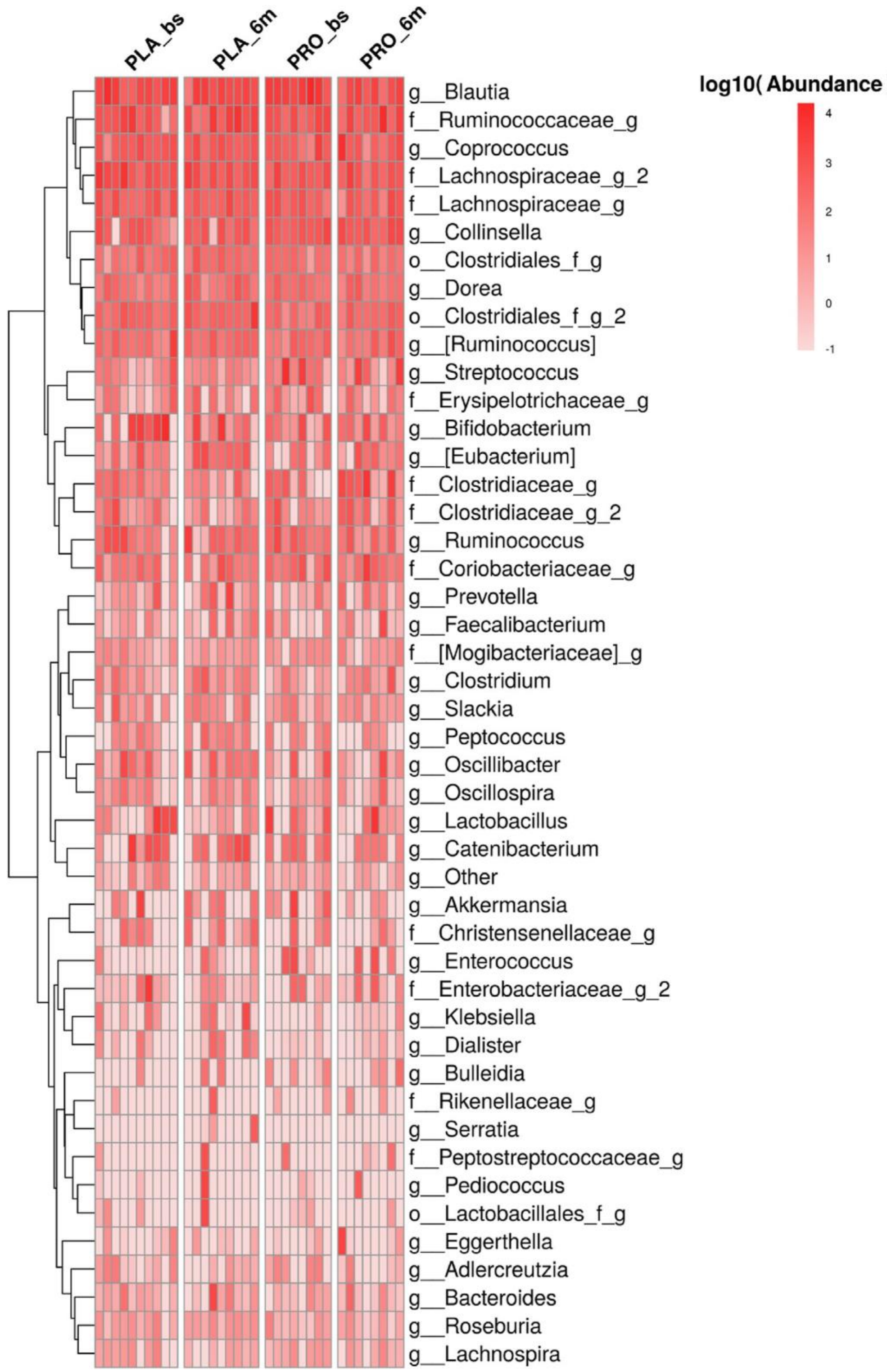


FIGURE 2. Heatmap displaying the log10-transformed abundance of most abundant bacterial genera across samples of NASH patients treated with probiotics (PRO) or placebo (PLA) for 6 months. plc_bs: placebo baseline, plc_6m: placebo after 6 months, probio_bs: probiotic baseline, probio_6m: probiotic after 6 months. '_f' and '_g' at the end of taxon denotes unclassified family and genus, respectively.

interventions) and in the profile of the patients, considering that all participants in our study were patients with NASH, a more advanced stage of NAFLD, and consequently, more difficult to reverse.

PROs or symbiotics to patients with NASH, no statistically significant changes in BMI were observed [14,37,41]. Shavakhi et al. [36] performed a study of patients with NASH using PROs plus metformin or metformin only (500 mg/d) for 6 mo and found significantly decreased BMI in both groups. Ferolla et al. [40], in a randomly assigned, controlled clinical trial involving 50 biopsy-proven patients with NASH, using Lactobacillus reuteri with guar gum and inulin plus nutritional counseling

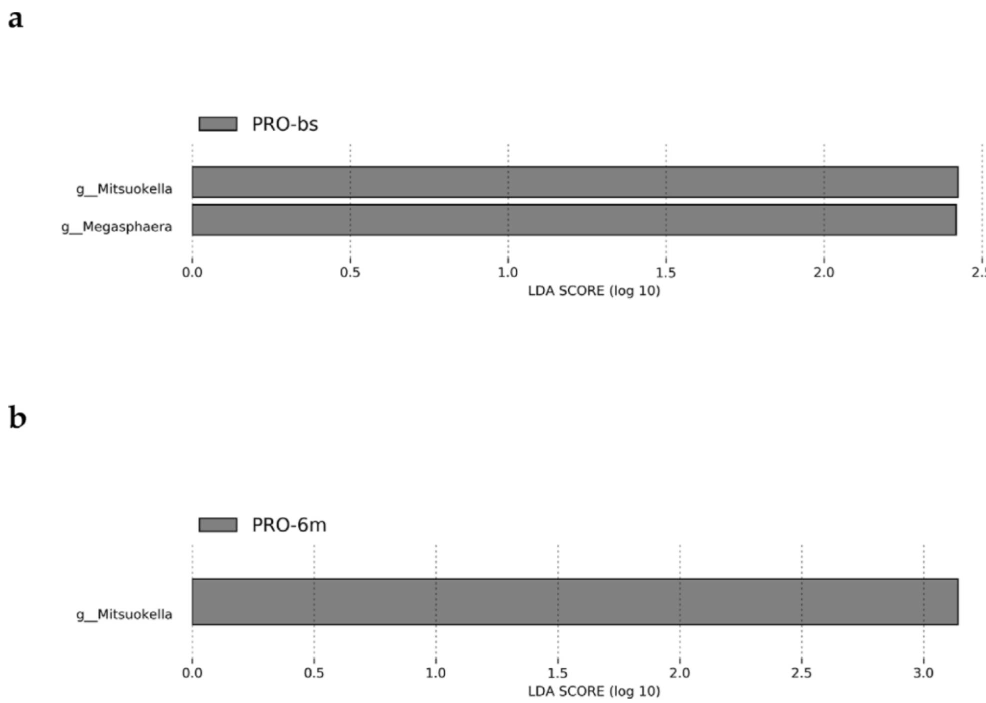


FIGURE 3. LEfSe analysis. Differential abundance of bacterial genera between probiotics (PROs) and a placebo (PLA) at (a) baseline and (b) 6 months.

compared with nutritional counseling alone for 3 mo, observed that the symbiotic group had reduced BMI. Sepideh et al. [42] observed that anthropometric indices were not significantly modified during the study or between groups after intervention.

The administration of PROs did not significantly change the microbiota composition of patients with NASH in our study. Intestinal dysbiosis is associated with various liver diseases, including NAFLD and NASH [43]. A review [44] analyzed studies that evaluated modifications in the intestinal microbiota associated with NAFLD/NASH. However, few studies to date have analyzed the relationship between the gut microbiome and NAFLD in humans, and these studies have yielded divergent results [45–49]. Although some strains of PROs cause few changes in the microbiota composition and diversity, PROs may have beneficial effects on health, with improvements in the biochemical and histological parameters depending on changes in feeding habits and the associated use of prebiotics and PROs [39,50–53]. It is possible that the potential benefits of PRO supplementation may occur with a longer duration of treatment or a combination of PROs and another intervention. Other analyzed studies performed interventions ranging from 2 to 7 mo [12–14,19,36–40,42] with divergent results depending on the PRO used and intervention length.

A possible limitation of this study is the presence of patients with cirrhosis in the sample, which may have impaired the reversal of fibrosis because advanced fibrosis is more aggressive and difficult to treat. In addition, most of the participants used statins and/or metformin because they were patients with metabolic syndrome; therefore, some metabolic markers, such as glucose, insulin, total cholesterol, HDL cholesterol, and LDL cholesterol, may have been influenced by medication. Supplementary analyses were performed to check for differences in these metabolic markers according to metformin and statin use, and no differences were found between or within groups (data not shown). Approximately 20% of the patients in each group used antibiotics for a short period of ≤6 mo prior to enrollment. It is possible that antibiotic use interferes with the action of PROs. However, there was no significant difference in the prevalence of antibiotic, metformin, or statin use between the groups.

The study participants showed heterogeneity in some hepatic parameters. Nevertheless, the groups did not show any statistically significant differences at baseline. It is important to note that the groups were compared in relation to the fibrosis stage, and no differences were found between them. Consequently, because heterogeneity was present in both groups, we believe that the effects of treatment were not hindered by such heterogeneity. This study reflected real-life patients routinely seen in outpatient services, usually with heterogeneity and many comorbidities. This study presented a robust design with a reasonable intervention length (6 mo) and concomitant evaluation of several parameters that were not tested in similar trials, including liver fibrosis assessment before and after intervention and gut microbiota alterations in patients with NASH. Therefore, it adds to the nutrition scientific literature by studying a carefully designed treatment that does not present all expected outcome changes.

In conclusion, in the within-group analyses, treating patients with NASH with PRO supplementation for 6 mo reduces APRI scores. Nevertheless, these results suggest that supplementation with PROs alone is not sufficient to improve enzymatic liver markers, inflammatory parameters, and gut microbiota in patients with NASH.

## Author contribution

The authors' responsibilities were as follows – GZP, CVT, and CB: contributed substantially to the conception and design of the study; GSE, GZP, SAF, AP, GPD, VPH, TVR, GP, and AM:

contributed to the acquisition of data or analysis and interpretation of data; all authors contributed to the drafting or making critical revisions related to important intellectual content; and all authors: read and approved the final manuscript.

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## Author disclosures

The authors report no conflicts of interest.

## Data availability

Data can be made available upon request.

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