UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Keila Lopes Mendes

Avaliação dos efeitos metabólicos do resveratrol associado a diferentes composições dietéticas de macronutrientes e ao *Lactococcus lactis*

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Tese apresentada ao Programa de Pós-graduação em Ciências em Saúde da Universidade Estadual de Montes Claros-Unimontes, como parte das exigências para a obtenção do título de Doutora em Ciências da Saúde.

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Orientador: Prof. Dr. Sérgio Henrique Sousa Santos

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“Que teu alimento seja teu remédio e que teu remédio seja teu alimento.”

Hipócrates, século V a.C.

RESUMO

Nos últimos anos, a prevalência da síndrome metabólica tem aumentado progressivamente, em todo o mundo. Nessa síndrome, são reunidas algumas patologias, como obesidade, estado proinflamatório e alterações hepáticas. A diminuição dos processos inflamatórios, por meio da modulação da expressão de citocinas inflamatórias, e da lipogênese, por meio da modulação dos genes que regulam essa via, poderiam se constituir em estratégias para a prevenção da síndrome metabólica. Nesse sentido, a dieta pode ter um papel fundamental, reduzindo processos inflamatórios e o ganho de gordura corporal. Atualmente, há muitas recomendações nutricionais sugerindo o aumento do consumo de nutracêuticos, como o resveratrol, um polifenol. O potencial uso do resveratrol na prevenção da síndrome metabólica é promissor, porém são necessários estudos associando o resveratrol a diferentes padrões dietéticos e aos probióticos, que também têm tido um crescente consumo populacional. Diante disso, esta pesquisa teve por objetivo conhecer o efeito do resveratrol, associado a diferentes composições dietéticas e ao *Lactococcus lactis*, sobre marcadores lipogênicos e proinflamatórios. O primeiro produto avaliou a associação do resveratrol a diferentes composições dietéticas. Para isso, camundongos FVB/N foram divididos em 5 grupos (Dieta padrão, Dieta hiperlipídica, Dieta hiperlipídica + resveratrol, Dieta hiperproteica, Dieta hiperproteica + resveratrol), tratados por 60 dias. Após o sacrifício, foram realizadas análises plasmáticas (glicose, colesterol total, HDL colesterol e triglicerídeos) e qPCR para análise da expressão de RNAm de marcadores lipogênicos (PPARy, SREBP1, FAS, ACC) no tecido adiposo perigonadal. O segundo produto avaliou a associação do resveratrol ao *Lactococcus lactis*. Para isso, camundongos C57BL/6 foram divididos em 4 grupos (Grupo padrão, *Lactococcus lactis*, Resveratrol, *Lactococcus lactis* + Resveratrol), tratados por 15 dias. Após o sacrifício, foram realizadas análises plasmáticas (glicose, colesterol total, triglicerídeos, ALT e AST), qPCR, para análise da expressão de RNAm de marcadores proinflamatórios (IL-6, TNFα) no fígado e imunohistoquímica, para análise da expressão proteica de quimiocinas (IL-8) e citocinas (TNFα) no fígado. Com relação à associação do resveratrol a diferentes composições dietéticas, os resultados encontrados mostraram que, com a dieta hiperproteica, o resveratrol diminuiu o peso e a adiposidade corporal, colesterol total e a expressão de marcadores lipogênicos (ACC e FAS) e aumentou o HDL colesterol. Já com a dieta hiperlipídica, o resveratrol diminuiu os níveis de colesterol total. Sobre a associação do resveratrol ao *Lactococcus lactis*, os resultados encontrados mostraram que, com a associação de *Lactococcus lactis* + Resveratrol, houve diminuição no peso corporal e lipídeos plasmáticos (colesterol total e triglicerídeos), e tanto com o tratamento com *Lactococcus lactis* quanto com a associação *Lactococcus lactis* + Resveratrol, houve diminuição da expressão de marcadores proinflamatórios (IL-6, TNF-α e IL-8) e parâmetros hepáticos (ALT e AST). Diante disso, conclui-se que o consumo dietético de resveratrol, em associação a diferentes composições dietéticas e ao *Lactococcus lactis*, melhora o perfil lipídico, diminui o peso corporal e diminui a expressão de marcadores lipogênicos e proinflamatórios, tendo grande potencial para uso na prevenção da síndrome metabólica.

Palavras-chave: Dieta. Polifenóis. Lipogênese. Inflamação. Probióticos.

ABSTRACT

In the last years, the prevalence of metabolic syndrome have increased progressively worldwide. In this syndrome, some disorders coexist, such as obesity, a proinflammatory state and hepatic alterations. The decrease in the inflammatory process, by modulating the expression of inflammatory cytokines, and lipogenesis through the modulation of genes that regulate this pathway could be a strategy for the prevention of metabolic syndrome. In this sense, the diet may have a key role in reducing the inflammatory process and the gain of body adiposity. Currently there are several nutritional recommendations that include the consumption of nutraceuticals, such as resveratrol, a polyphenol. The potential use of resveratrol in the prevention of the metabolic syndrome is promising, but further studies associating the resveratrol to different dietary patterns and probiotics, which have a growing population consumption, are needed. Thus, this study aimed to evaluate the effect of resveratrol, associated with different dietary compositions and *Lactococcus lactis*, on proinflammatory and lipogenic markers. The first paper evaluated the association of resveratrol and different dietary compositions. To achieve this goal, FVB/N mice were divided into 5 groups, as follows: Standard diet, high-fat diet, high-fat diet plus resveratrol, high-protein diet and high-protein diet plus resveratrol treated for 60 days. Following sacrifice, plasma analysis were performed (glucose, total cholesterol, HDL cholesterol and triglycerides) and qRT-PCR for mRNA expression analysis of lipogenic markers (PPARy, SREBP1, FAS, ACC) in the perigonadal adipose tissue. The second paper evaluated the association between resveratrol and *Lactococcus lactis*, a probiotic. In order to achieve that, C57BL/6 mice were divided into 4 groups, as follows: Standard, *Lactococcus lactis*, Resveratrol and *Lactococcus lactis* + Resveratrol, during a period of 15 days. Following the sacrifice, plasma analysis were performed (glucose, total cholesterol, triglycerides, ALT and AST), as well as qRT-PCR for the mRNA expression analysis of the proinflammatory markers (IL-6, TNFα) in the liver and immunohistochemistry for the protein expression analysis of chemokines (IL-8) and cytokines (TNFα) in the liver. Regarding the association between resveratrol and the different dietary compositions, the results found showed that with the high-protein diet, the resveratrol was able to decrease the body weight and total adiposity, the plasma levels of total cholesterol and the expression of the lipogenic markers (ACC and FAS) and increased the plasma levels of HDL cholesterol. In contrast, along with the high-fat diet, the resveratrol decreased the plasma levels of total cholesterol only. When associated to the probiotic *Lactoccocus lactis*, the results found showed that this association induced a decrease in body weight and in the plasma levels of lipids (total cholesterol and triglycerides). For both treatments, *Lactoccocus lactis* alone and *Lactococcus lactis* + Resveratrol, a decrease in the expression of the proinflammatory markers (IL-6, TNF-α e IL-8) and hepatic parameters (ALT and AST) was observed. In this perspective, it may be concluded that the dietary consumption of resveratrol, in association to different dietary compositions and to the probiotic *Lactoccocus lactis*, improves the lipid profile, decrease body weight and decreases the expression levels of lipogenic and proinflammatory markers, thus being considered to have a great potential to be used in the prevention of the metabolic syndrome.

Key words: Diet. Resveratrol. Lipogenesis. Inflammation. Probiotics.

LISTA DE ILUSTRAÇÕES

Figura 1: Esquema da via lipogênica..... 20
Figura 2: Etiologia do processo inflamatório..... 21
Figura 3: Estrutura química dos isômeros trans e cis-resveratrol..... 24
Figura 4: Mecanismos de ação anti-obesidade do resveratrol..... 26
Figura 5: Mecanismos de ação dos probióticos na microbiota intestinal..... 29
Figura 6: Microbiota intestinal e sua relação com o fígado..... 30

LISTA DE TABELAS

Tabela 1: Concentração de resveratrol encontrada em alimentos naturais..... 25

LISTA DE ABREVIATURAS E SIGLAS

ACC	Acetil-CoA carboxilase
ALT	Alanina transaminase
AST	Aspartato transaminase
BAL	Bactérias ácido-lácticas
DASH	Dietary Approach to Stop Hypertension
DM	*Diabetes mellitus*
DRI	Dietary Reference Intake
FAS	Sintase de ácido graxo
HDL	High density lipoprotein
HMG-CoA	3-hidroxi-3-methyl-glutaril-CoA
IL-1β	Interleucina 1β
IL-6	Interleucina 6
IL-8	Interleucina 8
LPS	Lipopolissacarídeo
OMS	Organização Mundial de Saúde
PPARγ	Peroxisome proliferator-activated receptor
RSV	Resveratrol
SIRT1	Sirtuína 1
SM	Síndrome Metabólica
SREBP-1c	Sterol regulatory element-binding protein 1
TGI	Trato gastrointestinal
TNFα	Fator de necrose tumoral α

SUMÁRIO

1 INTRODUÇÃO 14
2 OBJETIVOS 16
2.1 Objetivo Geral 16
2.2 Objetivos Específicos 16
3 REVISÃO DE LITERATURA 17
3.1 Síndrome metabólica..... 17
3.2 Lipogênese..... 19
3.3 Inflamação..... 20
3.4 Dieta e síndrome metabólica..... 22
3.5 Resveratrol..... 24
3.6 Bactérias probióticas: *Lactococcus lactis*..... 28
4 PRODUTOS 32
4.1 Artigo 1: Distinct metabolic effects of resveratrol on lipogenesis markers in adipose tissue of mice treated with high-polyunsaturated fat and high-protein diets..... 33
4.2 Artigo 2: Oral treatment with Resveratrol and *Lactococcus lactis* decrease body weight and total cholesterol, and improve liver proinflammatory markers in C57BL/6 mice..... 50
5 CONSIDERAÇÕES FINAIS..... 62
REFERÊNCIAS 63
ANEXOS 71
ANEXO A – Parecer da Comissão de Ética em Experimentação e Bem-estar Animal da UNIMONTES..... 72
ANEXO B – Parecer da Comissão de Ética em Experimentação e Bem-estar Animal da UNIMONTES..... 74
ANEXO C – Normas de formatação do produto 1, periódico Life Sciences..... 75
ANEXO D – Normas de formatação do produto 2, periódico Molecular and Cellular Endocrinology..... 83

1 INTRODUÇÃO

Nos últimos anos, a prevalência da síndrome metabólica tem aumentado progressivamente, em todo o mundo. Nessa síndrome, são reunidas algumas patologias, como obesidade, dislipidemia, hipertensão arterial, resistência à insulina, diabetes, estado proinflamatório e alterações hepáticas.

A obesidade, caracterizada pelo alto percentual de gordura corporal, é um fator de risco associado à síndrome metabólica. Na gênese da obesidade, encontra-se o processo da lipogênese, via relacionada à síntese de triglicerídeos, que podem vir a ser armazenados no tecido adiposo. Além da lipogênese, o estado proinflamatório é um achado comum na obesidade e está presente também na síndrome metabólica. A diminuição dos processos inflamatórios, por meio da modulação da expressão de citocinas inflamatórias, e da lipogênese, por meio da modulação dos genes que regulam essa via, poderia se constituir em estratégias para a prevenção da síndrome metabólica.

Nesse sentido, a dieta pode ter um papel fundamental, reduzindo processos inflamatórios e o ganho de gordura corporal. Atualmente, há muitas recomendações nutricionais sugerindo o aumento do consumo de probióticos e nutracêuticos. Esses têm recebido atenção crescente no meio científico. Dentre os nutracêuticos, o resveratrol, um polifenol, desponta como um componente dietético de grande importância, pois está relacionado a funções antioxidantes, anti-inflamatórias e de melhora do perfil lipídico. Além do resveratrol, o consumo de probióticos, como o *Lactococcus lactis*, tem sido considerado como benéfico na redução de fatores de riscos metabólicos associados à síndrome metabólica.

O potencial uso do resveratrol na prevenção da síndrome metabólica se constitui em um desafio promissor, uma vez que os estudos experimentais até o momento só associam os efeitos benéficos do resveratrol concomitantemente ao consumo de dietas hiperlipídicas, sendo necessários estudos associando o resveratrol a outros padrões dietéticos. Além disso, uma vez que as recomendações nutricionais sugerem tanto o consumo de probióticos como do resveratrol, seria interessante conhecer o efeito do consumo concomitante desses na prevenção de fatores de risco relacionados à síndrome metabólica.

Nesse contexto, os resultados da presente pesquisa permitem conhecer o efeito do resveratrol, associado a diferentes composições dietéticas e ao *Lactococcus lactis*, sobre marcadores lipogênicos e proinflamatórios, abrindo a perspectiva de desenvolvimento de novas alternativas para a prevenção de doenças cardiometabólicas.

2 OBJETIVOS

2.1 Objetivo geral

- Avaliar os efeitos do resveratrol, administrado em conjunto a diferentes composições dietéticas de macronutrientes e ao *Lactococcus lactis* subsp. *lactis*, sobre a expressão de marcadores lipogênicos e proinflamatórios.

2.2 Objetivos específicos

- Avaliar os efeitos do consumo do resveratrol associado a diferentes composições dietéticas sobre o peso e a adiposidade corporal.
- Analisar as alterações do perfil glicídico e lipídico relacionadas ao consumo do resveratrol associado a diferentes composições dietéticas.
- Avaliar o efeito do consumo do resveratrol associado a diferentes composições dietéticas sobre a expressão de marcadores lipogênicos no tecido adiposo branco.
- Avaliar os efeitos do resveratrol administrado em conjunto ao *Lactococcus lactis* subsp. *lactis* sobre o peso e a adiposidade corporal.
- Analisar as alterações do perfil glicídico e lipídico relacionadas ao tratamento com resveratrol e *Lactococcus lactis* subsp. *lactis*.
- Avaliar o efeito do resveratrol em conjunto ao *Lactococcus lactis* subsp. *lactis* sobre a expressão de marcadores proinflamatórios no fígado.

3 REVISÃO DE LITERATURA

3.1 Síndrome metabólica

Nos últimos anos, a síndrome metabólica (SM) tem emergido como um problema de saúde pública, pois a sua prevalência vem aumentando mundialmente (1). A síndrome metabólica é definida como um transtorno complexo representado por um conjunto de fatores de risco cardiovascular que está normalmente associado à resistência insulínica e à deposição central de gordura (2).

A síndrome metabólica teve sua origem em 1920, quando Kylin, um médico sueco, demonstrou a associação de hipertensão arterial, hiperglicemia e gota. Mais tarde, em 1947, Vague descreveu que a obesidade visceral estava comumente associada a alterações metabólicas encontradas em doenças cardiovasculares e diabetes. Depois disso, em 1965, um resumo foi apresentado na Associação Europeia para o Estudo da Diabetes, reunião anual, por Avogaro e Crepaldi, descrevendo uma síndrome que compreendia hipertensão, hiperglicemia e obesidade (3).

Em 1988, Reaven descreveu um conjunto de fatores de risco para diabetes e doenças cardiovasculares e denominou-o de "Síndrome X", identificando a resistência à insulina, definida como a menor captação da glicose pelos tecidos periféricos, como o substrato fisiopatológico comum da síndrome (4). Em 1989, Kaplan (5) renomeou a síndrome de "Quarteto Mortal", para a combinação de obesidade superior do corpo, intolerância à glicose, hipertrigliceridemia e hipertensão e, no entanto, em 1992, foi novamente rebatizado para "síndrome de resistência à insulina" (6). Outro sinônimo já foi utilizado para denominar essa constelação de fatores de risco, como síndrome plurimetabólica (7). Em 1998, a Organização Mundial da Saúde estabeleceu o termo unificado síndrome metabólica (7).

Essa síndrome é composta por obesidade abdominal, dislipidemia, hipertensão arterial, resistência à insulina, hiperinsulinemia, intolerância à glicose, estado proinflamatório e pró-trombótico, culminando em doenças cardiovasculares e diabetes (8, 9). Tem sido também frequentemente associada a alterações hepáticas mais graves (10).

A definição do diagnóstico da SM se dá pela combinação de três ou mais condições: obesidade central (com adequações conforme a etnia), baixos níveis de colesterol HDL (mulheres≤50 mg/dL homens≤40 mg/dL e/ou uso de medicações para o controle dessa alteração), elevados níveis de triglicérides (≥150 mg/dL e/ou uso de medicações para o controle dessa alteração), elevados níveis de glicemia jejum (≥100 mg/dL e/ou uso de medicações para o controle dessa alteração) e elevados níveis de pressão arterial (Sistólica ≥130mmHg e/ou Diastólica≥85 mmHg dL e/ou uso de medicações para o controle dessa alteração) (9).

Mundialmente, a prevalência da SM entre a população adulta, constatada por alguns estudos, varia de 25% a 45%, sendo a maior frequência em mulheres (11-14). Estudos mostram que a sua prevalência aproxima-se de 35% em países desenvolvidos, como os EUA (15). No Brasil, uma revisão sistemática de estudos realizada por Bortoletto et al. (16) evidenciou que a prevalência de SM variou de 7,1% (adultos jovens) a 56,9% (idosos) entre os estudos, tendo relação com o aumento da idade. Estimativas recentes apontam para a prevalência em torno de 24% (17), chegando a mais de 80% entre os pacientes com *Diabetes Mellitus* (DM) tipo 2 (18). A SM contribui, ainda, para a crescente oneração dos gastos com serviços de saúde, estimados em mais de US$ 2 bilhões anuais no Brasil (19).

Alguns fatores podem estar contribuindo para o aumento da prevalência da SM, entre os quais se destacam: hábitos alimentares (alimentação rica em gorduras e açúcares, e pobre em frutas e hortaliças), estilo de vida (sedentarismo, tabagismo e etilismo) e estresse. Esses fatores de risco comportamentais impactam nos principais fatores de riscos metabólicos, como excesso de peso/obesidade, pressão arterial elevada, aumento da glicose sanguínea, lipídios e colesterol (1, 9, 20).

A obesidade, caracterizada pelo alto percentual de gordura corporal, é um fator de risco associado à síndrome metabólica. Na gênese da obesidade, encontra-se o processo da lipogênese, via relacionada à síntese de triglicerídeos, que podem vir a ser armazenados no tecido adiposo.

3.2 Lipogênese

A lipogênese é um processo que ocorre principalmente no tecido adiposo, mas também no fígado. Essa via é utilizada para manter e controlar a homeostase de energia, por meio da comunicação contínua entre os órgãos e tecidos, especialmente o tecido adiposo (21). Na presença de um elevado teor de gordura ou carboidratos vindos da dieta, a lipogênese é estimulada e o excesso de gordura é armazenado como triglicerídeos (também chamados triacilgliceróis) (22).

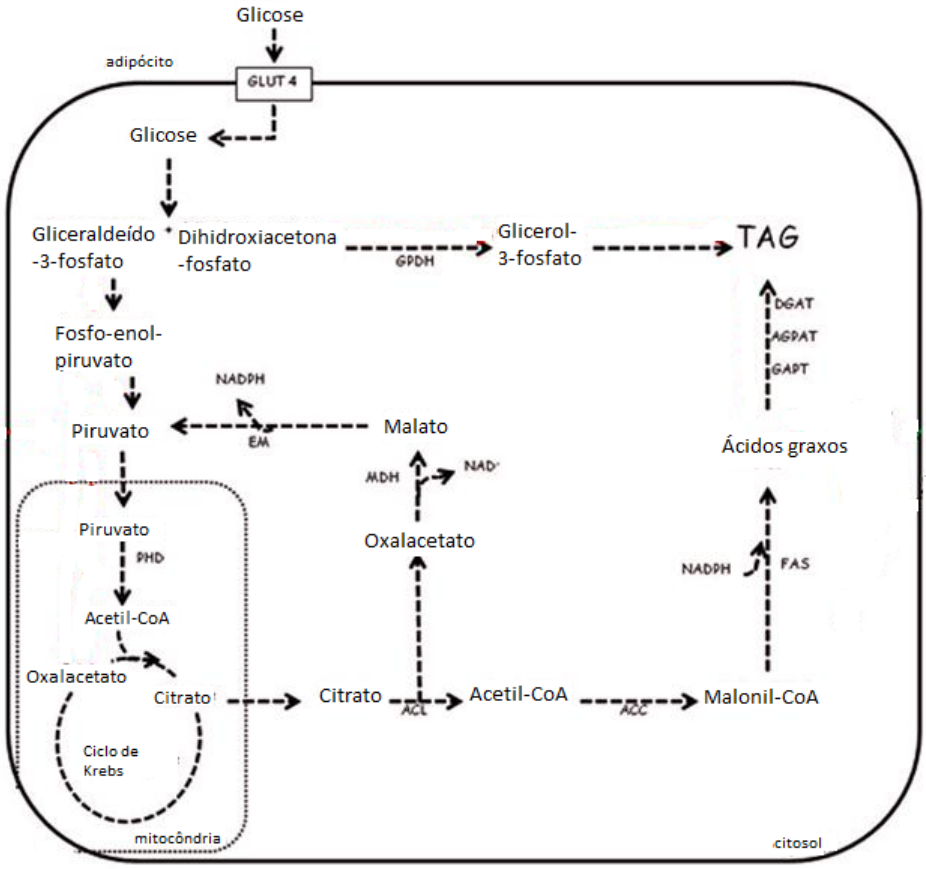
O processo de síntese de triglicerídeos pode se dar a partir do metabolismo de carboidratos e lipídeos, que culminam na formação de acetil-CoA (Figura 1). Esse sofre a ação da enzima acetil-CoA carboxilase (ACC), transformando-se em malonil-CoA. Esse último produto entra em uma complexa via de síntese de ácidos graxos, catalisada pela enzima ácido graxo sintase (FAS), gerando ácidos graxos livres. Esses se complexam à coenzima A, culminando na formação de acil-CoA, que reage com glicerol-3-fosfato, completando a biossíntese de triacilgliceróis. Esses podem, finalmente, ser incorporados à gotícula citoplasmática de gordura (21, 23).

Esse processo é controlado por diversos hormônios. A insulina estimula a síntese de lipídeos e a adipogênese, enquanto o glucagon e as catecolaminas promovem a fosforilação da ACC e inibem a síntese de ácidos graxos (21).

Alguns genes, como o SREBP1 e o PPARy, também exercem um papel crucial no controle da lipogênese. PPARγ é o principal regulador da adipogênese e exerce um papel na regulação do metabolismo lipídico e homeostase da glicose (24). A adipogênse é caracterizada por um aumento do número de adipócitos no tecido adiposo (hiperplasia) e se inicia com a diferenciação dos adipócitos a partir das células-tronco (25). Durante a última fase de diferenciação, os adipócitos mostram um grande aumento na lipogênese, por meio do aumento da expressão e atividade das enzimas ACC e FAS (26). Esse processo é controlado pelo SREBP-1c, o fator de transcrição mais importante na regulação da lipogênese (27).

Além da lipogênese, a inflamação está comumente presente tanto na obesidade quanto na síndrome metabólica, sendo necessário o estudo de alternativas para a prevenção da inflamação.

Figura 1: Esquema da via lipogênica no tecido adiposo branco. Para a biossíntese de triacilgliceróis (TAG), o acetil-CoA sofre a ação da enzima acetil-CoA carboxilase (ACC) transformando-se em malonil-CoA. Este último produto entra em uma complexa via de síntese de ácidos graxos, catalisada pela enzima ácido graxo sintase (FAS), que culmina na formação de acilCoA, que é utilizado para a esterificação com glicerol-3-fosfato, obtido como um produto da via glicolítica, completando a biossíntese de TAG, que é incorporado à gotícula citoplasmática de gordura.



Fonte: Adaptado de Proença et al. (28).

3.3 Inflamação

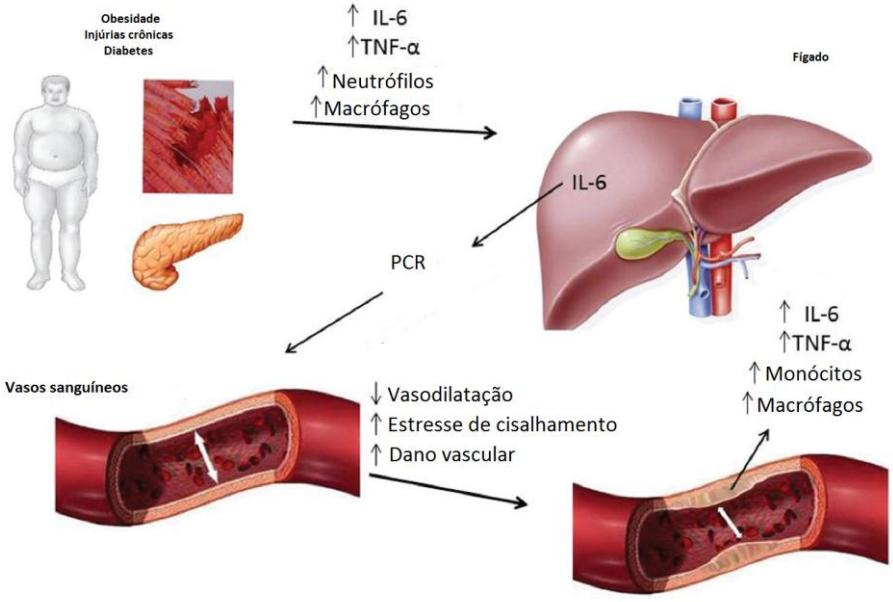
A inflamação é uma resposta biológica envolvida na manutenção da homeostase (29), e recebe atenção por seu papel em doenças crônicas, como a síndrome metabólica e a obesidade (30, 31). A obesidade está associada a uma resposta inflamatória crônica, caracterizada pela ativação de vias de sinalização proinflamatórias e produção anormal de adipocinas, induzindo a expressão de marcadores biológicos da inflamação (Figura 2) (32).

A inflamação aguda se inicia abruptamente e, geralmente, é de rápida resolução, envolvendo a estimulação do receptor Toll-like 4 (TLR4). Segundo Xiao e colaboradores (33), por meio

disso, ocorre uma reprogramação de cerca de 80% dos genes que modulam os leucócitos sanguíneos. Isso mostra que ocorre uma reprogramação gênica após uma resposta inflamatória aguda, enfatizando a determinação epigenética no processo inflamatório (34). A fase proinflamatória induz a expressão de alguns genes, tais como fator de necrose tumoral-alfa (TNF-α) e diversas interleucinas e, em seguida, reprograma diversos outros conjuntos de genes para apoiar a fase de adaptação (35), que dura muito mais tempo do que a fase inicial proinflamatória até chegar à homeostase (36).

O processo inflamatório apresenta uma característica muito importante, que é a disfunção endotelial, que inclui o recrutamento de moléculas de adesão, citocinas proinflamatórias, e enzimas que degradam a matriz (37). Os sinais responsáveis pela iniciação do processo inflamatório sistêmico agudo incluem subunidade p65 (RelA) do fator nuclear kappa β (NF-κβ), metilases, quinases de stresse (ERK, p38 e JNK), e acetil transferases (34). NF-κβ é um regulador mestre da resposta imune e inflamação (38), relacionado à síntese de várias citocinas, tais como TNF-α, IL-1β, interleucina-6 (IL-6), interleucina-8 (IL-8) e ciclo-oxigenase-2 (39).

Figura 2: Etiologia do processo inflamatório. Inflamação crônica de baixo grau, causada por diversos fatores, tais como diabetes e obesidade, aumenta concentração de marcadores e células e citocinas pró-inflamatórias, que leva ao aumento da produção de proteína c-reativa (PCR) no fígado, provocando diminuição da vasodilatação e aumento do dano vascular.



Fonte: Adaptado de Teixeira et al. (40).

TNF-α é uma citocina proinflamatória que promove a transcrição de vários genes inflamatórios, o aumento da migração de leucócitos e provoca a apoptose de células epiteliais intestinais (41). IL-6 é uma citocina produzida pelos linfócitos T sinoviais, fibroblastos, monócitos e macrófagos, cujos efeitos incluem a indução da produção de fase aguda por diferenciação dos linfócitos B, hepatócitos, e linfócitos T (42). Já a IL-8 é considerada uma quimiocina, que atua por meio de quimiotaxia, atraindo neutrófilos para o local da inflamação, promovendo a adesão de neutrófilos, a transmigração através do endotélio e estimulando as células a realizar a fagocitose (43).

Além disso, uma característica importante do processo inflamatório é a ativação de macrófagos e a infiltração nos tecidos residentes, processo conhecido por mediar a inflamação local, sendo característico da síndrome metabólica (44-46). Sob estímulo, os macrófagos infiltram o tecido, perpetuando a inflamação local e contribuindo para o desenvolvimento da resistência à insulina e alterações metabólicas (47).

A diminuição dos processos inflamatórios, por meio da modulação da expressão de citocinas inflamatórias, poderia ser uma estratégia para a prevenção da SM. Nesse sentido, a dieta pode ter um papel fundamental na prevenção da síndrome metabólica, reduzindo processos inflamatórios e o ganho de gordura corporal.

3.4 Dieta e síndrome metabólica

O papel da dieta no tratamento da síndrome metabólica é uma consideração importante que pode potencializar a perda de peso, diminuição da inflamação e uma diminuição do risco cardiometabólico. Portanto, a dieta ideal deve combinar todos os componentes da dieta que influenciam esses fatores (48). Mudanças de estilo de vida baseadas em alimentação saudável são essenciais para a prevenção e o tratamento da síndrome metabólica, principalmente as mudanças para redução da obesidade (9). A intervenção nutricional faz parte da terapia inicial para a prevenção e o tratamento da SM como integrante das alterações de estilo de vida (49).

Embora as recomendações gerais para adultos de Dietary Reference Intakes (DRI) incluam uma ingestão total de gordura de 20-35 % do consumo calórico diário, 10-35 % das calorias totais de proteína, carboidratos e oscilando de 45% para 65% (50), a população mundial está aumentando o seu consumo de dietas ricas em açúcares, carboidrato refinado, proteínas,

gorduras e alimentos de origem animal, ao passo que as dietas ricas em legumes, grãos e outros produtos hortícolas estão a diminuir em todo o mundo (51).

A gordura saturada e a frutose são mais suscetíveis de estimular a acumulação hepática de lipídeos, enquanto a gordura insaturada, antioxidantes e dietas ricas em proteínas parecem ter um efeito mais preventivo (52). De um modo geral, o consumo de dietas hiperlipídicas, hiperglicídicas e hipercalóricas, principalmente as ricas em gorduras saturadas e açúcares simples, é o fator dietético que mais predispõe à SM. No Brasil, um estudo realizado na população nipo-brasileira demonstrou que o consumo de gordura total aumenta, enquanto que o consumo do ácido graxo poliinsaturado linoleico reduz a chance para a presença da SM (53).

Dietas ricas em proteína e pobres em carboidratos, dietas ricas em carboidratos de baixo índice glicêmico e ingestão adequada de ácidos graxos ômega-3 são fatores nutricionais atualmente propostos para o tratamento da síndrome metabólica (48). O consumo de alimentos vegetais (frutas, hortaliças e grãos integrais) tem sido apontado como um hábito preventivo da SM. Em um estudo realizado na população iraniana, o consumo de grãos integrais foi negativamente associado à presença de hipertensão arterial, hipertrigliceridemia e SM (54).

Outro estudo realizado em pacientes brasileiros com DM tipo 2 demonstrou que o consumo de alimentos ricos em fibras solúveis, representados pelos grãos integrais e frutas, foi um fator de proteção para a presença da SM (55). Em indivíduos idosos, a ingestão diária de mais do que três porções de alimentos ricos em grãos integrais foi associada à menor frequência da SM e a um menor risco de mortalidade por doença cardiovascular (56).

Além desses estudos, que focalizam alguns nutrientes ou grupos alimentares, também têm sido conduzidos alguns trabalhos mostrando a influência de dietas já consagradas sobre a SM. A dieta mediterrânea (rica em grãos integrais, legumes, frutas, vegetais, nozes, azeite de oliva e peixes) foi comparada com a dieta recomendada pela *American Heart Association* (gordura total < 30% do valor energético total). Ao final de dois anos, o número de componentes da SM foi menor nos pacientes que seguiram a dieta mediterrânea (57).

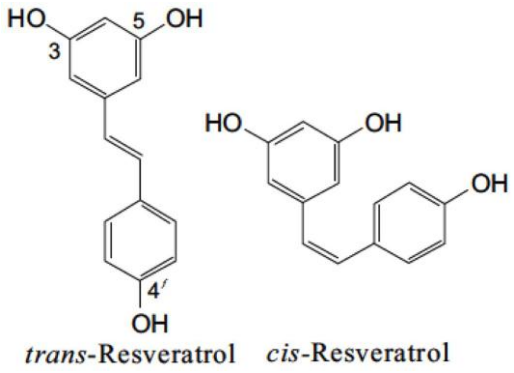
Já em um ensaio clínico de menor duração, a dieta DASH (Dietary Approach to Stop Hypertension) foi mais efetiva na melhora do perfil de todos os componentes da SM (reduções de cintura abdominal, peso, triglicerídeos e níveis pressóricos e aumento do HDL-colesterol), quando comparada com uma dieta controle e com uma dieta hipocalórica para perda de peso (58).

Além dessas dietas e nutrientes, atualmente há muitas recomendações nutricionais para o aumento do consumo de probióticos e nutracêuticos (59, 60). Esses têm recebido atenção crescente no meio científico. Dentre os estudos mais recentes, o resveratrol (RSV) desponta como um componente dietético de grande importância na SM, pois está relacionado a funções antioxidantes, anti-inflamatórias e de melhora do perfil lipídico.

3.5 Resveratrol

Resveratrol (3,5,4-trihidroxiestilbeno) (Figura 3) é um polifenol, pertencente ao grupo dos flavonoides, encontrado em mais de 70 espécies vegetais, principalmente na casca da uva e vinho tinto (61), mas também em cranberries e amendoins (62) (Tabela 1).

Figura 3: Estrutura química dos isômeros trans e cis-resveratrol.



Fonte: Rege et al. (63)

Videiras produzem quantidades aumentadas de resveratrol quando estão sujeitas a estresse em seu desenvolvimento, como ataques por organismos patogênicos. Nesse caso, o resveratrol age como uma molécula promotora de defesa, chamada fitoalexina (64).

Tabela 1: Concentração de resveratrol encontrada em alimentos naturais.

Fonte	Concentração de Resveratrol
Vinhos tintos	0,1-14,3 mg/L
Vinhos brancos	< 0,1-2,1 mg/L
Suco de uva	~ 0,50 mg/L
Suco de cranberry	~ 0,2 mg/L
Suco de uva branca	~ 0,05 mg/L
Casca da uva seca	~ 24,06 ug/g
Amendoim cozido	~ 5,1 mg/g
Uvas	0,16-3,54 ug/g
Amendoins	0,02-1,92 ug/g
Pistaches	0,09-1,67 g/g
Manteiga de amendoim	0,3-1,4 g/g
Amendoins torrados	~ 0,055 g/g
Mirtilos	~ 32 ng/g

Fonte: Adaptado de Mukherjee et al. (65).

O grande interesse nesse composto começou em um trabalho que notou menor mortalidade por doenças cardíacas em populações com alimentação rica em gorduras e com consumo regular de vinho, sugerindo que o vinho poderia ser cardioprotetor, sendo esse fenômeno conhecido como Paradoxo Francês (66). Mais tarde, foi identificado que o resveratrol era o componente presente no vinho tinto que conferia esse efeito cardioprotetor (67).

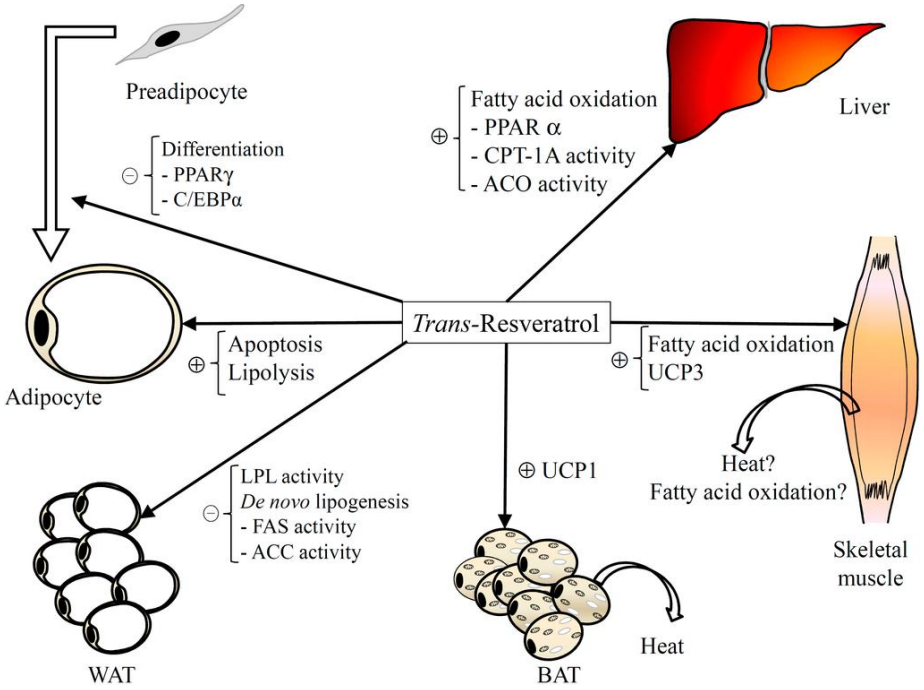
Atualmente, o resveratrol é bem conhecido por sua propriedade cardioprotetora, anticâncer, anti-inflamatória e antioxidante (26, 63, 68, 69), influenciando muitas enzimas que conferem diversos efeitos fisiológicos, como efeitos antidiabéticos, proteção microvascular, neuroproteção (70), e atividades antienvelhecimento, que se assemelham aos efeitos funcionais da restrição calórica (62). O resveratrol também promove a lipólise e a β oxidação dos ácidos graxos, e diminui a adipogênse e lipogênese, agindo, consequentemente, como um composto antiobesidade (Figura 4) (71). Várias vias metabólicas, enzimas e genes são alvos de modulação pelo resveratrol, tais como AMPK (72), sirtuínas, FoxO (73), PGC-1a (74), FAS, SREBP-1c, ACC, glicose-6-fosfato desidrogenase, HMG-CoA redutase (75), PPAR, CPT-1 e acil-CoA oxidase (75-80).

Pesquisas realizadas nos últimos anos têm demonstrado que o resveratrol é capaz de mimetizar a restrição calórica (81), o que poderia explicar o seu efeito no prolongamento da

vida útil em leveduras e em ratos. Esse efeito biológico está subtendido por vários mecanismos moleculares de ação do resveratrol no metabolismo celular, incluindo a interação com sirtuinas (82), principalmente a SIRT1.

As sirtuínas fazem parte de um grande grupo de enzimas (1 a 7) que compõe a família das desacetilases de histonas, cujo papel principal é reverter a acetilação regulatória das proteínas tipo histona, influenciando, diretamente, a estrutura dos nucleossomos e, consequentemente, a transcrição gênica (81). SIRT 1 é a sirtuina mais ativada por meio da restrição calórica e pelo resvetrarol e está envolvida em muitos processos vitais, como reparo de DNA, sobrevivência celular, gliconeogênese, diferenciação das células musculares, regulação do ciclo celular, metabolismo lipídico, transporte de gordura e sensibilidade insulínica (81). SIRT1 também regula proteínas que atuam na fisiopatologia das doenças metabólicas, sendo destacada a sua grande importância na atuação sobre os componentes da síndrome metabólica (83, 84).

Figura 4: Mecanismos de ação anti-obesidade do resveratrol. O resveratrol age em diferentes tecidos e órgãos, como fígado, tecido adiposo branco, tecido adiposo marrom e músculo, por diversas vias metabólicas, que culminam no efeito anti-obesidade do resveratrol.



Fonte: Aguirre et al. (26).

O resveratrol é também descrito por desenvolver um papel importante no metabolismo dos lipídeos. O resveratrol reduz a síntese do colesterol, por diminuir a expressão da enzima HMG-CoA redutase, aumentar a excreção de colesterol e aumentar o transporte reverso de

colesterol. Isso se dá por meio do aumento dos níveis de HDL, mediando o efluxo de colesterol a partir de macrófagos nas paredes das artérias (85). Jeon, Lee e Choi (86), em um estudo com animais alimentados com uma dieta aterogênica e resveratrol, evidenciaram que o resveratrol aumentou, significativamente, a concentração plasmática de HDL-C, em comparação com o controle.

As propriedades anti-inflamatórias do resveratrol têm sido descritas em diversas doenças, tais como artrite, pancreatite e colite experimental. Um estudo realizado por Stefan Bereswill et al. (87) demonstrou que a administração oral de resveratrol a camundongos melhorou a inflamação hiperaguda do intestino delgado. Além disso, os animais dos grupos que receberam o tratamento foram protegidos do desenvolvimento de imunopatologia e exibiram melhores condições clínicas, menor perda de peso corporal e encurtamento do comprimento do intestino delgado.

Panaro et al. (88) avaliaram o efeito do resveratrol sobre as respostas inflamatórias induzidas em células intestinais por lipopolissacarídeo (LPS). Curiosamente, os resultados demonstraram que o resveratrol foi capaz de reduzir, significativamente, a produção de óxido nítrico (NO). Além disso, foi recentemente observado, em estudos com camundongos, que o resveratrol tem um efeito benéfico significativo na colite crônica induzida experimentalmente, e que esse efeito protetor parece estar relacionado a uma modulação de mediadores proinflamatórios, incluindo uma redução da expressão de iNOS na mucosa do cólon.

Um dos mecanismos pelos quais o resveratrol pode atuar na diminuição da inflamação se dá por respostas imunes de regulação tipo Th1 e bloqueio da translocação bacteriana, mantendo a função de barreira intestinal (87). Assim, o resveratrol pode atenuar, significativamente, vários componentes da resposta das células intestinais a estímulos proinflamatórios, sugerindo um efeito potencial terapêutico no tratamento de doenças inflamatórias.

Uma questão conflitante diz respeito à quantidade de resveratrol recomendada para ingestão, pois ainda não há uma dose de referência estabelecida. Em estudos animais, uma variedade de doses já foi usada (0,1 a 1.500 mg/kg de peso corporal) (89). Em humanos, também são usadas distintas doses nos estudos (90), sendo que os autores concluem que baixas doses protegem a saúde de diferentes tipos de doenças (65). Assim, a quantidade de resveratrol recomendada para ingestão se constitui em uma lacuna do conhecimento.

Sabe-se que as bactérias intestinais são capazes de converter o resveratrol para hidro-resveratrol, que, pelo menos parcialmente, é absorvido e ainda metabolizado em formas conjugadas que podem ser excretados na urina. Estudos profundos sobre o metabolismo do resveratrol influenciado pela microbiota intestinal, no entanto, são escassos (91). Além disso, o potencial uso do resveratrol na prevenção da síndrome metabólica também se constitui em um desafio promissor, uma vez que os estudos experimentais até o momento só associam os efeitos benéficos do resveratrol concomitante ao consumo de dietas hiperlipídicas (83, 92-95), sendo necessários estudos associando o resveratrol a outros padrões dietéticos.

Além do resveratrol, outros alimentos funcionais de grande importância na prevenção da síndrome metabólica são as bactérias probióticas, como o *Lactococcus lactis*.

3.6 Bactérias probióticas: *Lactococcus lactis*

Os probióticos são considerados bactérias benéficas com vários efeitos positivos para os seres humanos (96). A definição internacional de probióticos é dada pela Organização Mundial de Saúde (OMS): são microrganismos vivos, administrados em quantidades adequadas, que conferem benefícios à saúde do hospedeiro (97).

Há dois principais membros desse grupo: bifidobactérias e lactobacilos (98). Várias espécies, *como Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus fermentum* e *Lactobacillus reuteri*, são conhecidas por suas propriedades probióticas (99).

As bactérias pertencentes ao gênero Lactobacillus são mais frequentemente empregadas como suplementos probióticos para alimentos, como é o caso do leite fermentado e iogurtes (97). Algumas bactérias do ácido lático (BAL) são bastante conhecidas por suas aplicações terapêuticas no tratamento e na prevenção de várias desordens, sendo, portanto, denominadas de probióticos. As BAL constituem um grupo de microrganismos gram-positivos, microaerófilos, não formadores de esporos e não móveis, capazes de converter açúcares (hexoses) em ácido lático (100).

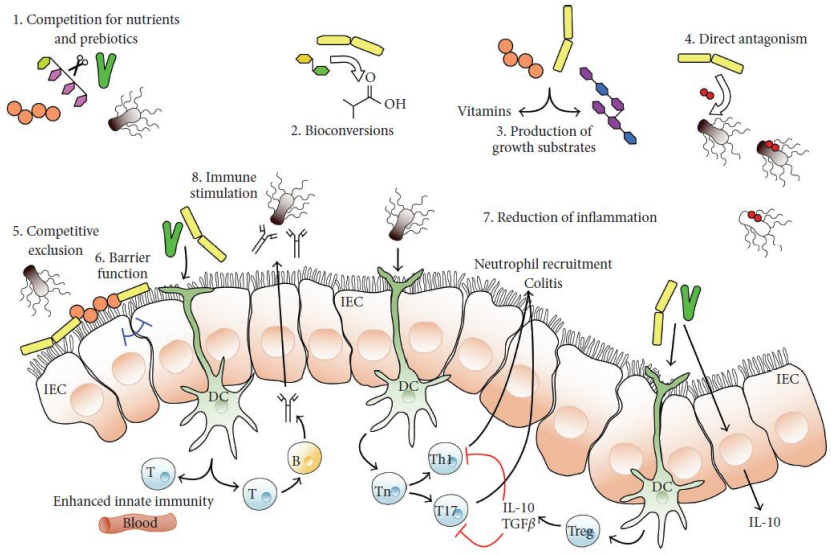
Lactococcus lactis pertence ao grupo das bactérias do ácido lático e é a principal constituinte da cultura iniciadora láctea utilizada em todo o mundo para a produção de produtos lácteos

(101). *Lactococcus lactis* é a espécie melhor caracterizada dentre as BAL, pois, além de sua importância econômica, possui seu genoma completamente sequenciado e diversas ferramentas genéticas e de expressão já foram desenvolvidas para essa espécie. Portanto, *Lactococcus lactis* é considerado um organismo modelo dentro do grupo (102).

Alguns autores admitem que *Lactococcus lactis* não colonizam o trato gastrointestinal (TGI), estando em constante trânsito no TGI após a sua ingestão (103), exercendo uma ação local e sistêmica (104). A influência benéfica dos probióticos sobre a microbiota intestinal humana inclui fatores, como efeitos antagônicos, competição e efeitos imunológicos. Assim, a utilização de culturas bacterianas probióticas estimula a multiplicação de bactérias benéficas, em detrimento à proliferação de bactérias potencialmente prejudiciais, reforçando os mecanismos naturais de defesa do hospedeiro (97).

Três possíveis mecanismos de atuação são atribuídos aos probióticos: modulação da microbiota intestinal - competição por sítios de adesão, competição por nutrientes e produção de compostos antimicrobianos; alteração do metabolismo microbiano - aumento ou diminuição da atividade enzimática e estímulo da imunidade do hospedeiro, além de outros possíveis efeitos (97). Esses e outros mecanismos estão ilustrados na Figura 5.

Figura 5: Mecanismos de ação dos probióticos na microbiota intestinal.



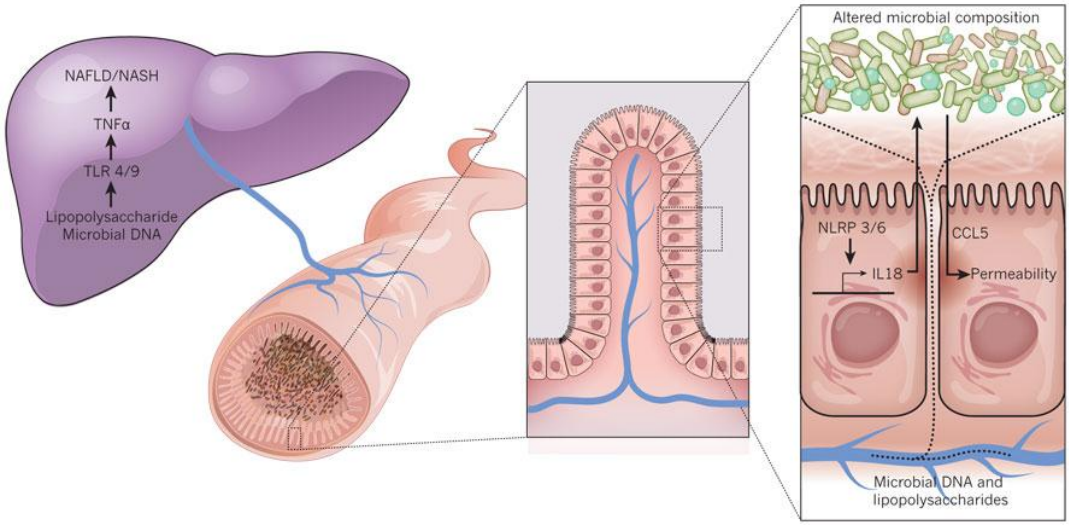
Fonte: O'Toole et al. (105).

Os microorganismos probióticos podem atuar diretamente na microbiota intestinal, diretamente com o muco e epitélio intestinal, e no sistema imune sistêmico e em outros órgãos, como o fígado (106), por meio da circulação portal (Figura 6).

Além dos benefícios naturais, outra aplicação bastante promissora para BAL é o seu uso como vetores vivos para entrega de antígenos e proteínas terapêuticas na superfície de mucosas (107). *Lactococcus lactis* tem se destacado como um microrganismo alternativo para a produção de moléculas de interesse biotecnológico, quando comparado ao uso de modelos clássicos, como a *Escherichia coli* e *Pichia pastoris*, uma vez que *Lactococcus lactis* não produz endotoxinas, LPS ou qualquer outro produto metabólico tóxico (102).

Entre os benefícios do consumo de probióticos estão o controle de desordens metabólicas, incluindo a obesidade e diabetes (98), efeitos anti-inflamatórios (108) e prevenção do câncer, por meio da manutenção do equilíbrio da microbiota intestinal (109). Um estudo de Wang et al. (110) avaliou o efeito de três bactérias probióticas em ratos alimentados com dieta hiperlipídica, evidenciando que os probióticos diminuíram, significativamente, os níveis séricos de triglicérides, colesterol total e LDL colesterol e aumentaram os níveis de HDL colesterol.

Figura 6: Microbiota intestinal e sua relação com o fígado. A composição alterada da microbiota pode afetar outros órgãos como o fígado.



Fonte: Tremaroli et al. (111).

Uma vez que as recomendações nutricionais sugerem tanto o consumo de probióticos como do resveratrol, seria interessante conhecer o efeito do consumo concomitante desses sobre o

metabolismo. Entretanto, na literatura, ainda não há estudos que façam tal avaliação associando o resveratrol e *Lactococcus lactis*, sendo necessários estudos nesse sentido, a fim de avaliar o efeito desses na prevenção de fatores de risco relacionados à síndrome metabólica.

4 PRODUTOS

Os produtos foram dois artigos científicos:

4.1 Produto 1: *Distinct metabolic effects of resveratrol on lipogenesis markers in adipose tissue of mice treated with high-polyunsaturated fat and high-protein diets,* formatado segundo as normas para publicação do periódico Life Sciences, enviado ao periódico.

4.2 Produto 2: *Oral treatment with Resveratrol and Lactococcus lactis decrease body weight and total cholesterol, and improve liver proinflammatory markers in C57BL/6 mice*, formatado segundo as normas para publicação do periódico Molecular and Cellular Endocrinology.

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4.1 Artigo 1

Distinct metabolic effects of high-dose resveratrol on lipogenesis markers in adipose tissue of mice treated with high-polyunsaturated fat and high-protein diets

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ABSTRACT

Objective: A healthy diet is essential for the prevention and treatment of metabolic syndrome. The present study evaluated the effect of resveratrol associated with high-polyunsaturated fat and high-protein diets on expression of adipogenic and lipogenic genes.

Research Methods & Procedures: FVB/N mice were divided into 6 groups (n = 7 each) and fed with experimental diets for 60 days: standard (ST), high-fat diet (HFD), and high-protein diet (HPD), with and

without resveratrol (RSV) (4g/kg diet). The body weight, food intake, and energy intake (kcal) were evaluated. Blood parameters (HDL-C, total cholesterol, glucose, and triglyceride levels) were assessed. Real-time PCR was performed to analyze the expression of adipogenesis and lipogenesis markers: PPARγ, SREBP-1c, ACC and FAS in samples from perigonadal adipose tissue.

Results: In the HPD+RSV group, resveratrol decreased body weight, body adiposity, adipose tissue weight, adipocyte area, total cholesterol, ACC and FAS expression, and increased HDL-cholesterol in comparison to HPD. In the HPD group there was a decrease in adipocyte area, as well as PPARγ, SREBP-1c and ACC expression in comparison to ST. While in HFD+RSV, resveratrol decreased levels of total cholesterol, and PPARγ, SREBP-1c and ACC expression in comparison to HFD. In the HFD group there was decrease in body weight, and PPARγ, SREBP-1c and ACC expression in comparison to ST.

Conclusions: The obtained results show that resveratrol decreases lipogenesis markers and metabolic parameters in the setting of a high-protein diet. Moreover, resveratrol decreased total cholesterol in both diets. These results point to the increased potential of resveratrol use in prevention or treatment of metabolic syndrome, acting on different dietary compositions.

Keywords: Diet; Resveratrol; Lipogenesis; Adipogenesis; Cholesterol.

Chemical compound studied in this article: Resveratrol (PubChem CID: 445154)

Introduction

Metabolic syndrome (MS) is a public health problem, and its consistent increase in prevalence is a worldwide phenomenon (Grundy, 2008), which is closely associated with the increase the in prevalence of obesity (Grundy, 2008) and sedentary lifestyles (Alberti et al. , 2009). MS is a complex of interrelated risk factors, that includes raised blood pressure, dysglycemia, low high-density lipoprotein cholesterol levels, elevated triglyceride levels, and obesity, contributing to the development of cardiovascular disease and diabetes (Alberti et al., 2009).

More attention must be given to lifestyle changes based on a healthy diet and an increase in physical activities in order to prevent and treat obesity and MS (Alberti et al., 2009). Although general recommendations for adults of Dietary Reference Intakes (DRI) include a total fat intake of 20–35% of daily caloric consumption, 10-35% of total calories as protein, and carbohydrates oscillating from 45% to 65% (2005), the world population is increasing its consumption of diets rich in sugars, refined carbohydrates, proteins, fats and animal-source foods, while diets rich in legumes, coarse grains, and other vegetables are decreasing everywhere (Popkin et al. , 2012).

A good strategy for the prevention of MS development is nutraceutical intake, such as resveratrol (RSV). Resveratrol (3,5,40-trihydroxystilbene) is one of the natural polyphenolic compounds mainly found in grape skins and red wine (Cho et al. , 2012). RSV is well known for its anti-cancer, anti-inflammatory, anti-obesity, cardioprotective, and antioxidant properties (Aguirre et al. , 2014, de la Lastra and Villegas, 2007, El-Mowafy and Alkhalaf, 2003, Hung et al. , 2000). RSV promotes lipolysis and fatty acid β oxidation, thus

decreasing adipogenesis and lipogenesis and consequently acting as an anti-obesity compound (Wang et al. , 2014).

Adipogenesis is characterized by an increase in the number of adipocytes in adipose tissue (hyperplasia), that starts with the differentiation of adipocytes from stem cells (Rosen and MacDougald, 2006). In this differentiation process, sterol regulatory element binding protein 1c (SREBP-1c) and peroxisome proliferator-activated receptor gamma (PPARγ), the latter being considered the main adipogenesis inducing regulator, are required to induce the well-known shape of the adipocytes, which is spherical, from a fibroblast cell shape (Aguirre et al., 2014). During the last phase of differentiation, the adipocytes show a great increase in lipogenesis, via increased expression and activity of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). This process is controlled by SREBP-1c (Aguirre et al., 2014).

The literature describes experimental animal studies involving the administration of resveratrol with a high saturated fat diet, and reports improvement in health and metabolic parameters of the animals studied (Andrade et al. , 2014a, Oliveira Andrade et al. , 2014). However, resveratrol may have distinct metabolic effects in other dietary compositions, besides the high saturated fat diet. There are only a few studies involving the concomitant consumption of resveratrol in other dietary patterns, such as high-polyunsaturated fat and high-protein diets. In this context, this study aims to evaluate the effect of high-dose of resveratrol associated with different dietary macronutrients on expression of adipogenic and lipogenic genes in the adipose tissue.

Materials and methods

Animals and experimental diets

Thirty-five female FVB/N mice, aged to 8 weeks, were divided into 6 groups that were fed with experimental diets for 60 days (n=7 per treatment). The mice from the State University of Montes Claros (Montes Claros, Minas Gerais, Brazil) were housed in cages, under a 12h:12h light-dark cycle (lights on from 7:00 to 19:00 h) at a controlled temperature of 25.0 ± 2.0ºC. Food and water were offered ad libitum. This study was approved by Ethics Committee of Experimentation and Animal Welfare of Unimontes, Montes Claros, Brazil (process nº 064/2013).

The experimental groups were: Standard diet (ST), Standard diet plus resveratrol (ST+RSV), High-fat diet (HFD), HFD plus resveratrol (HFD+RSV), High-protein diet (HPD), and HPD plus resveratrol (HPD+RSV). The ST+RSV group was included in the experimental phase, but was not used in the posterior analysis (plasma parameters, histology and real-time PCR) due to the absence of statistically significant differences regarding body composition (see Fig. 1A to 1I, and IK).

The experimental diets (HFD and HPD) were formulated as described in previous studies (Bianchetti et al. , 2010, de Pinho et al. , 2013, Duffy et al. , 2002) and were standardized and purchased from Rhoster®, Brazil. The high fat diet had the following composition: Cornstarch (40.57%), Casein (14%), dextrinized Starch (15.5%), Sucrose (10%), Soybean oil (10%), Cellulose - fiber (5%), Mineral mix AIN- 93M (3.5%), Vitamin Mix AIN -93 (1%), L- Cysteine (0.18%), Choline Bitartrate (0.25%), and Tert-butylhydroquinone (0.0008%). The high protein diet had the following composition: Cornstarch (32.57%), Casein (28%), dextrinized Starch (15.5%), Sucrose (10%), Soybean oil (4%), Cellulose - fiber (5%), Mineral mix AIN- 93M (3.5%), Vitamin Mix

AIN -93 (1%), L- Cysteine (0.18%), Choline Bitartrate (0.25%), and Tert-butylhydroquinone (0.0008%). Standard diet (Labina®) was produced by Purina®, Brazil. The centesimal composition of each diet is detailed in Table 1. In RSV groups, resveratrol powder (Sigma–Aldrich Co. LLC., Saint Louis, MO, EUA) was added to diet powder in the proportion of 4g/kg diet (Ringholm et al. , 2013, Tauriainen et al. , 2011), which corresponds to 300 mg/kg of body weight/day.

Measurements of body weight, food intake, tissue collection and plasma parameters

Body weight (BW), food intake, and energy intake (food intake in kcal) were recorded twice weekly. At the end of the experiment, the animals were fasted overnight (12h) and euthanized by decapitation. Samples of blood and adipose tissue (perigonadal, mesenteric and retroperitoneal) were collected, weighed, and stored immediately in liquid nitrogen and subsequently at −80 °C for posterior analysis. Body adiposity was calculated by the sum of perigonadal, mesenteric, and retroperitoneal adipose tissues. Blood samples were centrifuged (3000 rpm for 10 min) and the plasma was separated for the determination of glucose, triglycerides, high density lipoprotein (HDL), and total cholesterol levels, by enzymatic tests (Wiener Lab, Argentina).

Histology

Perigonadal adipose tissue samples were fixed in formaldehyde solution (10%) and embedded in paraffin serially sectioned at 5 mm, stained with hematoxylin and eosin (HE), and evaluated under a conventional light microscope using an Olympus FSX100 microscope (Tokyo, Japan). Images of fat tissue areas (10 ocular and 40 objective lenses) were captured with FSX-BSW software (Olympus, Tokyo, Japan). Adipocyte cell area was measured using ImageJ software (NIH, USA).

Reverse transcription and real-time PCR

Samples of perigonadal adipose tissue were prepared using Trizol reagent (Invitrogen Corp.VR, San Diego, CA, USA) and treated with DNAse (Invitrogen Corp.VR). Reverse transcription was carried out with M-MLV (Invitrogen Corp.VR) using random hexamer primers. Levels of genes of interest (Table 2) were determined by Real Time PCR (SYBR Green reagent) in Step One Plus equipment (Applied Biosystems-EUA). Gene expression was quantified using the relative comparative Ct (threshold cycle) method with GAPDH as the endogenous control (Livak and Schmittgen, 2001).

Statistical analysis

Analyses were performed using GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA, USA). Data were evaluated by one-way ANOVA, followed by Tukey post test. All data are given as means ± S.D. Statistical significance was accepted at $p < 0.05$.

Results

Resveratrol, diets, body composition and metabolic parameters

A decrease in the BW average was observed in HFD (21.12 ± 0.80g) and HFD+RSV (21.93 ± 1.37g) when compared to ST (25.08 ± 0.83g) (Fig. 1A). The group HPD+RSV (19.47 ± 1.63g) had lower BW than HPD (23.44 ± 1.00g) and ST (25.08 ± 0.83g) (Fig. 1B). Average food intake (Fig. 1C and 1D) and energy intake (Fig. 1E and 1F) were not statistically different between the groups.

Body adiposity was lower in HPD+RSV (0.019 ± 0.006g/BW) than in ST (0.040 ± 0.013g/BW) and HPD (0.038 ± 0.006g/BW) (Fig. 1H). The mass of perigonadal adipose tissue was significantly lower in HPD+RSV (0.008 ± 0.002g/BW) than in HPD (0.015 ± 0.005g/BW) (Fig. 1J). Mesenteric adipose tissue was higher in HPD (0.009 ± 0.004) than in HPD+RSV (0.004 ± 0.002). Retroperitoneal adipose tissue weight was not significantly different between the treatments.

The histological results showed that resveratrol reduced adipocyte area in HPD+RSV (1 053 000 ± 187 900 μm^2) group in comparison to HPD (1 396 000 ± 206 700 μm^2) and ST (1 765 000 ± 387 900 μm^2). In addition, HPD group showed reduced adipocyte area (μm^2) when compared to ST group (Fig. 2B). In the HFD and HFD+RSV groups the adipocyte areas were not significantly different (Fig. 2A).

Total cholesterol levels were lower in HFD+RSV (112 ± 41 mg/dL) than in HFD (222 ± 142 mg/dL) (Fig. 3A), and in HPD+RSV (117 ± 34 mg/dL) than in HPD (191 ± 32 mg/dL) and ST (182 ± 54 mg/dL) (Fig. 3B). HDL cholesterol was lower in HPD (66 ± 12 mg/dL) than in ST (100 ± 30 mg/dL) and HPD+RSV (93 ± 11 mg/dL) (Fig. 3D). Glucose and triglyceride levels were not significantly different between the treatments.

Resveratrol, diets and adipogenic gene expression

Real-time PCR was performed in order to analyze the expression of adipogenic genes, such as Peroxisome proliferator-activated receptor gamma (PPARγ), Sterol regulatory element-binding transcription factor 1-c (SREBP-1c), Acetyl-CoA carboxylase (ACC) and Fatty acid synthase (FAS).

PPARγ expression was higher in ST (1.00 ± 0.00) than in HFD (0.06 ± 0.10), HFD+RSV (0.03 ± 0.03), HPD (0.25 ± 0.33) and HPD+RSV (0.08 ± 0.13) (Fig. 4A and 4B). SREBP-1c expression was also higher in ST (1.00 ± 0.00) than in HFD (0.12 ± 0.11), HFD+RSV (0.21 ± 0.39), HPD (0.20 ± 0.16) and HPD+RSV (0.06 ± 0.05) (Fig. 4C and 4D).

The expression of ACC (Fig. 4E and 4F), was higher in ST (1.00 ± 0.00) than HFD (0.24 ± 0.29), HFD+RSV (0.12 ± 0.23), HPD (0.58 ± 0.26) and HPD+RSV (0.01 ± 0.01), as well as HPD that showed higher expression than HPD+RSV (Fig. 4F). Finally, FAS expression was higher in HPD (1.79 ± 0.66) when compared with HPD+RSV (0.55 ± 0.68) (Fig. 4H).

Discussion

The main findings of the present study showed distinct metabolic effects of resveratrol under different dietary compositions. In the literature, experimental studies with animals, involving resveratrol administered along with a high saturated fat diet are more easily found (Andrade et al., 2014a, Oliveira Andrade et al., 2014).

However, there are only a few studies that show the association between resveratrol and high-protein diet (Kim et al. , 2014a, Kim et al. , 2014b). Our study showed that animals treated with a high-protein diet and resveratrol had a decrease in BW, body adiposity, perigonadal adipose tissue, adipocyte area, total cholesterol, ACC and FAS expression, and had an increase in HDL cholesterol. Additionally, the effects of resveratrol in high-fat diet included a decrease in total cholesterol levels.

The macronutrient profile of a diet, in the treatment of MS, is an important consideration that may potentiate weight loss, and a decrease in cardiometabolic risk, thus the ideal diet should combine all of the dietary components that influence these factors (Abete et al. , 2010). High-protein and low-carbohydrate diets, low-glycemic index carbohydrates, and adequate omega-3 fatty acid intake are nutritional factors currently proposed for the treatment of MS (Abete et al., 2010). Saturated fat and fructose are more likely to stimulate hepatic lipid accumulation, whereas unsaturated fat, antioxidants and high-protein diets seem to have a more preventive effect (de Wit et al. , 2012).

Our study shows that resveratrol decreased body weight and body adiposity. Animals on a high-protein diet plus resveratrol had lower body weight average, perigonadal adipose tissue weight and body adiposity than animals on high-protein diet, which can be explained by the resveratrol anti-obesity effect (Wang et al., 2014), showing the important role of resveratrol in weight and fat loss. Resveratrol exerts its positive effects on MS by activating Sirt1, which regulates proteins that play important roles in the pathophysiology of metabolic diseases, such as the reduction of body fat adiposity (Perez-Torres et al. , 2013).

In contrast with other studies of our group, based on a high saturated fat diet (Andrade et al. , 2014b, Santos et al. , 2013), our findings showed that mice on a high-fat diet and high-fat diet plus resveratrol presented with lower body weight than mice on the standard diet, which can be explained by the composition of our high fat diet that is based on soybean oil, known to be rich in polyunsaturated fatty acids (PUFA). Studies show that PUFA is effective in increasing lean mass and in reducing body fat or the fat:lean ratio when compared with the high-fat lard control diet (Yepuri et al. , 2011).

Regarding the histological analysis of the perigonadal adipose tissue, the animals in the HPD and HPD+RSV groups showed shorter adipocytes when compared to ST group. In addition, more importantly, resveratrol was capable of decreasing the average area of adipocytes in HPD group, contributing to an improvement in overall adiposity. This result confirms that resveratrol is effective in reducing the size of adipose tissue (Macarulla et al. , 2009).

Resveratrol has demonstrated an important role in lipid metabolism. In our study, total cholesterol levels were lower in the groups HFD+RSV and HPD+RSV when compared with the HFD and HPD groups, respectively. Resveratrol reduces cholesterol synthesis by downregulating HMG-CoA reductase and enhances reverse cholesterol excretion and cholesterol transport through increasing HDL levels and the capacity of HDL to mediate cholesterol efflux from macrophages in arterial walls (Wang et al. , 2012). Another study by our group found the same result, in which low dose resveratrol (30 mg/kg/day) decreased total cholesterol in the setting of a high-fat diet (Andrade et al., 2014a). The same result was found in the study of Kim et al. (Kim et al. , 2011) using 0.4% of resveratrol, the same dose of our study. Additionally, HDL-cholesterol levels were higher in HPD+RSV than in HPD. Jeon, Lee and Choi (Jeon et al. , 2014) in a study with animals fed with an atherogenic diet and resveratrol, found that resveratrol significantly increased the plasma HDL-C concentration compared with the control.

The expression of adipogenic genes, PPARγ and SREBP-1c, was lower in all diets (HFD and HPD) than in ST and there was no difference with resveratrol treatment. However, a study showed that resveratrol decreased PPARγ and SREBP-1c expression in high-fat diet group (Andrade et al., 2014a). Kim et al. (Kim et al., 2011) showed that resveratrol (0.4%) significantly reversed the HFD-induced up-regulation of key adipogenic genes (PPARγ2, C/EBPα, SREBP-1c, LPL, aP2, and leptin) in the epididymal adipose tissues of mice. PPARγ is the master regulator of adipogenesis, and plays an important role in lipid metabolism and glucose homeostasis (Moran-Salvador et al. , 2011), while also being decreased by resveratrol (Calleri et al. , 2014). SREBP-1c is the most important transcription factor regulating de novo lipogenesis, and is also inhibited by resveratrol via the Sirt1–FOXO1 signaling pathway (Wang et al. , 2009).

On the other hand, expression of lipogenic enzymes, ACC and FAS, were reduced in HPD+RSV group, which lead us to see the effects of resveratrol on decreasing lipogenesis in high-protein diet. Another study of our group showed that resveratrol (30 mg/kg/day) decreased ACC expression in the setting of a high-fat diet (Andrade et al., 2014a). In the study of Kim et al. (Kim et al., 2011), resveratrol (0.4%) decreased the expression of FAS in mice treated with a high-fat diet.

Lipogenesis involves the synthesis of fatty acids, from acetyl CoA, used as substrates in the synthesis of triacylglycerols (Aguirre et al., 2014). High-protein diets rich in branched-chain amino acids can increase lipogenesis by converting amino acid into acetyl-CoA (Brosnan and Brosnan, 2006), and high-fat diets increase lipogenesis through fatty acid conversion to acetyl-CoA (Shi and Tu, 2015). Acetyl-CoA undergoes action of ACC and FAS, and increases fatty acids synthesis (Aguirre et al., 2014) (Fig. 4I). In the literature, resveratrol is described as acting by decreasing adipogenic gene expression, such as PPARγ and SREBP-1c, which control lipogenic enzymatic activity (Aguirre et al., 2014). In our study, resveratrol decreased ACC and FAS expression in high-protein diet, decreasing lipogenesis (Szkudelska and Szkudelski, 2010, Wang et al., 2014) (Fig. 4I). This decrease in lipogenesis should be considered as a main contributor to the reduction in body weight and body adiposity of resveratrol in high-protein diets.

Many studies show the role of resveratrol in decreasing lipogenesis in association with a high-fat diet (Aguirre et al., 2014Aguirre et al., 2014, Andrade et al., 2014a, Wang et al., 2014), but in our study this result was not found. This absence of effect of resveratrol in the high-fat diet is related to the composition of our diet, rich in polyunsaturated fat. On the other hand, the literature has no studies showing this effect on high-protein diet. Given this, to our knowledge, our study is the first to show the role of resveratrol in decreasing lipogenesis in animals treated with a high-protein diet.

Conclusion

In conclusion, the obtained results show that resveratrol decreases markers of lipogenesis and metabolic parameters in the setting of a high-protein diet. Moreover, resveratrol decreased the total cholesterol in both diets. These results indicate the increased potential of resveratrol use in prevention and treatment of metabolic syndrome, acting in different dietary compositions.

Competing interests

There are no conflicts of interests.

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Abbreviations

ST, standard; HFD, high-fat diet; HFD+RSV, HFD plus resveratrol; HPD, high-protein diet; HPD+RSV, HPD plus resveratrol; PPARγ, peroxisome proliferator-activated receptor gamma; SREBP-1c, sterol regulatory element-binding transcription factor 1-c; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; MS, Metabolic Syndrome.

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Table 1
Experimental diets composition

Diet	Carbohydrate (%)	Protein (%)	Lipid (%)	Calories (kcal/g)
Standard diet (ST)	65	23	12	3.95
High-fat diet (HFD)	69	11	20	3.84
High-protein diet (HPD)	59	31	10	3.65

Table 2
Sequence of primers used for Real-time PCR analysis

Gene	Primer Forward	Primer Reverse
GAPDH	AAC GAC CCC TTC ATT GAC CTC	CTT CCC ATT CTC GGC CTT GAC
SREBP1-c	TGC GTG GTT TCC AAC ATG AC	CCT CAT GTA GGA ATA CCC TCC TCA TA
FAS	CAT CCT AGG CAT CCG AGA CCT	ATC GTG TTC TCG TTC CAG GAT C
ACC	GAA CAT CCC CAC GCT AAA CAG A	CTG ACA AGG TGG CGT GAA GG
PPAR-y	TTA TGG GTG AAA CTC TGG G	CAA CCA TTG GGT CAG CTC

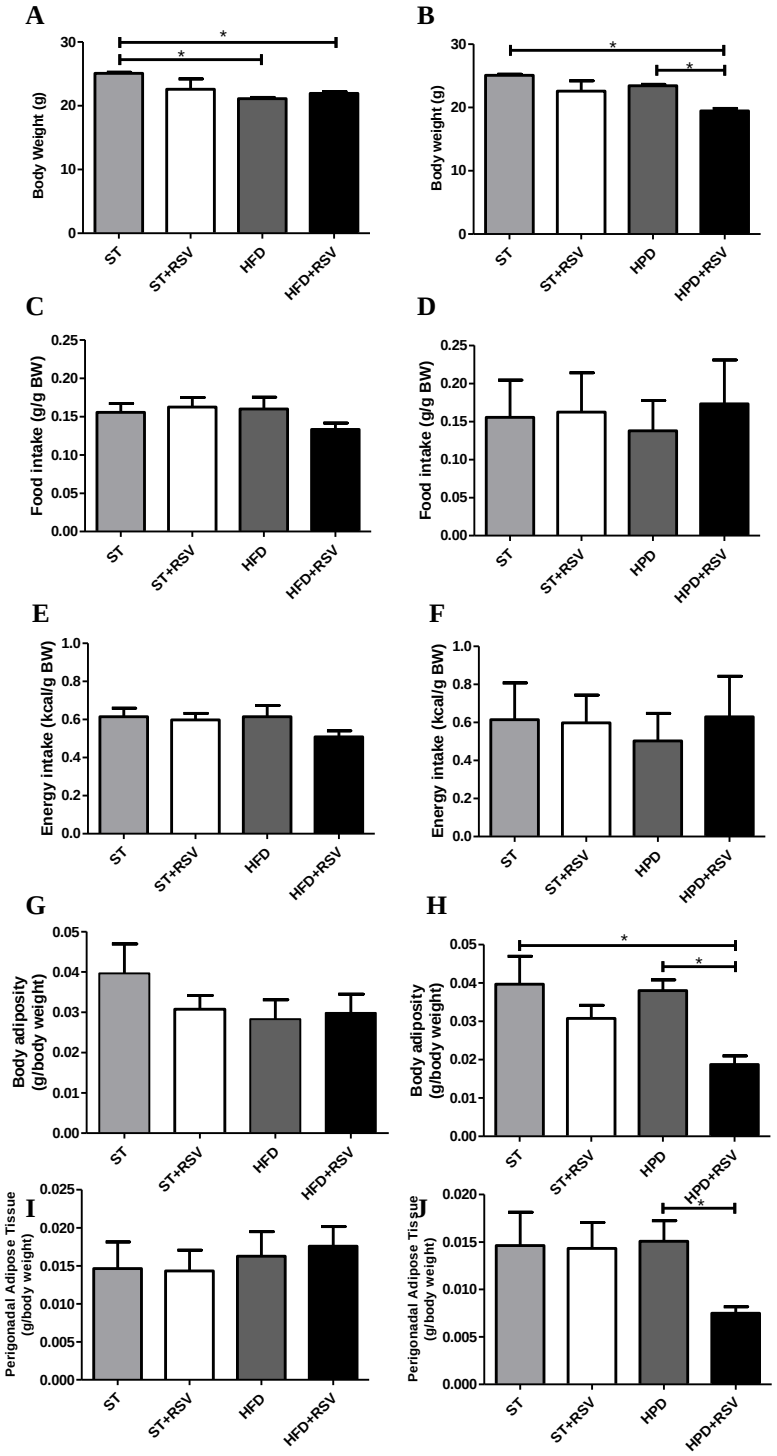

Fig. 1. Body composition in mice-fed standard (ST), standard plus resveratrol (ST+RSV), high fat diet (HFD), HFD plus resveratrol (HFD+RSV), high protein diet (HPD), and HPD plus resveratrol (HPD+RSV). Body Weight (BW) (g) in HFD **(A)** and HPD **(B).** Food intake (g/BW) in HFD **(C)** and HPD **(D).** Energy intake (kcal/BW) in HFD **(E)** and HPD **(F).** Body adiposity (g/BW) in HFD **(G)** and HPD **(H)**. Perigonadal adipose tissue (g/BW) in HFD **(I)** and HPD **(J)**. *p<0.05.

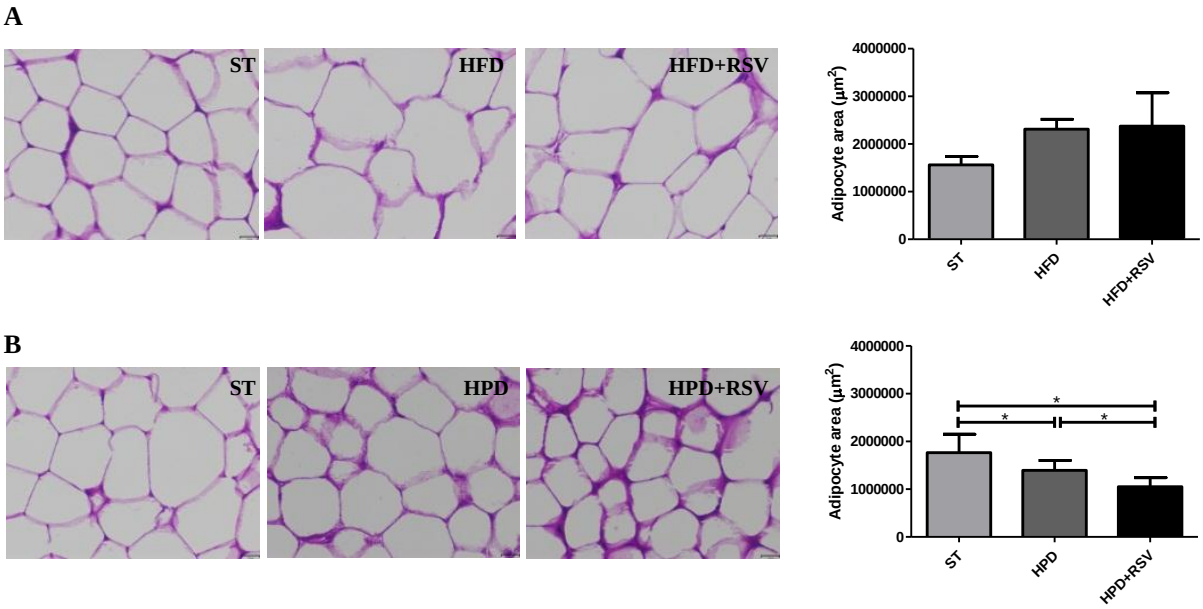

Fig. 2. Histological analysis in mice-fed standard (ST), high fat diet (HFD), HFD plus resveratrol (HFD+RSV), high protein diet (HPD), and HPD plus resveratrol (HPD+RSV). Hematoxylin-Eosin (HE) stained tissue sections of perigonadal adipose tissue and adipocyte area (μm^2) in HFD **(A)** and HPD **(B).** *$p<0.05$.

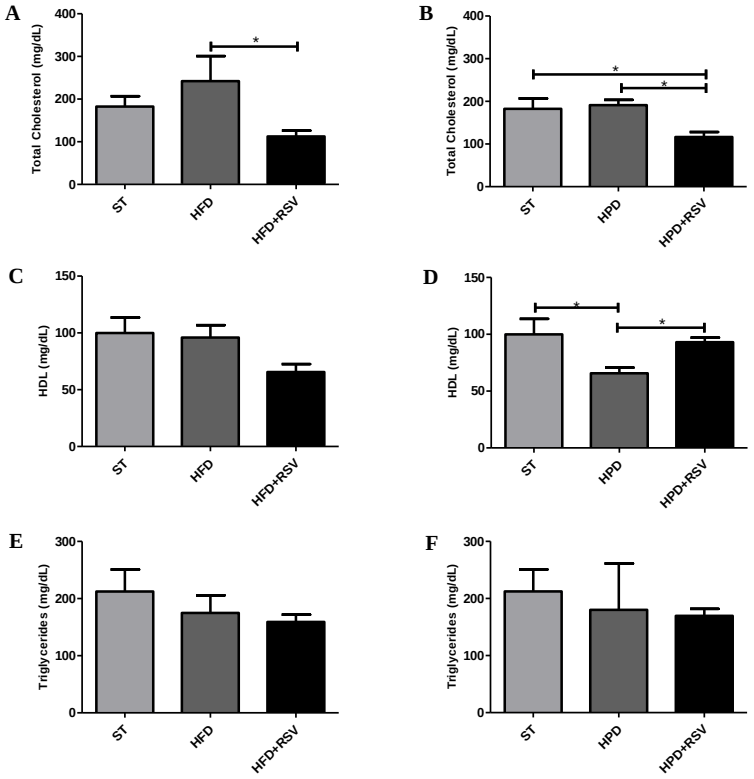

Fig. 3. Lipid profile in mice-fed standard (ST), high fat diet (HFD), HFD plus resveratrol (HFD+RSV), high protein diet (HPD), and HPD plus resveratrol (HPD+RSV). Total cholesterol (mg/dL) in HFD **(A)** and HPD **(B).** HDL cholesterol (mg/dL) in HFD **(C)** and HPD **(D)**. Triglycerides (mg/dL) in HFD **(E)** and HPD **(F)**. * $p<0.05$.

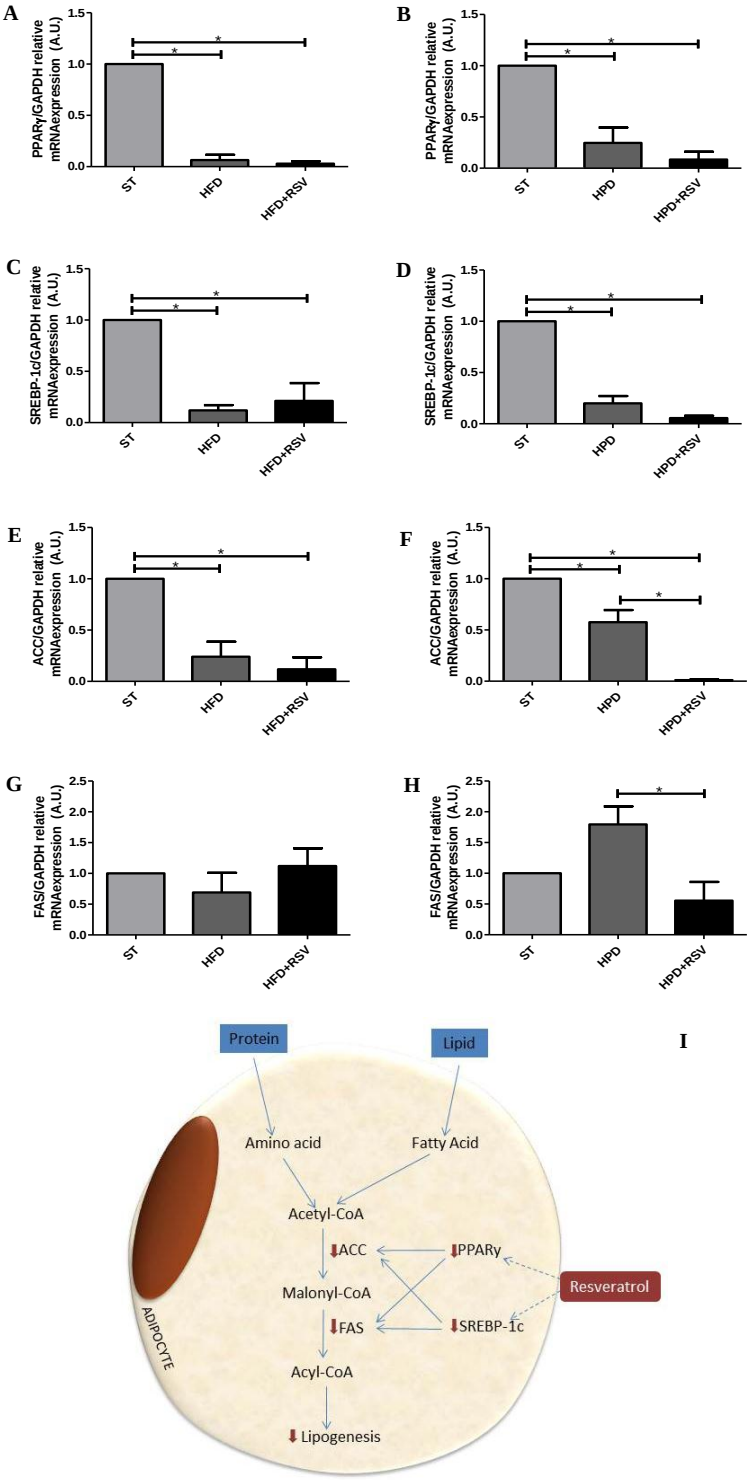

Fig. 4. Expression of adipogenic genes in perigonadal adipose tissue of mice-fed standard (ST), high-fat diet (HFD), HFD plus resveratrol (HFD+RSV), high-protein diet (HPD), and HPD plus resveratrol (HPD+RSV). Peroxisome proliferator-activated receptor gamma (PPARγ) (Arbitrary Unit) in HFD **(A)** and HPD **(B)**. Sterol regulatory element-binding transcription factor 1-c (SREBP-1c) (Arbitrary Unit) in HFD **(C)** and HPD **(D)**. Acetyl-CoA carboxylase (ACC) (Arbritary Unit) in HFD **(E)** and HPD **(F)**. Fatty acid synthase (FAS) (Arbritary Unit) in HFD **(G)** and HPD **(H).** Possible mechanism of resveratrol on high-fat and high-protein diets in adipogenesis and lipogenesis **(I)**. *p<0.05.

4.2 Artigo 2

Acute oral treatment with Resveratrol and *Lactococcus lactis* subsp. *lactis* decrease body weight and total cholesterol, and improve liver proinflammatory markers in C57BL/6 mice

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* Keila Lopes Mendes, Letícia Antunes Athayde, Ronize Viviane Jorge de Faria and Luiz Henrique da Silveira contributed to conduct the study, as well as to collect, analyze and perform the interpretation of the data.

* *Keila Lopes Mendes* wrote the manuscript.

* *Sérgio Henrique Sousa Santos* helped to revise the manuscript and contributed to the final approval of the version to be published. All authors have approved the final article.

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ABSTRACT

Diet plays an essential role in preventing inflammation and hepatic diseases. In this context, the present study aimed to evaluate the effect of resveratrol and *Lactococcus lactis* on metabolic parameters and expression of hepatic proinflammatory markers. C57BL/6 mice were divided into 4 groups: Standard (ST), *Lactococcus lactis* (LL), Resveratrol (RSV), and *Lactococcus lactis* together with resveratrol (LL+RSV). *Lactococcus lactis* and resveratrol were administered by orogastric gavage. Blood parameters were assessed. mRNA expression of IL-6

and TNF-α was evaluated by Real-time PCR and IL-8 and TNF-α protein expression by immunohistochemistry. The main findings showed that association of resveratrol and *Lactococcus lactis* decreased body weight, aspartate aminotransferase levels and total cholesterol levels. LL and LL+RSV decreased triglycerides levels and IL-6, TNF-α, and IL-8 expression. These results open a perspective of using resveratrol and *Lactococcus lactis* to improve metabolic parameters and *Lactococcus lactis* in preventing inflammation and the development of hepatic diseases.

Keywords: Diet; Resveratrol; Inflammation; *Lactococcus lactis*; Liver.

1 Introduction

Hepatic diseases is a significant global health problem and causes global increases of morbidity and mortality (Zhou, Chen, He et al., 2015). Inflammation, a biological response involved in the homeostasis maintenance, is considered one of the consequences of liver disease (Medzhitov, 2010). The inflammatory process includes the recruitment of adhesion molecules and proinflammatory cytokines (Lappas, 2012). The proinflammatory phase activates and inhibits genes in order to induce the inflammatory phase and reprograms other genes to support the adaptation phase (McCall, Yoza, Liu et al., 2010). A decrease of the inflammation may have beneficial effects on the pathophysiology of liver disease. In this regard, diet may play an important role in reducing inflammation.

In the last few years evidences support that nutrition plays an important role for a healthy life (Mukherjee, Dudley and Das, 2010). Currently, there are several nutritional recommendations that include the consumption of healthy foods, probiotics and nutraceuticals (Monteiro, Cannon, Moubarac et al., 2015,Ebner, Smug, Kneifel et al., 2014). Resveratrol (3,5,40-trihydroxystilbene), a poliphenol found in grape skin and red wine (Cho, Jung and Choi, 2012), it is known for its anti-cancer, anti-adiposity, cardioprotective, and antioxidant properties (Aguirre, Fernandez-Quintela, Arias et al., 2014,Novelle, Wahl, Dieguez et al., 2015).

Probiotics are considered beneficial bacteria in gastrointestinal tract with several good effects (Ho, Lu, Chang et al., 2014). There are two key members of this group: *lactobacilli* and *bifidobacteria* (Mallappa, Rokana, Duary et al., 2012). *Lactococcus lactis* belongs to lactic acid bacteria and it is the main constituent of dairy starter culture used worldwide for the production of dairy products (Cavanagh, Fitzgerald and McAuliffe, 2015). The benefits of probiotic consumption include the control of metabolic disorders, including obesity and diabetes (Mallappa et al., 2012), anti-inflammatory effects (Luerce, Gomes-Santos, Rocha et al., 2014) and prevention of cancer, by maintaining the balance of the microflora (Kahouli, Tomaro-Duchesneau and Prakash, 2013).

Some studies have evaluated the effect of the isolated consumption of resveratrol and probiotics on inflammation (Luerce et al., 2014,Wang, Sun, Li et al., 2013,Poulsen, Fjeldborg, Ornstrup et al., 2015). However, the associated consumption of probiotics and resveratrol were not evaluated. In this context, the present study aims to evaluate the effect of resveratrol and *Lactococcus lactis* on metabolic parameters and liver proinflammatory markers.

2 Materials and methods

Animals and experimental procedure

Twenty female C57BL/6 mice aged eight weeks were used for this experiment, and they were randomly divided into 4 groups (5 animals/group) as follows: Standard group (ST), *Lactococcus lactis* (LL), Resveratrol (RSV), and *Lactococcus lactis* together with resveratrol (LL+RSV). The mice, obtained from the Universidade Federal de Minas Gerais (Belo Horizonte, Minas Gerais, Brazil), were maintained in individual cages and exposed to a 12h:12h light cycle (lights on from 7:00 to 19:00 h) at a controlled temperature of 25.0 ± 2.0ºC. Food and water were offered *ad libitum*. The mice were fed for 15 days with standard diet (Labina®, Purina®, Brazil), which is composed of 65% of carbohydrates, 23% of proteins, 12% of lipids, with a total of 3.95 kcal/g of diet. This study was approved by the Ethics Committee of Experimentation and Animal Welfare of Unimontes, Montes Claros, Brazil (process nº 082/2014).

The experimental period lasted 15 days. In the first four days, the animals received *Lactococcus lactis* or the vehicle solution (brain-heart infusion broth) in the morning and resveratrol or its vehicle (saline) in the afternoon. In the next 11 days, the animals received only resveratrol or saline. All treatments were administered by orogastric gavage. Body weight (BW) and food intake were recorded every day.

At the end of the experiment (16th day), the animals were fasted overnight (12h) and euthanized by decapitation. Samples of blood and adipose tissue (perigonadal, mesenteric and retroperitoneal) and liver were collected and weighted. Body adiposity was calculated by the sum of perigonadal, mesenteric, and retroperitoneal adipose tissues. The liver was stored immediately in liquid nitrogen and subsequently at −80 °C for posterior analysis. Blood samples were centrifuged (3000 rpm for 10 min) and the plasma was separated for the determination of glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, and total cholesterol levels, by enzymatic tests (Wiener Lab, Argentina).

Lactococcus lactis preparation and administration

The bacterial strain used in this work was *Lactococcus lactis* subsp. *lactis*. Single colonies were inoculated in plates with brain-heart infusion (BHI) agar, remaining in an incubator at 30°C for 24 hours. After this period, the cells was diluted in BHI broth until it reach 1.9 of absorbance (spectrophotometer at 540 nm), which corresponds to $1x10^9$ cells/mL, as calibration curve made previously. The animals received 0.2 mL of this solution by orogastric gavage during 4 consecutive days. The control groups received BHI broth only.

Resveratrol preparation and administration

The animals received treatment with resveratrol (Sigma-Aldrich®, purity ≥ 99%) at 100 mg/kg of animal weight, diluted in physiological saline (sodium chloride 0.9%) and administered to animals by orogastric gavage (0.2 mL) during 15 consecutive days following the beginning of the treatment. The control groups received saline only.

Immunohistochemistry

For immunohistochemical reactions, 3 μm tissue sections were mounted on organosilane slides. All sections were then deparaffinized in xylene, and rehydrated with a series of alcohol. For antigen retrieval, sections were heated for 5 min at 125˚C in Tris-EDTA buffer. Endogenous peroxidase was blocked with 0.03% H_2O_2 in ethanol for 30 min. The primary antibody Interleukin 8 (IL-8) (1:100, Clone 6217, R&D Systems Inc) and Tumor necrosis factor alpha (TNF-α) (1:100, clone ab66579, ABCAM®, Cambridge, UK) were used and the incubation time was 18 h at 4˚C. The primary antibodies were detected using the LSAB kit (Dako, Denmark). Signals were revealed with 3'3-diaminobenzidine-tetrahydrocloridre for 5 min and counter-stained with Harris hematoxylin for 30 sec. As a positive control we used lymph node samples and the negative control of the reaction was obtained from the samples without incubation with the respective primary antibodies. The immunohistochemical expression of biomarkers was evaluated using an Olympus FSX100 microscope (Tokyo, Japan). Images (10 ocular and 40 objective lenses) were captured with FSX-BSW software (Olympus, Tokyo, Japan). The immunohistochemical analysis investigated the percentage of positively stained cells (ImageJ software, NIH, USA) and are expressed as mean ± standard deviation (mean ± SD) values.

Reverse transcription and real-time PCR

Total RNA from the liver samples was prepared using TRIzol reagent (Invitrogen Corp.VR, San Diego, CA, USA), treated with DNAse (Invitrogen Corp.VR) and reverse transcribed with M-MLV (Invitrogen Corp.VR) using random hexamer primers. The levels of the genes of interest were determined by qRT-PCR using SYBR Green reagent and the QuantStudio 6 Flex system (Applied Biosystems-EUA). Gene expression was quantified using the relative comparative Ct (threshold cycle) method (Livak and Schmittgen, 2001) and normalized to the endogenous β-actin *(FW: GGCTGTATTCCCCTCCATCG; RV: CCAGTTGGTAACAATGCCATGT).* The genes of interest and respective primers were: Interleukin 6 (IL-6) *(FW: TTGGGAGTGGTATCCTCTGTG; RV: TTCCATCCAGTTGCCTTCTTG)* and TNF-α *(FW: CATCTTCTCAAAATTCGAGTGACAA; RV: TGGGAGTAGACAAGGTACAACCC).*

Statistical analysis

Analyses were executed by GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA, USA). Data were evaluated by one-way ANOVA, followed by Tukey post test. All data are given as means ± S.D. Statistical significance was accepted at $p < 0.05$.

3 Results

Body composition and metabolic parameters

A decrease in the body weight (BW) average was observed in the LL+RSV group (20.93 ± 0,50g) compared to the groups LL (22.06 ± 0.49g) and RSV (21.74 ± 0.33g) (Fig. 1A). In addition, the BW was higher

in LL and RSV than in ST (21.05 ± 0.50g). Body adiposity and food intake were not statistically different among the groups (Fig. 1B and 1C). The liver mass was significantly lower in LL (0.039 ± 0.002g/BW) than in ST (0.046 ± 0.006g/BW) (Fig. 1D).

The plasma levels of alanine aminotransferase were lower in LL (30 ± 14.1 U/L), RSV (40 ± 14.1 U/L) and LL+RSV (25 ± 17.3 U/L) as compared to ST (85 ± 31.1 U/L) (Fig. 1E). Aspartate aminotransferase (U/L) plasma levels were lower in LL+RSV (237.5 ± 59.1 U/L) than in ST (520 ± 50 U/L), LL (383 ± 61.1 U/L) and RSV (460 ± 70.7 U/L) (Fig. 1F). The glucose plasma levels were lower in LL+RSV (56.4 ± 12.9 mg/dL) compared to LL (99 ± 43.2 mg/dL) (Fig. 1G). Total cholesterol plasma levels were lower in LL+RSV (85 ± 5.8 mg/dL) than in ST (110 ± 10 mg/dL), LL (116.7 ± 15.3 mg/dL) and LL+RSV (115 ± 7.1 mg/dL) (Fig. 1H). Triglycerides plasma levels were lower in LL (92.5 ± 22.2 mg/dL) and LL+RSV (87.5 ± 18.9 mg/dL) as compared to ST (135 ± 20.8 mg/dL) (Fig. 1I).

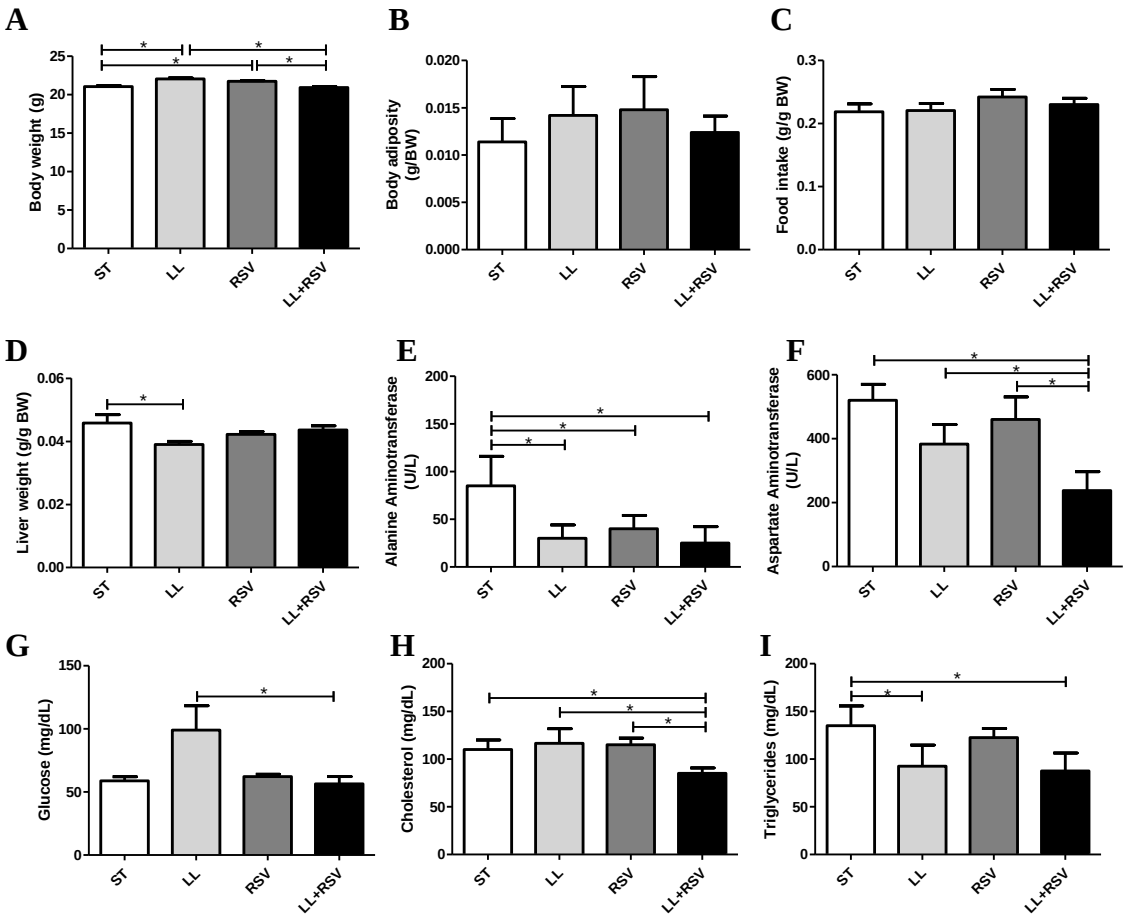

Figure 1 - Body composition and metabolic parameters of mice treated as follows: Standard (ST), *Lactococcus lactis* (LL), Resveratrol (RSV), and *Lactococcus lactis* together with resveratrol (LL+RSV). **(A)** Body Weight (BW) (g)**. (B)** Body adiposity (g/BW). **(C)** Food intake (g/BW)**. (D)** Liver weight (g/BW)**. (E)** Alanine Aminotransferase (ALT) (U/L)**. (F)** Aspartate Aminotransferase (AST) (U/L)**. (G)** Plasma glucose (mg/dL)**. (H)** Total cholesterol (mg/dL)**. (I)** Triglycerides (mg/dL)**.** * $p<0.05$.

Proinflammatory markers expression

qRT-PCR was performed in order to analyze the expression of inflammatory cytokines, such as IL-6 and TNF-α. IL-6 expression (Fig. 2A) was lower in LL (0.11 ± 0.09), RSV (0.48 ± 0.34) and LL+RSV (0.17 ±

0.25) than in ST (1.00 ± 0.00). The expression of TNF-α (Fig. 2B) was lower in LL (0.27 ± 0.26) than in ST (1.00 ± 0.00) and RSV (1.29 ± 0.22). In addition, LL+RSV (0.58 ± 0.24) showed lower expression of TNF-α as compared to RSV.

The immunohistochemical expression pattern of IL-8 and TNF-α is demonstrated in Fig. 2C to 2E. We observed that the frequency of cells expressing the IL-8 cytokine was significantly lower in LL (33.21 ± 2.42%), RSV (34.01 ± 1.38%) and LL+RSV (33.87 ± 1.6%) as compared to ST (40.57 ± 2.38%) (Fig. 2C and 2E). Additionally, the frequency of cells expressing TNF-α was significantly lower in LL (23.26 ± 1.0%) compared to ST (28.51 ± 4.61%) and RSV (30.97 ± 2.36%). In addition, LL+RSV had a significantly lower frequency (25.74 ± 1.5%) of positive TNF- α marked cells compared to the RSV group (Fig. 2D and 2E).

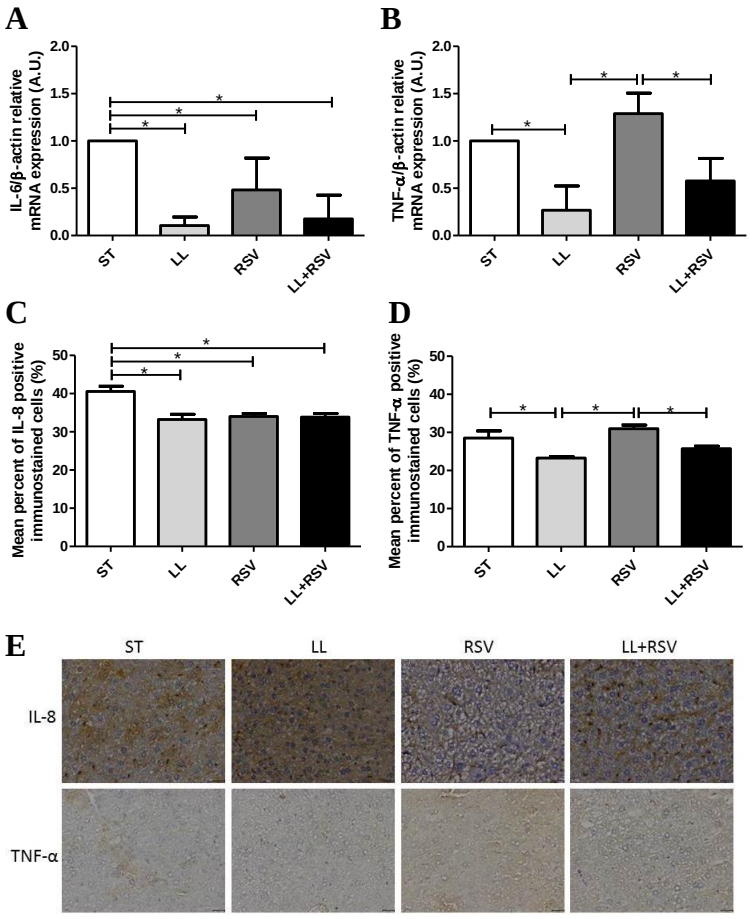

Figure 2 – Analysis of expression of inflammatory-related target genes in Standard (ST), *Lactococcus lactis* (LL), Resveratrol (RSV), and *Lactococcus lactis* together with resveratrol (LL+RSV) groups of mice. **(A)** mRNA expression of Interleukin 6 (IL-6) (Arbitrary Unit)**. (B)** mRNA expression of Tumor necrosis factor alpha (TNF-α) (Arbitrary Unit)**. (C)** Protein expression of Interleukin 8 (IL-8) (% of positive immunostained cells)**. (D)** Protein expression of Tumor necrosis factor alpha (TNF-α) (% of positive immunostained cells)**. (E)** Images of proinflammatory cytokines expression in liver**.** *p<0.05.

4 Discussion

The present study showed that in this experimental model the acute oral administration of *Lactococcus lactis* subsp. *lactis* and resveratrol was effective in decreasing the body weight, plasma levels of AST and plasma levels of total cholesterol and that both *Lactococcus lactis* subsp. *lactis* and the association of *Lactococcus lactis*

subsp. *lactis* and resveratrol were effective in reducing liver expression of inflammatory markers: mRNA expression of IL-6 and TNF-α and protein expression of IL-8 and TNF-α.

Resveratrol mimics the effects of calorie restriction (Timmers, Konings, Bilet et al., 2011), driven by the activation of Sirtuin 1 (Chung, Yao, Caito et al., 2010), a histone deacetylase. Various metabolic pathways, enzymes and genes are targets of modulation by resveratrol, such as AMP kinase (Pacholec, Bleasdale, Chrunyk et al., 2010), sirtuins and forkhead family of transcription factors (FOXO) (Mukhopadhyay, Pacher and Das, 2011), peroxisome proliferators-activated receptor g co-activator 1α (Um, Park, Kang et al., 2010), fatty acid synthase, sterol regulatory element-binding protein- 1c, acyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, and HMG-CoA reductase (Pan, Lai, Tsai et al., 2014), peroxisome proliferator-activated receptor a, carnitine palmitoyltransferase 1 and acyl-CoA oxidase (Pan et al., 2014,Shang, Chen, Xiao et al., 2008,Jeon, Jeong, Shin et al., 2012,Cho, Ahn, Kim et al., 2008,Gomez-Zorita, Fernandez-Quintela, Macarulla et al., 2012,Alberdi, Rodriguez, Macarulla et al., 2013).

Lactococcus lactis is in constant transit through the gastro intestinal tract after ingestion (Santos Rocha, Lakhdari, Blottière et al., 2012) and as a probiotic bacteria exerts a local and systemic action (Santos Rocha, Gomes-Santos, Garcias Moreira et al., 2014). Probiotic microorganisms can act by three ways: directly on the intestinal microbiota, directly on the intestinal mucus and epithelium, and on the other organs and systemic immune system, such as liver (Gerritsen, Smidt, Rijkers et al., 2011).

Our study showed that LL+RSV decreased body weight. This can be explained by the resveratrol anti-obesity effect, decreasing adipogenesis and increasing lipolysis (Wang, Moustaid-Moussa, Chen et al., 2014). In addition, some studies have shown that treatment with probiotics decreased body weight (Nunez, Galdeano, de LeBlanc Ade et al., 2014) and adiposity (Kadooka, Sato, Ogawa et al., 2013). Then, the association is beneficial in reducing the body weight.

The evaluation of hepatic parameters (liver weight, ALT, and AST) showed that the association between RSV and LL might be effective in reducing hepatic steatosis. The liver weight was lower in LL group, which is in agreement with other studies using probiotics in association with high-fat diet (Zheng, Lu, Wang et al., 2013) and high-cholesterol diet (Xie, Cui, Yin et al., 2011). Additionally, the levels of ALT were lower in LL, RSV and LL+RSV groups as compared to ST. Moreover, the AST levels were lower only in the LL+RSV group. Some studies show the role of probiotics in decreasing the level of these enzymes (Buss, Valle-Tovo, Miozzo et al., 2014), showing that probiotics can be used in the treatment of liver diseases. Concerning the use of resveratrol, some studies indicate that in diabetic animals resveratrol was effective in reducing the levels of these enzymes, but this effect was not observed in healthy animals (Schmatz, Perreira, Stefanello et al., 2012,Palsamy and Subramanian, 2011).

Regarding the glucose metabolism, our study shows that the association between resveratrol and *Lactococcus lactis* subsp. *lactis* was effective in decreasing fasting glucose in comparison to the *Lactococcus lactis* group. This effect may be attributed to resveratrol. One meta-analysis study showed that resveratrol intervention significantly reduced fasting glucose, insulin concentrations, HbA1c and HOMA-IR values (Liu, Zhou, Wang et al., 2014). Interestingly, our results from the LL group contrasts with the literature, as some studies showed a significant decrease of glucose after the consumption of probiotics, which was not observed in our study (Asemi, Zare, Shakeri et al., 2013,Mazloom, Yousefinejad and Dabbaghmanesh, 2013). The increase

of glucose in LL group can be attributed to BHI broth, a culture medium rich in nutrients, which can enhance the LL metabolism.

The evaluation of the lipid metabolism showed that the association between *Lactococcus lactis* and resveratrol was effective in decreasing the plasma levels of total cholesterol and triglycerides. In addition, the group treated with *Lactococcus lactis* only, was effective in decreasing the plasma levels of triglycerides. A study of Wang and cols. (Wang, Zhang, Chen et al., 2012) evaluated the cholesterol-lowering effects of 3 bacteria probiotics in rats fed a high-lipid diet, and found that the rats treated with probiotic had a significant reduction in serum levels of triglycerides, total cholesterol, and low-density lipoprotein cholesterol, and a significantly increase in the levels of high-density lipoprotein. Resveratrol is also described to develop an important role on lipid metabolism. A study carried out by our group found that resveratrol decreased total cholesterol levels and triacylglycerol levels in mice with hepatic steatosis (Andrade, Paraiso, de Oliveira et al., 2014). Resveratrol reduces cholesterol by downregulating HMG-CoA reductase (Wang, Yang, Qian et al., 2012) and decreases triglycerides levels by a reduction of the lipogenesis process (Wang et al., 2014).

The main findings of this study were that both the mRNA and protein expression of proinflammatory cytokines (IL-6 and TNF-α) and chemokines (IL-8), assessed by qRT-PCR and immunohistochemistry, respectively, were reduced by both *Lactococcus lactis* and *Lactococcus lactis* together with resveratrol. These results show that, in this experimental model, the inflammation decreasing can be attributed to *Lactococcus lactis*. IL-6 is a cytokine produced by T-lymphocytes, synovial fibroblasts, monocytes, and macrophages, whose effects includes induction of acute-phase ignited by B-lymphocyte differentiation, hepatocytes, and T-lymphocyte subset differentiation (Lin, 2015). A study of Yoon and cols. (Yoon, Yoon, Kim et al., 2014) with C57BL/6 mice show that bacteria probiotics were effective in decreasing IL-6 expression. With respect to resveratrol, Andrade and cols. (Andrade et al., 2014) in a study with FVB/N mice found that this compound decreased IL-6 and TNF-α expression in the liver, but in our study this result was not found.

IL-6 and TNF-α are involved in metabolic complications of liver diseases (Tilg and Moschen, 2010). TNF-α is a pro-inflammatory cytokine that promotes the transcription of several inflammatory genes, enhances leukocyte migration, and causes apoptosis of intestinal epithelial cells (Fausel and Afzali, 2015). Some studies show the role of probiotics in decreasing the expression levels of TNF-α (Ritze, Bardos, Claus et al., 2014,Sharma, Kapila, Dass et al., 2014,Huang, Lin, Liu et al., 2015). Another study, carried by Anukam and cols. (Anukam, Hayes, Summers et al., 2009) evaluated urine and serum of patients with acute infection and found that the use of probiotics was able to reduce the levels of IL-6, IL-8, and TNF-α. Additionally, although our study did not show this same result, studies have shown that resveratrol has an important role in decreasing TNF-α expression, being its anti-inflammatory function highly dependent of Sirt1(Zhu, Liu, Wang et al., 2011).

Lactococcus lactis and *Lactococcus lactis* together with resveratrol were also modulators of IL-8 expression. IL-8 plays a role of chemotaxis of neutrophils to the site of inflammation, promoting neutrophil adhesion and transmigration across the endothelium and stimulates the cells to perform phagocytosis (Jundi and Greene, 2015). A study showed that administration of *Lactococcus lactis* decrease the expression of TNF-α and IL-8 (Nishitani et al., 2009).

Importantly, this study performed an acute treatment in healthy animals, but according to the literature, there is still no consensus if resveratrol it is effective or not in Healthy subjects, without metabolic diseases (Novelle et al., 2015). This may explain why resveratrol showed different results from those found in the

literature. Future studies will be conducted to evaluate the effect of this association in animals with metabolic disorders.

5 Conclusion

In conclusion, the present results showed that association between *Lactococcus lactis* subsp. *lactis* and resveratrol was effective in decreasing body weight, plasma levels of AST and plasma levels of total cholesterol. Moreover, *Lactococcus lactis* subsp. *lactis* and the association of *Lactococcus lactis* subsp. *lactis* and resveratrol improved the hepatic proinflamatory profile. These results points to the increased potential of resveratrol and *Lactococcus lactis* in improving metabolic parameters and *Lactococcus lactis* in prevention of inflammation and development of hepatic diseases.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

ST, standard; LL, *Lactococcus lactis*; RSV, ; LL+RSV, *Lactococcus lactis* together with resveratrol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumor necrosis factor alpha.

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5 CONSIDERAÇÕES FINAIS

Nos últimos anos, a emergência da síndrome metabólica trouxe consigo a necessidade de estudos que objetivem descobrir alternativas viáveis para sua prevenção e tratamento. Atualmente, a prevenção e o tratamento dessa síndrome e de seus fatores de risco metabólicos preconizam mudanças de estilo de vida, baseadas principalmente em uma alimentação saudável. Nesse sentido, a dieta exerce um papel de extrema importância, pois está presente tanto na etiologia quanto no tratamento dessas doenças.

Neste estudo, procurou-se avaliar o efeito do consumo do resveratrol, associado a diferentes composições dietéticas em macronutrientes e a um probiótico, o *Lactococcus lactis* subsp. *lactis*. Com relação à associação do resveratrol a diferentes composições dietéticas, os resultados encontrados mostraram que, com a dieta hiperproteica, o resveratrol diminuiu o peso e a adiposidade corporal, colesterol total e a expressão de marcadores lipogênicos (ACC e FAS) e aumentou o HDL colesterol. Já com a dieta hiperlipídica, o resveratrol diminuiu os níveis de colesterol total. Sobre a associação do resveratrol ao *Lactococcus lactis* subsp. *lactis,* os resultados encontrados mostraram que, com a associação de *Lactococcus lactis* subsp. *lactis* e Resveratrol, houve diminuição no peso corporal e lipídeos plasmáticos (colesterol total e triglicerídeos), e com o tratamento com *Lactococcus lactis* subsp. *lactis* e com a associação *Lactococcus lactis* e Resveratrol, houve diminuição da expressão de marcadores proinflamatórios (IL-6, TNF-α e IL-8) e parâmetros hepáticos (ALT e AST).

Uma limitação de ambos os estudos é que esses foram realizados em animais saudáveis e sem qualquer patologia, por razões metodológicas. Dessa forma, foi visto o mecanismo de ação do resveratrol associado a diferentes composições dietéticas em macronutrientes e ao *Lactococcus lactis* subsp. *lactis.* Porém, são necessários estudos com animais que tenham desordens metabólicas, a fim de avaliar o potencial preventivo e terapêutico do resveratrol com essas associações.

Diante disso, conclui-se que o consumo dietético de resveratrol, em associação a diferentes composições dietéticas e ao *Lactococcus lactis* subsp. *lactis*, melhora o perfil lipídico, diminui o peso corporal e diminui a expressão de marcadores lipogênicos e proinflamatórios, tendo grande potencial na prevenção da síndrome metabólica.

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ANEXOS

ANEXO A – Parecer da Comissão de Ética em Experimentação e Bem-estar Animal da UNIMONTES.

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

Unimontes

PARECER CONSUBSTANCIADO

Montes Claros, 06 de Dezembro de 2013.

Processo° **064**

Titulo do Projeto de Pesquisa: Avaliação metabólica de camundongos submetidos a diferentes composições dietéticas e tratados com Resveratrol

Equipe técnica
Orientador: Prof. Dr. Sérgio Henrique Sousa Santos
Orientanda de Doutorado: Keila Lopes Mendes, Jamille Fernandes Lula, Simone Moreira Macedo.
Orientanda de Mestrado: João Marcus Oliveira Andrade, Alanna Paraíso.

Histórico

A Síndrome Metabólica (SM) tem sido alvo de muitos estudos nos últimos anos. Ela pode ser definida como um grupo de fatores de risco inter-relacionados, de origem metabólica, que diretamente contribuem para o desenvolvimento de doença cardiovascular (DCV) e/ou diabetes do tipo 2. São considerados como fatores
de risco metabólicos: dislipidemia aterogênica (hipertrigliceridemia, níveis elevados de apolipoproteína B, partículas de LDL-colesterol pequenas e densas e níveis baixos de HDL-colesterol), hipertensão arterial, hiperglicemia e um estado pró-infl amatório e pró-trombótico. A obesidade abdominal e a resistência à insulina parecem ter um papel fundamental na gênese desta síndrome. Seu tratamento deve ter como objetivo estimular mudanças no estilo de vida, que promovam a perda de peso. Ainda não se estabeleceu uma causa única ou múltiplas causas para o desenvolvimento da Síndrome Metabólica. Resveratrol (3, 4 ', 5 trihidroxiestilbeno) é uma fitoalexina ocorrem naturalmente produzidos por algumas espermatófitas, tais como videiras, em resposta a uma lesão. Dado que está presente na baga casca de uva, mas não em carne, vinho branco contém quantidades muito pequenas de resveratrol, em comparação com vinho tinto. As concentrações sob a forma de trans-e cis-isómeros aglicona e glucósidos estão sujeitos a numerosas variáveis. No vinho tinto, a concentração do isómero trans, que é a forma principal, geralmente, varia entre 0,1 e 15 mg / L. Como composto fenólico, o resveratrol contribui para o potencial antioxidante do vinho tinto e, assim, podem desempenhar um papel na prevenção de doenças cardiovasculares humanas. O resveratrol foi mostrado para modular o metabolismo de lipídeos, e para inibir a oxidação de lipoproteínas de baixa densidade e a agregação de plaquetas. Além disso, como fitoestrógeno, o resveratrol pode fornecer proteção cardiovascular. Este composto também possui propriedades anti-inflamatórias e anti-cancro. No entanto, a biodisponibilidade e vias metabólicas devem ser conhecidos antes de tirar conclusões sobre os benefícios do resveratrol dietético para a saúde. A Síndrome Metabólica traz consequências danosas tanto a nível individual quanto a nível populacional, como o aumento dos custos com internações, medicações e atendimentos ambulatoriais, além de cada vez mais superlotar os serviços de saúde. Diante deste quadro, a dieta tem um fator fundamental, tanto na prevenção quanto no tratamento deste quadro, pois faz parte das mudanças de estilo de vida ligadas a etiologia desta síndrome. Neste sentido, o resveratrol, um polifenol presente em alimentos vegetais, pode exercer um papel de grande importância, pois está relacionado a funções antioxidantes, anti-inflamatórias e de melhora do perfil lipídico.

Mérito

Trata-se de um estudo de caráter investigativo, utilizando 80 animais (Mus musculus / Camundongos da Linhagem FVB/N) divididos em 10 grupos, G1-Controle: Dieta normal (padrão); G2 - Controle: Dieta normal + Resveratrol, G3- Experimental: Dieta AIN-93M; G4- Experimental: Dieta AIN-93M + Resveratrol; G5- Experimental: Dieta Hiperglicídica; G6-Experimental: Dieta Hiperglicídica + Resveratrol; G7- Experimental: Dieta Hiperlipídica; G8- Experimental: Dieta Hiperlipídica + Resveratrol; G9- Experimental: Dieta Hiperproteica; G10- Experimental: Dieta Hiperproteica + Resveratrol. As dietas experimentais a ser utilizadas serão produzidas pela empresa Rhoster Indústria e Comércio Ltda,com Composição pre-estabelecidas. Tem como objetivo Avaliar o metabolismo glicêmico e lipídico de camundongos da linhagem FVB/N submetidos a diferentes composições dietéticas e tratados com resveratrol, avaliar a expressão de sirtuinas no tecido adiposo, avaliar o padrão de expressão dos marcadores inflamatórios IL1-β, IL-6, TNF-α e TGF-β, avaliar o padrão de expressão de adipocinas (leptina, resistina e adiponectina), estudar a regulação glicêmica por meio de testes de sensibilidade insulínica e tolerância a glicose, bem como avaliar os níveis glicêmicos durante o jejum, mensurar os níveis plasmáticos lipídicos, dosando especificamente os níveis de triglicérides, colesterol total e HDL. Serão realizados no Biotério da Universidade Federal de Minas Gerais. O animal não sofrerá dor ou estresse, não usará drogas analgésica e/ou anestésicas, usará imobilização do animal sem relaxante muscular, não será submetido a cirugias, na sua alimentação conterá dosagem pré-estabelecida de Resveratrol. Haverá extração de fluídos ou tecidos de sangue para dosagens. No final do experimento e eutanásia serão extraídos tecidos adiposos brancos abdominais, mesentéricos, retroperitoneal, tecido adiposo marrom, fígado, rim, pâncreas, baço, coração e músculos. A eutanásia será técnica de decapitação por guilhotina.

Parecer

A Comissão de Ética em Experimentação e Bem-Estar Animal da Unimontes analisou o processo 064 e entende que o mesmo, se justifica. Sendo assim, somos pela **APROVAÇÃO** do projeto de pesquisa.

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

ANEXO B – Parecer da Comissão de Ética em Experimentação e Bem-estar Animal da UNIMONTES.

UNIVERSIDADE ESTADUAL DE MONTES CLAROS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO E BEM-ESTAR ANIMAL

Unimontes

PARECER CONSUBSTANCIADO

Montes Claros, 07 de novembro de 2014.

Processo N.º **82**
Título do Projeto: Avaliação metabólica, imunológica e microbiológica de camundongos tratados com resveratrol, probiótico e álcool
Orientadora: Prof. Dr. Sérgio Henrique Sousa Santos
Co-orientador: -
Colaboradores:
1) Profa. Dra. Mariléia Chaves Andrade
2) Prof. Dr. Sérgio Avelino Mota Nobre
3) Keila Lopes Mendes
4) Letícia Antunes Athayde
5) Deryk Patrick Oliveira Amaral
6) Emily Dardiane Soares Barbosa

Histórico

O trato gastrointestinal é continuamente exposto a uma vasta quantidade de antígenos externos. O contato com estes inúmeros antígenos que entram pela via oral desencadeia uma robusta resposta imune com repercussões sistêmicas, de natureza fisiológica ou patológica. Algumas desordens gastrointestinais podem ocorrer quando os mecanismos de defesa são quebrados por agentes infecciosos e irritantes, doenças autoimunes, fumo, estresse, uso prolongado de anti-inflamatórios não esteroidais e ingestão de álcool. Uma grande variedade de estudos têm mostrado que a ingestão de álcool afeta diversos componentes do organismo gerando importantes repercussões locais e sistêmicas. Tais alterações desencadeadas pelo consumo de etanol podem levar a uma quebra nos mecanismos reguladores e na homeostase do TGI, além de afetar de forma marcante os componentes da imunidade celular e humoral.Atualmente, os tratamentos para danos gerados pelo álcool se restringem a intervenções psicoterapêuticas e psicofarmacológicas. Neste sentido, o uso de terapias alternativas seguras capazes de prevenir os efeitos do consumo de álcool a nível local e sistêmico surge como forte candidato no tratamento de alterações decorrentes do consumo. Diante disso, o tratamento com probióticos, como a bactéria *Lactococcus lactis*, e com polifenóis, como o Resveratrol pode ser uma estratégia efetiva para prevenir a reatividade exacerbada do sistema imune associada à ingestão aguda de etanol.

Mérito

Estudar os efeitos do tratamento sinérgico com *Lactococcus lactis* e Resveratrol, como estratégia profilática para modulação das alterações inflamatórias desencadeadas pela ingestão aguda de etanol em camundongos C57BL/6.

Partindo do princípio que é necessário também:
-Avaliar alterações metabólicas gerais desencadeadas pela ingestão de etanol, bem como o efeito modulador da administração prévia de *L. lactis* e resveratrol;
-Verificar alterações morfo-funcionais locais (trato gastrointestinal), após administração de etanol, e os efeitos moduladores da ingestão prévia de *L. lactis* e resveratrol;
-Analisar alterações imunológicas locais e sistêmicas desencadeadas pela administração de etanol, e o impacto imuno-modulador do pré-tratamento oral com *L. lactis* e resveratrol.

Parecer

A presidência do Comitê de Ética em Pesquisa da Unimontes analisou o processo **082,** e entende que o mesmo está dentro das normas do Comitê e das Resoluções do Conselho Nacional da Saúde/Ministério da Saúde. Sendo assim, somos pela **APROVAÇÃO** do projeto de pesquisa.

Prof. Orlando Rapahel Lopasso Júnior
Presidente da Comissão de Ética em Experimentação e Bem-Estar Animal da UNIMONTES

1

ANEXO C – Normas de formatação do produto 1, periódico Life Sciences

NEW SUBMISSIONS

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.
As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.
If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.
Divide the article into clearly defined sections.

Figures and tables embedded in text

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

Language

Please write your text in good English (American or British usage is accepted, but not a mixture of these). For language assistance, please see Language Services, above. Use decimal points (not decimal commas); use a space for thousands (10 000 and above).

Use of word processing software

Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier:https://www.elsevier.com/guidepublication). See also the

section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Organization of the manuscript

Beginning with the first page, present your manuscript in the order below:
1. Title: First letter capitalized, subsequent letters in lower case. Maximum length 150 characters including spaces. Avoid abbreviations.
2a. Names of all authors.
2b. Affiliations of all authors. If necessary, use superscripted lowercase letters after the author's name to distinguish affiliations.
3. Author to whom proofs and correspondence should be sent, including name, mailing address, telephone and fax numbers, and e-mail address.
4. A structured abstract has to be submitted for full length articles (not for reviews) of no more than 250 words. The following headings must be used:
Aims:
Main methods:
Key findings:
Significance:
5. Three or more key words for indexing purposes. In addition to key words from the title, please suggest other terms that help define the study. We encourage authors to test the relevance of their key words by using them for a database search and comparing the results with the topic of their own paper.

Word limits: In **full papers**, individual sections should be no longer than Abstract 250 words, Introduction 500 words, Discussion 1500 words, Conclusion 150 words. Materials and Methods and Results sections should be concise but there is no formal word limit.

Headings: Papers must include the major headings Abstract, Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgments, and References. Include subheadings as appropriate. Review articles must contain Abstract and Introduction, with subsequent headings and subheadings as appropriate.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature.

Conclusions
Present the conclusions of the study in a short Conclusions section.

The Graphical Abstract is optional for research articles, but mandatory for reviews. GAs should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Graphical abstracts should be submitted as a separate file in the online submission system. Refer to the following website for more informationhttp://www.elsevier.com/graphicalabstracts.

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Abbreviations must be explained the first time they are used, both in the Abstract and again in the main text.

Abbreviations used as names of cell lines do not need to be explained, but the species and tissue of origin should be made clear in text the first time the cell line is mentioned. Examples: "the human colonic adenocarcinoma cell line Caco-2" or "the porcine renal endothelial cell line LLC-PK1".

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Please note that funding information must appear under the Acknowledgments heading.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be

the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files.

A detailed guide on electronic artwork is available on our website:
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TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
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Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
• Supply files that are too low in resolution.
• Submit graphics that are disproportionately large for the content.

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Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

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Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

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One author has been designated as the corresponding author with contact details:

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• Keywords

• All figure captions

• All tables (including title, description, footnotes)

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)

Printed version of figures (if applicable) in color or black-and-white

• Indicate clearly whether or not color or black-and-white in print is required.

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ANEXO D – Normas de formatação do produto 2, periódico Molecular and Cellular Endocrinology

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As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.
If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.
Divide the article into clearly defined sections.

Figures and tables embedded in text
Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

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Use of word processing software
Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier:https://www.elsevier.com/guidepublication). See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

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Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself. The abstract should be no more than 150 words.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files.
Seehttps://www.elsevier.com/graphicalabstracts for examples.
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Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).
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Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first

page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork

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General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files.
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Formats
Regardless of the application used, when your electronic artwork is finalized, please 'save as'

or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
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Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
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