UNIVERSIDADE ESTADUAL DE MONTES CLAROS

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Avaliação dos Efeitos da Angiotensina-(1-7) Sobre o Tecido Adiposo, Inflamação e Microbiota Intestinal, e o Impacto do Uso de Probiotico na Expressão do Sistema Renina Angiotensina

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Orientador: Prof. Dr. Sérgio Henrique Sousa Santos

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Unimontes

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RESUMO

O tecido adiposo foi recentemente reconhecido como um importante órgão endócrino e metabólico responsável pela síntese de adipocinas que controlam o metabolismo, a homeostase corporal, e a inflamação. Evidências recentes sugerem o importante papel da Angiotensina-(1-7) no tratamento da obesidade e de distúrbios metabólicos devido a seu papel anti-inflamatório e por ser o principal contraregulador do eixo da Ang II do SRA. Além disso, é importante considerar outros reguladores metabólicos importantes, como a microbiota intestinal e sua modulação por meio de probioticos. Neste contexto, a presente tese como objetivo avaliar os efeitos da Ang-(1-7) sobre o tecido adiposo, inflamação e microbiota intestinal, e o impacto do uso de probiotico na expressão do sistema renina angiotensina. Para isso foram produzidos três produtos. No produto 1 realizamos uma revisão da literatura científica a cerca dos achados relacionados ao eixo ECA2/Ang-(1-7)/MasR com foco no seu papel na regulação da secreção de adipocinas e dos processos relacionados à inflamação aguda e crônica. É importante ressaltar o papel endócrino do tecido adiposo na síntese de adipocinas, modulando sua secreção e regulando diversos processos associados à inflamação aguda e crônica, além de sua relevância na homeostase do organismo. As vias moleculares envolvidas nos mecanismos de ação das adipocinas criaram a necessidade de novos estudos visando investigar intervenções terapêuticas, bem como novas ferramentas para esclarecer os mecanismos desconhecidos. A interação e relevância da Ang- (1-7) na expressão e inflamação das adipocinas foram discutidas em obesidade, diabetes tipo 2, doenças cardiovasculares, distúrbios renais e hepáticos, entre outros. Nesta perspectiva, concluímos que a inflamação pode ser modulada pelas adipocinas e Ang- (1–7) em diferentes órgãos e pode ser um alvo importante para o tratamento e a prevenção de respostas inflamatórias. No produto 2 avaliarmos os efeitos da administração oral com Ang-(1-7) sobre a microbiota intestinal em camundongos obesos. Camundongos Swiss machos foram divididos em quatro grupos: obesos e não-obesos / tratados e não tratados com ANG-(1-7). Foi observada uma diminuição significativa na glicemia de jejum, colesterol total, triglicérides e níveis de LDL e aumento de HDL em animais tratados com ANG-(1-7). A análise histológica mostrou redução da altura das vilosidades intestinais em camundongos tratados com ANG- (1-7). Adicionalmente, houve um aumento da abundância de *Bacteroidetes* e uma redução de *Firmicutes* (razão *Bacteroidetes/Firmicutes* aumentada) e *Enterobacter cloacae* foram observados no grupo HFD+ANG- (1-7). A expressão de RNAm intestinal do receptor TLR4 foi reduzida no grupo HFD+ANG- (1-7). Finalmente, a expressão intestinal do B0AT1 foi aumentada em animais obesos tratados com ANG-(1-7), mostrando um possível mecanismo associado à captação de triptofano. Os resultados do presente estudo sugerem, pela primeira vez, interação entre SRA e modulação da microbiota intestinal. Por fim, no produto 3 avaliamos o efeito da suplementação com *Bifidobacterium longum* nos parâmetros metabólicos e na expressão do sistema renina-angiotensina em camundongos obesos. Camundongos Swiis machos foram divididos em quatro grupos: obesos e não-obesos / tratados e não tratados com *B. longum*. Após quatro semanas de tratamento, os camundongos obesos que receberam o *B. longum* apresentaram diminuição significativa do peso corporal, da adiposidade, melhora na tolerância a glicose, redução dos níveis séricos de glicemia e colesterol total. Além disso, análises histológicas demonstraram redução do acúmulo de triglicérides hepático também no

grupo obeso tratado. Análises de mRNA mostraram aumento significativo na expressão da enzima-conversora de angiotensina 2 ECA2 e do RMAS nos camundongos obesos que receberam o *B. longum*. Nossos dados sugerem pela primeira vez a modulação do SRA pela cepa *B. longum*.

Palavras-chave: tecido adiposo; obesidade; enzima Conversora de Angiotensina 2; *Firmicutes*; *Bacteroidetes*; inflamação; intestino.

ABSTRACT

Adipose tissue has recently been recognized as an important endocrine and metabolic organ responsible for the synthesis of adipokines that control metabolism, body homeostasis, and inflammation. Recent evidence suggests the important role of Angiotensin- (1-7) in the treatment of obesity and metabolic disorders due to its anti-inflammatory action and for being the main counter-regulator of the AngII axis of the renin-angiotensin system (RAS). Also, it is important to consider other important metabolic regulators, such as the intestinal microbiota and its modulation using probiotics. In this context, the present thesis aims to evaluate the effects of Ang- (1-7) on adipose tissue, inflammation, and intestinal microbiota, as well as the impact of probiotic use on the expression of the RAS. For this, three products were produced.Regarding product 1, we conducted a review on the findings related to the ECA2/Ang-(1-7)/MasR axis focusing on its role in the regulation of adipokine secretion and processes related to acute and chronic inflammation. It is important to emphasize the endocrine role of adipose tissue in the synthesis of adipokines, modulating their secretion and regulating various processes associated with acute and chronic inflammation, as well as their relevance in the body's homeostasis. The importance of molecular pathways involved in the mechanisms of action of adipokines has created the need for new studies aimed at investigating therapeutic interventions, as well as new tools to clarify the unknown mechanisms. The interaction and relevance of Ang- (1-7) in the expression and inflammation of adipokines have been discussed in regards to obesity, type 2 diabetes, cardiovascular diseases, renal and hepatic disorders, among others. In this perspective, we conclude that inflammation can be modulated by adipokines and Ang- (1-7) in different organs and they may be important targets for the treatment and prevention of inflammatory responses. In relation to product 2, we evaluated the effects of oral administration with Ang- (1-7) on the intestinal microbiota of obese mice. Male Swiss mice were divided into four groups: obese and non-obese/treated and not treated with ANG- (1-7). A significant decrease in fasting glycemia, total cholesterol, triglycerides, and LDL levels, and an increase in HDL in animals treated with ANG- (1-7) were observed. Histological analysis showed a reduction of intestinal villus height in ANG-(1-7)treated mice. In addition, an increase in the abundance of *Bacteroidetes* and a reduction in*Firmicutes* (increased *Bacteroidetes*/*Firmicutes* ratio) and *Enterobacter cloacae* were observed in the HFD + ANG- (1-7) group. Intestinal mRNA expression of the TLR4 receptor was reduced in the HFD + ANG- (1-7) group. Finally, intestinal expression of B0AT1 was increased in obese animals treated with ANG- (1-7), showing a possible mechanism associated with tryptophan uptake. The results of the present study suggest, for the first time, an interaction between RAS and the modulation of the intestinal microbiota. Finally, concerning product 3, we evaluated the effect of *Bifidobacterium longum* supplementation on metabolic parameters and the expression of the renin-angiotensin system in obese mice. Male Swiis mice were divided into four groups: obese and non-obese/treated and not treated with *B. longum*. After four weeks of treatment, obese mice that received *B. longum* showed a significant decrease in body weight, adiposity, serum glycemia levels, and total cholesterol, as well as an improvement in glucose tolerance. Furthermore, histological analyses demonstrated a reduction in the accumulation of hepatic triglycerides in the obese-treatedgroup. Analyses of mRNA showed a significant increase in the

expression of angiotensin-converting enzyme 2 ECA2 and RMAS in the obese mice that received *B. longum*. Our data suggest for the first time the modulation of RAS by the *B. longum*strain.

Keywords: adipose tissue; obesity; angiotensin-converting enzyme 2; *Firmicutes*; *Bacteroidetes*; inflammation; intestine.

LISTA DE ABREVIATURAS E SIGLAS

AGT	Angiotensinogênio
ANG A	Angiotensina A
Ang I	Angiotensina I
Ang II	Angiotensina II
Ang-(1–7)	Angiotensina (1–7)
Ang-(1-9)	Angiotensina-(1-9)
AT1	Receptor da Angiotensina II Tipo 1
AT2	Receptor da Angiotensina II Tipo 2
B. longum	*Bifidobacterium longum*
CD-14	*Cluster* de diferenciação
CLG	Camundongo Livre de Germes
COX-2	Ciclo-oxigenase-2
DH	Dieta Hiperlipídica
ECA	Enzima Conversora da Angiotensina
ECA2	Enzima Conversora da Angiotensina
FAO	Organização das Nações Unidas para Alimentação e Agricultura
GRAS	Geralmente Reconhecido como Seguro
IL-1 β	Interleucina 1 Beta
IL-6	Interleucina 6
IL-6	Interleucina-6
LPS	Lipopolissacarídeos
IMC	Índice de Massa Corporal
Mas-KO	Camundongo *Knoukout* para o receptor Mas
MCP-1	Proteína De Quimioatração De Monócitos
METAHIT	Metagenoma do trato Gastrointestinal Humano
MrgD	Receptor D acoplado a proteína D relacionado com Mas
NOD-1	Proteína contendo domínio de oligomerização de ligação a nucleotídeo 1
NF-KB	Fator Nuclear Kappa B
OMS	Organização Mundial da Saúde
PAI-1	Inibidor do Ativador De Plasminogênio Tipo 1

PMH	Projeto Microbioma Humano
SRA	Sistema renina-angiotensina
TGF-β	Fator de crescimento transformador *Beta*
TGI	Trato Gastrointestinal
TNF-α	Fator de Necrose Tumoral Alfa
TRL4	Receptores do Tipo Tool 4

SUMÁRIO

1. INTRODUÇÃO

Obesidade

A Organização Mundial da Saúde (OMS) define a obesidade como uma condição de acúmulo anormal ou excessivo de gordura no tecido adiposo (1). A obesidade se tornou um problema de saúde mundial, sendo considerada uma pandemia. Estudos demonstram que, no ano de 2015 esta condição afetou cerca de 107,7 milhões de crianças e 603,7 milhões de adultos (2). Ainda segundo a OMS, em 2016, 39% dos adultos (com idade igual ou maior que 18 anos) apresentavam sobrepeso e 13% obesidade. Em números absolutos, mais de 1,5 bilhões de adultos apresenta sobrepeso e obesidade (3). Estima-se que em 2025, 18% dos homens e 21% das mulheres serão obesos (4). Ainda segundo a OMS, em 2030, a obesidade acometerá cerca de 20% da população adulta do mundo (5)**.**

A obesidade pode ser classificada de acordo com o índice de massa corporal (IMC), calculado pelo peso em quilogramas dividido pela altura ao quadrado em metros. Segundo as diretrizes da OMS, um indivíduo obeso é aquele que possui um IMC $\geq$ 30 kg/m^2. O excesso de peso é classificado como IMC 25,0 a < 30 kg/m^2, o peso normal varia de um IMC de 18,5 a < 25 kg/m^2 e um IMC abaixo de 18,5 kg/m^2 é considerado abaixo do peso (6, 7).

A etiologia da obesidade é complexa e multifatorial, causada principalmente pelo desequilíbrio entre o consumo e gasto energético (8), e em menor proporção por fatores genéticos e ambientais (9). O aumento da prevalência da obesidade contribui fortemente para múltiplas doenças cardio-metabólicas, como diabetes tipo 2, dislipidemia, doença arterial coronariana, acidente vascular encefálico, hipertensão arterial e vários tipos de câncer (10- 12).

A obesidade é considera uma doença crônica que compromete a qualidade dos indivíduos acometidos (13), além de aumentar de maneira significativa os gastos públicos (14). Além disso, a etiologia da obesidade ainda não foi totalmente esclarecida, o que torna necessária a realização de novos estudos que possam elucidar os mecanismos moleculares adjacentes ao desenvolvimento desta doença. Neste contexto, a angiotensina-(1-7) se destaca, uma vez que, vários estudos já descreveram seu papel na redução da obesidade e das doenças metabólicas por ela desencadeadas (15, 16).

Sistema Renina-angiotensina (SRA)

O Sistema Renina-Angiotensina (SRA) é uma cascata enzimática que se inicia com a produção de angiotensinogênio (AGT), que é clivado pela enzima renina em angiotensina I (Ang I). Em seguida, a enzima conversora de angiotensina (ECA) transforma a Ang I no octapeptídeo Ang II, que é o produto final ativo mais descrito do SRA, atuando através de dois isotipos de receptores Ang II, o Receptor de Angiotensina tipo I (AT1) e o Receptor de Angiotensina tipo II (AT2) (17). Altos níveis de Ang II estão associados a vários distúrbios metabólicos (18). Em contrapartida, a enzima conversora de angiotensina 2 (ECA2) reduz os níveis de Ang II, transformando-a em Ang-(1-7), que também pode ser produzido a partir da Ang I, através da angiotensina- (1-9) (Ang-1–9) pela ação das endopeptidases: prolil- endopeptidase e endopeptidase neutra. A Ang-(1-7) atua através do receptor Mas exercendo efeitos antagônicos a Ang II, e a ativação do eixo ECA2/ Ang-(1-7)/Mas melhora doenças metabólicas e crônicas (19). Além disso, a Ang-(1-7) pode sofrer descarboxilação formando a alamandina, que também pode ser formada a partir da hidrólise da angiotensina A (Ang A) por ação da ECA2 (20). A Alamandina age através do seu receptor (MrgD), e exerce efeitos similares a Ang-(1-7) (Figura 1) (21).

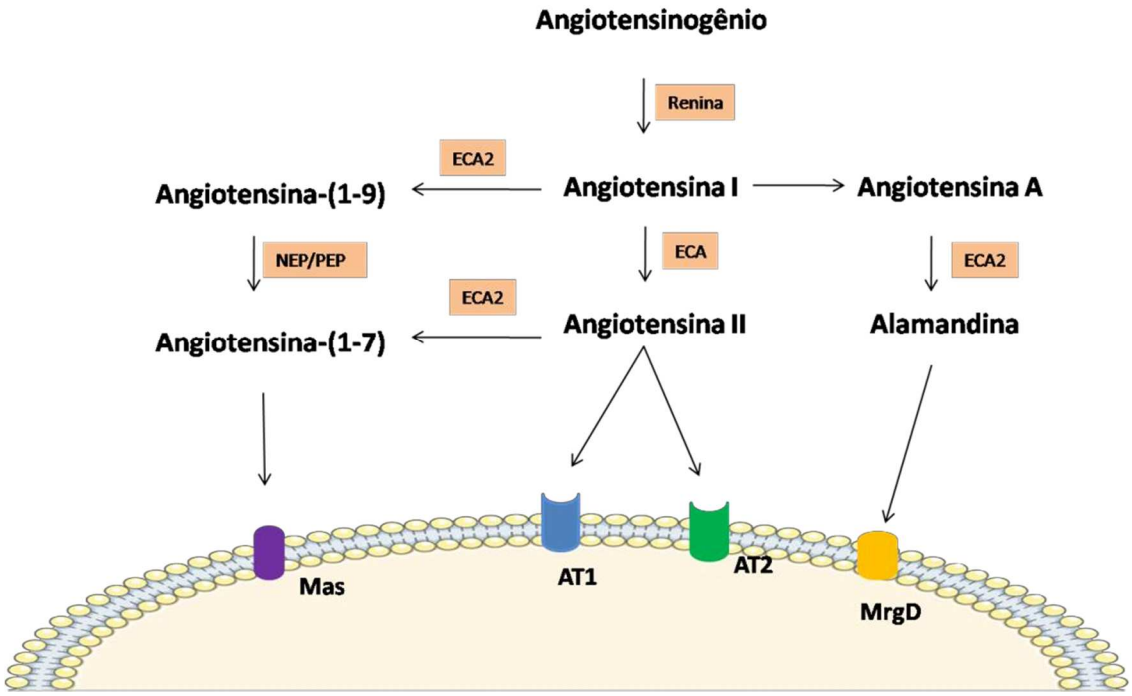

Figura 1: Visão geral do sistema renina-angiotensina (SRA). ECA, enzima conversora de angiotensina; ECA2, enzima conversora de angiotensina 2; AT1, receptor de angiotensina tipo 1; AT2, receptor de angiotensina tipo 2; Mas, Receptor Mas; MrgD, receptor D acoplado a proteína D relacionado com Mas, NEP, endopeptidase neutra; e PEP, prolil endopeptidase. Fonte: Adaptado de Qaradakhi, T., Apostolopoulos, V., Zulli, A., 2016 (21).

A maioria dos efeitos fisiológicos da Ang II são mediados pelos receptores AT1. A ligação da Ang II a esse receptor leva à inflamação, vasoconstrição, maior risco cardiovascular, aumento do estresse oxidativo e de fatores proliferativos, ativação do sistema nervoso, aumento da absorção de sódio, entre outros efeitos. Entretanto, os receptores AT2 embora encontrados em vários tecidos no período fetal, têm sua abundância diminuída após o nascimento. A ativação desses receptores está principalmente ligada a consequências antagônicas as dos receptores AT1, como vasodilatação, antiproliferação celular, melhora da função cardíaca e diminuição da absorção de sódio renal (22, 23).

A Ang-(1-7) é um heptapeptídeo com efeitos sistêmicos e locais significativos (24). A Ang- (1-7) é considerada o principal componente contrarregulatório do eixo Ang II/ECA/AT1 do SRA, atuando através do receptor Mas, que promove várias ações importantes, incluindo vasodilatação, redução do estresse oxidativo, efeitos antitrombóticos e melhora do metabolismo glicídico e lipídico (25).

As primeira evidências do papel metabólico da Ang-(1-7) e do seus receptor Mas vieram de ensaios com camundongos FVB/N com deficiência do receptor Mas (Mas-KO). Os Mas-KO apresentaram uma condição semelhante a síndrome metabólica, com hiperglicemia, intolerância à glicose, diminuição da sensibilidade à insulina e da captação de glicose via insulina em adipócitos, e diminuição do GLUT-4 no tecido adiposo branco. A deleção do receptor Mas também apresentaram dislipidemia, aumento da massa gorda, da expressão de insulina e leptina, e dos triglicerídeos musculares, além de, aumento da expressão do fator de crescimento transformador beta (TGF-β) e de AGT no tecido adiposo (26). Outros achados mostraram que a deleção do receptor Mas causa hipertensão arterial e disfunção endotelial (27). Além disso, a deficiência da ECA2 agrava a tolerância à glicose e a insensibilidade à insulina induzida por dieta (28).

Outros estudos revelaram que concentrações circulantes aumentadas de angiotensina (1-7), como resultado de expressão trangênica ou infusão crônica, melhora a hiperinsulinemia, a resistência à insulina e as respostas inflamatórias no tecido adiposo de ratos (29-31), e aumenta a sensibilidade à insulina e a tolerância à glicose em ratos com níveis normal de glicêmia (32). De maneira semelhante, o tratamento oral crônico com Ang-(1-7) diminui os níveis de glicemia, melhora a sensibilidade à insulina e previne a hiperinsulinemia em ratos diabéticos (31). Além dos efeitos metabólicos citados, é importante ressaltar que a ang- (1–7) tem um grande potencial

anti-inflamatório reduzindo as principais moléculas envolvidas nas doenças inflamatórias agudas e crônicas.

Tecido adiposo, adipocinas, Ang-(1-7) e inflamação

O tecido adiposo branco foi considerado por muitos anos um tecido cuja as principais funções eram reserva energética, na forma de triacilgliceróis, fornecimento de energia em um estado de jejum prolongado, proteção mecânica de órgãos e o isolamento térmico (33, 34). Entretanto, sabe-se hoje que o tecido adiposo é um importante órgão metabólico com função endócrino, responsável pela síntese e secreção de mais de 600 moléculas bioativas denominadas adipocinas (34).

As adipocinas estão envolvidas em vários processos metabólicos importantes, como regulação do apetite e saciedade, metabolismo energético, armazenamento de gordura, sensibilidade e secreção da insulina, regulação da pressão arterial, função endotelial, inflamação e homeostase (35, 36). Além disso, as adipocinas atuam em diferentes órgãos-alvo, como fígado, cérebro, músculo, coração, vasos, sistema imunológico e outros (35, 37-40).

Entre as principais adipocinas destaca-se a adiponectina, apelina, leptina, resistina, interleucina-1β (IL-1β), interleucina-6 (IL-6), fator de necrose tumoral-alfa (TNFα), proteína quimiotática de monócitos-1 (MCP-1), omentina, inibidor do ativador de plasminogênio tipo 1 (PAI-1), e os componentes do sistema renina-angiotensina, como o AGT, a AngII, ECA, ECA2, e Ang-(1-7) (34-36, 41).

No tecido adiposo, as adipocinas regulam as funções e o metabolismo dos adipócitos, a adipogênese e o recrutamento de células imunes (36, 37, 42). Assim como as adipocinas, o SRA também exerce efeitos importantes sob o tecido adiposo. Estudos experimentais em roedores e humanos ligaram a obesidade à ativação do eixo ECA/Ang II/AT1 (34, 43). O tecido adiposo expressa todos os componentes do SRA, e na obesidade torna-se o principal sítio produtor de AGT e Ang II (44). Por outro lado, o eixo Ang- (1-7)/MAS apresenta efeitos essenciais no armazenamento de gordura, captação de glicose e principalmente na regulação da produção de adipocinas (19, 45, 46), que por sua vez modulam a inflamação local e sistêmica (30, 31, 47).

Estudos em modelo experimental demonstraram que a administração oral de Ang- (1-7) foi capaz de prevenir a obesidade e a inflamação hepática através do bloqueio da via Resistina/Receptor tipo Toll 4/ Fator-Kappa Nuclear *Beta* (resistina / TLR4 / NF-kβ) em ratos (48), e reduzir a inflamação hepática induzida por dieta hipercalórica, através da redução da expressão das citocinas proinflamatórias Fator de Necrose Tumoral *Alfa* (TNF-α) e interleucina- 6 (IL-6) (48). Além disso, o aumento de Ang-(1-7) por meio de expressão transgênica em ratos exerce um efeito protetor contra a inflamação induzido pela obesidade, diminuindo a expressão da ciclo-oxigenase-2 (COX-2) e Interleucina-1- *Beta* (IL-1β) na gordura abdominal (31).

Microbiota intestinal e obesidade

Nos seres humanos, a microbiota intestinal é um complexo e dinâmico ecossistema que tem evoluído com seu hospedeiro (49). As comunidades microbianas em nosso intestino funcionam como um órgão com função metabólica, imunológica e endócrina (50). Atualmente, estima-se que o trato gastrointestinal (TGI) humano abriga aproximadamente 10^{14} micro-organismos, dez vezes mais do que o número de células no corpo humano, com 500-1000 espécies bacterianas distintas (51, 52). Estudos do Projeto Microbioma Humano (PMH) e do Metagenoma do trato Gastrointestinal Humano (*MetaHIT*) revelaram que a microbiota humana pode codificar pelo menos 10 milhões de genes [19], ou seja, 100 vezes mais genes do que o genoma humano (51).

A microbiota intestinal desempenha funções que o corpo humano sozinho não é capaz de realizar, resultando em uma relação simbiótica. Essa estreita relação é permite a manutenção de um TG intestinal normal, modulando processos como absorção de nutrientes a partir de alimentos ingeridos, motilidade e integridade da barreira intestinal, regulação do metabolismo do hospedeiro, principalmente o metabolismo glicídico e lipídico, e inflamação (53-55). Por exemplo, a microbiota fermenta componentes dos alimentos que não são digeridos pelo organismo, sintetiza proteínas e outros nutrientes essenciais, metaboliza toxinas alimentares e substâncias cancerígenas, assegura a maturação do sistema imunológico, afeta o crescimento e diferenciação de eritrócitos, regula a angiogênese intestinal, e protege contra patógenos entéricos (53).

Em geral, a microbiota intestinal é dominada por bactérias anaeróbicas pertencentes a três filos:

Bacteroidetes , *Firmicutes* e *Actinobacteria* (56-58). Geralmente, os *Firmicutes* e *Bacteroidetes* são mais abundantes, seguidos por *Proteobacteria* e *Actinobacteria, e em menos quantidade, Verrucomicrobia e Fusobacteria (57).*

A microbiota intestinal tem despertado o interesse da comunidade científica devido a sua relação direta com a saude humana. Desequilíbrios em sua composição (isto é, disbiose) têm sido associados a desordens imunológicas, suscetibilidade a infecções intestinais e, mais recentemente a várias doenças não-intestinais como doenças cardiovasculares, hepáticas, obesidade e diabetes (59-62). Uma vasta gama de estudos em modelos animais e em humanosrelataram a existência de uma relação direta entre a microbiota intestinal, a obesidade e seus distúrbios metabólicos associados (63-67). As primeiras evidências científicas sobre o papel da microbiota intestinal na regulação do peso corporal vieram de estudos conduzidos em camundongos livres de germes (CLG) (68).

Posteriormente, Backher et al. (69) descreveram um ganho total de 47% de massa gorda em camundongos selvagens quando comparados a CLG, mesmo quando estes apresentaram um maior consumo calórico. Além disso, quando o intestino de CLG foi colonizado por microbiota do ceco de camundongos selvagens, os camundongos CLG apresentaram um ganho de peso corporal de 60% (69). Também foi relatado que CLG alimentados com dieta ocidental rica em carboidratos ganharam menos peso que os camundongos selvagens, e não apresentaram alterações no metabolismo glicídico (70).

Estudos metagenômicos mostraram uma relação direta entre distúrbios metabólicos associados a obesidade e a disbiose intestinal (71, 72). Em camundongos e humanos, a diversidade microbiana e a proporção dos filos *Firmicutes* e *Bacteroidetes* estão associadas ao desenvolvimento da obesidade (72, 73). Esse achado foi corroborado por estudos realizados em camundongos ob/ob, modelo animal de diabetes tipo II, que apresentou uma menor razão *Bacteroidetes/Firmicutes* em relação ao tipo selvagem (74, 75). O aumento na abundância dos *Firmicutes* parece está associada alteração glicêmica com o aumento da captação de energia devido ao aumento do número de enzimas envolvidas na digestão do amido, sacarose e galactose (76). Ainda, CLG que receberam a microbiota intestinal de camundongos ob/ob tornaram-se obesos (77). Além disso, humanos obesos submetidos a restrição calórica apresentaram um aumento significativo na população de *Bacteroidetes*, demonstrando a relação direta desse filo com a perda de peso e com o fenótipo magro (71).

A dieta influência diretamente no desenvolvimento de disbiose intestinal, o consumo de uma dieta hiperlipídica (DH) aumenta a proporção de bactérias Gram-negativas no intestino (78), o que contribui para as doenças infecciosas e metabólicas (79). O aumento dessa classe de bactérias culmina em maior absorção de lipopolissacarídeos (LPS), principal componente da parede celular de micro-organismos Gram-negativos, que se acumula na circulação causando endotoxemia metabólica (80). A endotoxemia metabólica é caracterizada por um estado inflamatório crônico desencadeado pelo reconhecimento dos LPS microbianos e translocação bacteriana por interação com o receptor do tipo Toll 4(TLR4), Proteína contendo domínio de oligomerização de ligação a nucleotídeo 1(NOD-1), cluster de diferenciação 14 (CD-14), ativando a via do Fator Nuclear do tipo kappa beta(NF-κB) (80). Recentemente foi descrito que a DH altera a composição da microbiota intestinal, entretanto, para o desenvolvimento da obesidade, a presença de endotoxemia metabólica é obrigatória (81). Em camundongos, a endotoxemia causa aumento do peso corporal, hiperinsulinemia, e hiperglicemia (80).

Fei e Zhao (82) demonstraram que o transplante da bactéria *Enterobacter cloacae* B29 produtorada de endotoxina de indivíduo com obesidade mórbida para CLG foi associado à indução da obesidade, resistência insulínica com aumento concomitante dos níveis circulantes de endotoxina. Além disso, patógenos como *Bacteroides*, *Clostridium*, *Escherichia coli*, *Vibrio* e *Fusarium* podem produzir toxinas que induzem dano celular através da interrupção das interações entre as proteínas intracelulares aumentando a permeabilidade celular e, eventualmente, induzindo a morte celular (83). Como tal, o aumento dos níveis de bactérias produtoras de endotoxina prejudica o equilíbrio metabólico do hospedeiro (82).

A microbiota intestinal contribui para a obesidade induzida por dieta, controlando o processo de β-oxidação de ácidos graxos e armazenamento de triglicerídeos (69) e promovendo a captação de energia em uma dieta rica em carboidratos (70). Além disso, tem sido demonstrado que o consumo de dieta hiperlipídica e a composição da microbiota intestinal estão relacionados a mudanças na morfologia das vilosidades intestinais (84). Contudo, os dados referentes à microbiota intestinal e o SRA são escassos na literatura, e a interação entre a ang- (1-7) e a microbiota intestinal ainda não foi elucidada.

Ang-(1-7) e Microbiota intestinal

Estudos realizados em modelos animais já revelaram a expressão de marcadores do SRA no intestino (85-87). Componentes de RAS, como os receptores AT1, AT2 e ECA, foram encontrados na borda do epitélio do jejuno e íleo de ratos, onde o fluxo de fluidos e eletrólitos é regulado (88). O fato do jejuno de ratos expressar AGT, que é o precursor da Ang II e outras angiotensinas bioativas, indica que o enterócitos são capazes de sintetizar Ang II (88). Recentemente, evidências da associação do SRA e da microbiota intestinal via modulação do ECA2 foram descritas (89-91). Níveis aumentados de ECA2 foram detectados no trato gastrintestinal humano e, posteriormente, foi identificada a coletrina, um homólogo da ECA2, com atividade não catalítica (92).

A ECA2 é essencial para a expressão intestinal do principal transportador de aminoácidos neutros (B0AT1), um importante transportador de triptofano (Trp) (90). Níveis aumentados de Trp foram associados com o aumento da produção de peptídeos antimicrobianos, que são importantes moduladores da microbiota intestinal. A deficiência de transporte de Trp leva à secreção aberrante desses peptídeos antimicrobianos e consequente proliferação de cepas microbianas prejudiciais, conferindo suscetibilidade à inflamação intestinal (89).

Além disso, recentemente, Borges et al. (93) demonstraram que ratos pré-tratados com A779 (agonista do receptor Mas) e camundongos Mas-KO expostos a Ang- (1-7) apresentaram deficiência na absorção de Trp. Esses achados sugerem que a Ang- (1-7) estimula a absorção do triptofano através do receptor Mas, e esse efeito está diretamente associado ao aumento da expressão e atividade da ECA2. Apesar das evidências descritas, estudos sobre o efeito da Ang-(1-7) na microbiota intestinal são ausentes na literatura.

Probióticos

A OMS e a Organização das Nações Unidas para Alimentação e Agricultura (FAO) definem probioticos como micro-organismos vivos que quando administrados em quantidades adequadas, conferem benefícios a saude do hospedeiro (94). Os efeito benéficos mediados pelos probioticos se devem a sua capacidade de modular a microbiota intestinal, deslocando o

equilíbrio microbiano para o lado positivo (95), e melhorando a imunidade da mucosa (96- 98).

Entre os probióticos mais bem estudados estão às cepas bacterianas produtoras de ácido lático pertencentes aos gêneros *Bifidobacterium e Lactobacillus*, que têm um registro de segurança estabelecido e receberam o *status* GRAS (geralmente reconhecido como seguro) pela FAO (99), Atualmente, essas estirpes microbianas são amplamente utilizadas e estão incluídos em muitos alimentos funcionais e suplementos dietéticos (100-102).

Estudos revelaram que a administração de *Bifidobacterium* spp., inclusive do *B. longum*, reduz o peso corporal, melhora a tolerância à glicose e a resistência a insulina, protege da obesidade induzida por dieta mantendo a homeostase energética, reduzindo os níveis séricos de colesterol e triglicérides (103-106). Além disso, um ensaio clínico controlado duplo-cego randomizado demonstrou que os probióticos poderiam prevenir a produção de endotoxina e melhorar o metabolismo energético em indivíduos obesos (107).

2 OBJETIVOS

Objetivo geral

- Avaliar os Efeitos da Angiotensina-(1-7) Sobre o Tecido Adiposo, Inflamação e Microbiota Intestinal, e o Impacto do Uso de Probiotico na Expressão do Sistema Renina Angiotensina.

Objetivos específicos

- Realizar uma revisão da literatura científica a cerca dos achados relacionados ao eixo ECA2/ Ang-(1-7)/Mas com foco no seu papel na regulação da secreção de adipocinas e dos processos relacionados à inflamação aguda e crônica.
- Avaliar o efeito da angiotensina(1-7) sobre a microbiota intestinal de camundongos obesos.
- Avaliar o efeito da suplementação com *Bifidobacterium longum* no metabolismo e na expressão do sistema renina angiotensina hepático de camundongos obesos.

3 PRODUTOS

Produto 1: *Angiotensin-(1-7), Adipokines and Inflammation*, formatado segundo as normas para publicação do periódico Metabolism Clinical and Experimental. *Status:* publicado.

Produto 2: *Angiotensin-(1-7) Modulates Intestinal Microbiota Improving Metabolic Profile*, formatado segundo as normas para publicação do periódico Cellular Microbiology. *Status*: enviado para publicação.

Produto 3: *Bifidobacterium longum s*upplementation improves metabolic parameters and alters the expression of the renin-angiotensin system in obese mice, formatado segundo as normas para publicação do periódico Life Sciences. *Status*: enviado para publicação.

Produto 1

Angiotensin-(1-7), Adipokines and Inflammation

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Abstract

Nowadays the adipose tissue is recognized as one of the most critical endocrine organs releasing many adipokines that regulate metabolism, inflammation and body homeostasis. There are several described adipokines, including the renin-angiotensin system (RAS) components that are especially activated in some diseases with increased production of angiotensin II and several pro-inflammatory hormones. On the other hand, RAS also expresses angiotensin-(1–7), which is now recognized as the main peptide on counteracting Ang II effects. New studies have shown that increased activation of ACE2/Ang-(1–7)/MasR arm can revert and prevent local and systemic dysfunctions improving lipid profile and insulin resistance by modulating insulin actions, and reducing inflammation. In this context, the present review shows the interaction and relevance of Ang-(1–7) effects on regulating adipokines, and as one adipokine itself, modulating body homeostasis, with emphasis on its anti-inflammatory properties, especially in the context of metabolic disorders with focus on obesity and type 2 diabetes mellitus pandemic.

Keywords: Adipose tissue Angiotensin converting enzyme 2 (ACE2) Renin-angiotensin system Metabolism Mas receptor.

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1. Introduction

The angiotensin-(1–7) (Ang-(1–7)) is an heptapeptide with significant systemic and local effects, produced in the renin-angiotensin system (RAS) hormonal cascade, with pronounced effects counteracting the angiotensin II (Ang II) actions [1], especially considering

Ang II overproduction and high signaling present in several diseases, such as hypertension, diabetes, obesity, renal disorders and liver steatosis [2].

The RAS hormonal cascade initiates with the angiotensinogen (AGT) production, which is cleaved by the renin into angiotensin I (Ang I). Sequentially, the angiotensin converting enzyme (ACE) transforms the Ang I into the octapeptide Ang II, which is the most described active end product of the RAS, acting through two isotypes of Ang II receptors (AT1R and AT2R) [3]. High levels of Ang II produce AT1 hyperactivity and several metabolic disorders [4]. On the other hand, the angiotensin converting enzyme homolog 2 (ACE2) reduces Ang II levels by transforming it in Ang-(1–7), which also can be produced from Ang I passing through angiotensin-(1–9) (Ang-(1–9)) by the action of endopeptidases: prolyl-endopeptidase and neutral endopeptidase. The main described Ang-(1–7) effects are associated with Mas receptor activation, and the ACE2/Ang-(1–7)/Mas arm high activation has been effective in improving metabolic and chronic diseases [5]. Additionally, it is worth mentioning that Ang-(1–7) may be alternatively clived into alamandine that acts via its receptor MrgD, exerting similar actions to Ang-(1–7) [6].

The angiotensin II type I receptors modulate most of the Ang II physiological and pathophysiological effects. The Ang II binding to this receptor leads to vasoconstriction, inflammation, oxidative stress, proliferative factors augmentation, cardiovascular effects, nervous system activation, increased sodium absorption, among other effects. The Ang II type 2 receptors on the other hand, although found in several tissues in the fetal period, has its abundance decreased after birth. These receptors activation are mostly linked to beneficial consequences, including vasodilation, anti-proliferation (fibroblasts, endothelial cells and myocytes), cardiac function improvement and decreased sodium absorption in the proximal tubule [7,8].

The renin-angiotensin system activation is an important defense mechanism against hypovolemic hypotension, commonly observed during bleeding or salt privation. Aldosterone when bound to the mineralocorticoid receptor in epithelial cells of the renal collecting duct recruits sodium channels from the cytosol to the surface of the renal epithelial cells, thereby promoting increased sodium reabsorption, tubular potassium excretion and plasma volume expansion [9]. The aldosterone receptors are expressed in several tissues other than renal;

which when disturbed lead to vascular impairments. The aldosterone augments the Ang II actions, inducing vascular remodeling and inflammation, as well as the stimulation of mineralocorticoid receptors in the heart, kidneys, and brain. Moreover, the circulating aldosterone induces cardiac fibrosis and increased sympathetic activity [10].

The RAS upregulation in the central nervous system is characterized by an increased renin

activity and high aldosterone levels [11]. Moreover, it was also reported in the literature that in patients with visceral obesity, this hormone levels are normalized following weight loss [12]. In contrast, the renin-angiotensin aldosterone system blockage with ACE inhibitors and AT1R blockers is one of the most used approaches in the treatment of hypertension, congestive heart failure and coronary artery disease [13]. Studies suggest that the mineralocorticoids receptors in adipocytes promote the expression of inflammatory adipokines and facilitate the aldosterone pro-adipogenic effect. These receptors inhibition in experimental studies lead to decreased levels of proinflammatory factors in the adipose tissue and increased adiponectin expression in the heart and adipose tissue [14]. In summary, the increased amount of visceral adipose tissue induces the production of aldosterone and other hormones, which facilitates the appearance of chronic inflammation in the adipose tissue and consequent adipokines overexpression.

The Mas receptor (MasR) signaling activated by the Ang-(1–7) has several pathways, however, the most described via includes phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/ AKT) or forkhead box protein O1 (FOXO1) [15]. Another crucial signaling effect involves the direct activation of insulin receptor and insulin receptor substrate (IRS) [16,17]. Particularly in the adipose tissue, Ang-(1–7)/MasR axis presents essential effects modulating fat storage, glucose uptake and especially adipokines production regulation [5,18,19], which in turn alter local and systemic inflammation [20–22].

Several studies have shown that Ang-(1–7) exerts inhibitory effects on inflammation and cellular growth mechanisms [23,24]. Ang-(1–7) reduces key molecules signaling pathways thought to be relevant for acute and chronic inflammatory associated diseases. Excess or stressed adipose tissue may work as a systemic pump secreting pro-inflammatory adipokines and reducing the anti-inflammatory hormones [25]. In normal physiology the systemic AGT is mostly produced by the liver, however, in some diseases or hormonal disorders (such as obesity), the white adipose tissue assumes a pivotal role on AGT production, abnormally increasing the Ang II locally and also augmenting circulatory levels [26,27].

Here, we review findings related to the ACE2/Ang-(1–7)/Mas axis function with focus on the role on regulating adipokines secretion and modifying processes associated with acute and chronic inflammation. As most of the studies that involve the Ang-(1–7) signaling pathways were performed under an experimental context with animal models, we opted to discuss the human studies and their clinical importance at the end of each topic.

Adipokines, angiotensin-(1–7) and adipose tissue

The adipose tissue has long being recognized for its role in body energetic demands

supply in a prolonged fasting state, calories storage, body temperature control, and organs mechanical protection [28,29]. Recently, the adipose tissue was defined as an important endocrine organ, responsible for the synthesis and secretion of more than 600 bioactive molecules named adipokines [29]. In mammals there are two main types of adipose tissue, the white adipose tissue (WAT) and brown adipose tissue (BAT). These organs develop opposing roles, as while the WAT acts as by storing energy in the form of lipids, the BAT is responsible for the heat generation via energy consumption. Experimental studies with mice demonstrated that the BAT aids in the protection against the development of obesity and metabolic diseases via activation of thermogenesis [30].

The adipokines are involved in the satiety and appetite regulation, energetic metabolism, fat storage, insulin secretion and sensitivity, arterial pressure, endothelial function and homeostasis [31,32]. At the systemic level, the adipokines act in different target organs, such as liver, brain, muscle, heart, vessels, immunological system and others [31,33– 36].

The leptin and adiponectin levels might be used to distinguish the WAT and BAT, as the WAT expresses large amounts of these adipokines, while in BAT these molecules are little expressed, especially when the thermogenesis is active [37]. Similar to the WAT, the BAT also produces bioactive molecules called batokines [38,39]. These molecules may have different and/or opposing actions to the WAT adipokines, and act in different targets (e.g., central nervous system) in the control of energy expenditure. Experimental studies showed that several molecules synthetized by the BAT have already been described to exert autocrine, paracrine and endocrine actions, and the main examples are: Triiodothyronine, prostaglandins, angiotensinogen, interleukin-1α, insulinlike growth factor I, Interleukin-6, vascular endothelial growth factorA, fibroblast growth factor-2, nitric oxide, and fibroblast growth factor-21 [40–47].

Additionally, studies have reported a different type of adipocytes called beige. These adipocytes are brown-like cells (UCP+), but localized in the WAT [48, 49]. These cells are originated from the browning process, which is responsible for the dynamic conversion of white adipocytes into brown-like adipocytes due to the exposure to physiological, pharmacological or hormonal stimuli [50,51]. The white adipocytes browning is generally induced by the exposure to cold and physical exercise [50,52]. However, this process does not completely transform nor transdifferentiate white adipocytes into brown adipocytes; white adipocytes become only a phenotype resembling a brown adipocyte, which is also called a beige adipocyte [53,54]. The WAT browning exerts regulatory effects on the metabolism, such as increased energy expenditure, weight loss, insulin sensitivity and improved glucose tolerance, and although being performed on a rodent model, open perspectives as a potential target in the

prevention and treatment of metabolic diseases, such as diabetes and obesity[55]

Among the main adipokines it is possible to highlight the adiponectin (involved in insulin sensitivity regulation, antidiabetogenic, antiatherosclerotic and anti-inflammatory effects), apelin (insulin secretion inhibition), leptin (appetite and satiety control, energy intake, locomotor activity, energy expenditure, fertility, among others), resistin (related to obesity, insulin resistance and inflammation), proinflammatory adipokines (e.g., interleukin- 1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNFα), monocyte chemoattractant protein-1 (MCP-1), among others), and omentin (anti-inflammatory) [29,31,32].

In the adipose tissue, the adipokines modulate adipocyte functions and metabolism, adipogenesis and immune cells recruitment [32,33,56]. With local action we may cite bone morphogenic protein-4 (BMP-4) that regulates adipogenic cell precursors differentiation, bone morphogenic protein-7 (BMP-7) that stimulates the brown adipose tissue, regulates energy intake, and increases energy expenditure. Ghrelin inhibits BMP-4, BMP-7, and vascular endothelial growth factor (VEGF) that stimulate angiogenesis in the adipose tissue [29,31,32].

As well as the adipokines, the RAS was also described as a critical metabolic regulator. Experimental studies with rodents and humans, linked obesity with the ACE/Ang II/AT1 RAS axis activation [57,58]. The adipose tissue expresses all RAS components, and it is involved in the obesity effects and development due to its increased size, AGT and Ang II production [57]. On the other hand, animal studies evidenced that the RAS counterregulatory axis (ACE2/Ang-(1–7)/Mas) is capable of improving the lipid and glucose metabolism via decreased body adiposity, and directly at molecular signaling levels [27,59]. Mas receptor deficiency in mice was associated with a worse metabolic profile, with lower glucose tolerance, dyslipidemia, hypertension, increased leptin expression, decreased glucose uptake by adipocytes and increased adipose tissue size [27]. In the same way, transgenic rats with Ang-(1–7) overexpression presented improved metabolic efficiency, increased glucose tolerance and insulin sensitivity and higher glucose uptake via insulin [60]. It was also observed lipid parameters improvements with decreased triglycerides and cholesterol, as well as decreased abdominal fat mass in studies performed with rats [60,61].

It has been demonstrated that during adipogenesis, the apelin expression is increased in mice cell lines [62,63]. Some other in vitro studies showed that RAS blockage (Ang II/ACE) improves apelin expression and secretion in adipocytes from mice, which leads to reduced ROS and lipid accumulation in the adipocytes differentiation process [63]. AT1R blockage and consequent AT2R increase reduces TNFα expression and increases apelin expression in the

mice white adipose tissue [63, 64], suggesting an essential AT2R role in the browning regulation. In humans, apelin seems to induce white adipocyte browning, inhibiting AT1 via its interaction with the apelin receptor (APJ receptor) in vitro [65].

It is important to point that the adipokines may have an indirect effect on the RAS via modulation of insulin levels and lipid profile, thus evidencing an indirect relationship among these molecules. Nickenig and colleagues reported that insulin may upregulate AT1 via posttranscriptional mechanisms, thus linking hyperinsulinemia, hypertension and atherosclerosis [66]. The authors argue that the insulin influence on the RAS, more specifically on the AT1 receptor, takes place via tyrosine phosphorylation and MAP kinase– dependent intracellular pathways. Moreover, the lipid profile has also been associated with the RAS modulation (AT1 upregulation and increased AngII synthesis) [67]. The lipids influence on the AT1 expression and Ang II production, in contrast with the aforementioned insulin effects, seem to be via mRNA stabilization, and increased chymase system activity. As we know and will discuss throughout this review, the adipokines have an important influence on the glycemic and lipid profiles, and this influence may thus explain their relationship with the RAS modulation (Fig. 1).

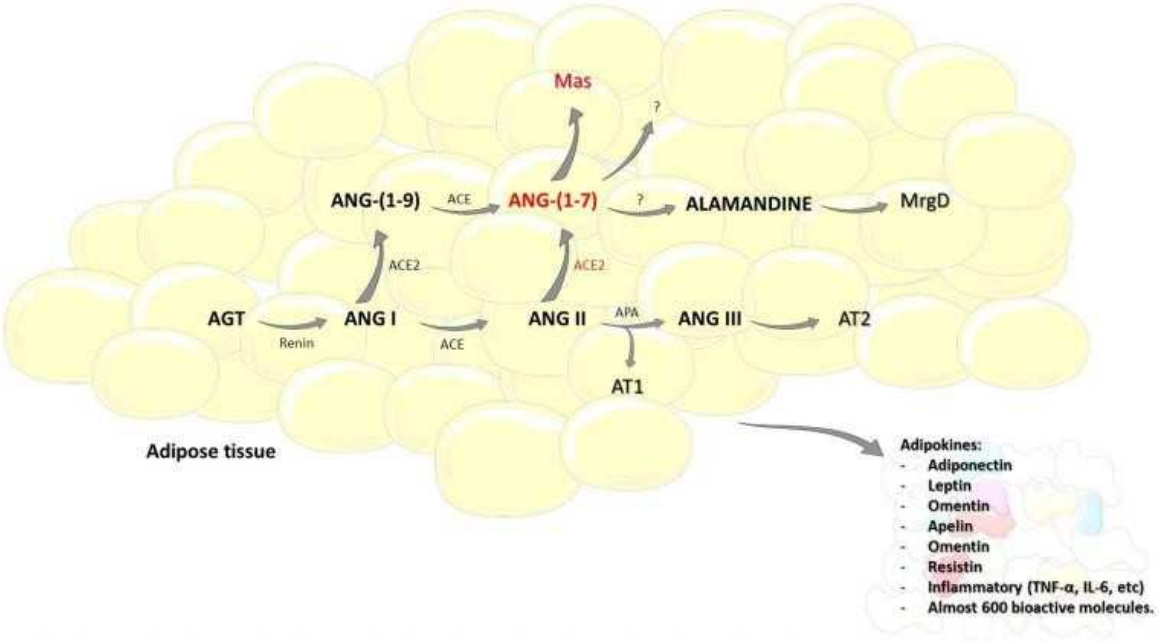

Fig. 1. Renin-angiotensin system and adipose tissue. AGT: angiotensinogen; ANG I: angiotensin I; ANG-(1–9): angiotensin 1–9; ANG-(1–7): angiotensin (1–7); ANG II: angiotensin II; ANG III: angiotensin III; Mas: Mas receptor; AT1: AT1 receptor; AT2: AT2 receptor; ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2; APA: aminopeptidase A; MrgD: Mas-related G-protein coupled receptor D; TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin 6.

The interaction between proinflammatory adipokines and RAS is also described in the literature. A study performed with adipose tissue samples from malnourished and obese mice and also confirmed in humans showed that the inflammatory state is shared between these two nutritional states and that the renin-angiotensin system modulates both profiles [68]. Interestingly, it has been reported in the literature that a possible strong influencer of the adipocytes expression/secretion profile is the muscle. The muscle is recognized as another important endocrine organ in our organism and expresses several important molecules, collectively called myokines, which counterbalance the adipokines released by the adipose tissue [69–71]. In this sense, the muscle influence might be also modulating the RAS expression in the adipose tissue.

1.2 Adipokines, Ang-(1–7) and inflammation

In the last years, Ang-(1–7) has been studied for its antiinflammatory properties in several disorders. Considering metabolic diseases, such as obesity and type 2 diabetes, it was evidenced that increased circulating Ang-(1–7) exerts a protective effect against the inflammatory process induced by obesity by decreasing ciclo-oxigenase2 (COX-2) and IL-1β expression in transgenic rats' abdominal fat [20]. Additionally, it was shown in another study that oral administration of Ang-(1–7) is capable of preventing obesity and liver inflammation via resistin/toll like receptor 4/Nuclear Factor-kappa β (Resistin/TLR4/NFkβ) pathway blockage in rats [72], and reduces diet-induced hepatic inflammation by decreasing TNF-α and IL-6 expression [72]. Interestingly, another possible mechanism by which Ang-(1–7) exerts its beneficial effects in inflammatory conditions associated to metabolic disorders in mice, is via Sirtuin 1 (SIRT1), a NAD-dependent deacetylase already described to be involved on improving several metabolic diseases [73].

Interestingly, Ang-(1–7) also ameliorates epicardial adipose tissue (EAT) inflammation induced by obesity. ACE2 knockout obese mice showed increased interferon gamma (IFN-y) expression in EAT, along with predominant CD11c+/F4/80+ Mf macrophage profile, while treatment with Ang-(1–7) reverted this inflammatory profile, mainly by decreasing TNF-α and IL-6 expression [74]. These findings are noticeable considering that epicardial fat is a current study target aiming to understand the association between obesity, metabolic diseases, and atherosclerosis.

In cardiovascular diseases, especially considering vascular inflammation, Ang-(1–7) diminished macrophage infiltration, MCP-1, IL-6, TNF-α, NFKβ, vascular cell adhesion protein

1 (VCAM-1), reactive oxygen species (ROS) levels, apoptosis and increased nitric oxide release, thus reducing atherosclerosis risks [75]. Furthermore, Ang-(1–7) was capable of resolving endothelial cell inflammation in vivo, thus preventing early atherosclerosis via decreased MCP-1, VCAM-1, IL-6 and atherosclerotic plaque inhibition in human cell lines and knockout APOE mice, both in vitro [76].

The Ang-(1–7) anti-inflammatory effects were also confirmed in rat pancreatic acinar cell lines, where this peptide attenuated caerulein (an acute pancreatitis inducer) induced inflammation by downregulating TLR4/NFKβ pathway [77]. In macrophage cell culture, Ang-(1–7) was capable of preventing proto-oncogene tyrosine-protein kinase Src activation, which are proteins necessary to the inflammatory response induced by lipopolysaccharide (LPS) [78].

In humans studies, endothelial cells culture, Ang II induced inflammation was prevented by Ang-(1–7) via reduced lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) expression, a novel scavenger receptor for oxidized LDL (oxLDL), a potent inflammatory activator [79]. In uropathies, which are also characterized by an inflammatory profile, Ang-(1–7) shown to be a protective compensatory molecule reducing inflammatory processes by decreasing macrophage infiltration and apoptosis [80]. The

renoprotective role of Ang-(1–7) is mostly discussed and reported by several studies showing neutrophil influx, and downregulation of chemokine (C-X-C motif) ligand 1 (CXCL), IL-6, TNF-α, endothelin 1 (ET-1), IL-1β and MCP-1.

These studies illustrate and confirm the Ang-(1–7) beneficial antiinflammatory effects in several inflammatory conditions, which are associated with adipokines disruption, such as obesity, type 2 diabetes, cardiovascular diseases, renal and hepatic disorders, among others (Fig. 2).

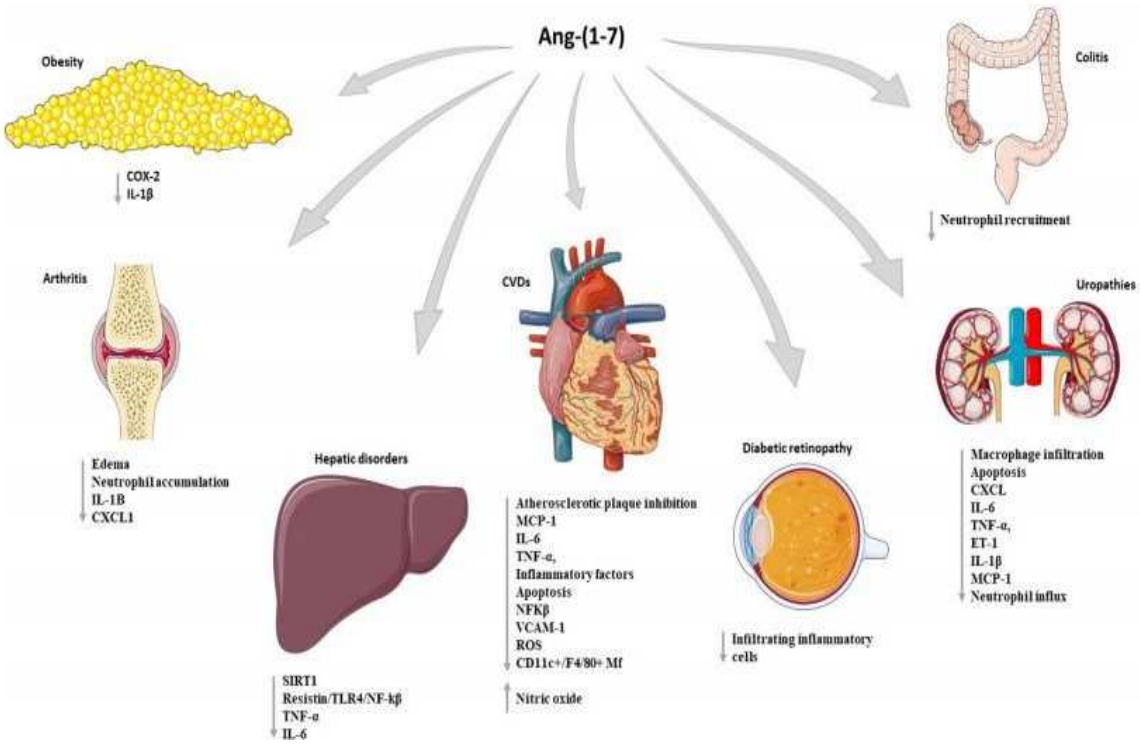

Fig. 2. Ang-(1–7) effects on inflammation. CVDs: cardiovascular diseases; ROS, reactive oxygen species; TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; IL-1β: interleukin- 1β; CXCL1: chemokine (C-X-C motif) ligand 1; SIRT1: Sirtuin 1; TLR4: Toll-like receptor 4; NF-κβ: factor nuclear kappa β; MCP-1: monocyte chemoattractant protein-1; VCAM-1: vascular cell adhesion molecule 1; ET-1: endothelin 1.

Angiotensin (1–7) and adipokines in different organs

The adipokines produced and released by the adipose tissue mediate the communication network between this tissue and other organs [35]. Modifications in the adipose tissue homeostasis alter the adipokines secretion to a pattern that induces metabolic dysfunctions [33, 34]. Angiotensin-(1–7) presents biological and pharmacological properties that are beneficial in the metabolic dysfunctions resolution [81], being relevant to explore the adipokines effects in different metabolic organs, highlighting Ang-(1–7) as a therapeutic tool.

Adiponectin

The adipokines released by the adipose tissue affect several biological processes involved in hepatic function, including angiogenesis, vasodilation, inflammation, and deposition of extracellular matrix proteins, thus modulating hepatic fibrogenesis [82]. In the liver, decreased adiponectin levels may predispose to steatosis and advanced hepatic lesion [82,83]. This adipokine acts by reducing the hepatic stellate cells activation, proliferation, and survivor [76]. Recent studies have evaluated adiponectin analog actions since it is not viable to

increase circulating adiponectin levels in humans. These agents attenuate hepatic fibrosis in animal models [84], and have potential to become new anti-fibrotic therapeutic drugs [85].

Taking into consideration the Ang-(1–7) employment for therapeutic purposes, a study from Tang et al. evidenced the Ang-(1–7) therapeutic role in preventing non-alcoholic fatty liver disease (NAFLD) in mice via an adiponectin-independent mechanism, which may be partially attributed to the mitogen activated protein kinases (MAPK) hepatic pathway. The study suggests that Ang-(1–7) treatment may stimulate AMPKα2 expression and 5′ AMP-activated protein kinase (AMPK) phosphorylation, which eventually triggered signaling cascades, being still necessary for further clarification [86]. The RAS positive regulation in hepatic diseases via AT1R/AT2R is directly associated with a pro-fibrotic process, findings evidenced in rat models [87–90]. In a cirrhotic mouse model, increased ACE2 expression inhibited hepatic fibrosis via increased Ang-(1–7), while ACE2 blocking exacerbated the fibrotic process, shedding light to the ACE2 therapeutic potential in the treatment of chronic hepatic lesion [91]. The ACE2/Ang-(1–7)/MasR exerts antifibrotic effects as observed in a hepatic fibrosis animal model where AVE 0991 (Ang-(1–7) agonist) reduced ACE, 1A1 collagen and α-actin expression and hydroxyproline levels, an important collagen component, which all together ameliorates fibrosis [92].

In the mice pancreas, adiponectin stimulates insulin secretion in vivo, and hypoadiponectinemia causes β cells dysfunction [93]. Consistent with these findings, mice without adiponectin exhibited dietinduced hepatic insulin resistance. Adiponectin exerts beneficial effects via activation of its two receptors: adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) [93,94].

The liver is also an important target in human studies, where hepatic stellate cells isolated from humans cirrhotic liver were shown to overexpress renin, ACE, and Ang II [95]. Increased hepatic stellate cells proliferation and extracellular matrix production via signaling pathways mediated by MAPK, phosphoinositol/Ca2+, and ROS generation are promoted by AT1R/ACE/Ang II axis activation [96–98].

Additionally, the cardiovascular system is another important investigation target in human studies. It is discussed that adiponectin might be involved in the chronic cardiac insufficiency (CCI) pathogenesis. A study performed by Kreth et al., characterized the adiponectin and its receptors expression in CCI to evaluate the impact of microRNAs in the cardiac adiponectin system. In CCI, AdipoR1 cardiac expression was four-fold increased, while the AdipoR2 increase was two times lower, showing the adiponectin association with its

receptors in the heart and that the microRNA-150 targeting might be a strategy to restore the cardioprotective adiponectin effects [99].

Leptin

Establishing the association between leptin and Ang-(1–7), a study performed by Uchiyama et al., investigated the perirenal visceral adipose tissue and visceral perirenal adipocyte isolated from Wistar male rats. The authors observed that Ang-(1–7) increased leptin secretion and expression, while alamandine decreased the leptin secretion and expression in the adipose tissue and serum [100]. Few studies are presenting the relation between leptin and Ang-(1–7), and among them, Schuchardet et al. demonstrated the Ang-(1–7) potential in regulating rats food intake and body weight. Ang-(1–7) also contributes to weight loss following AT1R blockage, since transgenic rats remained leptin responsible even with a chocolate and cookie diet. It is also suggested that Ang-(1–7) agonists might be pharmacological candidates in the obesity treatment and a new tool to treat metabolic disorders [101].

Mas double deficiency in ApoE-KO (DKO) mice leads to a lipodystrophy similar state, with increased hepatic lipid content and increased alanine aminotransferase levels. It was also observed increased cholesterol, triglycerides, and fasting glucose levels and decreased HDL and leptin levels [102].

Hamrick showed that leptin treatment in vivo increases myogenic genes expression in mice primary myoblasts. Leptin may centrally reduce medullar adipogenesis via its receptors in the hypothalamus, as well as directly through their receptors on bone marrow stem cells. Thus, aging seems to significantly alter the crosstalk mediated by leptin among organs and tissues [103].

The renal dysfunction in the chronic renal disease context is associated with high leptin levels, that also in NAFLD causes activation of renal mesangial cells and tubular inflammation via a NOX2 dependent pathway that positively regulates the pro-inflammatory miR21 molecule, findings from a mice model [104,105]. The Ang-(1–7)/MasR axis in the mice kidneys have vasodilatory, antiproliferative, antidiuretic and antinatriuretic activities [106,107].

In the heart, Carmo et al. recently determined that neuronal-SOCS3 deficiency in mice is a potential negative regulator of the leptin signaling pathway that amplifies the chronic leptin effects on food intake, energy expenditure, glucose and arterial pressure, protecting against the adverse cardiometabolic effects in obesity [108]. Oral administration of alamandine generated antihypertensive and antifibrotic effects in rats [109]. Furthermore, its subcutaneous application

also exerted antihypertensive effects, with improved cardiac hypertrophy and left ventricle function. Alamandine may exert its beneficial effects via the protein kinase A (PKA) signaling pathway modulation [110]. Interestingly, Wang et al. showed in rats that leptin is also produced by the skeletal muscle, and the leptin receptors are abundant in skeletal muscle and mesenchymal stem cells derived from bone (stroma), thus confirming this adipokine effects on the muscle cells [111].

The leptin influence on the human cardiovascular system and adipose metabolism has also been investigated and the main findings evidence a positive leptin effect produced by the pericardial adipose tissue and blood vessels [112]. An interesting study performed by Oral et al. showed that leptin administration in nine women with lipodystrophy and leptin serum levels below 4 ng/mL, significantly improved lipodystrophy besides insulin resistance [113].

Resistin

It was shown that Ang-(1–7) oral treatment improved the obese rats' metabolic profile (improved body weight, abdominal fat mass, insulin plasma levels, and circulating lipid levels) via decreased resistin, TLR4, ACE and increased ACE2 expression in the liver [72]. Santos and colleagues showed that Ang-(1–7) decreases MAPK phosphorylation, reducing IL-6 and TNF-α expression via resistin/TLR4/NF-kβ-pathway down-regulation in rats [72].

Resistin levels are increased in obesity. In a study performed in highfat-fed mice, the resistin levels normalization by antisense oligonucleotides reverses hepatic insulin resistance [114,115]. These proteins supposedly contribute to the visceral adiposity deleterious metabolic effects. Increased insulin secretion was observed in mice MasKO pancreatic islets treated with A776 (Mas antagonist) and stimulated with Ang- (1–7), although the insulin expression was not altered, indicating a smaller Ang-(1–7) not exclusively by Mas-dependent pathways [116].

Translational studies that investigated the resistin role in humans reported interesting findings. First, resistin expression was shown to be increased in fibrotic liver. In hepatic stellate cells, resistin seems to increase MCP-1 and interleukin-8 (IL-8) expression [117], besides contributing to the lipids uncontrolled uptake. The hepatic low-density lipoprotein receptor (LDL) negative regulation and de novo lipogenesis stimulation in hepatic cells are triggered by resistin and may augment dyslipidemia and hepatic steatosis [118].

In the pancreatic islets on the other hand, the literature regarding the resistin expression is scarce [119]. However, Alexandra et al. showed the resistin expression in human pancreatic islets via qRT-PCR and immunohistochemistry shedding new light on the resistin potential role in the pancreas [119].

Second, regarding the relationship between inflammation and the adipokines, Hollebeke

et al. studied the abdominal muscular density and area with inflammatory mediators associated with adiposity and resistin. The results showed that the muscular area was not associated with any inflammatory mediators studied, including resistin. It was yet not verified that higher densities of several muscle groups in the abdomen are significantly associated to lower IL-6 and resistin levels, independent from the muscle area in these groups [120].

Lastly, in the cardiovascular system, resistin levels are associated with coronary artery disease and heart failure severity. Turgay et al. evaluated if there is a relation between resistin levels and final diastolic pressure in the left ventricle and observed no correlation between resistin levels and left ventricular-end diastolic pressure, coronary artery disease severity, echocardiographic diastolic dysfunction parameters and constraint induced movement therapy. More studies are necessary to evaluate the resistin efficacy for clinical use [121].

Omentin

The association between omentin and Ang-(1–7) is still unknown in the literature, becoming a potential target for new studies. Omentin is an anti-inflammatory protein and improves insulin sensitivity [122]. If the omentin directly regulates the hepatic cells, biological function is still not clear in the literature. Unbalanced nitric oxide levels contribute to splanchnic vasodilation and hepatic vasoconstriction and consequently portal hypertension [123]. Although several mechanisms influence the vasodilation process, the RAS is one of the most studied over the years, and it is discussed that the ACE/Ang II/AT1 axis has been intimately associated with cardiovascular dysfunctions and metabolic disturbances [124].

Sit et al. evaluated the inflammatory response effects on serum omentin levels in acute and chronic pancreatitis and found that omentin levels elevation in rats at the early stage of pancreatitis was due to omentin's anti-inflammatory effects [125]. Castro et al. showed that both adipose tissue metabolism and adipokine secretion might be affected in diabetic rats, but omentin was no different between the groups [126].

The omentin role in human studies are more focused on metabolic diseases, where it was evidenced decreased omentin levels in type 1 and 2 diabetes mellitus, correlating with insulin resistance [127,128], which might be explained by the strong relationship between arterial pressure and insulin plasma concentration in hypertensive individuals with obesity, acting not only in the sympathetic nervous system, but also in renal function and arterial walls, leading to increased arterial pressure [129].

Moreover, Zorlu et al. compared serum omentin and obestatin levels in type 2 diabetic patients with normoalbuminuria and macroalbuminuria and observed that higher obestatin

serum levels were associated with macroalbuminuria, whereas serum omentin levels were similar between groups suggesting that obestatin may play a role in the underlying pathogenic mechanisms leading to diabetic nephropathy [130].

In the cardiovascular context, Fernándes-Trasancos et al. evaluated the omentin effects on the epicardial adipose tissue and vascular cells. This study showed that omentin treatment increased adiponectin levels induced by adipogenesis and reduced TNF-α levels in mature adipocytes. Omentin improved insulin activity in EAT and subcutaneous adipose tissue explants from patients with cardiovascular disease and decreased smooth muscle cells migration [131]. It is observed in the literature that omentin positively associates with adiponectin [129], which may justify the inflammation attenuation in the epicardial adipose tissue via ACE/Ang-(1–7)/Mas that reduces obesity-induced cardiac dysfunction by increasing adiponectin levels.

Apelin

Recently, Sabry et al. assessed the apelin treatment effects on diabetes mellitus type 2 induced by obesity and the possible interaction between the apelin/APJ and the renin-angiotensin system. The study showed that apelin-13 administration in rats resulted in improved insulin resistance, dyslipidemia, inflammation, oxidative stress, reduced AT1 gene expression and increased ACE2 expression in the adipose tissue, evidencing that the apelin beneficial effects are NO/ACE2/Ang-(1–7) dependent [132].

A review showed that apelin is the second catalytic substrate for ACE2 and acts as an inotropic and cardiovascular protective peptide. Chen et al. showed that microRNAs, linked to ACE2/apelin modulation, exhibit beneficial effects in the cardiovascular system and hypertension. The crosstalk between ACE2, the apelin system and microRNAs provides an important hypertension mechanist view [133]. Another review of the literature evidenced that the Apelin/APJ system is mainly expressed in vascular smooth muscle cells (VSMC). The study by Luo et al. was the first to demonstrate that the apelin/APJ system increases the VSMC proliferation by the extracellular signal–regulated kinase 1 (ERK1)/2-cyclin D1 signal pathway, thus being a promising target for the management of the vascular disease management [134]. The apelin effects on the cerebral arteries are unknown. Mughal, Sun and, O'Rourke, have demonstrated that apelin reduces the cerebral arteries nitric oxide-induced relaxation by inhibiting the calcium-activated high conductance K channels activation [135].

Recently, it was showed that the apelin/APJ system develops a critical role in kidney disease [136]. Guo et al. verified in mice that this system induces podocyte dysfunction in diabetic nephropathy via endoplasmic reticulum stress induced by decreased proteasome

activity in podocytes [137]. In this sense, the apelin-APJ system, in animal models, develops diverse roles in renal disease and might be a potential target in the renal disease treatment [138].

The inflammation develops an essential role in pancreatitis, and thus, Hans et al. showed that apelin inhibited the positive regulation of TNF-α, MIP-1α/β and IL-1β in mice with chronic pancreatitis, thus evidencing that apelin is involved in the inflammatory mediator's modulation in pancreatitis in this animal model [139].

The human studies involving the investigation of the apelin role on the other hand, are mainly focused on metabolism. It was reported that the physical exercise beneficial metabolic effects might be mediated by myokines. In this sense, Besse-Patim et al. studied the physical training effects and apelin expression in the human skeletal muscle. It was observed that physical training increases apelin expression in an obese individual via exercise-induced signaling pathways, thus being considered a new myokine with autocrine and paracrine actions. The physical activity beneficial effects on reducing arterial pressure in hypertensive individuals are linked, at least in part, to an increased apelin/APJ system expression. [140].

Interestingly, in vitro experiments with human cells evidenced that the angiotensin-converting enzyme 2 (ACE2) is a counterproductive regulator of the renin-angiotensin system (RAS), catalyzing the conversion of Ang II to Ang-(1–7). The apelin is a second catalytic substrate for ACE2 and functions as an inotropic peptide and cardioprotective [141].

Although an antagonistic relationship has been proposed between RAS and apelin, this functional interaction remains uncertain. In this perspective, we may conclude that adipokines, produced by the adipose tissue, have paracrine and autocrine actions that thus exert an effect in several organs and consequently different physiological and pathophysiological conditions (Fig. 3). These observations and evidence highlight the adipokines importance as molecular targets to be modulated in metabolic diseases treatment and prevention.

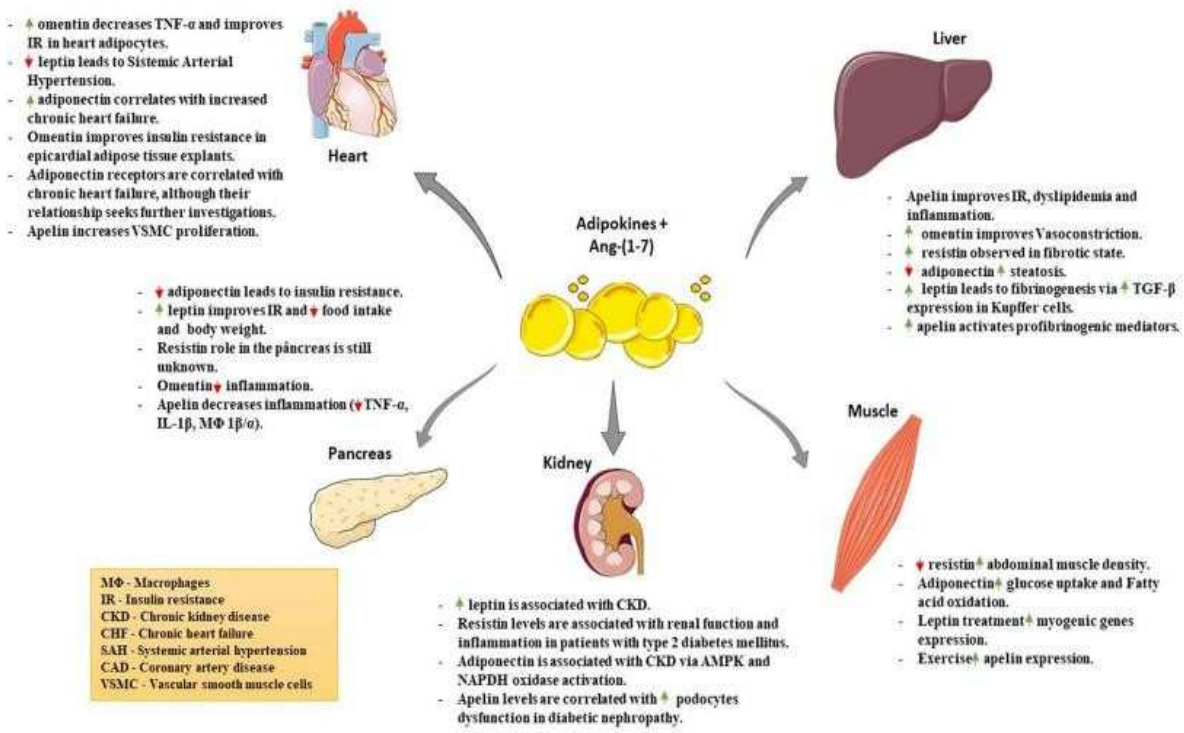

Fig. 3. Ang- (1–7) effects on adipokines: Ang-(1–7) has a protective effect despite the relationship between omentin and Ang-(1–7) being unknown. In the liver, the decrease of Adiponectin and increase of the other adipokines contribute to deleterious effects. In the pancreas there is an opposite effect to the liver and little is known about the effects of resistin. In the muscle, the decrease in resistin leads to an increase in abdominal muscle density and the increase in other adipokines has beneficial effects. Little is known about the impact of omentin on muscle. In the kidneys, the rise in adipokines contributes to the adverse effects. In the heart and blood vessels, the apelin/APJ system increases the proliferation of vascular smooth muscle cells while the other adipokines do not have favorable effects.

2. Adipokines and clinical implications

According to the Global report on diabetes from the World Health Organization, the diabetes prevalence almost quadrupled since 1980, reaching 422 million adults, which is in great part, due to the increased overweight/obesity (considered the main diabetes type 2 risk factor) prevalence. Type 2 diabetes mellitus may lead to macro and microvascular complications, and in 2012, was the direct death cause of 1.5 million people [142]. This growing prevalence and mortality generate social and economic implications in the health systems globally. It is necessary to find therapeutic targets aimed to set not only type 2 diabetes mellitus treatments but also risk factors attenuation [143]. In this context, deepening the knowledge of the adipokines endocrine effects in different target organs, their signaling pathways and links with RAS may contribute to developing new drugs, aiming at the diseases associated with broad-spectrum obesity therapy.

In obesity and type 2 diabetes mellitus, insulin resistance affects the perivascular adipose tissue endocrine function, altering vasoconstrictor and vasodilator molecules secretion and increasing the oxygen reactive species production. A study demonstrated that plasma adipokines or their receptors expression might improve insulin sensitivity and reduce cardiometabolic diseases morbimortality. In this perspective, the adipokines may constitute potential therapeutic targets for obesity and type 2 diabetes mellitus [144,145]. A study investigated the association between adipokines, anthropometric measures and biochemical parameters in type 2 diabetes mellitus, demonstrating that central obesity is correlated with adipokines synthesis unbalance, reinforcing their importance in type 2 diabetes mellitus [146]. Besides that, adipokines may be considered clinical biomarkers for the diagnosis and early interventions in pre-diabetic and T2DM patients [147].

Although several studies point to physiological and pathophysiological Ang-(1–7) properties in type 2 diabetes mellitus progression, its protective effects against hyperglycemia damage are still not completely elucidated. A clinical study also showed that intra-arterial infusion of Ang-(1–7) in obese patients exerted favorable effects by stimulating insulin-triggered vasodilation and inhibiting endothelin-1 vasoconstriction, which are comorbidities associated with obesity and insulin/glucose impaired metabolism [148].

Interestingly, a phase II, open-label pilot study reported that ACE2 infusion in patients with pulmonary arterial hypertension improved pulmonary hemodynamics and reduced oxidative stress and inflammation [149]. This study exemplifies the clinical application of RAS modulation in ameliorating disorders, such as hypertension. Recombinant ACE2 infusion in healthy individuals was also evaluated. The treatment was well tolerated and lead to significant changes in the RAS peptides concentrations [150].

Studies performed with animal models demonstrated that adiponectin exerts a fundamental role in obesity-associated diseases pathophysiology. Acting through its receptors (AdipoR1 and AdipoR2), it exerts direct effects on the liver, skeletal muscles, and vasculature (metabolic tissues), improving insulin sensitivity, lipid profile and producing anti-atherogenic and anti-inflammatory effects [151–154].

Leptin regulates energy homeostasis by inhibiting hunger (anorexigenic) and by increasing energy expenditure in over food conditions and increased fatty acid uptake. Leptin receptors are distributed in peripheral tissues, but the central nervous system is considered the main ingestion regulation site, which occurs via neurotensin (neurotransmitter). Recent findings demonstrated a positive association between circulating leptin and neurotensin, broadening the leptin-neurotensin peripheral and central mechanisms understanding, with new therapeutic

approaches perspectives [155].

Resistin was primarily known as a hormone secreted by adipocytes, but it is mainly expressed and secreted by macrophages. Increased resistin levels are associated with insulin resistance, endothelial dysfunction, and smooth muscle cells proliferation, promoting type 2 diabetes mellitus and mediating atherosclerosis pathogenesis [156].

Hepatic biopsies have shown that resistin plays an important role in the hepatic insulin resistance pathogenesis, aggravating NAFLD, being the most significant expression in patients with T2DM and dyslipidemia [157].

Apelin exerts significant effects on glycaemic and lipid metabolism, and it is mainly expressed and released by adipocytes. The apelin injection stimulated glucose uptake by the adipose tissue in mice and human [158,159]. Additionally, exogenous apelin administration in patients with central obesity resulted in insulin-stimulated vasodilation, thus showing the apelin effects on hemodynamic alterations associated with insulin impaired function conditions such as obesity [160].

Recently, ghrelin has been associated with several significant effects, besides its contribution as an orexigenic hormone, acting in the systemic metabolism. The ghrelin agonism may offer a therapeutic possibility for diabetic gastroparesis and anorexia, and its receptor antagonism may be used in the treatment of obesity and to improve glycaemic metabolism and type 2 diabetes mellitus [161,162].

3. Concluding remarks

In summary, it is important to highlight the adipose tissue endocrine significance on synthesizing adipokines, modulating its secretion and regulating several processes associated with acute and chronic inflammation, besides its relevance in whole body homeostasis. In the present review, we evidenced the main adipokines participation and their interaction with inflammation and the ACE2/Ang-(1–7)/MasR RAS axis. The molecular pathways involved in the adipokines action mechanisms raised the need for new studies aiming to investigate therapeutic interventions, as well as new tools to clarify the unknown mechanisms. The Ang-(1–7) interaction and relevance on the adipokines expression and inflammation were discussed in obesity, type 2 diabetes, cardiovascular diseases, renal and hepatic disorders, among others. In this perspective, we concluded that inflammation may be modulated by adipokines and Ang-(1–7) in different organs and might be an important target for the treatment and prevention of inflammatory responses.

Conflicts of interest

The authors declare no conflicts of interest.

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References

[1] Rein J, Bader M. Renin-angiotensin system in diabetes. Protein Pept Lett 2017;24: 833–40.
[2] Santos SHS. Editorial: renin-angiotensin system: role in chronic diseases. Protein Pept Lett 2017;24:782–3.
[3] Passos-Silva DG, Brandan E, Santos RA. Angiotensins as therapeutic targets beyond heart disease. Trends Pharmacol Sci 2015;36:310–20.
[4] Borem LMA, Neto JFR, Brandi IV, Lelis DF, Santos SHS. The role of the angiotensin II type I receptor blocker telmisartan in the treatment of non-alcoholic fatty liver disease: a brief review. Hypertens Res 2018;41:394–405.
[5] Santos SH, Andrade JM. Angiotensin 1–7: a peptide for preventing and treating metabolic syndrome. Peptides 2014;59:34–41.
[6] Qaradakhi T, Apostolopoulos V, Zulli A. Angiotensin (1–7) and alamandine: similarities and differences. Pharmacol Res 2016;111:820–6.
[7] Hernandez Schulman I, Zhou MS, Raij L. Cross-talk between angiotensin II receptor types 1 and 2: potential role in vascular remodeling in humans. Hypertension 2007;49:270–1.
[8] Kaschina E, Namsolleck P, Unger T. AT2 receptors in cardiovascular and renal diseases. Pharmacol Res 2017;125:39–47.
[9] Victor RG, Leimbach Jr WN, Seals DR, Wallin BG, Mark AL. Effects of the cold pressor test on muscle sympathetic nerve activity in humans. Hypertension 1987;9:429–36.
[10] Fyhrquist F, Saijonmaa O. Renin-angiotensin system revisited. J Intern Med 2008; 264:224–36.
[11] Engeli S, Negrel R, Sharma AM. Physiology and pathophysiology of the adipose tissue renin-angiotensin system. Hypertension 2000;35:1270–7.
[12] Fallo F, Pilon C, Urbanet R. Primary aldosteronism and metabolic syndrome. Horm Metab Res 2012;44:208–14.
[13] Nochioka K, Sakata Y, Shimokawa H. Combination therapy of renin angiotensin system inhibitors and beta-blockers in patients with heart failure. Adv Exp Med Biol 2018;1067:17–30.
[14] Stiefel P, Vallejo-Vaz AJ, Garcia Morillo S, Villar J. Role of the renin-angiotensin system and aldosterone on cardiometabolic syndrome. Int J Hypertens 2011;2011: 685238.
[15] Bader M, Alenina N, Andrade-Navarro MA, Santos RA. MAS and its related G protein-coupled receptors. Mrgprs Pharmacol Rev 2014;66:1080–105.
[16] Dominici FP, Burghi V, Munoz MC, Giani JF. Modulation of the action of insulin by angiotensin-(1–7). Clin Sci 2014;126:613–30.
[17] Santos SH, Giani JF, Burghi V, Miquet JG, Qadri F, Braga JF, et al. Oral administration of angiotensin-(1–7) ameliorates type 2 diabetes in rats. J Mol Med 2014;92: 255–65.

[18] Rubio-Ruiz ME, Del Valle-Mondragon L, Castrejon-Tellez V, Carreon-Torres E, DiazDiaz E, Guarner-Lans V. Angiotensin II and 1–7 during aging in metabolic syndrome rats. Expression of AT1, AT2 and Mas receptors in abdominal white adipose tissue. Peptides 2014;57:101–8.
[19] Liu C, Lv XH, Li HX, Cao X, Zhang F, Wang L, et al. Angiotensin-(1–7) suppresses oxidative stress and improves glucose uptake via Mas receptor in adipocytes. Acta Diabetol 2012;49:291–9.
[20] Santos SH, Fernandes LR, Pereira CS, Guimaraes AL, de Paula AM, CampagnoleSantos MJ, et al. Increased circulating angiotensin-(1–7) protects white adipose tissue against development of a proinflammatory state stimulated by a high-fat diet. Regul Pept 2012;178:64–70.
[21] Marcus Y, Shefer G, Sasson K, Kohen F, Limor R, Pappo O, et al. Angiotensin 1–7 as means to prevent the metabolic syndrome: lessons from the fructose-fed rat model. Diabetes 2013;62:1121–30.
[22] Antuna-Puente B, Feve B, Fellahi S, Bastard JP. Adipokines: the missing link between insulin resistance and obesity. Diabetes Metab 2008;34:2–11.
[23] Simoes e Silva AC, Silveira KD, Ferreira AJ, Teixeira MM. ACE2, angiotensin-(1–7) and Mas receptor axis in inflammation and fibrosis. Br J Pharmacol 2013;169: 477–92.
[24] Santos SH, Simoes e Silva AC. The therapeutic role of renin-angiotensin system blockers in obesity- related renal disorders. Curr Clin Pharmacol 2014;9:2–9.
[25] Patel VB, Basu R, Oudit GY. ACE2/Ang 1–7 axis: a critical regulator of epicardial adipose tissue inflammation and cardiac dysfunction in obesity. Adipocyte 2016;5:306–11.
[26] Giacchetti G, Faloia E, Mariniello B, Sardu C, Gatti C, Camilloni MA, et al. Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. Am J Hypertens 2002;15:381–8.
[27] Santos SH, Fernandes LR, Mario EG, Ferreira AV, Porto LC, Alvarez-Leite JI, et al. Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. Diabetes 2008;57:340–7.
[28] Kloting N, Bluher M. Adipocyte dysfunction, inflammation and metabolic syndrome. Rev Endocr Metab Disord 2014;15:277–87.
[29] Bluher M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. Best Pract Res Clin Endocrinol Metab 2013;27:163–77.
[30] Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med 2009;360: 1509–17.
[31] Bluher M, Mantzoros CS. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. Metabolism 2015;64:131–45.
[32] Blüher M. Importance of adipokines in glucose homeostasis. Diabetes Manage 2013;3:11.
[33] Bluher M. Adipokines - removing road blocks to obesity and diabetes therapy. Mol Metab 2014;3:230–40.
[34] Bluher M. Adipose tissue dysfunction in obesity. Exp Clin Endocrinol Diabetes 2009;117:241–50.
[35] Lehr S, Hartwig S, Sell H. Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders. Proteomics Clin Appl 2012;6:91–101.
[36] Van de Voorde J, Pauwels B, Boydens C, Decaluwe K. Adipocytokines in relation to cardiovascular disease. Metabolism 2013;62:1513–21.
[37] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev 2004;84:277–359.
[38] Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, et al.

Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. J Clin Invest 2013;123:215–23.
[39] Townsend K, Tseng YH. Brown adipose tissue: recent insights into development, metabolic function and therapeutic potential. Adipocyte 2012;1:13–24.
[40] Fernandez JA, Mampel T, Villarroya F, Iglesias R. Direct assessment of brown adipose tissue as a site of systemic tri-iodothyronine production in the rat. Biochem J 1987;243:281– 4.
[41] Portet R, de Marco F, Zizine L, Bertin R, Senault C. Perinatal variations of prostaglandins E2 and F alpha levels in brown adipose tissue of the rat; effects of ambient temperature. Biochimie 1980;62:715–8.
[42] Cassis LA. Role of angiotensin II in brown adipose thermogenesis during cold acclimation. Am J Physiol 1993;265:E860–5.
[43] Burysek L, Houstek J. Beta-adrenergic stimulation of interleukin-1alpha and interleukin- 6 expression in mouse brown adipocytes. FEBS Lett 1997;411:83–6.
[44] Gunawardana SC, Piston DW. Reversal of type 1 diabetes in mice by brown adipose tissue transplant. Diabetes 2012;61:674–82.
[45] Lindquist JM, Rehnmark S. Ambient temperature regulation of apoptosis in brown adipose tissue. Erk1/2 promotes norepinephrine-dependent cell survival. J Biol Chem 1998;273:30147–56.
[46] Kikuchi-Utsumi K, Gao B, Ohinata H, Hashimoto M, Yamamoto N, Kuroshima A. Enhanced gene expression of endothelial nitric oxide synthase in brown adipose tissue during cold exposure. Am J Physiol Regul Integr Comp Physiol 2002;282: R623–6.
[47] Chartoumpekis DV, Habeos IG, Ziros PG, Psyrogiannis AI, Kyriazopoulou VE, Papavassiliou AG. Brown adipose tissue responds to cold and adrenergic stimulation by induction of FGF21. Mol Med 2011;17:736–40.
[48] Cummins TD, Holden CR, Sansbury BE, Gibb AA, Shah J, Zafar N, et al. Metabolic remodeling of white adipose tissue in obesity. Am J Physiol Endocrinol Metab 2014; 307:E262–77.
[49] Armani A, Cinti F, Marzolla V, Morgan J, Cranston GA, Antelmi A, et al. Mineralocorticoid receptor antagonism induces browning of white adipose tissue through impairment of autophagy and prevents adipocyte dysfunction in high-fat-diet-fed mice. FASEB J 2014;28:3745–57.
[50] Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? Genes Dev 2013;27:234–50.
[51] Abdullahi A, Jeschke MG. White adipose tissue browning: a double-edged sword. Trends Endocrinol Metab 2016;27:542–52.
[52] Aldiss P, Betts J, Sale C, Pope M, Budge H, Symonds ME. Exercise-induced 'browning' of adipose tissues. Metabolism 2018;81:63–70.
[53] Bartelt A, Heeren J. Adipose tissue browning and metabolic health. Nat Rev Endocrinol 2014;10:24–36.
[54] Scheele C, Nielsen S. Metabolic regulation and the anti-obesity perspectives of human brown fat. Redox Biol 2017;12:770–5.
[55] Rao C, Huang D, Mao X, Chen R, Huang D, Huang K. The novel adipokine CTRP5 is a negative regulator of white adipose tissue browning. Biochem Biophys Res Commun 2019;510:388–94.
[56] Bluher M. Clinical relevance of adipokines. Diabetes Metab J 2012;36:317–27.
[57] Boustany CM, Bharadwaj K, Daugherty A, Brown DR, Randall DC, Cassis LA. Activation of the systemic and adipose renin-angiotensin system in rats with dietinduced obesity and hypertension. Am J Physiol Regul Integr Comp Physiol 2004; 287:R943–9.
[58] Cassis LA, Police SB, Yiannikouris F, Thatcher SE. Local adipose tissue reninangiotensin system. Curr Hypertens Rep 2008;10:93–8.

[59] Giani JF, Mayer MA, Munoz MC, Silberman EA, Hocht C, Taira CA, et al. Chronic infusion of angiotensin-(1–7) improves insulin resistance and hypertension induced by a high-fructose diet in rats. Am J Physiol Endocrinol Metab 2009;296:E262–71.
[60] Santos SH, Braga JF, Mario EG, Porto LC, Rodrigues-Machado Mda G, Murari A, et al. Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1–7). Arterioscler Thromb Vasc Biol 2010;30:953–61.
[61] Alberdi G, Rodriguez VM, Miranda J, Macarulla MT, Churruca I, Portillo MP. Thermogenesis is involved in the body-fat lowering effects of resveratrol in rats. Food Chem 2013;141:1530–5.
[62] Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, et al. Apelin, a newly identified adipokine up-regulated by insulin and obesity. Endocrinology 2005;146:1764–71.
[63] Hung WW, Hsieh TJ, Lin T, Chou PC, Hsiao PJ, Lin KD, et al. Blockade of the reninangiotensin system ameliorates apelin production in 3T3-L1 adipocytes. Cardiovasc Drugs Ther 2011;25:3–12.
[64] Kurata A, Nishizawa H, Kihara S, Maeda N, Sonoda M, Okada T, et al. Blockade of angiotensin II type-1 receptor reduces oxidative stress in adipose tissue and ameliorates adipocytokine dysregulation. Kidney Int 2006;70:1717–24.
[65] Siddiquee K, Hampton J, McAnally D, May L, Smith L. The apelin receptor inhibits the angiotensin II type 1 receptor via allosteric trans-inhibition. Br J Pharmacol 2013;168:1104–17.
[66] Nickenig G, Roling J, Strehlow K, Schnabel P, Bohm M. Insulin induces upregulation of vascular AT1 receptor gene expression by posttranscriptional mechanisms. Circulation 1998;98:2453–60.
[67] Borghi C, Urso R, Cicero AF. Renin-angiotensin system at the crossroad of hypertension and hypercholesterolemia. Nutr Metab Cardiovasc Dis 2017;27:115–20.
[68] Pinheiro TA, Barcala-Jorge AS, Andrade JMO, Pinheiro TA, Ferreira ECN, Crespo TS, et al. Obesity and malnutrition similarly alter the renin-angiotensin system and inflammation in mice and human adipose. J Nutr Biochem 2017;48:74–82.
[69] Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol 2012;8:457–65.
[70] Pedersen BK, Fischer CP. Beneficial health effects of exercise—the role of IL-6 as a myokine. Trends Pharmacol Sci 2007;28:152–6.
[71] Pedersen BK, Febbraio MA. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev 2008;88:1379–406.
[72] Santos SH, Andrade JM, Fernandes LR, Sinisterra RD, Sousa FB, Feltenberger JD, et al. Oral angiotensin-(1–7) prevented obesity and hepatic inflammation by inhibition of resistin/TLR4/MAPK/NF-kappaB in rats fed with high-fat diet. Peptides 2013; 46:47–52.
[73] Oliveira Andrade JM, Paraiso AF, Garcia ZM, Ferreira AV, Sinisterra RD, Sousa FB, et al. Cross talk between angiotensin-(1–7)/Mas axis and sirtuins in adipose tissue and metabolism of high-fat feed mice. Peptides 2014;55:158–65.
[74] Patel VB, Mori J, McLean BA, Basu R, Das SK, Ramprasath T, et al. ACE2 deficiency worsens epicardial adipose tissue inflammation and cardiac dysfunction in response to diet-induced obesity. Diabetes 2016;65:85–95.
[75] Yang G, Meng F, Wang J, Zhao Y, Zhao L. Angiotensin-(1–7)/Mas axis and vascular inflammation. Eur Sci J 2015;11:24.
[76] Zhang YH, Zhang YH, Dong XF, Hao QQ, Zhou XM, Yu QT, et al. ACE2 and Ang-(1–7) protect endothelial cell function and prevent early atherosclerosis by inhibiting inflammatory response. Inflamm Res 2015;64:253–60.
[77] Wang Y, Wang G, Cui L, Liu R, Xiao H, Yin C. Angiotensin 1–7 ameliorates

caeruleininduced inflammation in pancreatic acinar cells by downregulating Toll-like receptor 4/nuclear factor-kappaB expression. Mol Med Rep 2018;17:3511–8.
[78] Souza LL, Costa-Neto CM. Angiotensin-(1–7) decreases LPS-induced inflammatory response in macrophages. J Cell Physiol 2012;227:2117–22.
[79] Wang L, Hu X, Zhang W, Tian F. Angiotensin (1–7) ameliorates angiotensin IIinduced inflammation by inhibiting LOX-1 expression. Inflamm Res 2013;62: 219–28.
[80] Rocha NP, Bastos FM, Vieira ELM, Prestes TRR, Silveira KDD, Teixeira MM, et al. The protective arm of the renin-angiotensin system may counteract the intense inflammatory process in fetuses with posterior urethral valves. J Pediatr 2018. https://doi. org/10.1016/j.jped.2018.02.003.
[81] Lei Y, Xu Q, Zeng B, Zhang W, Zhen Y, Zhai Y, et al. Angiotensin-(1–7) protects cardiomyocytes against high glucose-induced injuries through inhibiting reactive oxygen species-activated leptin-p38 mitogen-activated protein kinase/extracellular signal-regulated protein kinase 1/2 pathways, but not the leptin-c-Jun Nterminal kinase pathway in vitro. J Diabetes Investig 2017;8:434–45.
[82] Buechler C, Haberl EM, Rein-Fischboeck L, Aslanidis C. Adipokines in liver cirrhosis. Int J Mol Sci 2017;18:1392.
[83] Duan XF, Tang P, Li Q, Yu ZT. Obesity, adipokines and hepatocellular carcinoma. Int J Cancer 2013;133:1776–83.
[84] Kumar P, Smith T, Rahman K, Thorn NE, Anania FA. Adiponectin agonist ADP355 attenuates CCl4-induced liver fibrosis in mice. PLoS One 2014;9:e110405.
[85] Wang H, Zhang H, Zhang Z, Huang B, Cheng X, Wang D, et al. Adiponectin-derived active peptide ADP355 exerts anti-inflammatory and anti-fibrotic activities in thioacetamide-induced liver injury. Sci Rep 2016;6:19445.
[86] Tang A, Li C, Zou N, Zhang Q, Liu M, Zhang X. Angiotensin-(1–7) improves nonalcoholic steatohepatitis through an adiponectin-independent mechanism. Hepatol Res 2017;47:116–22.
[87] Baik SK, Jo HS, Suk KT, Kim JM, Lee BJ, Choi YJ, et al. Inhibitory effect of angiotensin II receptor antagonist on the contraction and growth of hepatic stellate cells. Korean J Gastroenterol 2003;42:134–41.
[88] Herath CB, Warner FJ, Lubel JS, Dean RG, Jia Z, Lew RA, et al. Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1–7) levels in experimental biliary fibrosis. J Hepatol 2007;47:387–95.
[89] Paizis G, Cooper ME, Schembri JM, Tikellis C, Burrell LM, Angus PW. Up-regulation of components of the renin-angiotensin system in the bile duct-ligated rat liver. Gastroenterology 2002;123:1667–76.
[90] Shim KY, Eom YW, Kim MY, Kang SH, Baik SK. Role of the renin-angiotensin system in hepatic fibrosis and portal hypertension. Korean J Intern Med 2018;33:453–61.
[91] Osterreicher CH, Taura K, De Minicis S, Seki E, Penz-Osterreicher M, Kodama Y, et al. Angiotensin-converting-enzyme 2 inhibits liver fibrosis in mice. Hepatology 2009;50:929–38.
[92] Lubel JS, Herath CB, Tchongue J, Grace J, Jia Z, Spencer K, et al. Angiotensin-(1–7), an alternative metabolite of the renin-angiotensin system, is up-regulated in human liver disease and has antifibrotic activity in the bile-duct-ligated rat. Clin Sci 2009;117:375–86.
[93] Choi J, Kobayashi H, Okuda H, Harada KH, Takeda M, Fujimoto H, et al. β-Cell-specific overexpression of adiponectin receptor 1 does not improve diabetes mellitus in Akita mice. PLoS One 2018;13:e0190863.
[94] Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 2003; 423:762.
[95] Bataller R, Sancho-Bru P, Gines P, Lora JM, Al-Garawi A, Sole M, et al. Activated

human hepatic stellate cells express the renin-angiotensin system and synthesize angiotensin II. Gastroenterology 2003;125:117–25.
[96] Suk KT, Yoon JH, Kim MY, Kim CW, Kim JK, Park H, et al. Transplantation with autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: phase 2 trial. Hepatology 2016;64:2185–97.
[97] Bataller R, Gines P, Nicolas JM, Gorbig MN, Garcia-Ramallo E, Gasull X, et al. Angiotensin II induces contraction and proliferation of human hepatic stellate cells. Gastroenterology 2000;118:1149–56.
[98] Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. J Clin Invest 2003;112:1383–94.
[99] Kreth S, Ledderose C, Schütz S, Beiras A, Heyn J, Weis F, et al. MicroRNA-150 inhibits expression of adiponectin receptor 2 and is a potential therapeutic target in patients with chronic heart failure. J Heart Lung Transplant 2014;33:252–60.
[100] Uchiyama T, Okajima F, Mogi C, Tobo A, Tomono S, Sato K. Alamandine reduces leptin expression through the c-Src/p38 MAP kinase pathway in adipose tissue. PLoS One 2017;12:e0178769.
[101] Schuchard J, Winkler M, Stölting I, Schuster F, Vogt FM, Barkhausen J, et al. Lack of weight gain after angiotensin AT1 receptor blockade in diet-induced obesity is partly mediated by an angiotensin-(1–7)/Mas-dependent pathway. Br J Pharmacol 2015;172:3764–78.
[102] Silva AR, Aguilar EC, Alvarez-Leite JI, da Silva RF, Arantes RM, Bader M, et al. Mas receptor deficiency is associated with worsening of lipid profile and severe hepatic steatosis in ApoE-knockout mice. Am J Physiol Regul Integr Comp Physiol 2013; 305:R1323–30.
[103] Hamrick MW. Role of the cytokine-like hormone leptin in muscle-bone crosstalk with aging. J Bone Metab 2017;24:1–8.
[104] Zhu Q, Scherer PE. Immunologic and endocrine functions of adipose tissue: implications for kidney disease. Nat Rev Nephrol 2018;14:105.
[105] Alhasson FA, Das S, Chandrashekaran V, Dattaroy D, Seth RK, Nagarkatti M, et al. Adipokine leptin mediated NOX-2 promotes kidney inflammation in nonalcoholic fatty liver disease via miR21-dependent mesangial cell immune activation. Am Assoc Immnol2017.
[106] Pinheiro SV, Simoes e Silva AC, Sampaio WO, de Paula RD, Mendes EP, Bontempo ED, et al. Nonpeptide AVE 0991 is an angiotensin-(1–7) receptor Mas agonist in the mouse kidney. Hypertension 2004;44:490–6.
[107] Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, et al. Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci U S A 2003;100:8258–63.
[108] do Carmo JM, da Silva AA, Freeman JN, Wang Z, Moak SP, Hankins MW, et al. Neuronal SOCS3 (suppressor of cytokine signaling 3): role in modulating chronic metabolic and cardiovascular effects of leptin. Hypertension 2018;71:1248–57 [HYPERTENSIONAHA. 118.11127].
[109] Lautner RQ, Villela DC, Fraga-Silva RA, Silva N, Verano-Braga T, Costa-Fraga F, et al. Discovery and characterization of alamandine: a novel component of the reninangiotensin system. Circ Res 2013;112:1104–11.
[110] Liu C, Yang CX, Chen XR, Liu BX, Li Y, Wang XZ, et al. Alamandine attenuates hypertension and cardiac hypertrophy in hypertensive rats. Amino Acids 2018;50: 1071–81.
[111] Wang J, Liu R, Hawkins M, Barzilai N, Rossetti L. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. Nature 1998;393:684.
[112] Sawicka M, Janowska J, Chudek J. Potential beneficial effect of some adipokines

positively correlated with the adipose tissue content on the cardiovascular system. Int J Cardiol 2016;222:581–9.
[113] Oral EA, Simha V, Ruiz E, Andewelt A, Premkumar A, Snell P, et al. Leptinreplacement therapy for lipodystrophy. N Engl J Med 2002;346:570–8.
[114] Park HK, Ahima RS. Resistin in rodents and humans. Diabetes Metab J 2013;37: 404–14.
[115] Muse ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, et al. Role of resistin in diet-induced hepatic insulin resistance. J Clin Invest 2004;114:232–9.
[116] Sahr A, Wolke C, Maczewsky J, Krippeit-Drews P, Tetzner A, Drews G, et al. The angiotensin-(1–7)/Mas axis improves pancreatic beta-cell function in vitro and in vivo. Endocrinology 2016;157:4677–90.
[117] Bertolani C, Sancho-Bru P, Failli P, Bataller R, Aleffi S, DeFranco R, et al. Resistin as an intrahepatic cytokine: overexpression during chronic injury and induction of proinflammatory actions in hepatic stellate cells. Am J Pathol 2006; 169:2042–53.
[118] Melone M, Wilsie L, Palyha O, Strack A, Rashid S. Discovery of a new role of human resistin in hepatocyte low-density lipoprotein receptor suppression mediated in part by proprotein convertase subtilisin/kexin type 9. J Am Coll Cardiol 2012;59: 1697–705.
[119] Minn AH, Patterson NB, Pack S, Hoffmann SC, Gavrilova O, Vinson C, et al. Resistin is expressed in pancreatic islets. Biochem Biophys Res Commun 2003;310:641–5.
[120] Van RH, Cushman M, Schlueter EF, Allison MA. Abdominal muscle density is inversely related to adiposity inflammatory mediators. Med Sci Sports Exerc 2018; 50:1495–501.
[121] Yıldırım ÖT, Yıldırır A, Sade LE, Hasırcı SH, Kozan H, Özçalık E, et al. Is there a relationship between resistin levels and left ventricular end-diastolic pressure? Anadulu Kardiyoloji Dergisi: AKD 2018;19:267.
[122] Yang R-Z, Lee M-J, Hu H, Pray J, Wu H-B, Hansen BC, et al. Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action. Am J Physiol 2006;290:E1253–61.
[123] Tsukamoto H, Matsuoka M, French SW. Experimental models of hepatic fibrosis: a review. Semin Liver Dis 1990:56–65.
[124] Costa MA, Lopez Verrilli MA, Gomez KA, Nakagawa P, Pena C, Arranz C, et al. Angiotensin-(1–7) upregulates cardiac nitric oxide synthase in spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 2010;299:H1205–11.
[125] Sit M, Aktas G, Yilmaz EE, Alcelik A, Terzi EH, Tosun M. Effects of the inflammatory response on serum omentin levels in early acute and chronic pancreatitis. Clin Ter 2014;165:e148–52.
[126] Castro CA, da Silva KA, Buffo MM, Pinto KNZ, Duarte FO, Nonaka KO, et al. Experimental type 2 diabetes induction reduces serum vaspin, but not serum omentin, in Wistar rats. Int J Exp Pathol 2017;98:26–33.
[127] Devaraj S, Cheung AT, Jialal I, Griffen SC, Nguyen D, Glaser N, et al. Evidence of increased inflammation and microcirculatory abnormalities in patients with type 1 diabetes and their role in microvascular complications. Diabetes 2007;56:2790–6.
[128] Gualillo O, Gonzalez-Juanatey JR, Lago F. The emerging role of adipokines as mediators of cardiovascular function: physiologic and clinical perspectives. Trends Cardiovasc Med 2007;17:275–83.
[129] Moreno-Navarrete JM, Catalan V, Ortega F, Gomez-Ambrosi J, Ricart W, Fruhbeck G, et al. Circulating omentin concentration increases after weight loss. Nutr Metab 2010;7:27.
[130] Zorlu M, Kiskac M, Guler EM, Gultepe I, Yavuz E, Celik K, et al. Serum obestatin and omentin levels in patients with diabetic nephropathy. Niger J Clin Pract 2017;20: 182–7.
[131] Fernandez-Trasancos A, Agra RM, Garcia-Acuna JM, Fernandez AL,

GonzalezJuanatey JR, Eiras S. Omentin treatment of epicardial fat improves its antiinflammatory activity and paracrine benefit on smooth muscle cells. Obesity (Silver Spring) 2017;25:1042–9.
[132] Sabry MM, Mahmoud MM, Shoukry HS, Rashed L, Kamar SS, Ahmed MM. Interactive effects of apelin, renin-angiotensin system and nitric oxide in treatment of obesityinduced type 2 diabetes mellitus in male albino rats. Arch Physiol Biochem 2018:1–11.
[133] Chen LJ, Xu R, Yu HM, Chang Q, Zhong JC. The ACE2/apelin signaling, microRNAs, and hypertension. Int J Hypertens 2015;2015:896861.
[134] Luo X, Liu J, Zhou H, Chen L. Apelin/APJ system: a critical regulator of vascular smooth muscle cell. J Cell Physiol 2018;233:5180–8.
[135] Mughal A, Sun C, O'Rourke ST. Apelin reduces nitric oxide-induced relaxation of cerebral arteries by inhibiting activation of large-conductance, calcium-activated K channels. J Cardiovasc Pharmacol 2018;71:223–32.
[136] Sekerci R, Acar N, Tepekoy F, Ustunel I, Keles-Celik N. Apelin/APJ expression in the heart and kidneys of hypertensive rats. Acta Histochem 2018;120:196–204.
[137] Guo C, Liu Y, Zhao W, Wei S, Zhang X, Wang W, et al. Apelin promotes diabetic nephropathy by inducing podocyte dysfunction via inhibiting proteasome activities. J Cell Mol Med 2015;19:2273–85.
[138] Huang Z, Wu L, Chen L. Apelin/APJ system: a novel potential therapy target for kidney disease. J Cell Physiol 2018;233:3892–900.
[139] Han S, Englander EW, Gomez GA, Greeley Jr GH. Apelin regulates nuclear factorkappaB's involvement in the inflammatory response of pancreatitis. Pancreas 2017;46:64–70.
[140] Besse-Patin A, Montastier E, Vinel C, Castan-Laurell I, Louche K, Dray C, et al. Effect of endurance training on skeletal muscle myokine expression in obese men: identification of apelin as a novel myokine. Int J Obes (Lond) 2014;38:707.
[141] Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol Chem 2002;277:14838–43.
[142] Organization WH. Global report on diabetes; 2016.
[143] Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, et al. IDF diabetes atlas: global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Res Clin Pract 2017;128:40–50.
[144] Sorisky A. Effect of high glucose levels on white adipose cells and adipokines-fuel for the fire. Int J Mol Sci 2017;18.
[145] El Husseny MW, Mamdouh M, Shaban S, Ibrahim Abushouk A, Zaki MM, Ahmed OM, et al. Adipokines: potential therapeutic targets for vascular dysfunction in type II diabetes mellitus and obesity. J Diabetes Res 2017;2017:8095926.
[146] Harke SM, Khadke SP, Ghadge AA, Manglekar AS, Shah SS, Diwan AG, et al. Adipocytokines and anthropometric measures in type 2 diabetics. Diabetes Metab Syndr 2017;11(Suppl. 1):S273–6.
[147] Ashoori MR, Rahmati-Yamchi M, Ostadrahimi A, Fekri Aval S, Zarghami N. MicroRNAs and adipocytokines: promising biomarkers for pharmacological targets in diabetes mellitus and its complications. Biomed Pharmacother 2017;93:1326–36.
[148] Schinzari F, Tesauro M, Veneziani A, Mores N, Di Daniele N, Cardillo C. Favorable vascular actions of angiotensin-(1–7) in human obesity. Hypertension 2018;71:185–91.
[149] Hemnes AR, Rathinasabapathy A, Austin EA, Brittain EL, Carrier EJ, Chen X, et al. A potential therapeutic role for angiotensin-converting enzyme 2 in human pulmonary arterial hypertension. Eur Respir J 2018;51.

[150] Haschke M, Schuster M, Poglitsch M, Loibner H, Salzberg M, Bruggisser M, et al. Pharmacokinetics and pharmacodynamics of recombinant human angiotensin-converting enzyme 2 in healthy human subjects. Clin Pharmacokinet 2013;52:783–92.
[151] Achari AE, Jain SK. Adiponectin, a therapeutic target for obesity, diabetes, and endothelial dysfunction. Int J Mol Sci 2017;18.
[152] Matsuda M, Shimomura I, Sata M, Arita Y, Nishida M, Maeda N, et al. Role of adiponectin in preventing vascular stenosis. The missing link of adipo-vascular axis. J Biol Chem 2002;277:37487–91.
[153] Okamoto Y. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. Circulation 2002;106:2767–70.
[154] Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002;8:1288–95.
[155] Barchetta I, Ciccarelli G, Cimini FA, Ceccarelli V, Orho-Melander M, Melander O, et al. Association between systemic leptin and neurotensin concentration in adult individuals with and without type 2 diabetes mellitus. J Endocrinol Invest 2018;41:1159–63.
[156] Park HK, Kwak MK, Kim HJ, Ahima RS. Linking resistin, inflammation, and cardiometabolic diseases. Korean J Intern Med 2017;32:239–47.
[157] Gierej P, Gierej B, Kalinowski P, Wroblewski T, Paluszkiewicz R, Kobryn K, et al. Expression of resistin in the liver of patients with non-alcoholic fatty liver disease. Pol J Pathol 2017;68:225–33.
[158] Castan-Laurell I, Boucher J, Dray C, Daviaud D, Guigne C, Valet P. Apelin, a novel adipokine over-produced in obesity: friend or foe? Mol Cell Endocrinol 2005;245:7–9.
[159] Dray C, Knauf C, Daviaud D, Waget A, Boucher J, Buleon M, et al. Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. Cell Metab 2008;8:437–45.
[160] Schinzari F, Veneziani A, Mores N, Barini A, Di Daniele N, Cardillo C, et al. Beneficial effects of Apelin on vascular function in patients with central obesity. Hypertension 2017;69:942–9. [161] Poher AL, Tschop MH, Muller TD. Ghrelin regulation of glucose metabolism. Peptides 2018;100:236–42.
[162] Collden G, Tschop MH, Muller TD. Therapeutic potential of targeting the ghrelin pathway. Int J Mol Sci 2017;18.

Produto 2

Angiotensin-(1-7) Modulates Intestinal Microbiota Improving Metabolic Profile

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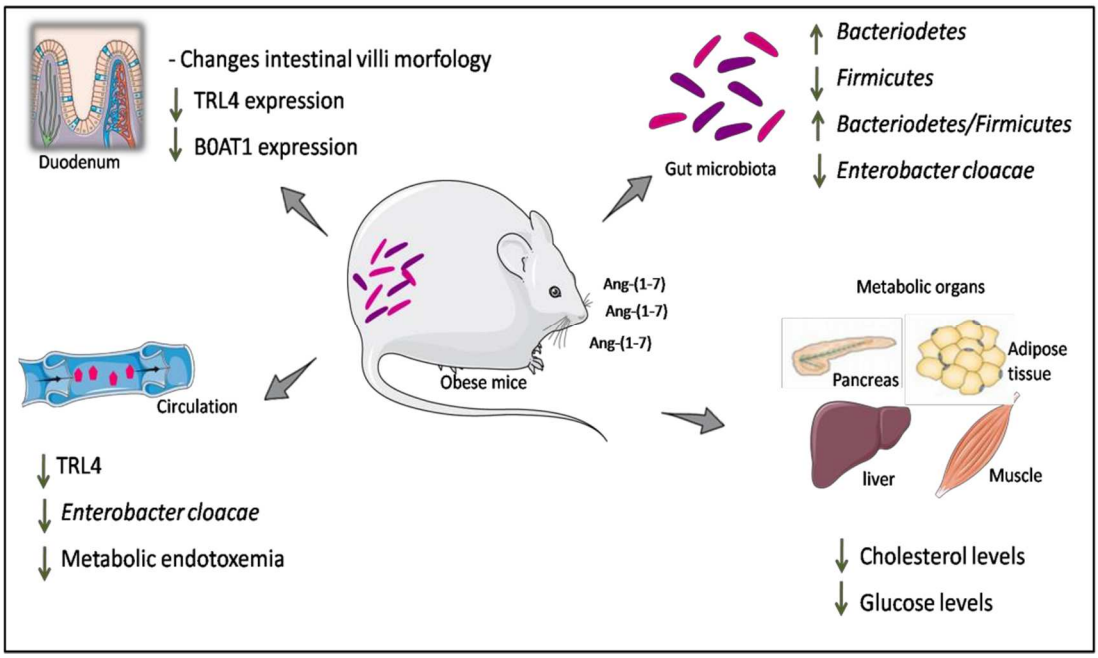
Abbreviations: **ACE**, Angiotensin-converting enzyme; **ACE2**, Angiotensin-converting enzyme 2; **AGT**, Angiotensinogen; **ANG-(1-7)**, Angiotensin-(1-7); **ANGII**, Angiotensin II; **AT_1**, Type 1 Angiotensin II receptor; **AT_2**, Type 2 Angiontensin II receptor; **B0AT1**, Neutral amino acid transporter; **CD-14**, Cluster of differentiation 14; **GAPDH**, Glyceraldehyde 3-phosphate dehydrogenase; **gDNA**, Genomic DNA; **GF**, Germ- free; **GLP-1**, Glucagon-like peptide-1; **HDL**, High-density lipoprotein; **HFD**, High-fat diet; **HPβCD**, Hydroxypropyl-β-cyclodextrin; **KO**, Knockout; **LDL**, Low-density lipoprotein; **LPS**, Lipopolysaccharides; **MAP3K7**, Mitogen-activated protein kinase kinase kinase 7; **Mas**, Mas Receptor; **MYD88**, Myeloid differentiation primary response gene 88; **NF-KB**, Factor nuclear kappa B; **NOD1,** Nucleotide-binding oligomerization domain-containing protein 1, **qPCR**, Real time-PCR; **SCFAs**, Short-chain fatty acids; **SEM**, Standard error; **ST**, Standard; **TIS**, Interactions total score; **TRAF6**, TNF receptor-associated factor 6; **TRL4**, Toll-like receptor 4; **TRP,** Tryptophan; **UBE2N**, Ubiquitin-Conjugating Enzyme E2 N; **WNL**, Weighted number of links; **WT**, Wild-type.

Graphical Abstract



Abstract

The renin-angiotensin system (RAS) and especially angiotensin-(1-7) [ANG-(1-7)] were recognized as essential agents on modulating energy homeostasis and body weight. Moreover, the gut microbiota also received attention when recent studies showed its ability to reverse obesity and metabolic disorders. Therefore, the present study aimed to evaluate the ANG-(1-7) oral administration effects on intestinal microbiota in obese mice. Mice were divided into 4 groups: obese and non-obese/ treated and non-treated with ANG-(1-7). Was observed a significant decrease in the fasting plasma glucose, total cholesterol, triglycerides, and LDL levels and increased HDL in animals treated with ANG-(1-7). The histological analysis showed intestinal villi height reduction in mice treated with ANG-(1-7). Additionally, increased *Bacteroidetes* and decreased *Firmicutes* (increased *Bacteroidetes/Firmicutes* ratio) and *Enterobacter cloacae* populations were observed in the HFD+ANG-(1-7) group. Receptor toll-like 4 (TLR4) intestinal mRNA expression was reduced in the HFD+ANG-(1-7) group. Finally, the intestinal expression of Neutral Amino Acid Transporter (B0AT1) was increased in animals treated with ANG-(1-7), showing a possible mechanism associated with tryptophan uptake. The results of the

present study suggest, for the first time, interaction between RAS and intestinal microbiota modulation.

1. Introduction

Obesity has become a pandemic and a severe public health problem. Compared to the 1980's, there was an increase of more than twice the number of obese individuals, and estimates indicate that by 2030, 57.8%, about 3.3 billion people, will be overweight or obese (Kelly, Yang, Chen, Reynolds, & He, 2008). The increase in the prevalence of obesity is directly related to metabolic alterations, characterized by arterial hypertension, dyslipidemia, hyperglycemia and hyperinsulinemia (Alberti, Zimmet, & Shaw, 2006).

Obesity is associated with several metabolic disarrangements, including an altered expression of the renin-angiotensin system (RAS), a hormonal cascade composed by several important bioactive peptides that modulates the whole-body metabolism (Santos et al., 2013; Santos et al., 2008; Santos et al., 2012). The ANG-(1-7)/ACE2/Mas axis counterbalance the ANGII/ACE/AT1 arm harmful effects (Santos et al., 2010), which when overexpressed induces significant glycemic and lipid alterations, besides the vascular damage (Santos et al., 2010; Santos et al., 2012). Santos et al.(Santos et al., 2012) described an improved lipid and glycemic profile in transgenic rats with increased circulating ANG- (1-7). Furthermore, the Mas receptor suppression in FVB/N mice induced harmful metabolic alterations in glycemic and lipid metabolism, promoting fat mass gain and a metabolic syndrome-like state (Santos et al., 2008). These findings point to the RAS as an important therapeutic target for the treatment of obesity-associated disorders.

Another important player in whole-body metabolism and obesity is the intestinal microbiota that modulates energy homeostasis and body weight (Tremaroli & Backhed, 2012). In mice and humans, the microbial diversity and the *Firmicutes* and *Bacteroidetes* phylum proportion are associated with obesity development (Ley et al., 2005; Ley, Turnbaugh, Klein, & Gordon, 2006). The intestinal microbiota contributes to diet-induced obesity by controlling the fatty acid *β*-oxidation process and triglycerides storage (Backhed et al., 2004) and promoting energy uptake in a high- carbohydrate diet (Backhed, Manchester, Semenkovich, & Gordon, 2007). Germ-free (GF) mice that received microbiota from *wild-type* (WT) donors presented increased adiposity and glycemic and lipid metabolism alterations

(Backhed et al., 2004). Subsequently, Riduara et al. (Ridaura et al., 2013) showed that microbiota transplant from the obese to the lean twin mice was not capable of inducing obesity, evidencing the protective effects of the lean individual's microbiota.

Data regarding intestinal microbiota and RAS are scarce in the literature, and the interaction between ANG-(1-7) and intestinal microbiota was not yet elucidated. Some authors suggested a possible interaction between the beneficial RAS axis (ACE2/ANG-(1-7)/Mas) and the intestinal microbiota via ACE2 modulation (J. M. O. Andrade, de Farias Lelis, Mafra, & Cota, 2017; Hashimoto et al., 2012).. Hashimoto et al. (Hashimoto et al., 2012) revealed that ACE2 deficiency in mice resulted in highly increased susceptibility to intestinal inflammation, and suggested that an altered intestinal microbiota composition and increased susceptibility to colitis may be driven by the RAS via ACE2 modulation. Furthermore, ACE2 exerts an essential role in the intestinal tryptophan transport, modulating the activity of the main neutral aminoacids transporter, B0AT1, localized in the located on the edge of the small intestine brush (Cole-Jeffrey, Liu, Katovich, Raizada, & Shenoy, 2015). ACE2- KO mice presented reduced serum neutral aminoacids levels and a deficient uptake of tryptophan (Trp). ACE2 deficiency was also correlated with decreased antimicrobial peptides levels and consequent altered intestinal microbiota ecology, which is restored after tryptophan administration (Hashimoto et al., 2012).

In this perspective, the present study aimed to evaluate the ANG-(1-7) effects on the intestinal microbiota of mice. To achieve that goal, we assessed the ANG-(1-7) oral administration effects on obese mice microbiota and associated metabolic morphologic parameters and performed bioinformatics analysis to explore the possible links between the RAS and intestinal microbiota.

2. Methods

Drug

In order to guarantee the oral absorption and effect of ANG-(1-7) through the gastrointestinal tract, the peptide was formulated using [hydroxypropyl-β-cyclodextrin/ANG-(1-7)- HPβCD/ANG-(1-7)], which consist on an ANG-(1-7) molecule included in acyclic oligosaccharides (cyclodextrin) (Lula et al., 2007). HPβCD/ANG-(1–7) was donated by the National Institute of Science and Technology - INCT-NanoBiofar (UFMG/Brazil). The daily dose (concentration of 100 μg/kg) was based on a previous study (J. M. Andrade et al., 2014), and ANG- (1-7) was mixed in the animal's diet.

Animals and Diets

The experiment was conducted with 32 male Swiss (four weeks old) divided into 4 groups (n=8 each) and fed the following experimental diets, respectively, for 4 weeks: Standard Diet *ad libitum* ST+HPβCD (ST), ST+ANG-(1–7)/HPβCD (ST+ANG-(1-7)), High-fat diet+HPβCD (HFD), and HFD + ANG-(1–7)/HPβCD(HFD+ANG-(1–7)). Obesity was induced by HFD (24.55% of carbohydrate, 14.47% of protein, and 60.98% fat, presenting a total of 5.28 kcal/g of diet). The control group was fed ST (50.30% of carbohydrate, 41.90% of protein, and 7.80% of fat with a total of 2.18 kcal/g of diet) (Haslam & James, 2005; Rocha & Libby, 2009). All experimental procedures were approved by the Ethics Committee of the State University of Montes Claros and were conducted by following the regulations described in the Committee's Guiding Principles Manual (Protocol number 103/2016).

Measurements of Body Weight, Food Intake, and Tissue Collection

The body weight and food intake were measured three times a week during all experimental procedure. Overnight fasted mice were killed by decapitation and samples of blood, adipose tissues (epididymal, mesenteric and retroperitoneal) and duodenum were collected, weighed, immediately frozen in liquid nitrogen and stored at -80ºC for subsequent analysis.

Determination of Blood Measurements

Serum was obtained after centrifugation(3200 rpm for 10 minutes at 4°C). Glucose, total cholesterol, triglycerides and high-density lipoprotein (HDL) were assayed using enzymatic kits (Wiener®, Argentina). Measurements were made on a Wiener BT-3000 plus ChemistryAnalyzer (Wiener®, Argentina). Low-density lipoprotein (LDL) was calculated based on the Friedewald formula (Friedewald, 1972).

Histology Staining

Duodenum samples were fixed in 10% neutral-buffered formalin at 4ºC overnight, dehydrated through a graded alcohol series, xylene, and paraffin, and then embedded in paraffin. Sections of 5μm were prepared for Hematoxylin & Eosin staining. The slides were analyzed in an FSX100 Inverted Microscope (São Paulo, Brazil).On each slide, the length of the intestinal villi was evaluated (measured from the base of the intestinal villi to the lamina muscularis mucosa)(Navarrete, Vasquez, & Del Sol,

2015) using the Image J software (Wayne Rasb and, National Institutes of Health, Bethesda, MD).

Gut Microbiota Analyzes

Stool samples collected from the mice large intestine immediately after the sacrifice were used. The samples were stored and frozen at -80ºC for subsequent analyses. Genomic DNA (gDNA) was extracted from 50mg of each sample, by silica particles, following washing for impurities removal and resuspension in Tris-EDTA. The gDNA amplification was performed by Real Time-PCT (qPCR) following the method described by Lee (2014), sequences of primers described in Table 1. Relative comparative CT method was applied to compare DNA concentrations between groups, using the 2−ΔΔCT equation (Livak & Schmittgen, 2001).

Table 1. Specific mice primers used in this study.

Primer target	Primer name	Primer sequences (5'-3')	Reference
Total bacteria	341F 543R	CCTACGGGAGGCAGCAG ATTACCGCGGTGCTGG	Lee et al., 2014 [1].
Bacteriodetes	Bact934F Bact1060R	GGA RCA TGT GGT TTA ATT CGA TGA T AGC TGA CGA CAA CCA TGC AG	Lee et al., 2014 [1].
Firmicutes	Firm934F Firm1060R	GGA GYA TGT GGT TTA ATT CGA AGC A AGC TGA CGA CAA CCA TGC AC	Lee et al., 2014 [1].
Lactobacillus spp.	F R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	Lee et al., 2014 [1].
Enterobacter clocae	F R	CGAGAGCCTGUTGCTG GAT TGGCTGACCCAAT	Anbazhagan et al., 2010 [2].
TRL-4	F R	TGGCTGGTTTACACATCCATCGGT TGGCACCATTGAAGCTGAGGTCTA	Qin et al., 2015 [3].
B0AT1	SLC6A19F SLC6A19 R	TTC ACA TCT GTG TAT GCG GCC A AGT GGC ATT GCA CCA CTG TT	Yangzom et al., 2015 [4].

Reverse transcription and qRT-PCR

Total RNA from the duodenum was prepared using TRIzol reagent (Invitrogen Corp.®, San Diego, California, USA), treated with DNAse and reverse transcribed with M-MLV (Invitrogen

Corp.®). The endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Toll-Like Receptor 4 (TRL-4) and Neutral Amino Acid transporter (B0AT1) were amplified using specific primers and SYBR green reagent (Applied Biosystems®, USA) in a plus-one platform (Applied Biosystems®). Relative comparative CT method was applied to compare gene expression levels between groups, using the $2-\Delta\Delta CT$ equation (Livak & Schmittgen, 2001).

Bioinformatics analysis

The bioinformatics was performed as previously described (Santos, 2016). The critical genes involved in the RAS modulation of the intestinal microbiota were identified through a search on the Gene Cards database. The keywords were "renin-angiotensin system" and "microbiota" and "liver". The word “liver” was chosen as one of the key-words because of its important role as a metabolic organ and accessory organ of the intestine. A list composed by potential "gene candidates" that are associated with the keywords chosen was retrieved. Following that, the gene list was expanded in the web-available software STRING (version 9.1), mapping the interaction network among the protein- coding genes. Direct and indirect gene interactions were considered with a high confidence degree (over 0.9, interval 0-0.99); the number of clusters was obtained when mathematical convergence was achieved, and the leader genes were identified. Topological analyses were performed in the Cytoscape software.

Statistical Analysis

All data were transferred to GraphPad Prism software (Version 5.0®, San Diego, California, USA) and analyzed with 95% confidence ($p<0.05$). Data are expressed as the mean ± SEM. The statistical significance of differences in mean values between mice groups was assessed by One-Way ANOVA followed by Tukey post-test, and Student t-test. Differences among various classes based on the weighted number of links (WNL) versus the global connectivity Interactions Total Score (TIS) were assessed by the Kruskal-Wallis test. Statistical significance was set at $p<0.05$.

3. Results

Body Weight, Food Intake, and Tissue Collection

First, we assessed the body and fat weight. Differences in body weight were not observed between the HFD and HFD+ANG-(1-7) groups (HFD, 59.35 g ± 1.93; HFD+ANG-(1-7), 56.30 g ± 0.61). As expected, the ST and ST+ANG-(1-7) groups adiposity (sum of the adipose tissues: epididymal,

mesenteric and retroperitoneal) was significantly smaller as compared to the HFD groups (ST, 0.023 g/BW± 0.004; ST+ANG-(1-7), 0.023 g/BW±0.002;HFD, 0.041 g/BW± 0.008; HFD+ANG-(1-7), 0.043 g/BW±0.008), and no differences were observed between HFD and HFD+ANG-(1-7). Also, differences in food intake (ST, 0.1987 g ± 0,008; ST+ANG-(1-7), 0,191 g ± 0.007; HFD, 0,107g ± 0.006, HFD+ANG-(1-7), 0,109 g ± 0.009) and energy intake (ST, 0.441 Kcal ±0.017; ST+ANG-(1-7), 0.400Kcal ±0.021; HFD, 0.561 Kcal ±0.043, HFD+ANG-(1-7), 0.601 Kcal ±0.061) were not found between the animals treated with similar diets.

HFD+ANG-(1-7) mice exhibited significantly decreased glucose levels (HFD, 155.7 mg/dl±3.93 vs. HFD+ANG-(1-7), 108.0mg/dl±5.05) as compared to the HFD group (Figure 1A). Total cholesterol (HFD, 147.5 mg/dl±12.52; HFD+ANG-(1-7), 95.20±1.37) and triglyceride (HFD,128.3 mg/dl±9.30 versus HFD+ANG-(1-7), 79 mg/dl±13.46) levels were also decreased in the HFD-ANG-(1-7) treated group (Figure 1B and 1C). The serum HDL levels were increased in the animals treated with ANG-(1-7) as compared to HFD group (HFD, 55.33 mg/dl±5.55; HFD+ANG-(1– 7), 87.80 mg/dl±7.65(Figure 1D).Also, serum LDL levels were increased in the HFD group as compared to ST (ST, 14.15 mg/dl±2.945; HFD, 43.65 mg/dl±12.24), while the HFD+ANG-(1-7) group presented reduced levels of LDL as compared to HFD (HFD, 43.65 mg/dl±12.24; HFD+ANG- (1-7), 15.63 mg/dl±4.151) (Figure 1E).

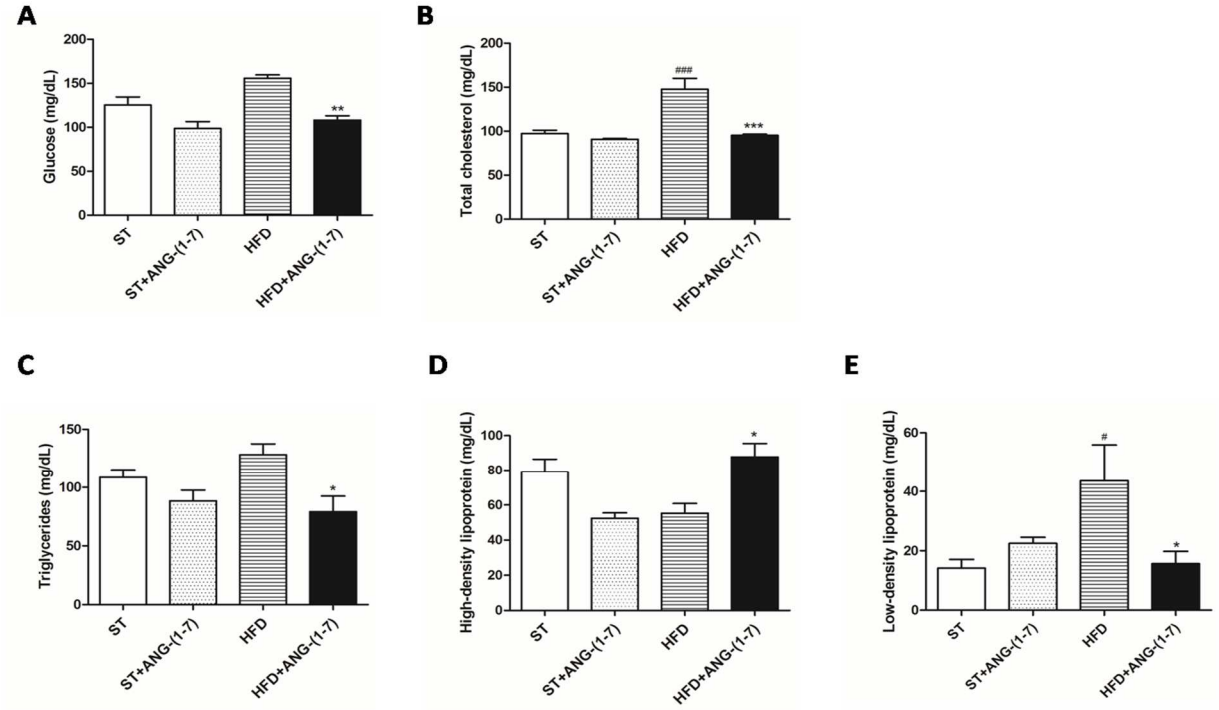


Figure 1. Oral treatment with angiotensin-(1-7) improves the plasmatic parameters of obese mice. **A**. Glucose levels **B.** Total cholesterol. **C**. Triglycerides. **D**. High-density lipoprotein. Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as $^{\#}p< 0.05$ $^{\#\#\#}p<0.001$ in comparison to the ST group, and *p<0.05, **p<0.01, ***p<0.001 in comparison to the HFD group (one-way ANOVA).

The duodenum histological analyses performed to examine the ANG-(1-7) effects in villi size evidenced a substantial decrease in the villus height in relation to HFD (HFD, 452.9 µm±11.83; HFD+ANG-(1-7), 405.1 µm±10.00) (Figure 2A and B).

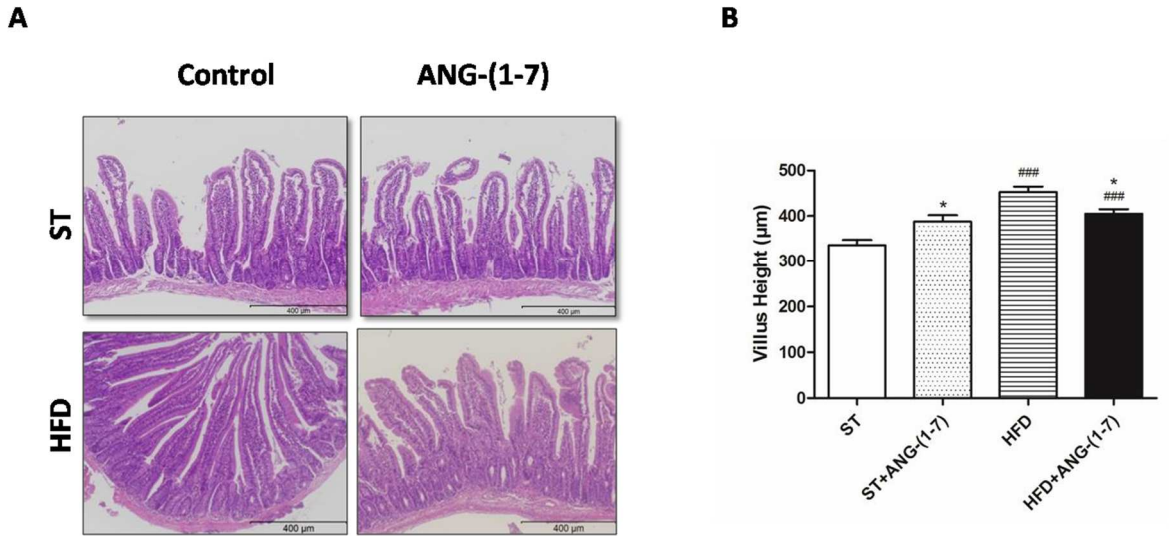


Figure 2. Angiotensin-(1-7) alters the height of intestinal villi. A. Duodenum villi Hematoxylin & Eosin (HE) staining. **B.** ImageJ analysis of villi Height and thickness. Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as $^{\#}p<0.05$ $^{\#\#\#}p<0.001$ in comparison to the ST group, and *p< 0.05, **p< 0.01, ***p< 0.001 in comparison to the HFD group (one-way ANOVA).

The gut microbiota analyses evidenced that ANG-(1-7) modulates the microbial populations that colonize the intestine. The HFD+ANG-(1-7) on the other hand, displayed a slight increase *Bacteroidetes as* compared to HFD (HFD, 0,0420±0.004 vs. HFD+ANG-(1-7), 0,790±0.298) (Figure 3A). Additionally, the *Firmicutes* population was decreased in the ST + ANG-(1-7) (ST, 0.38±0.086 versus ST + ANG- (1-7), 0.03±0.033) and HFD + ANG- (1-7) (HFD, 2.35±0.36 versus HFD + ANG-(1-7), 1.10 ±0.40) groups (Figure 3B).The *Bacteroidetes/Firmicutes* ratio (Figure 3C) confirmed the significant *Bacteroidetes* increase and *Firmicutes* decrease in the treatment groups (HFD+ANG-(1-7) as compared to HFD (HFD, 0.167±0.003; HFD+ANG-(1-7), 0.743±0.172).

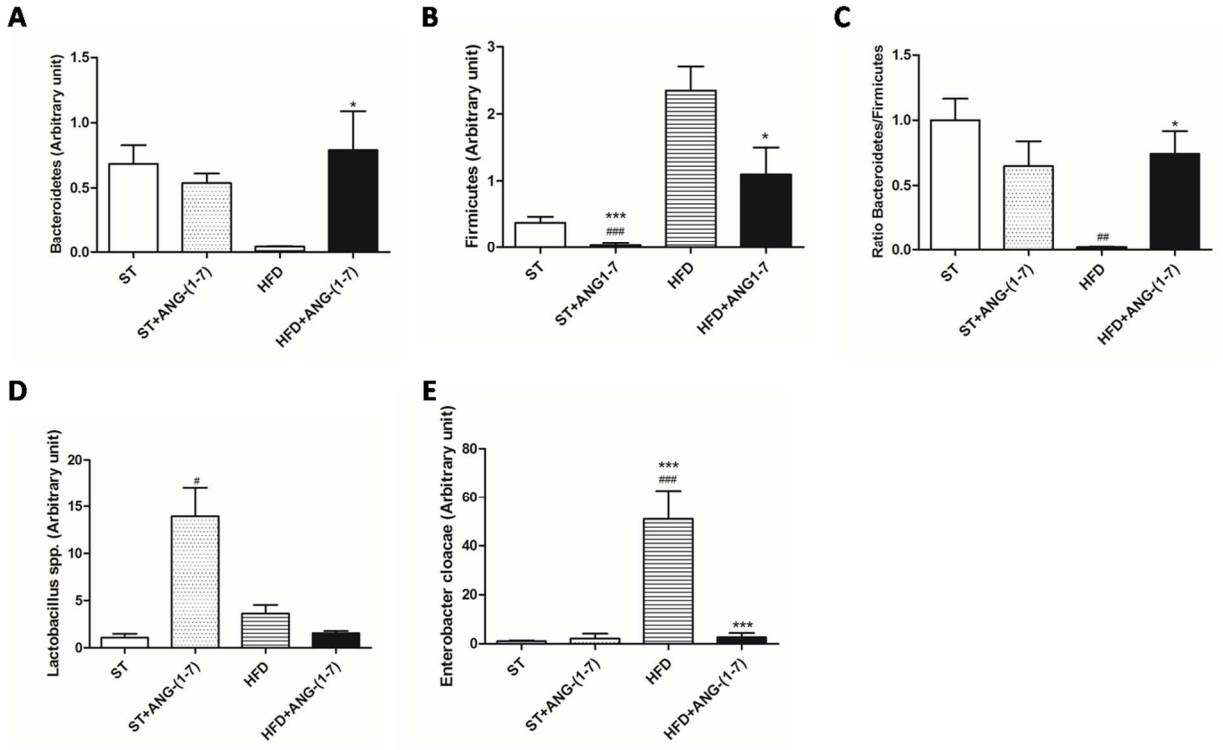


Figure 3. Angiotensin-(1-7) modulates the intestinal microbiota. **A**. *Bacteroidetes*. **B.** *Firmicutes.* **C.** Bacteroidetes/Firmicutes ratio. **D.** *Lactobacillus spp.* and **E.** *Enterobacter cloacae*. Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as $^{\#}p < 0.05$ $^{\#\#\#}p<0.001$ in comparison to the ST group, and *p<0.05, **p< 0.01, ***p< 0.001 in comparison to the HFD group (one-way ANOVA).

We also evaluated the ANG-(1-7) oral administration effects on *Lactobacillus* gDNA expression (Figure 3D), where a significant increase in the ST+ANG-(1-7) fed animals wasobserved (ST, 1,080±0.400; ST+ANG-(1-7), 13,92 ±3.138). Significant differences were not found between the HFD and HFD+ANG-(1-7) groups. As expected, the HFD group presented a significantly higher expression of *Enterobacter cloacae* in the HFD group(Figure 3E), and interestingly, the treatment with ANG-(1-7) was capable of decreasing this microbial population in the HFD+ANG-(1-7) treated animals (HFD, 50.98±11.62; HFD+ANG-(1-7), 2.65±1.72).

The Gene Cards and String data base analyses included four genes related to RAS and microbiota; all scored over 7.5. Figure 4A displays the interaction map obtained. The genes that showed a higher weighted number of links (WNL) and lower total interaction score (TIS) were considered leaders TRAF6 (TNF receptor-associated factor 6), TRL4 (Toll-like receptor 4), MAP3K7

(mitogen-activated protein kinase kinase kinase 7), MYD88 (Myeloid differentiation primary response gene 88) and *UBE2N* (Ubiquitin-Conjugating Enzyme E2 N) (Fig. 4B).

To confirm the main bioinformatic findings, we assessed the TRL4 expression by qRT-PCR in our study. Differences between ST versus ST+ANG-(1-7) were not found (ST, 1.00±0.0; ST+ANG-(1-7), 0.41±0.027). The HFD group, on the other hand, presented a significantly higher expression as compared to HFD+ANG-(1-7) group (HFD, 10.18±2.73 and HFD+ANG-(1-7), 1.37±.070) (Figure 4C). To establish a link between the ANG-(1-7) effects on microbiota and inflammation, we evaluated the B0AT1 intestinal expression, which is an essential mediator of the microbiota effects. We observed that ingestion of HFD with ANG-(1-7) promoted a significant increase of B0AT1 in duodenal enterocytes (HFD, 0.05± 0.03; HFD+ANG-(1-7), 0.18± 0.03) (Fig. 4D).

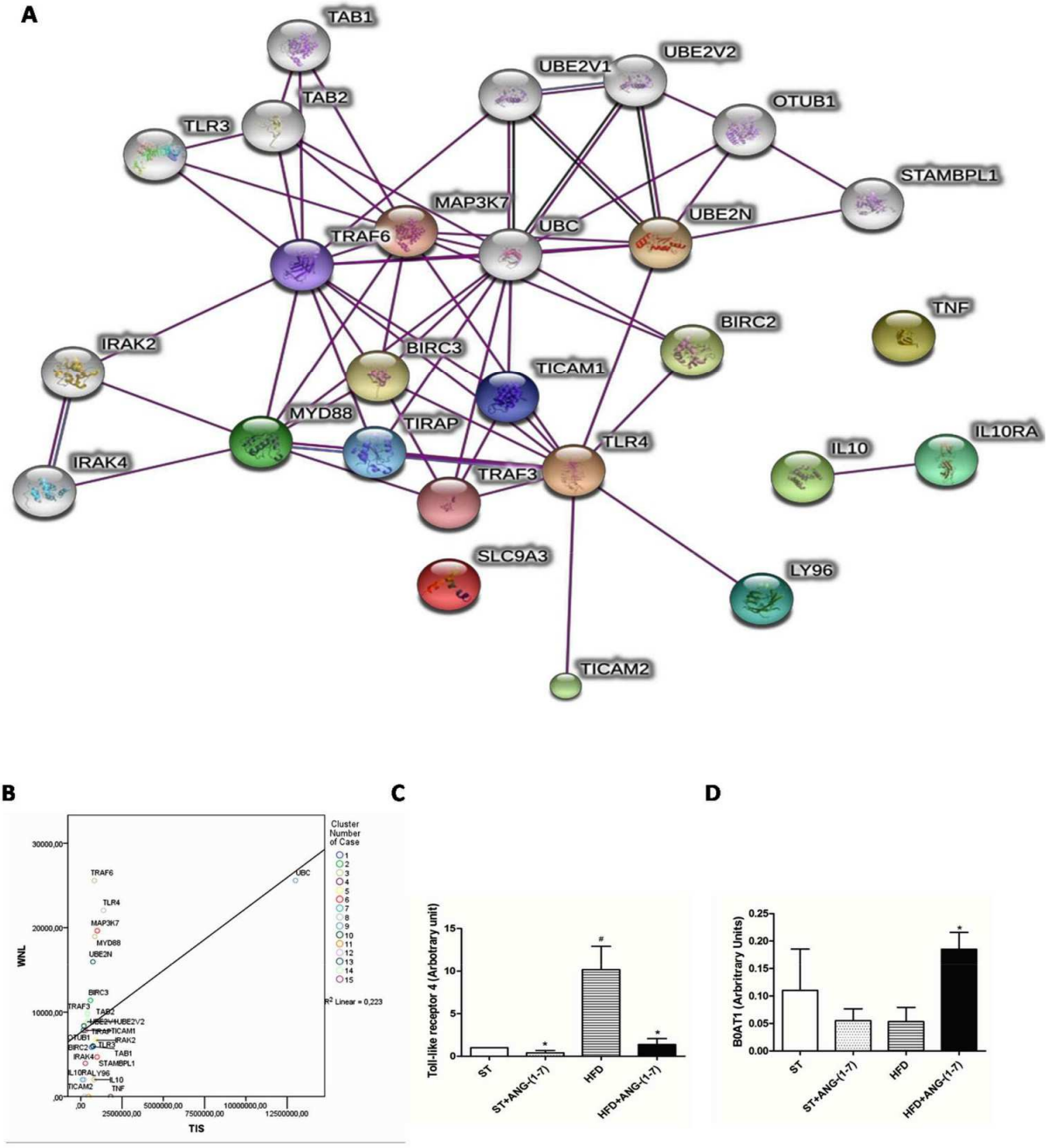


Figure 4. Bioinformatics and RT-PCR analyzes demonstrated that the renin-angiotensin system and the intestinal microbiota regulate inflammation via TRL4, and increases intestinal expression of B0AT1 mRNA in obese mice. A. STRING network **B.** Diagram showing condition- related connectivities (WNL, weighted number of links) versus the global connectivities (TIS Interactions Total Score).The leader genes and clusters. TRAF6 (TNF receptor-associated factor 6), TRL-4 (Toll-like receptor 4), MAP3K7 (mitogen-activated protein kinase 7), MYD88 (Myeloid differentiation primary response gene 88) and UBE2N (Ubiquitin-Conjugating Enzyme E2 N) genes presented higher WNL and lowered TIS. The results of the Kruskal-Wallis test. Statistical significance was set at a p-value. Statistical significance was set at a p-value <0.05. **C**. Duodenum TRL4 mRNA expression performed by qRT-PCR analyses. **D.** Duodenum B0AT1 mRNA expression performed by qRT-PCR analyses. Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as #p<0.05 in comparison to the ST group, and *p< 0.05 in comparison to the HFD group (one-way ANOVA) and Student's t-test.

4. Discussion

The present study reported for the first time that the oral administration of ANG-(1-7) modulated the intestinal villi size and improved glucose and lipid metabolic parameters along with other metabolic homeostasis critical regulators. Furthermore, previous studies have already demonstrated beneficial ANG-(1-7) chronic (long-term treatments) effects on reducing body weight and fat mass (Oliveira Andrade et al., 2014; Santos et al., 2013).

The fasting glucose, total cholesterol, and triglyceride levels reduction and the high HDL levels observed in obese treated animals might be strongly related to the RAS expression markers regulation. Mas receptor deficiency in FVB/N (Mas-KO) mice leads to severe glucose and lipid metabolism changes, inducing a condition similar to the metabolic syndrome, displaying increased fasting glucose levels, glucose intolerance, reduced insulin sensitivity, dyslipidemia, and angiotensinogen increased expression(Santos et al., 2008). On the other hand, a transgenic rat model with increased chronic ANG-(1-7) plasma levels evidenced increased glucose tolerance, improved insulin sensitivity and consequently, increased glucose uptake. Decreased cholesterol and triglyceride levels were also detected, as well as decreased abdominal fat mass associated with unaltered food intake. These alterations were accompanied by reduced angiotensinogen levels in the adipose tissue (Santos et al., 2010).

HFD fed animals presented increased intestinal villi height (Navarrete et al., 2015; Soares, Beraldi, Ferreira, Bazotte, & Buttow, 2015), which suggest associated digestibility adaptations (Santoro et al., 2003)and the HFD consistency (Pluske, Hampson, & Williams, 1997), or increased food retention in the duodenum or proximal jejunum caused by a decrease in intestinal motility (Fu et al., 2014). Petit et al. (Petit et al., 2007) and Wit et al. (de Wit et al., 2008) suggested that such variances are associated with increased cell proliferation, and consequent intestinal mass increase via

Ki-67 (cell nuclear proliferation marker) increased expression in enterocytes and cell cycle regulation via apoptosis. The ANG-(1-7) seems to modulate the mechanisms involved in intestinal villi morphology alterations induced by high-fat diets.

In addition to diet, the intestinal microbiota also contributes to changes in the villi and intestinal

crypts morphology. Sharma et al. (Sharma, Schumacher, Ronaasen, & Coates, 1995) demonstrated that rats transplanted with lean human microbiota displayed reduced villi and intestinal crypts as compared to non-transplanted rats. Other studies showed that germ-free mice presented a reduced total intestinal area (Gordon & Bruckner-Kardoss, 1961) and villi thickness (Reinhardt et al., 2012) due to reduced cell regeneration (Banasaz, Norin, Holma, & Midtvedt, 2002), and extended cell cycles (Alam, Midtvedt, & Uribe, 1994).

The intestinal microbiota comprises approximately 100-500 species, predominating the *Firmicutes* and *Bacteroidetes* phyla, but also presenting other phyla such as *Actinobacteria*, *Proteobacteria*, *Verrumicrobia*, *Fusobacteria* and *Cyanobacteria* (Qin et al., 2010; "Structure, function and diversity of the healthy human microbiome," 2012). The intestinal microbiome exerts a vital role in energetic metabolism, where individuals with obesity and insulin resistance are also accompanied by intestinal dysbiosis (Karlsson, Tremaroli, Nielsen, & Backhed, 2013; Ley et al., 2006). Ley et al. (Ley et al., 2006) described several microbial groups that may contribute to obesity development. Metagenomic studies show that the *Firmicutes* proportion is bigger in obese humans and animals as compared to lean controls (Kien, Schmitz-Brown, Solley, Sun, & Frankel, 2006; Turnbaugh et al., 2006). Studies performed in ob/ob mice demonstrated that this strain has a smaller *Bacteroidetes/Firmicutes* ratio as compared to WT animals (Fleissner et al., 2010; Villanueva-Millan, Perez-Matute, & Oteo, 2015).

Moreover, *Firmicutes*-rich ob/ob mice appear to present glycemic alterations, with increased energy harvesting due to an increased number of enzymes involved in the starch, sucrose and galactose digestion (Arora & Sharma, 2011).A low proportion of *Bacteroidetes/Firmicutes* was associated with increased lipopolysaccharides (LPS) release in the circulation, contributing to the low- grade systemic chronic inflammation observed in obesity (Caricilli et al., 2011). Germ-free animals that received intestinal microbiota from ob/ob mice become obese, confirming the association between microbiota and obesity (Alang & Kelly, 2015; Turnbaugh et al., 2006).Obese individuals submitted to calorie restriction had a significant increase in the *Bacteroidetes* population, suggesting this phyla importance on weight loss and lean phenotype (Karlsson et al., 2013).

In our study, describing for the first time the modulation of the intestinal microbiota via ANG-(1-7), it was observed that ANG- (1-7) attenuated the increase in *Firmicutes* and increased the

Bacteroidetes / Firmicutes ratio, which may be directly related to the metabolic efficiency improvement. The *Bacteroidetes* and intestinal microbiota diversity expansion are also associated with short-chain fatty acids (SCFAs) that induce Glucagon-like peptide-1 (GLP-1) release by intestinal L cells, improving insulin sensitivity and diabetes (Drucker & Nauck, 2006).

The *Lactobacillus* spp. the population was increased in the ST+ANG-(1-7) fed animals. *Lactobacillus* strains that produce SCFAs, especially the conjugated linoleic acid (Lee et al., 2006),promoted body weight loss and decreased adiposity, improved glucose tolerance modulating the leptin expression and fatty acid synthetase. Other studies suggest that *Lactobacillus* improve the lipid profile via fatty acids oxidation (Kim, Park, Kim, Kim, & Hyun, 2013)or lipoprotein lipase inhibition (Aronsson et al., 2010). *Lactobacillus* strains solidify the junctions between epithelial cells, resulting in reduced epithelial permeability, improving the intestinal barrier integrity and facilitating tissue repair after injury (Cario, Gerken, & Podolsky, 2007).

Increased gram-negative bacteria population was found in the intestine from obese individuals, leading to increased LPS absorption that when accumulated in the circulation causes "metabolic endotoxemia." Endotoxemia in murine models is associated with metabolic alterations similar to those induced by HFD, such as body weight gain, hyperinsulinemia, and hyperglycemia(Cani et al., 2007).It was recently described that HFD alters the intestinal microbiota composition, however, for the obesity development, the presence of metabolic endotoxemia is mandatory (de La Serre et al., 2010).

The ANG-(1-7) decreased the *Enterobacter cloacae* B29 population in HFD fed animals. This bacteria strain was described as an endotoxin producer, inducing obesity and insulin resistance in germ-free mice that received this isolated strain from individuals with obesity degree III. Energy balance alterations in metabolic disease are associated with circulating endotoxins. Although the

mechanism by which the ANG-(1-7) promotes the *Enterobactercloacae* B29 decrement in the obese microbiota were still not described, the metabolic endotoxemia reduction might be a therapeutic target in the metabolic syndrome treatment (de La Serre et al., 2010; Fei & Zhao, 2013).

The intestinal metabolic endotoxemia is characterized by a chronic inflammation state

unleashed by microbial LPS recognition, and bacteria translocation via intestinal receptors (NOD1, CD-14, and TLR-4) (Amar et al., 2011).The bacteria antigen interaction and its receptors activate NF- KB and AP-1, transcription factors responsible for increasing the proinflammatory cytokine transcription. The binding of LPS to TRL-4 is mediated by the CD14 pattern recognition receptor, which also mediates MyD88-dependent TNFα expression, inducing TRIF-mediated IFN expression (Gioannini et al., 2004; Kagan & Medzhitov, 2006). Interestingly, in CD-14 KO animals, HFD or LPS administration did not induce metabolic alterations or metabolic endotoxemia, demonstrating the TRL4 role on body weight regulation and glucose tolerance (Cani et al., 2007).

This association was confirmed by bioinformatics and TLR4 expression analyses, which demonstrated a possible interaction among intestinal microbiota, RASand signaling inflammatory pathways (TRAF6, TRL-4, and Myd88). Santos et al. (Santos et al., 2013)shown that HFD+ANG-(1-7) fed rats presented decreased hepatic inflammation via decreased TRL4 and ACE expression, and increased ACE2 expression.

RAS components such as ACE, AT1 and AT2 receptors, and angiotensinogen were found in the border of rats jejunal and ileum epithelium, where the fluids and electrolytes flux is regulated (Wong, Debnam, & Leung, 2007). The fact that the rat jejunum expresses angiotensinogen, which is a precursor of Ang II and other bioactive angiotensins, indicates that enterocytes are capable of synthesizing Ang II (Wong et al., 2007). Recently, evidence of the RAS and intestinal microbiota association via ACE2 modulation have been published (J. M. O. Andrade et al., 2017; Camargo et al., 2009; Cole-Jeffrey et al., 2015). Increased ACE2 levels were detected in the human gastrointestinal tract, and posteriorly, collectrin,an ACE2 homolog, was identified, suggesting a non-catalytic ACE2 activity (Zhang et al., 2001). ACE2 is essential for B0AT1 intestinal expression, an important tryptophan (Trp) transporter (Camargo et al., 2009). Hashimoto et al. (Hashimoto et al., 2012)

demonstrated that ACE2 deficiency results in increased intestinal inflammatory susceptibility and germ-free mice transplanted with ACE2 KO mice acquired colitis.

Borges et al. (Borges et al., 2017) demonstrated that rats pre-treated with A779 (Mas antagonist), and Mas-KO mice exposed to ANG-(1-7) presented a deficiency in Trp absorption. These

findings suggest that the ANG-(1-7) stimulates the tryptophan absorption via the Mas receptor, and this effect was directly associated with an increased ACE2 expression and activity.

A possible mechanism by which angiotensin-(1-7) modulates the intestinal microbiota. It was already described that ANG-(1-7) administration improves tryptophan transportation in the intestinal mucosa (Borges et al., 2017) via B0AT1 (Hashimoto et al., 2012). In our study, B0AT1 expression was increased in ANG-(1-7) treated animals, suggesting a possible interaction between ANG-(1-7) and B0AT1. Previous studies describe that increased Trp levels were associated with increased antimicrobial peptides production, modulating the intestinal microbiota. Trp transport deficiency leads to aberrant secretion of antimicrobial peptides and consequent proliferation of malefic microbial strains, thus conferring intestinal susceptibility to inflammation (Hashimoto et al., 2012).

We have shown that the oral administration of ANG-(1-7) has altered the glucose and lipid levels and intestinal villi morphology, possibly via intestinal microbiota, promoting the proliferation of beneficial microbial classes and reducing metabolic endotoxemia triggered by *Enterobacter cloacae*, and TLR4 intestinal expression. The results of the present study suggest, for the first time, the interaction between RAS and intestinal microbiota. B0AT1 appears to be one of the mediators, since uptake of Trp may play an important role in intestinal microbiota modulation mediated by ANG- (1-7) (Borges et al., 2017). It is worth mentioning that there are many questions to be addressed in future studies such as: How does ANG-(1-7) interact with B0AT1? Is there a direct correlation between ANG-(1-7) and the synthesis of specific microbial metabolites that alter the host metabolism? In conclusion, the present study describes a significant ANG-(1-7) effect, a major RAS component, on the obese mice intestinal microbiota.

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Author contributions

RA.S.S, S.H.S.S, A.S.M, J.R.O. and D.F.L. contributed to the design of the study, acquisition of the data, and analysis and interpretation of the data. J.M.O.A., D.F.L., S.H.S.S. and I.V.B. contributed

to drafting the article and revising it critically for important intellectual content, and A.M.B.P., A.L.S.G. and B.M.A.C. contributed to the final approval of the version to be submitted. All authors read and approved the final manuscript.

Conflict(s) of Interest/Disclosure(s)

The authors declare that they have no competing interest/ disclosure(s).

References

Alam, M., Midtvedt, T., & Uribe, A. (1994). Differential cell kinetics in the ileum and colon of germfree rats. *Scand J Gastroenterol, 29*(5), 445-451.

Alang, N., & Kelly, C. R. (2015). Weight gain after fecal microbiota transplantation. *Open Forum Infect Dis, 2*(1), ofv004. doi:10.1093/ofid/ofv004

Alberti, K. G., Zimmet, P., & Shaw, J. (2006). Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med, 23*(5), 469-480. doi:10.1111/j.1464-5491.2006.01858.x

Amar, J., Chabo, C., Waget, A., Klopp, P., Vachoux, C., Bermudez-Humaran, L. G., . . . Burcelin, R. (2011). Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment. *EMBO Mol Med, 3*(9), 559-572. doi:10.1002/emmm.201100159

Andrade, J. M., Lemos Fde, O., da Fonseca Pires, S., Millan, R. D., de Sousa, F. B., Guimaraes, A. L., . . . Santos, S. H. (2014). Proteomic white adipose tissue analysis of obese mice fed with a high-fat diet and treated with oral angiotensin-(1-7). *Peptides, 60*, 56-62. doi:10.1016/j.peptides.2014.07.023

Andrade, J. M. O., de Farias Lelis, D., Mafra, V., & Cota, J. (2017). The Angiotensin Converting Enzyme 2 (ACE2), Gut Microbiota, and Cardiovascular Health. *Protein Pept Lett*. doi:10.2174/0929866524666170728145333

Aronsson, L., Huang, Y., Parini, P., Korach-Andre, M., Hakansson, J., Gustafsson, J. A., . . . Rafter, J. (2010). Decreased fat storage by Lactobacillus paracasei is associated with increased levels of angiopoietin-like 4 protein (ANGPTL4). *PLoS One, 5*(9). doi:10.1371/journal.pone.0013087

Arora, T., & Sharma, R. (2011). Fermentation potential of the gut microbiome: implications for energy homeostasis and weight management. *Nutr Rev, 69*(2), 99-106. doi:10.1111/j.1753-4887.2010.00365.x

Backhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., . . . Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A, 101*(44), 15718-15723. doi:10.1073/pnas.0407076101

Backhed, F., Manchester, J. K., Semenkovich, C. F., & Gordon, J. I. (2007). Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci U S A, 104*(3), 979-984. doi:10.1073/pnas.0605374104

Banasaz, M., Norin, E., Holma, R., & Midtvedt, T. (2002). Increased enterocyte production in gnotobiotic rats mono-associated with Lactobacillus rhamnosus GG. *Applied and environmental microbiology, 68*(6), 3031-3034.

Borges, E. L., Lima, P. B., Peluso, A. A. B., Sampaio, W. O., Oliveira, J. S. d., Oliveira, M. L. d., . . . Santos, R. A. S. (2017). Angiotensin-(1-7) Influences Tryptophan Absorption in the Rat and Mouse Intestine. *British Journal of Medicine & Medical Research, 4*(19), 1-9. doi:10.9734/BJMMR/2017/30329

Camargo, S. M., Singer, D., Makrides, V., Huggel, K., Pos, K. M., Wagner, C. A., . . . Verrey, F. (2009). Tissue-specific amino acid transporter partners ACE2 and collectrin differentially interact with hartnup mutations. *Gastroenterology, 136*(3), 872-882. doi:10.1053/j.gastro.2008.10.055

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., . . . Burcelin, R. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes, 56*(7), 1761-1772. doi:10.2337/db06-1491

Caricilli, A. M., Picardi, P. K., de Abreu, L. L., Ueno, M., Prada, P. O., Ropelle, E. R., . . . Saad, M. J.

(2011). Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice. *PLoS Biol, 9*(12), e1001212. doi:10.1371/journal.pbio.1001212
Cario, E., Gerken, G., & Podolsky, D. K. (2007). Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. *Gastroenterology, 132*(4), 1359-1374. doi:10.1053/j.gastro.2007.02.056
Cole-Jeffrey, C. T., Liu, M., Katovich, M. J., Raizada, M. K., & Shenoy, V. (2015). ACE2 and Microbiota: Emerging Targets for Cardiopulmonary Disease Therapy. *J Cardiovasc Pharmacol, 66*(6), 540-550. doi:10.1097/FJC.0000000000000307
de La Serre, C. B., Ellis, C. L., Lee, J., Hartman, A. L., Rutledge, J. C., & Raybould, H. E. (2010). Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *Am J Physiol Gastrointest Liver Physiol, 299*(2), G440-448. doi:10.1152/ajpgi.00098.2010
de Wit, N. J., Bosch-Vermeulen, H., de Groot, P. J., Hooiveld, G. J., Bromhaar, M. M., Jansen, J., . . . van der Meer, R. (2008). The role of the small intestine in the development of dietary fat-induced obesity and insulin resistance in C57BL/6J mice. *BMC Med Genomics, 1*, 14. doi:10.1186/1755-8794-1-14
Drucker, D. J., & Nauck, M. A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet, 368*(9548), 1696-1705. doi:10.1016/S0140-6736(06)69705-5
Fei, N., & Zhao, L. (2013). An opportunistic pathogen isolated from the gut of an obese human causes obesity in germfree mice. *ISME J, 7*(4), 880-884. doi:10.1038/ismej.2012.153
Fleissner, C. K., Huebel, N., Abd El-Bary, M. M., Loh, G., Klaus, S., & Blaut, M. (2010). Absence of intestinal microbiota does not protect mice from diet-induced obesity. *Br J Nutr, 104*(6), 919-929. doi:10.1017/S0007114510001303
Fu, X. Y., Li, Z., Zhang, N., Yu, H. T., Wang, S. R., & Liu, J. R. (2014). Effects of gastrointestinal motility on obesity. *Nutr Metab (Lond), 11*(1), 3. doi:10.1186/1743-7075-11-3
Gioannini, T. L., Teghanemt, A., Zhang, D., Coussens, N. P., Dockstader, W., Ramaswamy, S., & Weiss, J. P. (2004). Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc Natl Acad Sci U S A, 101*(12), 4186-4191. doi:10.1073/pnas.0306906101
Gordon, H. A., & Bruckner-Kardoss, E. (1961). Effect of normal microbial flora on intestinal surface area. *Am J Physiol, 201*, 175-178.
Hashimoto, T., Perlot, T., Rehman, A., Trichereau, J., Ishiguro, H., Paolino, M., . . . Penninger, J. M. (2012). ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. *Nature, 487*(7408), 477-481. doi:10.1038/nature11228
Haslam, D. W., & James, W. P. (2005). Obesity. *Lancet, 366*(9492), 1197-1209. doi:10.1016/S0140-6736(05)67483-1
Kagan, J. C., & Medzhitov, R. (2006). Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell, 125*(5), 943-955. doi:10.1016/j.cell.2006.03.047
Karlsson, F., Tremaroli, V., Nielsen, J., & Backhed, F. (2013). Assessing the human gut microbiota in metabolic diseases. *Diabetes, 62*(10), 3341-3349. doi:10.2337/db13-0844
Kelly, T., Yang, W., Chen, C. S., Reynolds, K., & He, J. (2008). Global burden of obesity in 2005 and projections to 2030. *Int J Obes (Lond), 32*(9), 1431-1437. doi:10.1038/ijo.2008.102
Kien, C. L., Schmitz-Brown, M., Solley, T., Sun, D., & Frankel, W. L. (2006). Increased colonic luminal synthesis of butyric acid is associated with lowered colonic cell proliferation in piglets. *J Nutr, 136*(1), 64-69.
Kim, S. W., Park, K. Y., Kim, B., Kim, E., & Hyun, C. K. (2013). Lactobacillus rhamnosus GG improves insulin sensitivity and reduces adiposity in high-fat diet-fed mice through enhancement of adiponectin production. *Biochem Biophys Res Commun, 431*(2), 258-263. doi:10.1016/j.bbrc.2012.12.121
Lee, H. Y., Park, J. H., Seok, S. H., Baek, M. W., Kim, D. J., Lee, K. E., . . . Park, J. H. (2006). Human originated bacteria, Lactobacillus rhamnosus PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *Biochim Biophys Acta, 1761*(7), 736-744. doi:10.1016/j.bbalip.2006.05.007

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A, 102*(31), 11070-11075. doi:10.1073/pnas.0504978102

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Microbial ecology: human gut microbes associated with obesity. *Nature, 444*(7122), 1022-1023. doi:10.1038/4441022a

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*(4), 402-408. doi:10.1006/meth.2001.1262

Lula, I., Denadai, A. L., Resende, J. M., de Sousa, F. B., de Lima, G. F., Pilo-Veloso, D., . . . Sinisterra, R. D. (2007). Study of angiotensin-(1-7) vasoactive peptide and its beta-cyclodextrin inclusion complexes: complete sequence-specific NMR assignments and structural studies. *Peptides, 28*(11), 2199-2210. doi:10.1016/j.peptides.2007.08.011

Navarrete, J., Vasquez, B., & Del Sol, M. (2015). Morphoquantitative analysis of the Ileum of C57BL/6 mice (Mus musculus) fed with a high-fat diet. *Int J Clin Exp Pathol, 8*(11), 14649-14657.

Oliveira Andrade, J. M., Paraiso, A. F., Garcia, Z. M., Ferreira, A. V., Sinisterra, R. D., Sousa, F. B., . . . Santos, S. H. (2014). Cross talk between angiotensin-(1-7)/Mas axis and sirtuins in adipose tissue and metabolism of high-fat feed mice. *Peptides, 55*, 158-165. doi:10.1016/j.peptides.2014.03.006

Petit, V., Arnould, L., Martin, P., Monnot, M. C., Pineau, T., Besnard, P., & Niot, I. (2007). Chronic high-fat diet affects intestinal fat absorption and postprandial triglyceride levels in the mouse. *J Lipid Res, 48*(2), 278-287. doi:10.1194/jlr.M600283-JLR200

Pluske, J. R., Hampson, D. J., & Williams, I. H. (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science, 51*(1), 215-236. doi:http://dx.doi.org/10.1016/S0301-6226(97)00057-2

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., . . . Wang, J. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature, 464*(7285), 59-65. doi:http://www.nature.com/nature/journal/v464/n7285/suppinfo/nature08821_S1.html

Reinhardt, C., Bergentall, M., Greiner, T. U., Schaffner, F., Ostergren-Lunden, G., Petersen, L. C., . . . Backhed, F. (2012). Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodelling. *Nature, 483*(7391), 627-631. doi:10.1038/nature10893

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., . . . Gordon, J. I. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science, 341*(6150), 1241214. doi:10.1126/science.1241214

Rocha, V. Z., & Libby, P. (2009). Obesity, inflammation, and atherosclerosis. *Nat Rev Cardiol, 6*(6), 399-409. doi:10.1038/nrcardio.2009.55

Santoro, S., Velhote, M. C. P., Malzoni, C. E., Mechenas, A. S. G., Strassmann, V., & Scheinberg, M. (2003). Digestive adaptation: a new surgical proposal to treat obesity based on physiology and evolution. *risk, 19*, 20.

Santos, S. H., Andrade, J. M., Fernandes, L. R., Sinisterra, R. D., Sousa, F. B., Feltenberger, J. D., . . . Santos, R. A. (2013). Oral Angiotensin-(1-7) prevented obesity and hepatic inflammation by inhibition of resistin/TLR4/MAPK/NF-kappaB in rats fed with high-fat diet. *Peptides, 46*, 47-52. doi:10.1016/j.peptides.2013.05.010

Santos, S. H., Braga, J. F., Mario, E. G., Porto, L. C., Rodrigues-Machado Mda, G., Murari, A., . . . Santos, R. A. (2010). Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1-7). *Arterioscler Thromb Vasc Biol, 30*(5), 953-961. doi:10.1161/ATVBAHA.109.200493

Santos, S. H., Fernandes, L. R., Mario, E. G., Ferreira, A. V., Porto, L. C., Alvarez-Leite, J. I., . . . Santos, R. A. (2008). Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. *Diabetes, 57*(2), 340-347. doi:10.2337/db07-0953

Santos, S. H., Fernandes, L. R., Pereira, C. S., Guimaraes, A. L., de Paula, A. M., Campagnole-Santos, M. J., . . . Santos, R. A. (2012). Increased circulating angiotensin-(1-7) protects white adipose tissue against development of a proinflammatory state stimulated by a high-fat diet. *Regul Pept, 178*(1-3), 64-70. doi:10.1016/j.regpep.2012.06.009

Sharma, R., Schumacher, U., Ronaasen, V., & Coates, M. (1995). Rat intestinal mucosal responses to a microbial flora and different diets. *Gut, 36*(2), 209-214.

Soares, A., Beraldi, E. J., Ferreira, P. E., Bazotte, R. B., & Buttow, N. C. (2015). Intestinal and neuronal myenteric adaptations in the small intestine induced by a high-fat diet in mice. *BMC Gastroenterol, 15*, 3. doi:10.1186/s12876-015-0228-z

Structure, function and diversity of the healthy human microbiome. (2012). *Nature, 486*(7402), 207-214. doi:http://www.nature.com/nature/journal/v486/n7402/abs/nature11234.html#supplementary-information

Tremaroli, V., & Backhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature, 489*(7415), 242-249. doi:10.1038/nature11552c

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature, 444*(7122), 1027-1031. doi:10.1038/nature05414

Villanueva-Millan, M. J., Perez-Matute, P., & Oteo, J. A. (2015). Gut microbiota: a key player in health and disease. A review focused on obesity. *J Physiol Biochem, 71*(3), 509-525. doi:10.1007/s13105-015-0390-3

Wong, T. P., Debnam, E. S., & Leung, P. S. (2007). Involvement of an enterocyte renin-angiotensin system in the local control of SGLT1-dependent glucose uptake across the rat small intestinal brush border membrane. *J Physiol, 584*(Pt 2), 613-623. doi:10.1113/jphysiol.2007.138578

Zhang, H., Wada, J., Hida, K., Tsuchiyama, Y., Hiragushi, K., Shikata, K., . . . Makino, H. (2001). Collectrin, a collecting duct-specific transmembrane glycoprotein, is a novel homolog of ACE2 and is developmentally regulated in embryonic kidneys. *J Biol Chem, 276*(20), 17132-17139. doi:10.1074/jbc.M006723200

Produto 3

Title: Bifidobacterium longum supplementation improves metabolic parameters and alters the expression of the renin-angiotensin system in obese mice.

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Abbreviations: **ACE**, Angiotensin-converting enzyme; **ACE2**, Angiotensin-converting enzyme 2; **AGT**, Angiotensinogen; **ANG-(1-7)**, Angiotensin-(1-7); **ANGII**, Angiotensin II; **AT_1**, Type 1 Angiotensin II receptor; **AT_2**, Type 2 Angiontensin II receptor; ***B. longum***, *Bifidobacterium longum*; **CNS**, Central Nervous System**; FAO**, Food and Agriculture Organization**; GAPDH**, Glyceraldehyde 3-phosphate dehydrogenase; **GLP-1**, Glucagon-like peptide-1; **GPR43**, short chain fatty acid receptor; **GLUT-4**, *glucos**e*** transporter type 4; **HDL**, High-density lipoprotein; **HFD**, High-fat diet; **IKK-β**, IκB kinase beta; **LPS**, Lipopolysaccharides; **Mas**, Mas Receptor; **NF-KB**, Factor nuclear kappa B; **NKT**, Natural Killer Cells; **RT-PCR**, Real time-PCR; **PYY**, peptide YY**; SCFAs**, Short-chain fatty acids; **SCFAs**, short chain fatty acid; **SEM**, Standard error; **ST**, Standard; **TNF-*α***, Tumor Necrosis Factor alfa; **TRL4**, Toll-like receptor 4; **WHO**, World Health Organization.

Abstract

The beneficial effects mediated by probiotics are due to their ability to modulate the intestinal microbiota. Recently, the important role of the microbiota in metabolic regulation has been described, and the use of probiotics has become increasingly common. However, the mechanisms involved in the control of obesity by *Bifidobacterium longum* and its effect on the renin-angiotensin system (RAS) have not yet been described. Thus, the objective of the present

study was to evaluate the effect of *Bifidobacterium longum* supplementation on metabolic parameters and RAS expression in obese mice. Methods: Mice were divided into four groups: obese and non-obese/treated and not treated with *Bifidobacterium longum*. Results: After four weeks of treatment, obese mice that received *B. longum* showed a significant decrease in body weight, adiposity,serum glycemia and total cholesterol levels, as well as an improvement in glucose tolerance. In addition, histological analyses demonstrated a reduction in the accumulation of hepatic triglycerides in the obese-treated group. Analyses of mRNA showed a significant increase in the expression of the angiotensin-converting enzyme 2 (ECA2) and the Mas receptor (MASR) in the obese mice that received *B. longum*. Conclusion: Our data suggest for the first time the modulation of RAS by the*B. longum* strain. These findings may contribute tobetter understand the metabolic effect mediated by probiotics. However, the mechanisms that involve the possible role of *B. longum* in the ECA2/Ang- (1-7)/MASS axis of the RAS activation in the liver need to be further investigated.

Introduction

Obesity has become a global health problem and is considered a pandemic (1). According to the World Health Organization (WHO), in 2030, this condition will affect about 20% of the world's adult population (2). The etiology of obesity is multifactorial, being caused mainly by the imbalance between energyconsumption and expenditure (3). Obesity is a chronic disease that contributes heavily to multiple cardio-metabolic diseases, such as type 2 diabetes, dyslipidemia, coronary artery disease, stroke, hypertension, and various types of cancer (4-6).

Recent evidence has shown that the intestinal microbiota plays an important role in metabolic regulation, functioning as an organ with metabolic, immunological, and endocrine function (7), performing essential functions that the human body alone is not able to perform, resulting in a symbiotic relationship. This close link is essential for the absorption of energy from ingested food, motility and integrity of the intestinal barrier, regulation of host metabolism, especially the metabolism of glucose, lipid, and inflammation (8-10).

The intestinal microbiota consists of three main phyla: *Bacteroidetes* (*Porphyromonas*, *Prevotella*, *Bacteroides*), *Firmicutes* (*Ruminococos*, *Clostridium*,*Lactobacillus*,and *Eubacteria*), and *Actinobacteria* (*Bifidobacteria*) (11) with the majority of intestinal microbiota being represented by *Bifidobacterium* and *Bacteroides* (12). The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) define probiotics as live microorganisms which, when administered in adequate amounts, confer benefits to the host's health (13). The beneficial effects mediated by probiotics are due to their ability to modulate the intestinal microbiota and the mucosal immune system (14-16). Among the most well-studied probiotics are lactic acid-producing bacterial strains belonging to the*Bifidobacterium*and *Lactobacillus*genera, which have an established safety record and have received

the GRAS status (generally recognized as safe) by the US Food and Drug Administration (17), and are widely used and included in many functional foods and dietary supplements (18-20). Studies have shown that the administration of *Bifidobacterium* spp., including *B. longum*, reduces body weight, improves glucose tolerance and insulin resistance, and protects from diet-induced obesity by maintaining energy homeostasis, as well as reducing serum cholesterol levels and triglycerides (21-23). Another important metabolic regulator is the renin-angiotensin system (RAS), an enzymatic cascade composed of two main axes: the ECA/AngII/AT1R axis, which is over-expressed in obesity conditions and exerts deleterious effects, and the ECA2/Ang-(1-7)/MASRaxis, which counter-regulates the AngII axis by improving metabolic efficiency (24-26).

Considering the aforementioned, we hypothesized that the supplementation of obese animals with probiotic *B. longum* can modulate the ECA2/Ang-(1-7)/MASR axis of the renin-angiotensin system by improving the metabolic changes observed in obesity. Thus, the objective of the present study was to evaluate the effect of *Bifidobacterium longum* supplementation on metabolic parameters and the RAS expression in obese mice.

Materials and Methods Animals and Diets

The experiment was conducted with 32 four-week-old male Swiss mice divided into 4 groups (n=8 each) and fed, for 4 weeks, the following experimental diets, respectively: Standard Diet *ad libitum* (ST), Standard Diet *ad libitum* + *Bifidobacterium longum* (ST + *B. Longum*), High-fat diet (HFD), andHigh-fat diet+ *Bifidobacterium longum*(HFD + *B. longum*). The control group was fed an SD (50.30% carbohydrate, 41.90% protein, and 7.80% fat, totaling 2.18 kcal/g) (27, 28). Obesity was induced with an HFD (24.55% carbohydrate, 14.47% protein, and 60.98% fat, totaling 5.28 kcal/g), for eight weeks. The*B. longum*probiotic was administered by gavageat a dose of 50 billion bacteria/kg body wt^{-1} daily (29). Animals in control groups received a saline solution. All experimental procedures were approved by

the Ethics Committee of the State University of Montes Claros and were conducted by following the regulations described in the Committee Guiding Principles Manual (Protocol number 103/2016).

Measurements of Body Weight, Food Intake, and Tissue Collection

The body weight, food intake, and energy intake were measured three times a week during all experimental procedures. Mice were fasted overnight and killed bydecapitation.Samples of blood, adipose tissues (epididymal, mesenteric, and retroperitoneal) and liver were collected, weighed, immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent analyses.

Histology Staining

Epididymal adipose tissue and liver samples were fixed in 10% neutral-buffered formalin at 4ºC overnight, dehydrated by escalating grades of alcohol, xylene, and paraffin, and then embedded in paraffin. Sections of 5 μm were prepared for Hematoxylin & Eosin staining. The slides were analyzed in an FSX100 Inverted Microscope (Sao Paulo, Brazil). All samples were analyzed by densitometry, using the Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Glucose tolerance and insulin sensitivity tests

For the glucose-tolerance test, D-glucose (2 mg/g body weight) was intraperitoneally injected into overnight fasted mice. Glucose levels from tail blood samples were monitored at 0, 15, 30, 60, and 120 minutes after injection. Insulin sensitivity tests were performed with the animals in the fed state, after intraperitoneal injection of insulin (0.75 U/kg body weight), and tail blood samples were taken at the time points 0, 15, 30, and 60 minutes after injection for the measurement of blood glucose levels.

Determination of Blood Measurements

Serum was obtained after centrifugation (3200 rpm for 10 minutes at 4°C). Glucose, total cholesterol, triglycerides, and high-density lipoprotein (HDL) were assessed using enzymatic kits (Wiener®, Argentina). Measurements were performed on a Wiener BT-3000 plus ChemistryAnalyzer (Wiener®, Argentina).

Reverse transcription and qRT-PCR

Total RNA extracted from the liver was prepared using TRIzol reagent (Invitrogen Corp.®, San Diego, California, USA), treated with DNAse and reverse transcribed with M-MLV (Invitrogen Corp.®). The endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Angiotensin-converting enzyme (ACE), Angiotensin-converting enzyme 2 (ACE2), and Mas receptor (MASR) were assessed using specific primers and SYBR green reagent (AppliedBiosystems®, USA) in a plus-one platform (Applied Biosystems®). Relative comparative CT method was applied to compare gene expression levels between groups, using the $2^{-\Delta\Delta CT}$ equation (30), sequences of primers described in Table 1.

Table 1 – Specific mouse primers used in this study.

Primer target	Primer name	Primer sequences (5'-3')
ACE	F	CTC CTG GGA CTT CTA CAAC
	R	CTC CAT GTT CAC AGA GGT ACA CT
ACE2	F	GGA TAC CTA CCC TTC CTA CAT CAG C
	R	CAT CCC CAC ATA TCA CCA AGCA
MASR.	F	ACT GCC GGG CGG TCA TCA TC
	R	GGT GGA GAA AAG CAA GGA GA

Statistical Analysis

All data were analyzed with GraphPad Prism software (Version 5.0®, San Diego, California, USA), at a 95% confidence ($p<0.05$). Data are expressed as the mean ± SEM. The statistical significance between mice groups was assessed by One-Way ANOVA followed by Tukey post-test, and Student t-test. Statistical significance was set at $p<0.05$.

RESULTS

Food intake, energy intake, body weight, tissue weight, and adiposity

The analyses of food consumption (ST, 0.193±0.010; ST+*B.longum*; 0.180±0.009; HFD, 0.106±0.007; HFD+*B. Longum*, 0.110±0.007) and energy consumption (ST, 0.442±0.014; ST+*B.longum*; 0.404±0.011; HFD, 0.495±0.032; HFD+*B. Longum*, 0.5491±0.032) showed no statistically significant differences between the mice fed the same type of diet **(Fig. 1a and 1b)**. In the analysis of area under the body weight curve, a statistically significant reduction was observed in the probiotic treated group (HFD + *B. Longum*) when compared to the HFD group (HFD, 5758 ± 189.3; HFD + *B. Longum*, 4942 ± 125.4) **(Fig. 1c)**.

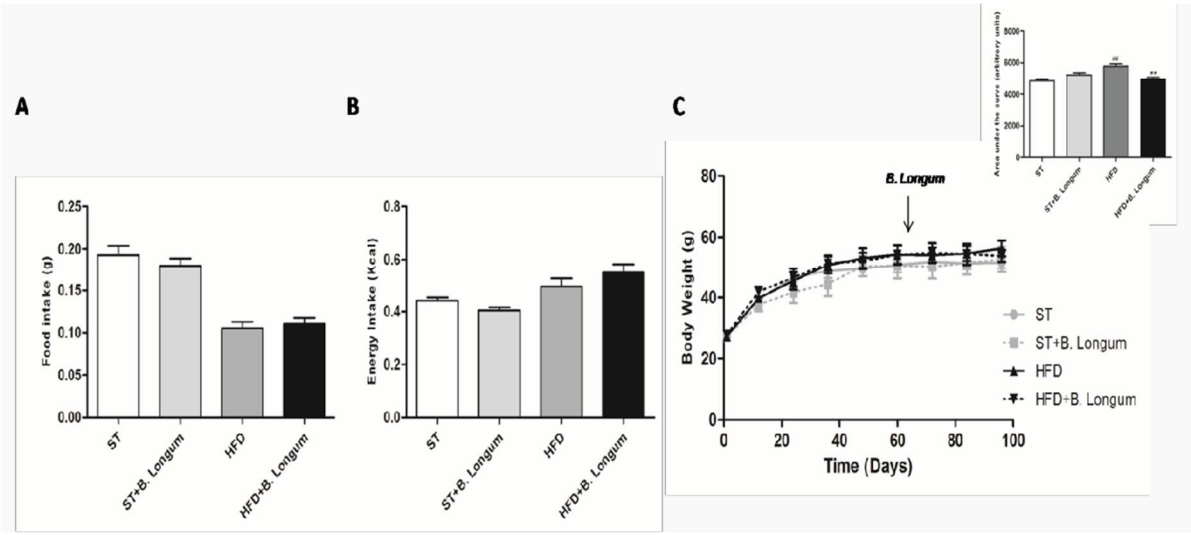

Figure 1. ***B. logum*** **reduces body weight of mice with obesity induced by the hyperlipid diet.** Food intake (a), energy intake (b), daily body weight and area under the curve (c). Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as $^{\#}p< 0.05$ $^{\#\#}$ p< 0.01 $^{\#\#\#}p<0.001$ in comparison to the ST group, and *p<0.05, **p<0.01, ***p<0.001 in comparison to the HFD group (one-way ANOVA).

The weight of the epididymal adipose tissue ((HFD, 0.029±0.007; HFD+*B. Longum*, 0.017±0.003), retroperitoneal (HFD, 0.01075±0.001; HFD+*B. Longum*, 0.005±0.001), mesenteric (HFD, 0.007±0.002; HFD+*B. Longum*, 0.005±0.001) and adiposity (HFD, 0.047±0.005; HFD+*B. Longum*, 0.028±0.004) were reduced in obese mice treated with *B. longum* **(Fig 2a-d).**

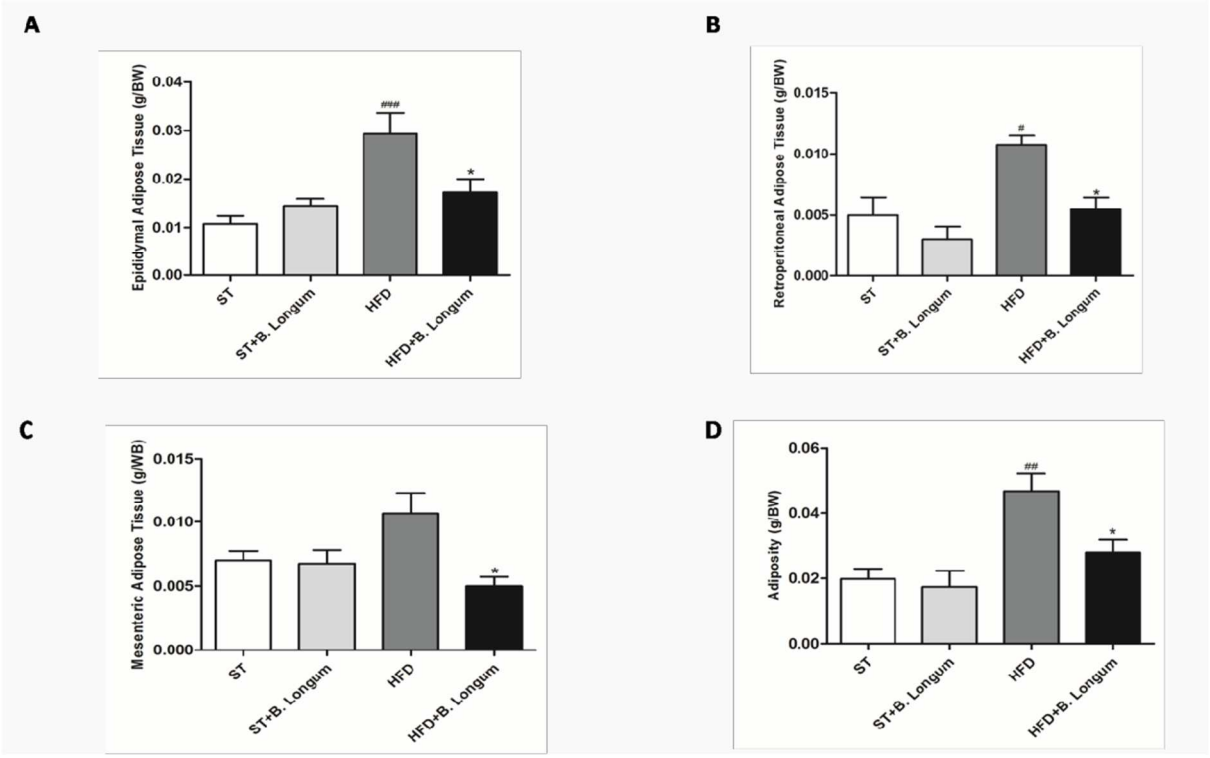

Figure 2. *B. longum* supplementation reduces adiposity of mice with obesity induced by HFD. Epididymal adipose tissue weight (a), retroperitoneal adipose tissue weight (b), mesenteric adipose tissue weight (c), adiposity (d). Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as $^{\#}p< 0.05$ $^{\#\#}$ p< 0.01 $^{\#\#\#}p<0.001$ in comparison to the ST group, and *p<0.05, **p<0.01, ***p<0.001 in comparison to the HFD group (one-way ANOVA).

Tolerance test, insulin sensitivity, and biochemical analyses

The area analysisunder the curve of the glucose tolerance test showed that obese animals treated with *B. longum*presented significantly reduced levels of glucose when compared to their respective control (HFD, 40543 ± 5215 vs. HFD +*B. Longum*, 26178 ± 2882) **(Fig. 3a)**. However, no differences were found between the groups in the insulin resistance test (4B)(ST, 6428± 183.0 vs. ST+*B. Longum*, 6928 ± 384.2; HFD, 6885± 195.2 vs. HFD+*B. Longum*, 6635±351.0) **(Fig. 3b)**. In comparison to the HFD group, the mice fed the HFD + *B. Longum* presented reduced levels of fasting glycemia (HFD, 143.8 mg/dL ± 12.24 vs. HFD + *B. Longum*, 77.00 mg/dL ± 13.8) **(Fig. 3c)** and total cholesterol (HFD, 156.7 mg/dL ± 23.73 vs. HFD + *B. Longum*, 105.7 mg/dL ± 5.78) **(Fig. 3d)**. However, the levels of triglycerides(ST,111.8 mg/dL ± 6.275 vs. ST+ *B. Longum*, 80.80 mg/dL ± 6.264; HFD, 134.7

mg/dL ± 9.528 vs. HFD +*B. Longum*,141,7 mg/dL ± 27.28) **(Fig. 3e)** and (ST,60.53 mg/dL ± 3.135 vs. ST+ *B. Longum*, 45.67 mg/dL ± 2.038; HFD, 55.33 mg/dL ± 5.549 vs. HFD +*B. Longum*,55.63 mg/dL ± 8.074) **(Fig. 3f)** did not differ statistically in comparison to their respective controls.

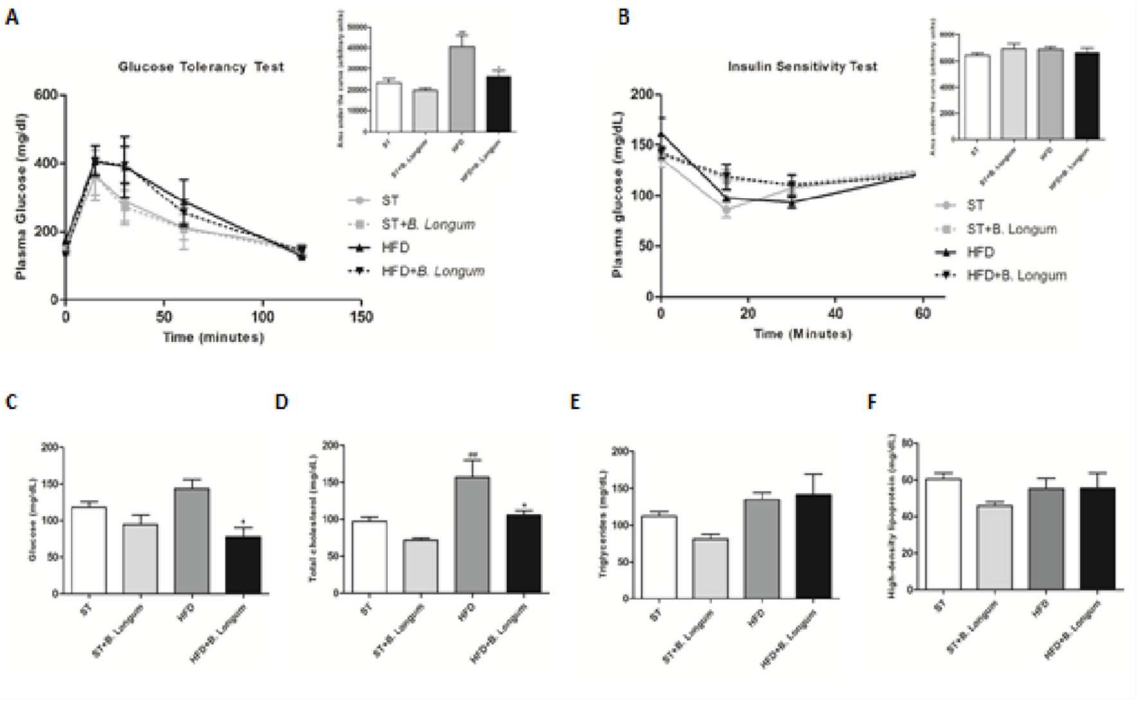

Figure 3. *B. longum* improves glucose tolerance and blood glucose and cholesterol levels in obese mice. Glucose tolerance teste and area under the curve (a), insulin sensitivity teste and area under the curve (b), glucose levels (c), total choresterol (d), triglycerides (e), and High-density lipoprotein (f). Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as $^{\#}p< 0.05$ $^{\#\#}$ p< 0.01 $^{\#\#\#}p<0.001$ in comparison to the ST group, and *p<0.05, **p<0.01, ***p<0.001 in comparison to the HFD group (one- way ANOVA).

Liver weight, hematoxylin-eosin staining, and PCR analisys

No statistically significant differences were found regarding the liver weight of the mice **(Fig. 4a).** Hepatic triglyceride decreased in the group of obese animals that received *B. longum* when compared to the obese control (HFD, 359865 ± 43636; HFD+ *B. longum*, 190024 ± 60260) **(Fig 4b-c)**.

Quantitative real-time PCR (qRT-PCR) analysis showed increased expression of ACE2 (HFD, 3.143 ± 1.244 vs. HFD +*B. Longum*, 7.158 ± 1.315) and MASR (HFD, 1.597 ± 0,584 vs. HFD +*B. Longum*,3.137 ± 0.186) in the group fed the HFD+*B. Longum* versus control HFD mice. No significant differences in the ACE (HFD, 2.171 ± 0.2619 vs. HFD +*B.*

Longum, 3.708 ± 0.510) expression were observed between groups and their respective controls (**Fig 4d-f)**.

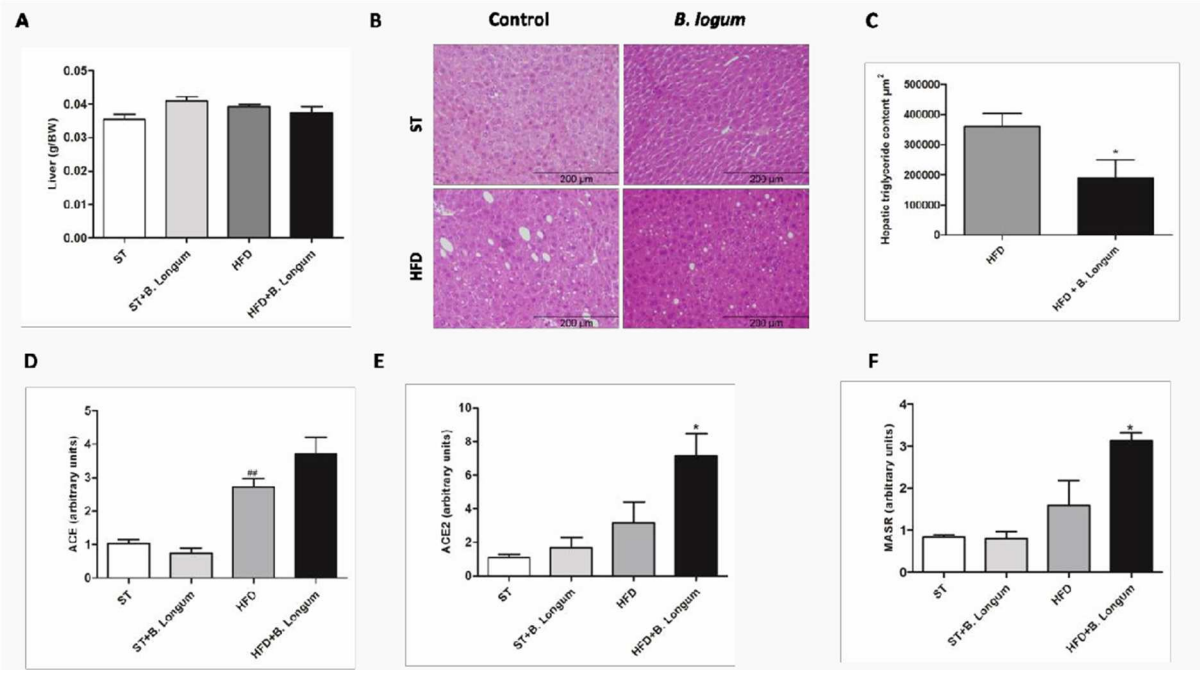

Figure 4. *B. longum* reduces the deposition of hepatic fat and modulates the expression of the axis ACE2/Ang-(1-7)/MasR of the rennin-angiotensin system. Liver weigh (a), hepatic Hematoxylin & Eosin (HE) staining (b), ImageJ analysis of hepatic triglycerides content (c), hepatic angiotensin-converting enzyme (ACE) mRNA expression (d), hepatic angiotensin-converting enzyme 2 (ACE2) mRNA expression (e), and Mas receptor (MASR) mRNA expression (f). Data are presented as means ± SEM. Statistically significant differences between the groups are indicated as $^{\#}p<0.05$ $^{\#\#\#}p<0.001$ in comparison to the ST group, and *p< 0.05, **p< 0.01, ***p< 0.001 in comparison to the HFD group (one-way ANOVA). HE ImagJ analysis was performed using Student's t-test).

Discussion

Experimental and clinical studies have shown that supplementation with probiotics exerts different metabolic effects and that these effects are strain-dependent (31, 32). There are several mechanisms by which bacterial strains perform their functions (33-35). Here, we demonstrate for the first time that probiotic supplementation with *Bifidobacterium longum* alters the expression of the RAS and the metabolism of obese mice.

Our results demonstrated an anti-obesity effect of the *B. longum* strain in obese mice. Chen et al. (36) also reported that the administration of *B. longum* improved the metabolic profile of mice fed a hyperlipidic diet, reducing body weight, adipocyte size, leptin levels, and increasing adiponectin levels. Leptin is an adipokine that acts as a global messenger of the

central nervous system (CNS), controlling food intake, energy expenditure, satiety, and appetite (37). Obesity and metabolic syndrome are characterized by a resistance to leptin due to the increase in adipokine levels (38). On the other hand, adiponectin regulates insulin sensitivity and exerts antidiabetogenic, anti-atherosclerotic, and anti-inflammatory effects, with an enhancement of metabolic efficiency when their levels are increased (39-41).

Improvement in glucose tolerance and reduction in serum glucose and total cholesterol levels of obese animals that received *B. longum* corroborate with the results of other studies (36, 42-44), and may be directly associated with particularities of the interaction of this microorganism with the intestinal microbiota of the host (17). As described previously, *Bifidobacterium* belongs to the *Bacteroidetes* phylum, and the increase in its abundance is directly related to the lean phenotype and metabolic improvement (8, 45). Microorganisms colonizing the intestinal microbiota have the ability to degrade polysaccharides and dietary fiber, producing short-chain fatty acids (SCFAs). In the intestine, SCFAs bind to the short-chain fatty acid receptor (GPR43) leading to secretion of anorexigenicpeptides, including glucagon-like peptide-1 (GLP-1) and YYpeptide (PYY), resulting in improved glucose tolerance and increased energy use (17).

Obesity is characterized by a low-grade inflammatory state (46-48) that causes increased intestinal permeability, allowing the diffusion of bacterial fragments, such as lipopolysaccharides (LPS), from the intestine to the bloodstream, resulting in metabolic endotoxemia. Individuals fed a high-fat diet have increased plasma levels of LPS (49, 50), which stimulate inflammation through interaction with TRL4 receptors (51, 52), leading to increased weight gain and fat mass, hepatic triglycerides accumulation, insulin resistance, type 2 diabetes, and atherosclerosis (44, 53). These changes were associated with a significant reduction in the population of *Lactobacillus* spp., *Bifidobacterium* spp., and *Bacteroides - Prevotella* spp in the intestine.

In addition, prebiotic fibers were added to the hyperlipid diet of rats and, as a result, an increased abundance of intestinal *Bifidobacteria* was observed, and consequently improved glucose tolerance, increased glucose-induced insulin secretion, normalized low-grade inflammation, and decreased endotoxemia and proinflammatory cytokines (44, 54-57). Studies in humans revealed that a lower *Bifidobacterium* abundance was found in overweight, obese or type 2 diabetic patients, whereas this abundance was higher in lean individuals(58). In addition, *B. longum* correlated negatively with serum levels of LPS, raising the possibility that this microorganism could be involved in the improvement of metabolic endotoxemia in human beings (58, 59).

Recent studies have shown that bacteria producing lactic acid, including *Bifidobacterium longum* (60), have hypocholesterolemic effects in rats and humans. Some possible mechanisms have been suggested such as (61-63): (1) fermentation products of lactic acid bacteria inhibit enzymes involved in the synthesis of cholesterol and thus reduce their production; (2) lactic acid bacteria facilitate the elimination of cholesterol in feces; (3) bacteria inhibit the absorption of cholesterol back into the body by binding to cholesterol; (4) bacteria interfere with the process of recycling bile salt (a metabolic product of cholesterol) and facilitate its elimination, which increases the demand for bile salt produced from cholesterol and therefore results in the consumption of body cholesterol; and (5) the assimilation of lactic acid.

Obese animals that received *B. longum* had a lower deposition of liver fat. Recently, Xu et al. demonstrated that *Bifidobacterium longum* supplementation attenuated the accumulation of hepatic fat in a model of non-alcoholic fatty liver disease (64). In addition, supplementation with VSL#3, a preparation composed of a mixture of four strains of *Lactobacillus* (*L. casei, L. plantarum, L. acidophilus, and L. delbrueckii subsp. bulgaricus*), three strains of *Bifidobacterium* (*B. longum, B. breve,* and *B. infantis*), and *Streptococcus thermophilus*, improved the fat-induced dietary hepatic steatosis and insulin resistance in mice by modulating hepatic natural killer T cells (NKT cells), suppressing the TNF-α/IKK-β signaling pathway, and reducingthe inflammatory signaling (65). It has also been suggested in some studies that *B. longum* can mitigate hepatic injuries caused by endotoxin-induced activation of macrophages (66, 67).

Increased hepatic ECA2 and MasR expression observed in obese animals treated with *B. longum* describes for the first time a modulation of the ECA2/Ang- (1-7)/MasR axis by a probiotic strain. In addition, the metabolic improvement observed in treated animals is also associated with RAS, since the increased expression of ACE2 and the Mas receptor is directly related to the increase in Ang- (1-7) (68). The first study showing the metabolic potential of Ang-(1-7)/MasR was performed by Santos et al. (26), who demonstrated a worsening of metabolic efficiency, as wel as dyslipidemia, insulin resistance, and decreased expression of adiponectin and GLUT-4 in transgenic mice with Mas receptor suppression. Later, it was revealed that circulating Ang- (1-7) overexpression improved the metabolic profile by lowering lipids and reducing adipose tissue mass in TGR-L3292 rats (68).

Regarding hepatic homeostasis,an oral administration of Ang-(1-7) may decrease gluconeogenesis in the liver, improving glucose and lipid metabolism (69). In addition, in HFD-fed rats, inflammatory marker levels were decreased by regulation of the

resistin/TLR4/NF-KB pathway (68). Another remarkable finding was that obese mice treated with an oral formulation of Ang-(1-7) showed a significant reduction in levels of TNF-β and IL-6, as well as decreased markers related to adipogenesis, such as acetyl CoA carboxylase (ACC), PPAR-Y, and sterol-1c binding regulatory proteins (SREBP-1c) (70).

In summary, we showed that oral supplementation with *B. longum* improved the metabolic profile of mice with hyperlipidemic diet-induced obesity, decreasing body weight, adiposity, glucose tolerance, serum glucose, and total cholesterol levels. Analysis of hepatic tissue revealed that treated-obese mice had lower triglyceride deposition in the liver. Additionally, increased ECA2/Ang- (1-7)/MASR axis expression was observed in obese-treated animals. The results of the present study suggest, for the first time, the modulation of the RAS by the *B. longum*strain. These findings may contribute to better understand the metabolic effects mediated by probiotics. However, the mechanisms that involve the possible role of *B. longum* in the activation of the ECA2/Ang-(1-7)/MASR axis of the RAS in the liver need to be further investigated.

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Author contributions

RA.S.S, S.H.S.S, A.S.M, J.R.O. and D.F.L. contributed to the design of the study, acquisition of the data, and analysis and interpretation of the data. J.M.O.A., D.F.L., S.H.S.S. and I.V.B. contributed to drafting the article and revising it critically for important intellectual content, and A.M.B.P. and A.L.S.G. contributed to the final approval of the version to be submitted. All authors read and approved the final manuscript.

Conflict(s) of Interest/Disclosure(s)

The authors declare no competing interest/ disclosure(s).

References

1. GBDO C, Afshin A, Forouzanfar MH, Reitsma MB, Sur P, K. Estep ea. Health effects of overweight and obesity in 195 countries over 25 years. N Engl J Med. 2017;377:13-27.
2. Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. International journal of obesity. 2008;32(9):1431-7.
3. Hill JO, Wyatt HR, Peters JC. Energy balance and obesity. Circulation. 2012;126(1):126-32.
4. Fontaine KR, Redden DT, Wang C, Westfall AO, Allison DB. Years of life lost due to obesity. Jama. 2003;289(2):187-93.
5. Bluher M. Adipose tissue inflammation: a cause or consequence of obesity-related insulin resistance? Clinical science. 2016;130(18):1603-14.
6. Rosso N, Chavez-Tapia NC, Tiribelli C, Bellentani S. Translational approaches: from fatty liver to non-alcoholic steatohepatitis. World journal of gastroenterology. 2014;20(27):9038-49.
7. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO reports. 2006;7(7):688-93.
8. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-20.
9. Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annual review of nutrition. 2002;22:283-307.
10. Boulange CL, Neves AL, Chilloux J, Nicholson JK, Dumas ME. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome medicine. 2016;8(1):42.
11. Azad MAK, Sarker M, Li T, Yin J. Probiotic Species in the Modulation of Gut Microbiota: An Overview. BioMed research international. 2018;2018:9478630.
12. Hentges DJ. The anaerobic microflora of the human body. Clinical infectious diseases
: an official publication of the Infectious Diseases Society of America. 1993;16 Suppl 4:S175-80.
13. FAO/WHO. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report. 2001.
14. Walsh CJ, Guinane CM, O'Toole PW, Cotter PD. Beneficial modulation of the gut microbiota. FEBS letters. 2014;588(22):4120-30.
15. Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. The British journal of nutrition. 2014;111(3):387-402.
16. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013;341(6145):569-73.
17. Mazloom K, Siddiqi I, Covasa M. Probiotics: How Effective Are They in the Fight against Obesity? Nutrients. 2019;11(2).
18. Frick JS, Schenk K, Quitadamo M, Kahl F, Koberle M, Bohn E, et al. Lactobacillus fermentum attenuates the proinflammatory effect of Yersinia enterocolitica on human epithelial cells. Inflammatory bowel diseases. 2007;13(1):83-90.
19. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature reviews Immunology. 2004;4(6):478-85.
20. Gourbeyre P, Denery S, Bodinier M. Probiotics, prebiotics, and synbiotics: impact on

the gut immune system and allergic reactions. Journal of leukocyte biology. 2011;89(5):685-95.

21. Musso G, Gambino R, Cassader M. Gut microbiota as a regulator of energy homeostasis and ectopic fat deposition: mechanisms and implications for metabolic disorders. Current opinion in lipidology. 2010;21(1):76-83.
22. An HM, Park SY, Lee DK, Kim JR, Cha MK, Lee SW, et al. Antiobesity and lipid-lowering effects of Bifidobacterium spp. in high fat diet-induced obese rats. Lipids in health and disease. 2011;10:116.
23. Karimi G, Jamaluddin R, Mohtarrudin N, Ahmad Z, Khazaai H, Parvaneh M. Single-species versus dual-species probiotic supplementation as an emerging therapeutic strategy for obesity. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2017;27(10):910-8.
24. Santos SH, Andrade JM, Fernandes LR, Sinisterra RD, Sousa FB, Feltenberger JD, et al. Oral Angiotensin-(1-7) prevented obesity and hepatic inflammation by inhibition of resistin/TLR4/MAPK/NF-kappaB in rats fed with high-fat diet. Peptides. 2013;46:47-52.
25. Santos SH, Braga JF, Mario EG, Porto LC, Rodrigues-Machado Mda G, Murari A, et al. Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1-7). Arteriosclerosis, thrombosis, and vascular biology. 2010;30(5):953-61.
26. Santos SH, Fernandes LR, Mario EG, Ferreira AV, Porto LC, Alvarez-Leite JI, et al. Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. Diabetes. 2008;57(2):340-7.
27. Haslam DW, James WP. Obesity. Lancet. 2005;366(9492):1197-209.
28. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nature reviews Cardiology. 2009;6(6):399-409.
29. Rashid SK, Idris-Khodja N, Auger C, Alhosin M, Boehm N, Oswald-Mammosser M, et al. Probiotics (VSL#3) prevent endothelial dysfunction in rats with portal hypertension: role of the angiotensin system. PloS one. 2014;9(5):e97458.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
31. Million M, Angelakis E, Paul M, Armougom F, Leibovici L, Raoult D. Comparative meta-analysis of the effect of Lactobacillus species on weight gain in humans and animals. Microbial pathogenesis. 2012;53(2):100-8.
32. Dong H, Rowland I, Yaqoob P. Comparative effects of six probiotic strains on immune function in vitro. The British journal of nutrition. 2012;108(3):459-70.
33. Firouzi S, Barakatun-Nisak MY, Ismail A, Majid HA, Nor Azmi K. Role of probiotics in modulating glucose homeostasis: evidence from animal and human studies. International journal of food sciences and nutrition. 2013;64(6):780-6.
34. Parvaneh K, Ebrahimi M, Sabran MR, Karimi G, Hwei AN, Abdul-Majeed S, et al. Probiotics (Bifidobacterium longum) Increase Bone Mass Density and Upregulate Sparc and Bmp-2 Genes in Rats with Bone Loss Resulting from Ovariectomy. BioMed research international. 2015;2015:897639.
35. karimi G, Jamaluddin R, Parvaneh K. The Effects of Probiotics on Body Weight and Biomarkers of Animal. Pakistan Journal of Nutrition. 2013;12:793-9.
36. Chen JJ, Wang R, Li XF, Wang RL. Bifidobacterium longum supplementation improved high-fat-fed-induced metabolic syndrome and promoted intestinal Reg I gene expression. Experimental biology and medicine. 2011;236(7):823-31.
37. Zhang PR, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994;372(6505):425-32.
38. Ekmen N, Helvaci A, Gunaldi M, Sasani H, Yildirmak ST. Leptin as an important link between obesity and cardiovascular risk factors in men with acute myocardial infarction. Indian heart journal. 2016;68(2):132-7.

39. Bluher M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. Best practice & research Clinical endocrinology & metabolism. 2013;27(2):163-77.
40. Bluher M, Mantzoros CS. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. Metabolism: clinical and experimental. 2015;64(1):131-45.
41. Blüher M. Importance of adipokines in glucose homeostasis. Diabetes Manage. 2013;3(5):389–400.
42. Backhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(3):979-84.
43. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(31):11070-5.
44. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56(7):1761-72.
45. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444(7122):1022-3.
46. Kuwahara A. Contributions of colonic short-chain Fatty Acid receptors in energy homeostasis. Frontiers in endocrinology. 2014;5:144.
47. Tilg H, Kaser A. Gut microbiome, obesity, and metabolic dysfunction. The Journal of clinical investigation. 2011;121(6):2126-32.
48. Boroni Moreira AP, Fiche Salles Teixeira T, do CGPM, de Cassia Goncalves Alfenas
R. Gut microbiota and the development of obesity. Nutricion hospitalaria. 2012;27(5):1408-14.
49. Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. The American journal of clinical nutrition. 2007;86(5):1286-92.
50. Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, et al. Energy intake is associated with endotoxemia in apparently healthy men. The American journal of clinical nutrition. 2008;87(5):1219-23.
51. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet- induced obesity and diabetes in mice. Diabetes. 2008;57(6):1470-81.
52. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004;118(2):229-41.
53. Cani PD. Human gut microbiome: hopes, threats and promises. Gut. 2018;67(9):1716- 25.
54. Tuohy KM, Rouzaud GC, Bruck WM, Gibson GR. Modulation of the human gut microflora towards improved health using prebiotics--assessment of efficacy. Current pharmaceutical design. 2005;11(1):75-90.
55. Everard A, Cani PD. Diabetes, obesity and gut microbiota. Best practice & research Clinical gastroenterology. 2013;27(1):73-83.
56. O'Mahony D, Murphy S, Boileau T, Park J, O'Brien F, Groeger D, et al. Bifidobacterium animalis AHC7 protects against pathogen-induced NF-kappaB activation in vivo. BMC immunology. 2010;11:63.
57. Furet JP, Kong LC, Tap J, Poitou C, Basdevant A, Bouillot JL, et al. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes. 2010;59(12):3049-57.

58. Wu X, Ma C, Han L, Nawaz M, Gao F, Zhang X, et al. Molecular characterisation of the faecal microbiota in patients with type II diabetes. Current microbiology. 2010;61(1):69-78.

59. Schwiertz A, Taras D, Schafer K, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity. 2010;18(1):190-5.

60. Xiao JZ, Kondo S, Takahashi N, Miyaji K, Oshida K, Hiramatsu A, et al. Effects of milk products fermented by Bifidobacterium longum on blood lipids in rats and healthy adult male volunteers. Journal of dairy science. 2003;86(7):2452-61.

61. Beena A, Prasad V. Effect of yogurt and bifidus yogurt fortified with skim milk powder, condensed whey and lactose-hydrolysed condensed whey on serum cholesterol and triacylglycerol levels in rats. The Journal of dairy research. 1997;64(3):453-7.

62. Fukushima M, Nakano M. The effect of a probiotic on faecal and liver lipid classes in rats. The British journal of nutrition. 1995;73(5):701-10.

63. Kim SJ, Park SH, Sin HS, Jang SH, Lee SW, Kim SY, et al. Hypocholesterolemic Effects of Probiotic Mixture on Diet-Induced Hypercholesterolemic Rats. Nutrients. 2017;9(3).

64. Xu RY, Wan YP, Fang QY, Lu W, Cai W. Supplementation with probiotics modifies gut flora and attenuates liver fat accumulation in rat nonalcoholic fatty liver disease model. Journal of clinical biochemistry and nutrition. 2012;50(1):72-7.

65. Ma X, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. Journal of hepatology. 2008;49(5):821-30.

66. Liu Q, Duan ZP, Ha DK, Bengmark S, Kurtovic J, Riordan SM. Synbiotic modulation of gut flora: effect on minimal hepatic encephalopathy in patients with cirrhosis. Hepatology. 2004;39(5):1441-9.

67. Fuller R. Probiotics in human medicine. Gut. 1991;32(4):439-42.

68. Santos SH, Andrade JM. Angiotensin 1-7: a peptide for preventing and treating metabolic syndrome. Peptides. 2014;59:34-41.

69. Bilman V, Mares-Guia L, Nadu AP, Bader M, Campagnole-Santos MJ, Santos RA, et al. Decreased hepatic gluconeogenesis in transgenic rats with increased circulating angiotensin-(1-7). Peptides. 2012;37(2):247-51.

70. Feltenberger JD, Andrade JM, Paraiso A, Barros LO, Filho AB, Sinisterra RD, et al. Oral formulation of angiotensin-(1-7) improves lipid metabolism and prevents high-fat diet-induced hepatic steatosis and inflammation in mice. Hypertension. 2013;62(2):324-30.

4 CONCLUSÕES

Conclui-se que a ang-(1-7) é um importante mediador negativo de processos inflamatórios agudos e crônicos, regulando a secreção e ação das adipocinas, e funcionando muitas vezes como uma adipocina, com ação em diferentes órgãos. Além disso, a administração oral de ANG- (1-7) melhora a hiperglicemia, dislipidemia e altera a morfologia das vilosidades intestinais, possivelmente através da modulação da microbiota intestinal, com consequente melhora da disbiose intestinal e da endotoxemia metabólica. Além disso, mostramos que a suplementação oral com o probiotico *B. longum* melhorou o perfil metabólico e diminuiu a deposição de triglicerídeos no fígado de camundongos com obesos. Adicionalmente, foi observado um aumento da expressão do eixo ECA2/Ang-(1-7)/MASR.

Em conjunto, esses achados sugerem para o promissor papel da ang-(1-7) e do probiótico *B longum* no tratamento dos distúrbios metabólicos associados à obesidade por meio da regulação da inflamação e da microbiota intestinal.

REFERÊNCIAS

1. Organization WH. Obesity: preventing and managing the global epidemic Report of a WHO consultation. World Health Organ Tech Rep Ser. 2000;894:1-253.
2. GBDO C, Afshin A, Forouzanfar MH, Reitsma MB, Sur P, K. Estep ea. Health effects of overweight and obesity in 195 countries over 25 years. N Engl J Med. 2017;377:13-27.
3. Organization WH. Obesity and overweight. 2018.
4. NCDRF C. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. Lancet. 2016;387:1377-96.
5. Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. International journal of obesity. 2008;32(9):1431-7.
6. Organization WH. Obesity: preventing and managing the global epidemic Report of a WHO Consultation (WHO Technical Report Series 894). Tech Rep Ser. 2000;894:1-253.
7. Expert Panel on the Identification E, and Treatment of Overweight and Obesity in Adults, (Ed.) NIoH. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults - the evidence report - NIH PUBLICATION NO. National Institutes of Health Bethesda. 1998:98-4083.
8. Hill JO, Wyatt HR, Peters JC. Energy balance and obesity. Circulation. 2012;126(1):126-32.
9. Walley AJ, Blakemore AI, Froguel P. Genetics of obesity and the prediction of risk for health. Human molecular genetics. 2006;15 Spec No 2:R124-30.
10. Fontaine KR, Redden DT, Wang C, Westfall AO, Allison DB. Years of life lost due to obesity. Jama. 2003;289(2):187-93.
11. Bluher M. Adipose tissue inflammation: a cause or consequence of obesity-related insulin resistance? Clinical science. 2016;130(18):1603-14.
12. Rosso N, Chavez-Tapia NC, Tiribelli C, Bellentani S. Translational approaches: from fatty liver to non-alcoholic steatohepatitis. World journal of gastroenterology. 2014;20(27):9038-49.
13. Heo M, Allison DB, Faith MS, Zhu S, Fontaine KR. Obesity and quality of life: mediating effects of pain and comorbidities. Obesity research. 2003;11(2):209-16.
14. Tremmel M, Gerdtham UG, Nilsson PM, Saha S. Economic Burden of Obesity: A Systematic Literature Review. International journal of environmental research and public health. 2017;14(4).
15. Lelis DF, Freitas DF, Machado AS, Crespo TS, Santos SHS. Angiotensin-(1-7), Adipokines and Inflammation. Metabolism: clinical and experimental. 2019;95:36-45.
16. Yu J, Wu Y, Zhang Y, Zhang L, Ma Q, Luo X. [Role of ACE2-Ang (1-7)-Mas receptor axis in heart failure with preserved ejection fraction with hypertension]. Zhong nan da xue xue bao Yi xue ban = Journal of Central South University Medical sciences. 2018;43(7):738-46.
17. Passos-Silva DG, Brandan E, Santos RA. Angiotensins as therapeutic targets beyond heart disease. Trends in pharmacological sciences. 2015;36(5):310-20.
18. Borem LMA, Neto JFR, Brandi IV, Lelis DF, Santos SHS. The role of the angiotensin II type I receptor blocker telmisartan in the treatment of non-alcoholic fatty liver disease: a brief review. Hypertension research : official journal of the Japanese Society of Hypertension. 2018;41(6):394-405.
19. Santos SH, Andrade JM. Angiotensin 1-7: a peptide for preventing and treating metabolic syndrome. Peptides. 2014;59:34-41.

20. Lautner RQ, Villela DC, Fraga-Silva RA, Silva N, Verano-Braga T, Costa-Fraga F, et al. Discovery and characterization of alamandine: a novel component of the renin-angiotensin system. Circulation research. 2013;112(8):1104-11.
21. Qaradakhi T, Apostolopoulos V, Zulli A. Angiotensin (1-7) and Alamandine: Similarities and differences. Pharmacological research. 2016;111:820-6.
22. Hernandez Schulman I, Zhou MS, Raij L. Cross-talk between angiotensin II receptor types 1 and 2: potential role in vascular remodeling in humans. Hypertension. 2007;49(2):270-1.
23. Kaschina E, Namsolleck P, Unger T. AT2 receptors in cardiovascular and renal diseases. Pharmacological research. 2017;125(Pt A):39-47.
24. Ribeiro-Oliveira A, Jr., Nogueira AI, Pereira RM, Boas WW, Dos Santos RA, Simoes e Silva AC. The renin-angiotensin system and diabetes: an update. Vascular health and risk management. 2008;4(4):787-803.
25. Shim KY, Eom YW, Kim MY, Kang SH, Baik SK. Role of the renin-angiotensin system in hepatic fibrosis and portal hypertension. The Korean journal of internal medicine. 2018;33(3):453-61.
26. Santos SH, Fernandes LR, Mario EG, Ferreira AV, Porto LC, Alvarez-Leite JI, et al. Mas deficiency in FVB/N mice produces marked changes in lipid and glycemic metabolism. Diabetes. 2008;57(2):340-7.
27. Xu P, Costa-Goncalves AC, Todiras M, Rabelo LA, Sampaio WO, Moura MM, et al. Endothelial dysfunction and elevated blood pressure in MAS gene-deleted mice. Hypertension. 2008;51(2):574-80.
28. Takeda M, Yamamoto K, Takemura Y, Takeshita H, Hongyo K, Kawai T, et al. Loss of ACE2 exaggerates high-calorie diet-induced insulin resistance by reduction of GLUT4 in mice. Diabetes. 2013;62(1):223-33.
29. Giani JF, Mayer MA, Munoz MC, Silberman EA, Hocht C, Taira CA, et al. Chronic infusion of angiotensin-(1-7) improves insulin resistance and hypertension induced by a high-fructose diet in rats. American journal of physiology Endocrinology and metabolism. 2009;296(2):E262-71.
30. Marcus Y, Shefer G, Sasson K, Kohen F, Limor R, Pappo O, et al. Angiotensin 1-7 as means to prevent the metabolic syndrome: lessons from the fructose-fed rat model. Diabetes. 2013;62(4):1121-30.
31. Santos SH, Fernandes LR, Pereira CS, Guimaraes AL, de Paula AM, Campagnole-Santos MJ, et al. Increased circulating angiotensin-(1-7) protects white adipose tissue against development of a proinflammatory state stimulated by a high-fat diet. Regulatory peptides. 2012;178(1-3):64-70.
32. Santos SH, Braga JF, Mario EG, Porto LC, Rodrigues-Machado Mda G, Murari A, et al. Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1-7). Arteriosclerosis, thrombosis, and vascular biology. 2010;30(5):953-61.
33. Kloting N, Bluher M. Adipocyte dysfunction, inflammation and metabolic syndrome. Reviews in endocrine & metabolic disorders. 2014;15(4):277-87.
34. Bluher M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. Best practice & research Clinical endocrinology & metabolism. 2013;27(2):163-77.
35. Bluher M, Mantzoros CS. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. Metabolism: clinical and experimental. 2015;64(1):131-45.
36. Blüher M. Importance of adipokines in glucose homeostasis. Diabetes Manage. 2013;3(5):389-400.
37. Bluher M. Adipokines - removing road blocks to obesity and diabetes therapy. Molecular metabolism. 2014;3(3):230-40.

38. Bluher M. Adipose tissue dysfunction in obesity. Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association. 2009;117(6):241-50.
39. Lehr S, Hartwig S, Sell H. Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders. Proteomics Clinical applications. 2012;6(1-2):91-101.
40. Van de Voorde J, Pauwels B, Boydens C, Decaluwe K. Adipocytokines in relation to cardiovascular disease. Metabolism: clinical and experimental. 2013;62(11):1513-21.
41. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. The Journal of clinical endocrinology and metabolism. 2004;89(6):2548-56.
42. Bluher M. Clinical relevance of adipokines. Diabetes & metabolismjournal. 2012;36(5):317-27.
43. Cassis LA, Police SB, Yiannikouris F, Thatcher SE. Local adipose tissue renin-angiotensin system. Current hypertension reports. 2008;10(2):93-8.
44. Yvan-Charvet L, Quignard-Boulange A. Role of adipose tissue renin-angiotensin system in metabolic and inflammatory diseases associated with obesity. Kidneyinternational. 2011;79(2):162-8.
45. Rubio-Ruiz ME, Del Valle-Mondragon L, Castrejon-Tellez V, Carreon-Torres E, Diaz-Diaz E, Guarner-Lans V. Angiotensin II and 1-7 during aging in Metabolic Syndrome rats. Expression of AT1, AT2 and Mas receptors in abdominal white adipose tissue. Peptides. 2014;57:101-8.
46. Liu C, Lv XH, Li HX, Cao X, Zhang F, Wang L, et al. Angiotensin-(1-7) suppresses oxidative stress and improves glucose uptake via Mas receptor in adipocytes. Acta diabetologica. 2012;49(4):291-9.
47. Antuna-Puente B, Feve B, Fellahi S, Bastard JP. Adipokines: the missing link between insulin resistance and obesity. Diabetes & metabolism. 2008;34(1):2-11.
48. Santos SH, Andrade JM, Fernandes LR, Sinisterra RD, Sousa FB, Feltenberger JD, et al. Oral Angiotensin-(1-7) prevented obesity and hepatic inflammation by inhibition of resistin/TLR4/MAPK/NF-kappaB in rats fed with high-fat diet. Peptides. 2013;46:47-52.
49. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science. 2008;320(5883):1647-51.
50. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO reports. 2006;7(7):688-93.
51. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science. 2005;308(5728):1635-8.
52. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312(5778):1355-9.
53. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-20.
54. Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annual review of nutrition. 2002;22:283-307.
55. Boulange CL, Neves AL, Chilloux J, Nicholson JK, Dumas ME. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome medicine. 2016;8(1):42.
56. Petriz BA, Castro AP, Almeida JA, Gomes CP, Fernandes GR, Kruger RH, et al. Exercise induction of gut microbiota modifications in obese, non-obese and hypertensive rats. BMC genomics. 2014;15:511.
57. Sun J, Chang EB. Exploring gut microbes in human health and disease: Pushing the envelope. Genes & diseases. 2014;1(2):132-9.
58. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy

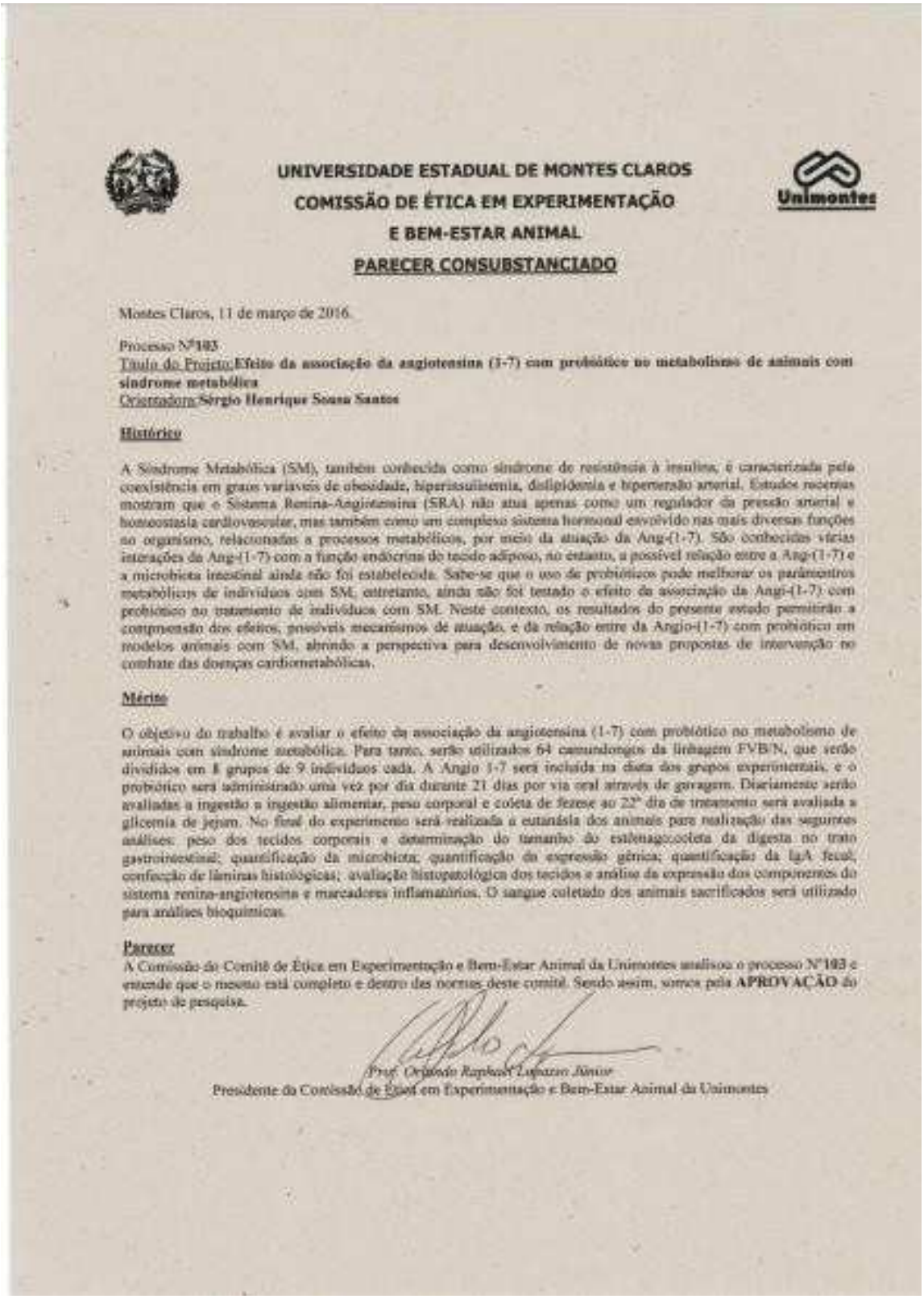
metabolism. Journal of lipid research. 2013;54(9):2325-40.
59. Backhed F, Fraser CM, Ringel Y, Sanders ME, Sartor RB, Sherman PM, et al. Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. Cell host & microbe. 2012;12(5):611-22.
60. Duca F, Gerard P, Covasa M, Lepage P. Metabolic interplay between gut bacteria and their host. Frontiers of hormone research. 2014;42:73-82.
61. Le Roy T, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, et al. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. Gut. 2013;62(12):1787-94.
62. Mayer EA, Tillisch K, Gupta A. Gut/brain axis and the microbiota. The Journal of clinical investigation. 2015;125(3):926-38.
63. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature. 2011;473(7346):174-80.
64. Murphy EF, Cotter PD, Healy S, Marques TM, O'Sullivan O, Fouhy F, et al. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. Gut. 2010;59(12):1635-42.
65. Sanz Y, Santacruz A, Gauffin P. Gut microbiota in obesity and metabolic disorders. The Proceedings of the Nutrition Society. 2010;69(3):434-41.
66. Million M, Lagier JC, Yahav D, Paul M. Gut bacterial microbiota and obesity. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2013;19(4):305-13.
67. Baothman OA, Zamzami MA, Taher I, Abubaker J, Abu-Farha M. The role of Gut Microbiota in the development of obesity and Diabetes. Lipids in health and disease. 2016;15:108.
68. Wostmann BS, Larkin C, Moriarty A, Bruckner-Kardoss E. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Laboratory animal science. 1983;33(1):46-50.
69. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(44):15718-23.
70. Backhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(3):979-84.
71. Karlsson F, Tremaroli V, Nielsen J, Backhed F. Assessing the human gut microbiota in metabolic diseases. Diabetes. 2013;62(10):3341-9.
72. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444(7122):1022-3.
73. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(31):11070-5.
74. Fleissner CK, Huebel N, Abd El-Bary MM, Loh G, Klaus S, Blaut M. Absence of intestinal microbiota does not protect mice from diet-induced obesity. The British journal of nutrition. 2010;104(6):919-29.
75. Villanueva-Millan MJ, Perez-Matute P, Oteo JA. Gut microbiota: a key player in health and disease. A review focused on obesity. Journal of physiology and biochemistry. 2015;71(3):509-25.
76. Arora T, Sharma R. Fermentation potential of the gut microbiome: implications for energy homeostasis and weight management. Nutrition reviews. 2011;69(2):99-106.
77. Alang N, Kelly CR. Weight gain after fecal microbiota transplantation. Open forum infectious diseases. 2015;2(1):ofv004.

78. Candido FG, Valente FX, Grzeskowiak LM, Moreira APB, Rocha D, Alfenas RCG. Impact of dietary fat on gut microbiota and low-grade systemic inflammation: mechanisms and clinical implications on obesity. International journal of food sciences and nutrition. 2018;69(2):125-43.
79. Singh DP, Singh J, Boparai RK, Zhu J, Mantri S, Khare P, et al. Isomalto-oligosaccharides, a prebiotic, functionally augment green tea effects against high fat diet-induced metabolic alterations via preventing gut dysbacteriosis in mice. Pharmacological research. 2017;123:103-13.
80. Cani PD, Osto M, Geurts L, Everard A. Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. Gut microbes. 2012;3(4):279-88.
81. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. American journal of physiology Gastrointestinal and liver physiology. 2010;299(2):G440-8.
82. Fei N, Zhao L. An opportunistic pathogen isolated from the gut of an obese human causes obesity in germfree mice. The ISME Journal. 2013;7:880–4.
83. StielerStewart A, ShannonPratt-Phillips, M.Gonzalez L. Alterations in Intestinal Permeability: The Role of the “Leaky Gut” in Health and Disease. Journal of Equine Veterinary Science. 2017;52:10-22.
84. Soares A, Beraldi EJ, Ferreira PE, Bazotte RB, Buttow NC. Intestinal and neuronal myenteric adaptations in the small intestine induced by a high-fat diet in mice. BMC gastroenterology. 2015;15:3.
85. Zamolodchikova TS, Shoibonov BB, Tolpygo SM. Local Renin-Angiotensin System of Small Intestine. Eksperimental'naia i klinicheskaia gastroenterologiia = Experimental & clinical gastroenterology. 2016;12(12):97-104.
86. Koga H, Yang H, Haxhija EQ, Teitelbaum DH. The role of angiotensin II type 1a receptor on intestinal epithelial cells following small bowel resection in a mouse model. Pediatric surgery international. 2008;24(12):1279-86.
87. Fandriks L. The renin-angiotensin system and the gastrointestinal mucosa. Acta physiologica. 2011;201(1):157-67.
88. Wong TP, Debnam ES, Leung PS. Involvement of an enterocyte renin-angiotensin system in the local control of SGLT1-dependent glucose uptake across the rat small intestinal brush border membrane. The Journal of physiology. 2007;584(Pt 2):613-23.
89. Hashimoto T, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, et al. ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. Nature. 2012;487(7408):477-81.
90. Camargo SM, Singer D, Makrides V, Huggel K, Pos KM, Wagner CA, et al. Tissue-specific amino acid transporter partners ACE2 and collectrin differentially interact with hartnup mutations. Gastroenterology. 2009;136(3):872-82.
91. Cole-Jeffrey CT, Liu M, Katovich MJ, Raizada MK, Shenoy V. ACE2 and Microbiota: Emerging Targets for Cardiopulmonary Disease Therapy. Journal of cardiovascular pharmacology. 2015;66(6):540-50.
92. Zhang H, Wada J, Hida K, Tsuchiyama Y, Hiragushi K, Shikata K, et al. Collectrin, a collecting duct-specific transmembrane glycoprotein, is a novel homolog of ACE2 and is developmentally regulated in embryonic kidneys. The Journal of biological chemistry. 2001;276(20):17132-9.
93. Borges EL, Lima PB, Peluso AAB, Sampaio WO, Oliveira JSd, Oliveira MLd, et al. Angiotensin-(1-7) Influences Tryptophan Absorption in the Rat and Mouse Intestine. British Journal of Medicine & Medical Research. 2017;4(19):1-9.

94. FAO/WHO. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report. 2001.
95. Fooks LJ, Gibson GR. Probiotics as modulators of the gut flora. The British journal of nutrition. 2002;88 Suppl 1:S39-49.
96. Walsh CJ, Guinane CM, O'Toole PW, Cotter PD. Beneficial modulation of the gut microbiota. FEBS letters. 2014;588(22):4120-30.
97. Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. The British journal of nutrition. 2014;111(3):387-402.
98. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013;341(6145):569-73.
99. Mazloom K, Siddiqi I, Covasa M. Probiotics: How Effective Are They in the Fight against Obesity? Nutrients. 2019;11(2).
100. Frick JS, Schenk K, Quitadamo M, Kahl F, Koberle M, Bohn E, et al. Lactobacillus fermentum attenuates the proinflammatory effect of Yersinia enterocolitica on human epithelial cells. Inflammatory bowel diseases. 2007;13(1):83-90.
101. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature reviews Immunology. 2004;4(6):478-85.
102. Gourbeyre P, Denery S, Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. Journal of leukocyte biology. 2011;89(5):685-95.
103. Musso G, Gambino R, Cassader M. Gut microbiota as a regulator of energy homeostasis and ectopic fat deposition: mechanisms and implications for metabolic disorders. Current opinion in lipidology. 2010;21(1):76-83.
104. An HM, Park SY, Lee DK, Kim JR, Cha MK, Lee SW, et al. Antiobesity and lipid-lowering effects of Bifidobacterium spp. in high fat diet-induced obese rats. Lipids in health and disease. 2011;10:116.
105. Karimi G, Jamaluddin R, Mohtarrudin N, Ahmad Z, Khazaai H, Parvaneh M. Single-species versus dual-species probiotic supplementation as an emerging therapeutic strategy for obesity. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2017;27(10):910-8.
106. Chen JJ, Wang R, Li XF, Wang RL. Bifidobacterium longum supplementation improved high-fat-fed-induced metabolic syndrome and promoted intestinal Reg I gene expression. Experimental biology and medicine. 2011;236(7):823-31.
107. Lee SJ, Bose S, Seo JG, Chung WS, Lim CY, Kim H. The effects of co-administration of probiotics with herbal medicine on obesity, metabolic endotoxemia and dysbiosis: a randomized double-blind controlled clinical trial. Clinical nutrition. 2014;33(6):973-81.

ANEXOS

ANEXO A – Parecer do Comitê de Ética e Pesquisa



UNIVERSIDADE ESTADUAL DE MONTES CLAROS
COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO
E BEM-ESTAR ANIMAL
PARECER CONSUBSTANCIADO

Montes Claros, 11 de março de 2016.

Processo Nº103
Título do Projeto: **Efeito da associação da angiotensina (1-7) com probiótico no metabolismo de animais com síndrome metabólica**
Orientadora: **Sérgio Henrique Sousa Santos**

Histórico

A Síndrome Metabólica (SM), também conhecida como síndrome de resistência à insulina, é caracterizada pela coexistência em graus variáveis de obesidade, hiperinsulinemia, dislipidemia e hipertensão arterial. Estudos recentes mostram que o Sistema Renina-Angiotensina (SRA) não atua apenas como um regulador da pressão arterial e homeostasia cardiovascular, mas também como um complexo sistema hormonal envolvido nas mais diversas funções no organismo, relacionadas a processos metabólicos, por meio da atuação da Ang-(1-7). São conhecidas várias interações da Ang-(1-7) com a função endócrina do tecido adiposo, no entanto, a possível relação entre a Ang-(1-7) e a microbiota intestinal ainda não foi estabelecida. Sabe-se que o uso de probióticos pode melhorar os parâmetros metabólicos de indivíduos com SM, entretanto, ainda não foi testado o efeito da associação da Angi-(1-7) com probiótico no tratamento de indivíduos com SM. Neste contexto, os resultados do presente estudo permitirão a compreensão dos efeitos, possíveis mecanismos de atuação, e da relação entre da Angio-(1-7) com probiótico em modelos animais com SM, abrindo a perspectiva para desenvolvimento de novas propostas de intervenção no combate das doenças cardiometabólicas.

Mérito

O objetivo do trabalho é avaliar o efeito da associação da angiotensina (1-7) com probiótico no metabolismo de animais com síndrome metabólica. Para tanto, serão utilizados 64 camundongos da linhagem FVB/N, que serão divididos em 8 grupos de 9 indivíduos cada. A Angio 1-7 será incluída na dieta dos grupos experimentais, e o probiótico será administrado uma vez por dia durante 21 dias por via oral através de gavagem. Diariamente serão avaliadas a ingestão a ingestão alimentar, peso corporal e coleta de fezese ao 22º dia de tratamento será avaliada a glicemia de jejum. No final do experimento será realizada a eutanásia dos animais para realização das seguintes análises: peso dos tecidos corporais e determinação do tamanho do estômago;coleta da digesta no trato gastrointestinal; quantificação da microbiota; quantificação da expressão gênica; quantificação da IgA fecal; confecção de lâminas histológicas; avaliação histopatológica dos tecidos e análise da expressão dos componentes do sistema renina-angiotensina e marcadores inflamatórios. O sangue coletado dos animais sacrificados será utilizado para análises bioquímicas.

Parecer
A Comissão de Comitê de Ética em Experimentação e Bem-Estar Animal da Unimontes analisou o processo Nº103 e entende que o mesmo está completo e dentro das normas deste comitê. Sendo assim, somos pela **APROVAÇÃO** do projeto de pesquisa.

Prof. Orlando Raphael Lopazzo Júnior
Presidente da Comissão de Ética em Experimentação e Bem-Estar Animal da Unimontes

ANEXO B - Normas para publicação no periódico *Cellular Microbiology*

Author Guidelines

Cellular Microbiology is published monthly and is covered by Wiley Blackwell's *Accepted Articles* and *Early View* services.

Cellular Microbiology publishes outstanding original scientific contributions on the intersection of microbial and host cell biology. The focus is the host cell responses elicited by the interaction of micro-organisms. Equal emphasis is placed on responses to prokaryotic, viral and eukaryotic micro-organisms. In addition to mammalian systems, papers addressing other hosts such as plants and insects are strongly encouraged.

The Journal is a member of the Committee on Publication Ethics (COPE):
http://www.publicationethics.org.uk

Editorial Policy
The scope of *Cellular Microbiology* includes the host cell responses and interactions elicited by prokaryotic, viral and eukaryotic microorganisms, including helminths, protozoa and fungi, that illuminate exploitation of host cell components, function and signalling pathways, and may include modulation or alteration of immune responses. Mechanistic insight obtained through robust biochemical, genetic, cellular, and bioimaging technologies are expected to support observational data. In addition to mammalian and plant systems, papers addressing other hosts such as invertebrate systems are encouraged. Because viruses are inherently dependent upon the host cell, virology-based submissions to *Cellular Microbiology* should advance knowledge of the interaction of viruses and host cells. In addition to the principal content of full length and short research papers, issues may include Editorials/Opinions, Microreviews and Technoreviews as regular features.

Research Papers
Full length or shorter communications of original research will be published (Breaking Reports). Only complete reports will be published; notes or preliminary communications will not be considered.

Breaking Reports
Cellular Microbiology now offers the opportunity to publish ‘Breaking Reports’. Such submissions will be handled expeditiously through the review process. Breaking Reports are short-format papers that report a single, key message highlighting a ground-breaking advance. These reports should not exceed 5-6 printed pages of the journal (approximately 5,000 words) including a maximum of three display items and no more than 30 references. The report should be divided into sections as described for full articles.

Microreviews
In addition to primary research articles, short Microreviews are published in areas of particular interest and current importance in the field of *Cellular Microbiology*. The content of these reviews should appeal broadly to the readership and highlight both recent advances and areas that require additional research. Reviews should focus on cell biological themes rather than being organism specific and should include a broad range of microbial-host interactions.

Technoreviews
Short reviews dealing with technical advances that provide new approaches for examination of host-pathogen interactions are welcome. Technoreviews should provide an overview of the development and/or application of the methodology including its relative strengths, advantages and potential limitations.

Submission of Manuscripts
All manuscripts should be submitted online at **http://mc.manuscriptcentral.com/cell-micro**. Any articles submitted as hardcopy will be returned to the authors for re-submission. A user ID and password are required and can be obtained on the first use of the site. Any articles submitted as hardcopy will be returned to the authors for re-submission. Full instructions are provided.

Full help and support are provided by e-mail (support@scholarone.com), the web (**http://mc.manuscriptcentral.com/cell-micro**). If you cannot submit online please contact the Central Editorial Office:

Cellular Microbiology Central Editorial Office, Wiley-Blackwell
9600 Garsington Road Oxford OX4 2DQ
UK
Fax: +44 1865 714591
E-mail: cell-micro-editorial-office@wiley.com

Manuscripts will be directed by subject category to the appropriate Editor.

- Manuscripts addressing mechanisms of pathogenesis and bacterial-cellular interactions will be directed to either Sergio Grinstein, Feng Shao, or Thierry Soldati.

- Manuscripts concerning parasitology will be directed to Artur Scherf.

- Mycology manuscripts will be handled by Neil Gow.

- Virology manuscripts will be directed to Jacomine Krijnse Locker.

- Microreviews and Technoreviews will be directed to Elizabeth Hartland.

In the event of a potential conflict of interest with a particular editor, authors may request another editor to handle their manuscript.

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By submitting a manuscript to or reviewing for this publication, your name, email address, and affiliation, and other contact details the publication might require, will be used for the regular operations of the publication, including, when necessary, sharing with the publisher (Wiley) and

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Accelerated decision procedure
Every effort will be made to ensure rapid publication. Authors will normally receive reviewers' comments within four weeks of submission. To ensure the best service to authors and reviewers, manuscripts may be immediately returned to authors without scientific review following editorial assessment if it is deemed not suitable for *Cellular Microbiology* or if extensive revisions are required.

To maintain current scientific reviews, revisions of manuscripts should be resubmitted within 3 months of the editor's decision. Additional time for resubmission required to conduct specific experimental protocols may be requested in advance.

No page charges will be levied. Papers must be submitted exclusively to *Cellular Microbiology* and are accepted on the understanding that they have not been, and will not be, published elsewhere. If any part of a study submitted to *Cellular Microbiology* is resubmitted at a later date, the manuscript must be directed to the Editor who originally handled it.

If accepted, papers become copyright of the Journal. Authors must give signed consent to publication in a letter sent with the paper, but permission to use material elsewhere (e.g. in review articles) will normally be granted on request.

The Publisher will dispose of all hardcopy or electronic material submitted two months after publication.

Expedited Review
Cellular Microbiology offers an expedited review track for manuscripts that have been previously reviewed by certain highly selective journals. If you feel that the previous reviews would support publication of the manuscript in *Cellular Microbiology* (in its present form, or after minor revision), you are welcome to provide a copy of the reviews with your submission (providing that you have first obtained appropriate permission from the reviewer to share their review with us).

Manuscripts should be re-formatted according to the *Cellular Microbiology* guidelines and submitted via the ScholarOne manuscript submission system. Please include:

- a cover letter requesting expedited review,
- a PDF file of the originally submitted manuscript uploaded to Supplementary Files,
- the previous decision letter including peer reviews, and any manuscript correspondence,
- in addition, please include for the editor a point-by-point response to the previous critiques

We will not give any of this information to reviewers. We believe this makes the review process more efficient, transparent and reasonable.

Manuscripts considered for expedited review are circulated to the entire board of editors, and advice of

an outside editorial advisor may also be obtained. In many cases, expedited review can result in manuscripts being accepted for publication (depending on any additional minor revisions that might be needed) within 3-10 days.

PRESENTATION OF MANUSCRIPTS (IMPORTANT: for review articles please see below)
Papers should be as concise as possible, compatible with clarity and completeness. For guidance, eight printed pages corresponds to 17 pages of double line-spaced manuscript with three figures/tables, 15 pages of manuscript with six figures/tables, or 14 pages of manuscript with eight figures/tables. The text should be double spaced.

Figures included with online submissions should be suitable for onscreen viewing and desktop printing. High resolution images (digital only) should be provided on request or on manuscript acceptance.

Figures
Please supply high quality digital versions of figures in EPS or TIFF format to be used in production. Photomicrographs should include a scaled bar and indicate the size (descriptions of magnification alone are not sufficient). Submitted photographic images should be scaled to publication size and must have an image resolution of 300 dpi or greater in TIFF format. Powerpoint files are no longer accepted, and TIFF files should not be produced by transferring images from a previous Powerpoint file, as this results in major loss in resolution. Annotated photographs, line graphs and bar charts should be generated in EPS format for best quality of reproduction. For more detailed guidelines, please refer to **http://authorservices.wiley.com/bauthor/illustration.asp**

Provide methodological details on image acquisition and image processing, including software and operations such as colourizing and other modifications. The Editors remind authors that it is not acceptable scientific conduct to modify any separate element within an image. Sometimes adjustments of the entire image in brightness, contrast and colour balance are justified if they do not misrepresent the original, observed data. Composite figures composed of grouped images such as insets from different fields or separate parts of gels must be explained in the figure legend and differentiated by use of dividing lines or other means to make composites unambiguous.

Please ensure that electronic artwork is prepared such that, after reduction to fit across one or two columns or two-thirds page width (80mm, 169mm or 110mm respectively) as required, all lettering will be clear and easy to read, i.e. no labels should be too large or too small. Avoid using tints if possible; if they are essential to the understanding of the figure, try to make them coarse. No artwork should be incorporated into the text files.
In the full-text online edition of the Journal, figure legends may be truncated in abbreviated links to the full-screen version. Therefore, the first 100 characters of any legend should inform the reader of key aspects of the figure.

Video files
Our preferred file format for movies is .mp4.

Presentation of Manuscripts (REVIEW ARTICLES)
Reviews should be 4–7 pages in length (approximately 5000 words including references) plus one or two figures. *Cellular Microbiology* encourages the publication of colour figures if warranted in Reviews. Colour illustrations should be used to highlight the major concepts, models, or techniques

described in the body of the text. However, additional supplemental material may be submitted for publication online only. Please include the word count on the title page before submission. In general, authors should avoid the use of unpublished work, or references to such materials, that will not be directly accessible to the readers. Reviews will be subjected to peer review in order to assure they contain a broad and fair perspective of the topic. As the majority of Reviews are invited by the editors, the review process is designed primarily as a mechanism to improve the content and presentation of the manuscript.

Figures
We strongly encourage authors to develop one or two models that capture the major findings of their review topics. Your submitted artwork will be revised by an in-house artist who will provide an illustration in the unified style for the journal. This will be sent to you for final approval before publication.

*Important guidelines: please submit colour artwork in either high-resolution PDF format or TIFF format (image resolution of 300 dpi or greater). All figures should have a maximal width of 169 mm and a maximal height of 228 mm. The minimal font size should be 8 and that the font should be Helvetica.

The following applies to all manuscript types:

Title page
Should include the author's name(s), affiliations and the address to which all correspondence and should be sent. Telephone and fax numbers should also be supplied, along with an e-mail address if available. Present addresses of authors should appear as a footnote. A running title of not more than 50 characters should be provided.

Nomination of reviewers
Authors should nominate six reviewers to be used and their contact information, and may suggest up to two reviewers not to use (additional justification should be provided in the covering letter). However the Editors reserve the right to select expert reviewers at their discretion.

Summary
All papers must normally include a summary not exceeding 200 words. The main text should be subdivided into Introduction, Results, Discussion and Experimental procedures. The Results and Discussion sections may be combined and can include additional subheadings. Experimental procedures should be sufficiently detailed to enable the experiments to be reproduced. (For review papers, the main text should be subdivided into logical headings that summarize the main points of the review).

All pages must be numbered consecutively. Tables, figure legends and acknowledgements should follow the main text, each on a separate page. Footnotes should be avoided.

Standard abbreviations should be as recommended in Quantities, Units, and Symbols (The Royal Society, 1988). Abbreviations of non-standard terms should follow, in parentheses, their first full usage.

Manuscript Text
The manuscript text file should be uploaded as a separate Word document. Files should be formatted double-spaced with no hyphenation and automatic word-wrap (no hard returns within paragraphs).

Please type your text consistently, e.g. take care to distinguish between '1' (one) and 'l' (lower-case L) and '0' (zero) and 'O' (capital O), etc.

Tables
Tables should be typed as text, using either 'tabs' or a table editor for layout. Do not use graphics software to create tables.

Mathematics
In-line equations should be typed as text. The use of graphics programmes and 'equation editors' should be avoided. Displayed equations are rekeyed by our typesetter.

APA Reference style

Manuscripts should use the APA referencing style. Detailed guide and examples can be found here: **https://www.apastyle.org/**

Genetic Nomenclature
Standard genetic nomenclature should be used. For more detailed information, authors should consult Bachman (*Microbiol Rev* 47: 180-230, 1983) for *E. coli* K-12; Sanderson and Roth (*Microbiol Rev*47: 310-453, 1983) for *Salmonella typhimurium*; Holloway *et al.* (*Microbiol Rev* 43: 73-102, 1979) for *Bacillus subtilis*; Perkins *et al.* (*Microbiol Rev* 46: 426-570, 1982) for *Neurospora crassa*; and the Handbook of Genetics Vol. 1 (R. C. King, ed., Plenum Press, 1974) for *Saccharomyces cerevisiae*.

Restriction Enzymes
Cellular Microbiology has adopted the revised convention of naming restriction enzymes without italics. The previous style was *Eco*RI, *Kpn*I, *Hind*III, *Sac*II, etc. These should now be written EcoRI, KpnI, HindIII, SacII. For more information on the updated guidelines to naming restriction enzymes please consult Roberts *et al.* (*Nucleic Acids Res* 31: 1805-1812).

Sequence Data Submission
Nucleotide sequence data can be submitted in electronic form to any of the three major collaborative databases: DDBJ/EMBL/GenBank. It is only necessary to submit to one database, without regard to where the sequence data will be published. Data are exchanged between DDBJ, EMBL and GenBank on a daily basis. The suggested wording for referring to accession number information is: 'These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number U12345'. Addresses for these databases can be obtained from the *Cellular Microbiology* Web site (**http://www.cell-micro.com**).

(New) Automated Data Repository Deposition
Cellular Microbiology is part of a new initiative to streamline submission, deposition, and permanent archival of data files in association with manuscripts. This is a simple, free, and low-effort service for all *Cellular Microbiology* authors. This service is specifically tailored for data files that are not necessarily suitable for deposition into larger, government/institutional repositories, i.e. small CSVs, images, PDFs, etc. Often, these smaller datasets, while valuable, are submitted as Supporting Information; this new service does not replace Supporting Information, although *Cellular Microbiology* encourages authors to classify files that would have previously been submitted as Supporting Information as “Data Files” during the submission process. If you decide to take advantage of this service, the following will happen, assuming your manuscript is accepted for publication:

1. Files submitted and categorized as “Data Files” will be permanently deposited into the *Cellular Microbiology* figshare repository: live examples are available **here**.

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For additional information/FAQs, please refer to this **page**.

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Wiley Blackwell requires that all authors disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise, that might be perceived as influencing an author’s objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker’s fees from a company. The existence of a conflict of interest does not preclude publication in this journal.

If the authors have no conflict of interest to declare, they must also state this at submission. It is the responsibility of the corresponding author to review this policy with all authors and to collectively list in the cover letter to the Editor, in the manuscript (under the Acknowledgment section), and in the online submission system ALL pertinent commercial and other relationships.

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Accepted Articles
Cellular Microbiology offers Accepted Articles. Accepted Articles have been accepted for publication and undergone full peer review but have not been through the copyediting, typesetting, pagination and proofreading process. Accepted Articles are published online a few days after final acceptance, appear in PDF format only, are given a Digital Object Identifier (DOI), which allows them to be cited and tracked, and are indexed by PubMed.

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Distribution of Strains
The publication of an article in *Cellular Microbiology* is subject to the understanding that authors will distribute freely any strains, clones or antibodies described therein for use in academic research.

Cellular Microbiology

are welcomed by the Editors. It is preferable, but not essential, that these should be related to submitted papers. A free pdf offprint of the cover will be provided to the author whose photograph is reproduced thereon.

ANEXO C - Normas para publicação no periódico *Life Sciences*

Your Paper Your Way

We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article.
To find out more, please visit the Preparation section below.

Introduction

Life Sciences is an international journal publishing articles that emphasize the molecular, cellular, and functional basis of therapy. All articles are rigorously reviewed. The Journal favors publication of full-length papers where modern scientific technologies are used to explain molecular, cellular and physiological mechanisms. Articles that merely report observations are rarely accepted. Articles should be written at a level accessible to readers who are non-specialists in the topic of the article themselves, but who are interested in the research.
The Journal welcomes reviews on topics of wide interest to investigators in the life sciences. We particularly encourage submission of focused reviews containing high-quality artwork and mechanistic diagrams.

IMPORTANT INFORMATION

- Submission of a paper will be held to imply that the manuscript contains original unpublished work and is not being submitted for publication elsewhere.
- Manuscripts should present novel findings addressing significant biological questions. Studies that fail to do so may be rejected without review.
- Quantitative conclusions must be based on truly quantitative methods.
- *Life Sciences* does not publish work on the actions of biological extracts of unknown chemical composition. Compounds studied must be of known chemical structure and concentration.
- The study must be reproducible; materials used must be available to other researchers so they can repeat the experiment.

For more details on how to write a world class paper, please visit our Pharmacology Author Resources page.
Please include word count and figure/table count on the cover page of your manuscript.

Authors are encouraged to submit video material or animation sequences to support and enhance your scientific research. For more information please see the paragraph on video data below.

Types of article

- Original research articles
- Reviews

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
- E-mail address
- Full postal address

All necessary files have been uploaded:
Manuscript:
- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided
- Indicate clearly if color should be used for any figures in print *Graphical Abstracts / Highlights files* (where applicable) *Supplemental files* (where applicable)

Further considerations
- Manuscript has been 'spell checked' and 'grammar checked'
- All references mentioned in the Reference List are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Internet)
- A competing interests statement is provided, even if the authors have no competing interests to declare
- Journal policies detailed in this guide have been reviewed
- Referee suggestions and contact details provided, based on journal requirements For further

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Before You Begin

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Studies in humans and animals

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

Conflict of Interest Policy

The Journal requires full disclosure of all potential conflicts of interest. At the end of the manuscript text, under a subheading "Conflict of Interest statement", all authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. If there are no conflicts of interest, the authors should state: "The authors declare that there are no conflicts of interest." See also https://www.elsevier.com/conflictsofinterest. A signed Conflict of Interests Policy Form is required upon submission. The corresponding author is responsible for completing the form, and signing it on behalf of all authors.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Author contributions

For transparency, we encourage authors to submit an author statement file outlining their individual contributions to the paper using the relevant CRediT roles: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Authorship statements should be formatted with the names of authors first and CRediT role(s) following. More details and an example

Authorship
All authors listed on your paper must have made significant contributions to the study. To ensure clarity, you are required upon submission to enter the specific details of each author's contribution, which must substantiate the inclusion of each person on the manuscript. This information is required to be filled in on the Conflict of Interests Policy and Author Statement Form.

Changes to authorship
Authors are expected to consider carefully the list and order of authors **before**submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.
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