UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia de Alimentos

GESSIKA CRISTINA BORGES CASTRO CARVALHO

EFEITO DAS GORDURAS *TRANS* NA MICROBIOTA INTESTINAL E NO SISTEMA *HEAT SHOCK PROTEINS* DE CAMUNDONGOS ALIMENTADOS COM PROTEÍNA DO SORO DO LEITE HIDROLISADA

EFFECT OF *TRANS* FAT ON GUT MICROBIOTA AND THE HEAT-SHOCK PROTEINS SYSTEM IN MICE FED WHEY PROTEIN HYDROLYSATE

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Dissertação apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Alimentos e Nutrição, na área de Nutrição Experimental aplicada à Tecnologia de Alimentos.

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Orientador: JAIME AMAYA-FARFAN

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A Ata de defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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RESUMO

Embora a gordura *trans* artificial tenha sido banida em alguns países, em outros, como o Brasil, alimentos industrializados contendo esse tipo de gordura continuam sendo produzidos e consumidos. Os ácidos graxos *trans* estão presentes na gordura parcialmente hidrogenada e seu consumo tem sido associado com diversos distúrbios na saúde. Mudanças no perfil da microbiota intestinal têm sido associadas com dietas hiperlipídicas e desordens metabólicas. Além disso, tem sido reportado que a indução das proteínas do estresse (*heat shock proteins* – HSPs) é capaz de melhorar a tolerância à glicose em animais obesos induzidos por uma dieta hiperlipídica. As proteínas do soro do leite hidrolisadas (PSLH) já mostraram aumentar a expressão das HSPs em ratos alimentados com uma dieta normocalórica e também atenuar efeitos deletérios causados por uma dieta hiperlipídica contendo banha de porco como fonte lipídica. O objetivo do presente trabalho foi avaliar o efeito metabólico de uma dieta hiperlipídica contendo apenas óleo vegetal parcialmente hidrogenado (possuindo ácidos graxos *trans*) como principal fonte lipídica na expressão das HSPs, perfil da microbiota intestinal, parâmetros antioxidantes e marcadores inflamatórios, além de verificar os possíveis efeitos protetores da proteína do soro do leite hidrolisada. Quarenta camundongos C57BL/6 machos foram divididos em cinco grupos: controle (AIN 93-G), dieta hiperlipídica contendo óleo não hidrogenado com caseína (OCAS) ou proteína do soro do leite hidrolisada (OWPH), dieta hiperlipídica contendo óleo parcialmente hidrogenado com caseína (HOCAS) ou proteína do soro do leite hidrolisada (HOWPH). Os resultados indicaram que o consumo de PSLH aumentou a expressão das HSP90, HSP60 e HSP25 nas dietas hiperlipídicas com óleo não hidrogenado, enquanto nenhuma influência foi notada com o óleo hidrogenado. Em contraste com o óleo não hidrogenado, o hidrogenado inibiu a fosforilação de JNK nas dietas experimentais com caseína, embora nenhuma alteração tenha sido observada no IKK. As fontes lipídicas ou proteicas não influenciaram os seguintes parâmetros inflamatórios: TLR4, CD14, MyD88, NF-κB e TNF-α. O consumo de PSLH nas dietas com ambos os óleos causou intolerância à glicose, mas não alterou os níveis de insulina, glicose basal e translocação de GLUT-4. Poucas alterações foram observadas no perfil da microbiota intestinal, sendo que nem a fonte lipídica nem a fonte proteica causaram inversão da taxa de *Bacteroidetes/Firmicutes*. Portanto, conclui-se que a alta ingestão de ácidos graxos *trans* não desencadeia inflamação, enquanto antagoniza o efeito protetor da PSLH associada à expressão de HSPs.

ABSTRACT

Although the non-natural *trans* fats has been banned in some countries, in others, like Brazil, industrialized foods containing *trans* fatty acids continue to be produced and consumed. *Trans* fatty acids are present in partially hydrogenated oil and its consumption has been associated with several health disorders. Changes in the gut microbiota profile have been associated with hyperlipidic diets and metabolic disorders. In addition, it has been reported that the heat shock proteins (HSPs) induction are able to improve glucose tolerance in obese animals induced by high-fat diet. Whey protein hydrolysate (WPH) has been shown to increase HSPs expression in rats fed a normocaloric diet and also attenuate some deleterious effects caused by a high-fat diet containing lard as lipid source. The aim of this study was to investigate the metabolic effect of a hyperlipidic diet containing only-vegetable *trans*-fatty acids on the HSP system, the gut microbiota profile, antioxidant parameters, inflammatory markers, and also verify the possible tissue-protective effects of co-ingesting WPH. Forty male C57BL/6 mice were divided into five groups: control (AIN 93-G), high-fat diet containing unhydrogenated oil with casein (OCAS) or whey protein hydrolysate (OWPH), high-fat diet containing partially hydrogenated oil with casein (HOCAS) or whey protein hydrolysate (HOWPH). The results indicated that that WPH increased HSP90, HSP60 and HSP25 expressions in the hyperlipidic-unhydrogenated oil diet, while no influence was noticed when the oil was hydrogenated. In contrast to the unhydrogenated, the hydrogenated oil inhibited JNK phosphorylation in the experimental casein diets though no alteration was observed in IKK. Neither oil nor protein was found to influence the pro-inflammatory TLR4, CD14, MyD88, NF-κB and TNF-α biomarkers. The consumption of WPH in both partially hydrogenated and unhydrogenated oils-diet showed impaired glucose tolerance, although without altering insulin levels, basal glucose and GLUT-4 translocation. Few changes were observed in the gut microbiota and neither the lipid nor the protein caused inversion of the *Bacteroidetes:Firmicutes* ratio. We conclude that high intake of *trans* fatty acids did not triggered inflammation, antagonized the protection of WPH-induced HSPs expression.

LISTA DE ABREVIATURAS E SIGLAS

BCAAs	Sigla na língua inglesa para aminoácidos de cadeia ramificada
EROs	Espécies reativas de oxigênio
HDL-c	Colesterol associado à lipoproteína de alta densidade
HOCAS	Sigla da dieta na língua inglesa, correspondendo à dieta hiperlipídica com 35% de gordura parcialmente hidrogenada e caseína como fonte proteica
HOWPH	Sigla da dieta na língua inglesa, correspondendo à dieta hiperlipídica com 35% de gordura parcialmente hidrogenada e proteína do soro do leite hidrolisada como fonte proteica
HSF	*Heat shock factor*, fator de transcrição
HSP	*Heat shock protein*, proteína do estresse
HSPs	*Heat shock proteins*, proteínas do estresse
GPx	Glutationa peroxidase
IKK-β	κβ - quinase subunidade beta
JNK	c-Jun NH_2-terminal quinase
LDL-c	Colesterol associado à lipoproteína de baixa densidade
Lp(a)	Lipoproteína
LPS	Lipopolissacarídeos
OCAS	Sigla da dieta na língua inglesa, correspondendo à dieta hiperlipídica com 35% de óleo não hidrogenado e caseína como fonte proteica
OMS	Organização Mundial da Saúde
OWPH	Sigla da dieta na língua inglesa, correspondendo à dieta hiperlipídica com 35% de óleo não hidrogenado e proteína do soro do leite hidrolisada como fonte proteica
PSL	Proteína(s) do soro do leite
PSLH	Proteína do soro do leite hidrolisada
PSLI	Proteína do soro do leite isolada
SOD	Superóxido dismutase
VIGITEL	Sistema de vigilância de fatores de risco e proteção para doenças crônicas por inquérito telefônico
WHO	World Health Organization

SUMÁRIO

INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

Os ácidos graxos *trans* artificiais estão presentes na gordura parcialmente hidrogenada. Muito tem sido falado sobre esse tipo de ácido graxo, pois a ingestão de alimentos contendo os mesmos tem sido associada a inúmeros danos ao homem, como o aumento do risco de doenças cardiovasculares (MOZAFFARIAN et al., 2006), infertilidade (CHAVARRO et al., 2007), cálculos biliares (TSAI et al., 2005), Alzheimer (MORRIS et al., 2003) e diabetes (SALMERÓN et al., 2001).

Em virtude dos efeitos deletérios causados pelas gorduras *trans* na saúde, os Estados Unidos da América (EUA) proibiram a presença desse tipo de gordura em alimentos industrializados (USFDA, 2015), enquanto no Brasil ainda permite-se sua presença em alimentos ultraprocessados desde que sua quantidade seja declarada no rótulo (BRASIL, 2003).

O conjunto de micro-organismos que vivem no lúmen gastrointestinal é chamado de microbiota intestinal (REA; DINAN; CRYAN, 2016). Esta última é formada por diversas espécies de micro-organismos, sendo que essa diversidade bem como a predominância pode variar ao longo do comprimento do trato gastrointestinal, modificando-se de acordo com o desenvolvimento do organismo e idade, podendo ser influenciada pelo genótipo, imunidade do hospedeiro além de influências ambientais, como a dieta ou transmissão direta de micro-organismos (DAVID et al., 2014; COX; BLASER, 2015).

De maneira geral, a relação entre os micro-organismos presentes no intestino e o hospedeiro é simbiótica, todavia a predominância de algumas bactérias constituintes da flora intestinal pode trazer malefícios ao homem, causando infecções e/ou contribuindo para o surgimento de doenças (ALONSO; GUARNER, 2013).

A dieta desempenha papel fundamental na saúde, uma vez que os alimentos, dependendo da sua composição, podem influenciar de maneira positiva ou negativa o corpo. O consumo de alimentos com alto teor lipídico pode ser prejudicial ao organismo, visto que a ingestão de uma dieta hiperlipídica por um longo período tem mostrado causar obesidade (HAMILTON et al., 2015), que por sua vez pode acarretar um quadro de inflamação (DE LA SERRE et al., 2010).

A obesidade e a dieta são moduladores do perfil da microbiota intestinal, podendo culminar em desordens metabólicas prejudiciais ao hospedeiro (LEY et al., 2006; CANI et al., 2008; TURNBAUGH et al., 2008; MURPHY et al., 2010; HAMILTON et al., 2015).

Sabe-se que uma dieta rica em gordura saturada modula de forma prejudicial o

perfil da microbiota intestinal (HILDEBRANDT et al., 2009; MURPHY, 2010), entretanto é escasso na literatura estudos sobre o efeito de uma dieta hiperlipídica contendo gorduras *trans* na microbiota, sendo este um dos objetivos deste trabalho.

As *heat shock proteins* (HSPs), ou proteínas do estresse, estão presentes em todos os tecidos do organismo desempenhando papel crucial, uma vez que compreendem um sistema natural de defesa capaz de proteger e reparar danos celulares ocasionados por variadas condições de estresse (DILLER, 2006; LIANOS et al., 2015).

Recentemente tem sido reportado que a obesidade, além de levar a intolerância à glicose, pode reduzir a expressão da HSP70 (CHUNG et al., 2008). Assim, há uma constante busca por novas estratégias capazes de elevar a expressão das HSPs, pois já se demonstrou que quando se restaura/aumenta esta expressão em modelo experimental de obesidade, ocorre uma melhora na sensibilidade à insulina e à tolerância à glicose (CHUNG et al., 2008; GUPTE et al., 2009a,b).

Estudos do nosso grupo de pesquisa demonstraram que o consumo da proteína do soro do leite hidrolisada (PSLH), como fonte proteica da dieta, foi capaz de elevar a expressão da HSP70 e HSP90 em uma dieta normocalórica (DE MOURA et al., 2013; MOURA et al., 2014) e amenizou os efeitos nocivos na microbiota e no organismo como um todo, causados por uma dieta rica em gordura saturada: banha de porco (MONTEIRO et al., 2016). Diante do exposto, o presente estudo objetivou verificar se estes efeitos se repetiriam no caso de uma dieta hiperlipídica contendo ácidos graxos *trans*, mas isenta de banha de porco. Assim, hipotetizou-se que a inserção de PSLH em uma dieta hiperlipídica amenizaria os efeitos adversos causados pela ingestão de uma dieta rica em gordura.

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REVISÃO BIBLIOGRÁFICA

2. REVISÃO BIBLIOGRÁFICA

2.1. Hidrogenação de óleos vegetais

O processo de hidrogenação, usando níquel como catalisador, foi descoberto pelo químico francês Paul Sabatier, todavia foi o químico alemão Wilhelm Normann que demonstrou que óleos líquidos poderiam ser hidrogenados e, então, patenteou o processo no início do século 20 (ECKEL et al., 2007; SCHEEDER, 2007). Assim, ao longo deste século foram ocorrendo modificações no processo e na formulação dos óleos vegetais hidrogenados, os quais mostraram ser uma boa alternativa à gordura animal devido ao seu baixo custo, maior estabilidade oxidativa e, consequentemente, maior vida de prateleira (ASCHERIO; WILLETT, 1997; ECKEL et al., 2007; OKIE, 2007).

A hidrogenação ocorre em tanques herméticos onde o gás hidrogênio é misturado ao óleo na presença do catalisador (geralmente níquel), a altas temperaturas, à determinada pressão, e posteriormente todos os traços do catalisador são removidos por filtração (COENEN, 1976). A técnica de hidrogenação de um óleo pode ser total ou parcial, sendo que o primeiro é obtido quando todas as duplas ligações são saturadas no processo, caso contrário, tem-se a hidrogenação parcial (GHOTRA; DYAL; NARINE, 2002). Ao longo do processo de hidrogenação parcial de óleos vegetais ocorre a conversão aleatória de uma dupla ligação de ácidos graxos insaturados, contendo pelo menos duas duplas ligações, para a configuração *trans*, de onde deriva a denominação "gordura *trans*" (MOZAFFARIAN et al., 2006).

Além da hidrogenação industrial, os ácidos graxos *trans* também podem ocorrer pela ação de bactérias no rúmen, processo conhecido como "biohidrogenação" e, portanto, podem ser encontrados naturalmente em alimentos derivados de animais ruminantes (WILLIAMS, 2000; THOMPSON; MINIHANE; WILLIAMS, 2011). No entanto, o teor de *trans* encontrado nos alimentos contendo gordura parcialmente hidrogenada é bem superior ao dos oriundos dos ruminantes, sendo cerca de 60% e 6%, respectivamente (STENDER; ASTRUP; DYERBERG, 2008).

O isômero *trans* predominante em gorduras provenientes de animais ruminantes é o ácido vacênico (C18:1,t11) e na gordura vegetal hidrogenada comercial é o ácido elaídico (C18:1,t9) (CRAIG-SCHMIDT, 2006).

Gagliardi, Mancini-Filho e Santos (2009) relatam que, a partir da década de 1960, as pessoas buscavam consumir outro tipo de gordura frente à gordura animal, já que havia muitas recomendações contra o consumo desta por ser rica em gorduras saturadas e colesterol.

Dessa maneira, foi a partir de então que o consumo de gorduras parcialmente hidrogenadas acelerou-se.

Inicialmente, havia pouca preocupação com os efeitos fisiológicos da gordura hidrogenada em humanos, visto que se restringiam à sua digestibilidade e biodisponibilidade, sendo que tais efeitos se mostraram positivos (SCHEEDER, 2007). Ainda, Eckel et al. (2007) salientam que além dos poucos estudos relacionando ácidos graxos *trans* e a saúde humana existentes naquela época, a maioria deles eram contraditórios ou apresentavam resultados inconsistentes. Porém, em 1947 já havia alertas pela busca de estudos adicionais verificando o potencial impacto das gorduras hidrogenadas na saúde, e em 1990 foram reportados efeitos deletérios dos ácidos graxos *trans* das gorduras industrialmente hidrogenadas nos lipídios do sangue (SCHEEDER, 2007).

Mensink e Katan (1990) observaram que, assim como os ácidos graxos saturados, a elevada ingestão de ácidos graxos *trans* também aumentava os níveis da lipoproteína de baixa densidade-colesterol (LDL-c). Não obstante, estes autores também verificaram que os ácidos graxos *trans* reduziam os níveis da lipoproteína de alta densidade-colesterol (HDL-c). Posteriormente, estudos como o de Zock e Katan (1992), Judd et al. (1994) e Sundram et al. (1997) confirmaram estes resultados.

Portanto, as alterações provenientes da alta ingestão de ácidos graxos *trans* podem ser consideradas negativas, pois provocam uma modificação expressiva da razão entre LDL-c e HDL-c, que, segundo Jukema et al. (2005), é utilizada como um importante indicador do risco de doenças cardiovasculares. Ascherio et al. (1999) notaram que a substituição da ingestão de gordura insaturada *cis,* na quantidade de 2% da energia, por gordura *trans* causa um aumento de 0,13 na relação LDL-c/HDL-c. Stampfer et al. (1991) ressaltam que o aumento de uma unidade em tal relação está associado a uma elevação média de 53% no risco de doenças cardiovasculares. Já Mozaffarian et al. (2006) sugeriram que o aumento de 2% no consumo de energia a partir de ácidos graxos *trans* está associado com um aumento de 23% na incidência de doenças coronarianas.

Além disso, alguns estudos evidenciam os efeitos dos ácidos graxos *trans* em aumentar a lipoproteína [Lp(a)] e os níveis de triacilgliceróis plasmáticos, sendo que altos níveis de Lp(a) têm sido associados com maior propensão a doenças cardiovasculares (NESTEL et al., 1992; ARO, et al., 1997; ASCHERIO et al., 1999). Salmerón et al. (2001), por sua vez, avaliaram a relação entre ingestão de gordura e o risco de diabetes tipo 2 em mulheres e reportaram que a ingestão de ácidos graxos *trans* aumentou o risco de desenvolvimento da referida doença. Hu et al. (1997) constataram que uma maior ingestão de

gordura saturada ou insaturada *trans* está relacionada com o aumento do risco de doenças coronarianas.

Adicionalmente, o consumo de gorduras *trans* foi associado com o aumento do risco de doenças coronarianas e do sistema circulatório (MOZAFFARIAN et al., 2006), formação de cálculos biliares (TSAI et al., 2005), doença de Alzheimer (MORRIS et al., 2003), infertilidade (CHAVARRO et al., 2007) e desenvolvimento de diabetes mellitus tipo 2 (SALMERÓN et al., 2001).

Devido aos efeitos deletérios oriundos da ingestão de gorduras *trans*, foi proibida, nos Estados Unidos, a utilização de óleo parcialmente hidrogenado em alimentos industrializados, uma vez que tal óleo representa a principal fonte dietética de gorduras *trans* artificial nos alimentos. Os fabricantes de alimentos têm até 2018 para se adequar a essa norma (USFDA, 2015).

São raros os dados na literatura a respeito da quantidade de ácidos graxos *trans* ingerida pelos brasileiros (HISSANAGA; PROENÇA; BLOCK, 2012). Porém, é recomendado pela Organização Mundial de Saúde (OMS) que a ingestão de gordura *trans* não ultrapasse 1% do valor calórico da dieta (WHO, 2013).

Atualmente, o consumidor brasileiro pode conferir a quantidade de gorduras *trans* presente em cada alimento, uma vez que a Agência Nacional de Vigilância Sanitária impôs, por meio da Resolução RDC nº 360 de 2003, que a partir de 31 de julho de 2006 todos os alimentos comercializados deveriam declarar o teor de ácidos graxos *trans* em sua rotulagem nutricional (BRASIL, 2003). Todavia, são considerados como "zero *trans*" os alimentos que apresentem teor de gorduras *trans* inferior ou igual a 0,1g por porção (BRASIL, 2012). Sendo assim, muitos alimentos alegam não conter gorduras *trans*, porém as mesmas estão "mascaradas" dentro da tolerância por porção. Adicionalmente, Kliemann et al. (2015) analisaram 2.020 alimentos industrializados presentes no mercado brasileiro, e verificaram que apesar de declarado na informação nutricional que os produtos não continham gorduras *trans*, as mesmas estavam presentes em alimentos descritos na lista de ingredientes, tais como: gordura vegetal hidrogenada, gordura vegetal parcialmente hidrogenada, óleo vegetal parcialmente hidrogenado, óleo vegetal hidrogenado.

2.2. Proteínas do soro do leite

O leite bovino é constituído por duas classes principais de proteínas: caseína e proteínas do soro do leite (*whey proteins*). A primeira representa cerca de 80% das proteínas

totais, já a segunda, aproximadamente, 20% (AIMUTIS, 2004).

O soro do leite é um subproduto obtido durante a produção de queijo, que antes era considerado apenas um resíduo de baixo valor comercial, porém, atualmente, destaca-se em virtude dos benefícios que este produto pode trazer à saúde e valor comercial agregado (SOUSA et al., 2012; PAL; RADAVELLI-BAGATINI, 2013). Apesar de sua composição variar de acordo com o tipo de leite e/ou queijo e seu respectivo processo produtivo, o soro do leite é composto principalmente pelas proteínas β-lactoglobulina, α-lactalbumina, albumina de soro bovino, glicomacropeptídeos, lactoferrina, imunoglobulinas, bem como lactose e minerais solúveis remanescentes (MARSHALL, 2004; PAL; RADAVELLI-BAGATINI, 2013). Ainda, o soro do leite é considerado uma fonte de proteínas completas, uma vez que possuem todos os aminoácidos essenciais (ADAMS; BROUGHTON, 2016).

As proteínas do soro do leite (PSL) são ricas em aminoácidos como a glutamina e principalmente em aminoácidos de cadeia ramificada (BCAAs), sendo atribuídos a estes últimos o efeito anti-adipogênico e proteção do músculo (PAL; RADAVELLI-BAGATINI, 2013).

São evidentes as vantagens para a saúde advindas da ingestão de PSL, tais como o aumento da absorção de minerais, saciedade, redução dos níveis de glicose e lipídios no sangue e efeitos sobre o sistema imune (KRISSANSEN, 2007; SOUSA et al., 2012). Adicionalmente, as PSL são capazes de favorecer o anabolismo muscular (HULMI; LOCKWOOD; STOUT, 2010).

Os suplementos alimentares comerciais que contêm majoritariamente proteínas do soro do leite são conhecidos como *whey protein*, destacando-se pelos seus efeitos nutricionais, e são divididos em três formas principais de acordo com seu teor proteico ou grau de pureza: *whey protein* concentrado, hidrolisado e isolado. Esses suplementos apresentam teor de proteínas variando entre 25% e 80%, aproximadamente 80%, igual ou superior a 90%, respectivamente (El SALAM; EL-SHIBINY; SALEM, 2009). Todavia, apesar dessas variações, infere-se que as mesmas parecem não afetar significativamente a taxa de esvaziamento gástrico ou a absorção de aminoácidos, posto que todas são facilmente digeríveis e rapidamente absorvidas (HULMI; LOCKWOOD; STOUT, 2010).

De Moura et al. (2013) verificaram que o consumo da proteína do soro do leite hidrolisada (PSLH), como fonte proteica em uma dieta normocalórica para ratos, mostrou-se capaz de elevar a expressão da HSP70 em diversos tecidos, evidenciando a capacidade protetora dessa fonte proteica. Ainda, estudo posterior, também com uma dieta normocalórica, demonstrou que o consumo da PSLH elevou a expressão da HSP90, mas não provocou

alteração na expressão da HSP25 e HSP60 (MOURA et al., 2014).

Monteiro et al. (2016) investigaram o efeito da ingestão da proteína do soro do leite, nas formas concentrada e hidrolisada, como fonte proteica em uma dieta hiperlipídica em camundongos, constatando que ambas proteínas foram capazes de atenuar os efeitos nocivos causados pelo excesso de gordura saturada na dieta.

Tranberg et al. (2013) estudaram os efeitos da ingestão de proteína do soro do leite isolada (PSLI) em camundongos. Dois grupos foram alimentados com dieta hiperlipídica, diferindo apenas da fonte proteica – caseína ou PSLI, sendo a dieta do terceiro grupo caracterizada pelo baixo teor de gordura e caseína. Observou-se diferença na composição da microbiota entre os grupos que ingeriram diferentes teores de gordura, mas não foi apresentada diferença em relação à fonte proteica. Os autores afirmam que o *whey* foi capaz de melhorar a sensibilidade à insulina e de reduzir o colesterol no plasma.

McAllan et al. (2014) notaram que camundongos alimentados com dieta hiperlipídica e PSLI na concentração de 20% apresentaram composição da microbiota distinta dos demais animais alimentados com dieta com o mesmo teor lipídico e outras concentrações de PSLI ou dieta normocalórica com caseína. Além disso, os autores também concluíram que em uma dieta com o mesmo teor calórico, a PSLI é capaz de reduzir a massa gorda e aumentar a massa magra quando comparada à caseína, e também diminuir a leptina no plasma e o nível de triacilglicerol no fígado.

Kobayashi et al. (2011) reportaram que a proteína do soro do leite é capaz de alterar beneficamente a composição da microbiota de porcos submetidos a uma dieta normocalórica.

Assim, os dados discrepantes e inconclusivos a respeito da habilidade do *whey* em modular a microbiota intestinal podem ser resultantes da diferença no tempo de suplementação com o referido alimento, dosagem, além dos diferentes métodos empregados para averiguação da composição microbiana e análise. Então, fazem-se necessários mais estudos para melhor elucidar esse assunto e também entender a capacidade das PSL em atuar como alimento funcional.

Não foram encontradas evidências na literatura sobre o efeito da PSLH em animais obesos induzidos com uma dieta hiperlipídica contendo gorduras *trans*.

2.3. *Heat shock proteins* – proteínas do estresse

As *heat shock proteins* (HSPs) foram descobertas em estudo realizado por

Feruccio Ritossa, no qual se notou um perfil diferente nos cromossomos de glândulas salivares de larvas de *Drosophila melanogaster* submetidas a um tratamento de choque térmico subletal, onde posteriormente se verificou que havia um dramático aumento na síntese de rRNA responsável pela expressão dessa classe de proteínas (RITOSSA, 1962). As HSPs são também chamadas de proteínas do estresse, sendo classificadas em seis famílias principais de acordo com sua massa molecular: HSP100, HSP90, HSP70, HSP60, HSP40 e HSPs de baixo peso molecular (sHSP – *small* HSPs) (BAKTHISARAN; TANGIRALA; RAO, 2015). Dentre esta última família, destaca-se a HSP27, que é presente em humanos, sendo a HSP25 o homólogo desta proteína em ratos (NAGARAJA et al., 2012).

As HSPs estão presentes em todo o organismo, protegendo e reparando danos celulares oriundos de diversas condições de estresse, compreendendo, portanto, um mecanismo natural de defesa (CHUNG et al., 2008; LIANOS et al., 2015). Essas proteínas garantem à célula maior tolerância e resistência contra diversos agentes agressores, no intuito de que haja a preservação da integridade e estrutura celular, possibilitando a sobrevivência das células durante períodos de estresse (GARRIDO et al., 2001; WISCHMEYER, 2002; DILLER, 2006).

A expressão das HSPs é induzida por diferentes estressores, incluindo hipóxia, isquemia, privação de glicose, exposição a toxinas celulares (como metais pesados, endotoxinas e espécies reativas de oxigênio – EROs), estresses térmico e oxidativo, inflamação, além de outras (SNOECKX et al., 2001; ÅKERFELT; MORIMOTO; SISTONEN, 2010; SILVER; NOBLE, 2012). Em decorrência da exposição a tais condições de estresse, é possível que ocorra problemas na conformação de proteínas, que podem culminar nas perdas de sua estrutura original e função, todavia as HSPs estão envolvidas no correto enovelamento e reenovelamento de proteínas, sendo capaz, portanto, de reparar as que perderem sua estrutura (WISCHMEYER, 2002; NIFOROU; CHEIMONIDOU; TROUGAKOS, 2014). Assim, as HSPs ligam-se a diferentes substratos e realizam variações na estrutura de proteínas a fim de se atingir o correto enovelamento ou evitar sua agregação (NIFOROU; CHEIMONIDOU; TROUGAKOS, 2014).

A longevidade pode estar relacionada ao estresse, nesse sentido as HSPs são altamente benéficas, já que além de serem induzidas em resposta a fatores intrínsecos e extrínsecos de estresse, elas também são mediadores importantes do organismo no que se refere à resistência ao estresse, que por sua vez está associada com a promoção de um gene HSP capaz de ativar a expressão HSP durante o envelhecimento, aumentando a resistência ao estresse e, consequentemente, a longevidade (PURANDHAR et al., 2014).

Os *heat shock factors* (HSFs) – fatores de transcrição – são mediadores da expressão das proteínas do estresse, sendo divididos basicamente em quatro grupos: HSF1, HSF2, HSF3 e HSF4. No entanto, o HSF1 é o regulador mais importante no processo de transcrição das HSPs, visto que é considerado o fator estresse-responsivo mais eficaz, pois além de ser rapidamente ativado, está presente na maioria dos eucariontes e em diversos tecidos (SANTORO, 2000; ÅKERFELT; MORIMOTO; SISTONEN, 2010).

Evidências indicam que a obesidade pode reduzir a expressão da HSP70 (CHUNG et al., 2008). Contudo, já se reportou que a restauração/aumento na expressão das HSPs, em modelo experimental de obesidade, culmina em melhora na sensibilidade à insulina e tolerância à glicose (CHUNG et al., 2008; GUPTE et al., 2009a,b).

A HSP70 é capaz de inibir a fosforilação das quinases como as c-Jun NH_2-terminal quinase (JNK) e kB quinase-B (IKKβ) as quais estão aumentadas em animais obesos insulino resistentes. A ativação dessas quinases provoca alterações na via de sinalização da insulina, as quais interfere na fosforilação do substrato receptor 1 (IRS1), reduzindo a sua interação com as proteínas subsequentes como a fosfatidilinositol–3–quinase (PI3K), prejudicando a via de sinalização da insulina e consequentemente a captação de glicose para dentro da célula. A capacidade das HSPs em inibir essas quinases aumentadas durante a obesidade é um mecanismo sugerido pelo qual o aumento das HSPs melhora a resistência à insulina e a tolerância à glicose em animais obesos induzidos por dieta hiperlipídica (GUPTE et al., 2009b).

Nesse sentido, ressalta-se a importância de descobrir novos compostos alimentares capazes de induzir o aumento e/ou restauração na expressão das HSPs em modelos experimentais de obesidade.

2.4. Microbiota intestinal

O número de micro-organismos no interior do trato gastrointestinal é tipicamente dez vezes superior ao de todas as células do corpo humano (PALMER et al., 2007). Gaboriau-Routhiau et al. (2009) afirmam que dos trilhões de bactérias que habitam o corpo humano, 90% residem no intestino. Já Palmer et al. (2007) especificam que normalmente há no trato intestinal humano 10^{11} – 10^{12} micro-organismos/ml de conteúdo luminal. O conjunto de micro-organismos que vivem em um ambiente específico é denominado microbiota (FOND et al., 2015). Assim, são constituintes da microbiota intestinal as células de leveduras, fúngicas, protozoárias e bacterianas (COX; BLASER, 2015).

A relação entre os micro-organismos presentes no intestino humano e o hospedeiro é simbiótica e, portanto, benéfica para ambos, uma vez que os micróbios se alojam em um ambiente propício para seu crescimento. Além disso, a microbiota também está relacionada com diversas funções importantes para o organismo humano, pois é capaz de sintetizar vitaminas essenciais, auxiliar na liberação de nutrientes a partir de substratos alimentares inacessíveis, contribuir no desenvolvimento da imunidade, além de consumir, armazenar e redistribuir energia (STAPPENBECK, 2002; BÄCKHED et al., 2005; COSTELLO et al., 2012; KAMADA et al., 2013*;* COX; BLASER, 2015). Todavia, algumas bactérias presentes na flora intestinal, quando dominantes, podem ser prejudiciais ao corpo humano, visto que podem acarretar na manifestação de doenças (ALONSO; GUARNER, 2013).

A microbiota é composta de, aproximadamente, 1100 espécies prevalentes, com pelo menos 160 espécies por indivíduo, sendo os filos dominantes: *Firmicutes, Bacteroidetes, Proteobacteria* e *Actinobacteria* (MORGAN; SEGATA; HUTTENHOWER, 2013; POWER et al., 2014). Contudo, vários fatores podem interferir na diversidade da flora intestinal, dentre eles estão a dieta e a imunidade do hospedeiro (DAVID et al., 2014). Assim, pode-se dizer que cada indivíduo possui uma flora microbiana singular.

Há um equilíbrio saudável na composição da microflora em indivíduos normais, entretanto tal balanço é rompido em casos de doença (ZHOU; ZHI, 2016). A disbiose intestinal pode ser definida como um desequilíbrio na microbiota intestinal, que produz efeitos prejudiciais, sendo que várias doenças estão associadas com a mesma, tais como: má nutrição, obesidade, doenças inflamatórias na pele, boca e trato intestinal (TURNBAUGH et al., 2006; COSTELLO et al., 2012; KARIN; JOBIN; BALKWILL, 2014). Segundo Walker et al. (2011), a identificação das comunidades microbianas presentes no intestino humano é de suma importância na avaliação da influência sobre a saúde.

A obesidade pode ser definida como o acúmulo normal ou excessivo de gordura no tecido adiposo que apresenta risco à saúde, pois pode conduzir a várias doenças crônicas, como diabetes, doenças cardiovasculares e alguns tipos de câncer. Entre as principais causas da ocorrência da obesidade pode-se citar o desbalanço energético entre a energia consumida e a despendida, uma vez que se elevou a ingestão de alimentos com maior valor energético e ricos em gordura, porém, em contraste, houve um aumento na inatividade física (WHO, 2016). Dados da Organização Mundial da Saúde (OMS) mostram que mais de 1,9 bilhões de adultos no mundo apresentavam sobrepeso em 2014 e destes, mais de 600 milhões eram obesos. De maneira simplificada, estima-se que 13% da população mundial era obeso em

2014. Além disso, o sobrepeso e a obesidade têm sido associados a mais óbitos no mundo inteiro quando comparados à desnutrição (WHO, 2016). Os dados também são preocupantes no Brasil, visto que segundo levantamento do VIGITEL (Vigilância de fatores de risco e proteção para doenças crônicas por inquérito telefônico) 52,5% dos brasileiros apresentavam excesso de peso e 17,9% estavam obesos no ano de 2014 (BRASIL, 2014).

O constante consumo de uma dieta rica em lipídios pode resultar em obesidade (HAMILTON et al., 2015). Reporta-se que a composição da microbiota intestinal difere entre indivíduos obesos e não obesos (LEY et al., 2005; HAMILTON et al., 2015).

Evidências indicam que a microbiota intestinal é capaz de ser modulada pela obesidade e pela dieta, podendo acarretar em desordens metabólicas maléficas ao hospedeiro (LEY et al., 2006; CANI et al., 2008; TURNBAUGH et al., 2008; MURPHY et al., 2010; HAMILTON et al., 2015). Reporta-se que as bactérias presentes no intestino humano são capazes de afetar o metabolismo do hospedeiro e a homeostase energética, podendo influenciar negativamente o metabolismo da glicose, com aumento do armazenamento de lipídios, incidência de inflamação e redução da atividade insulínica (CARVALHO; SAAD, 2013).

Vrieze et al. (2012) analisaram a transferência da microbiota intestinal em humanos, de magros para obesos, e constataram efeito benéfico, uma vez que houve um aumento na sensibilidade à insulina nos indivíduos obesos.

Já Ridaura et al. (2013) investigaram o transplante da microbiota de gêmeos (humanos), dos quais um apresentava fenótipo obeso e o outro magro, para camundongos com intestino estéril (*germ free*). O estudo mostrou que possivelmente o fenótipo foi transferido juntamente com a microbiota, uma vez que o tecido adiposo nos camundongos que receberam a microbiota dos gêmeos obesos foi significativamente maior do que o apresentado pelos que receberam a microbiota dos não-obesos. Adicionalmente, notou-se maior peso da gordura epididimal nos animais com a microbiota dos indivíduos obesos. Não obstante, esta diferença não foi atribuída à dieta, visto que ambos os grupos receberam dietas iguais. Enfim, conclui-se que a microbiota pode levar ao fenótipo de obesidade, independente da dieta consumida.

A obesidade pode acarretar no aumento da permeabilidade do epitélio intestinal e, consequentemente, reduzir a função de barreira facilitando a passagem de componentes microbianos que podem ocasionar inflamação (DE LA SERRE et al., 2010).

A inflamação, hiperplasia e hipertrofia do tecido adiposo em indivíduos obesos apresenta sinais como estresse do retículo endoplasmático (que pode resultar em apoptose

celular), hipóxia, alta produção de espécies reativas de oxigênio (EROs) e elevada produção de citocinas pró-inflamatórias (FURUKAWA et al., 2004; ÖZCAN et al., 2004; HOSOGAI et al., 2007; KAWASAKI et al., 2012).

A dieta hiperlipídica pode elevar a liberação de lipopolissacarídeos (LPS) no plasma, culminando em endotoxemia metabólica, que por sua vez promove a ativação de mastócitos e *Toll-like receptors* – TLRs (proteínas transmembranases responsáveis por detectar presença de patógenos invasores no organismo) e impulsionam a produção de citocinas inflamatórias, as quais desencadeiam a cascata inflamatória (CANI et al., 2007; DE LA SERRE, 2010; LEE, 2013). É raro na literatura estudos associando o consumo de gordura parcialmente hidrogenada, a qual possui o isômero *trans*, e alterações na flora intestinal, pois a maioria dos trabalhos que associa dieta *high-fat* e microbiota utiliza óleo de soja e/ou gordura saturada (banha de porco) como fonte lipídica.

Por conseguinte, tem sido explorada a busca por novas terapias a partir de compostos alimentares que favoreçam a microbiota intestinal a fim de reduzir a inflamação.

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ARTIGO

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3. ARTIGO

HSP expression, inflammation and gut microbiota in mice fed whey protein hydrolysate and high levels of vegetable *trans*-fat

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Abstract

Although industrialized *trans* fats are being phased out in some countries, many will continue to produce *trans*-fat-containing foods and little is known about the impact of their consumption on metabolic stress and dysbiosis. The aim of this study was to evaluate the effects of a whey protein hydrolysate (WPH) on HSP expression, inflammation and gut microbiota of mice consuming a hyperlipidic diet containing partially hydrogenated oil. Mice were divided into five groups: control (AIN 93-G) diet, two receiving hyperlipidic-hydrogenated-oil diets, but containing either casein or WPH (HOCAS, HOWPH) as sole protein source, and two other groups receiving hyperlipidic-unhydrogenated-oil diets with either casein or WPH (OCAS, OWPH). Results indicated that WPH increased HSP90, HSP60 and HSP25 expressions in animals consuming the hyperlipidic-unhydrogenated oil, while no influence was noticed when the oil was hydrogenated. Contrasting with the unhydrogenated, the hydrogenated oil inhibited JNK phosphorylation in the experimental casein diets though no alteration was observed in IKK. Neither lipid nor protein was found to influence the pro-inflammatory TLR4, CD14, MyD88, NF-κB and TNF-α biomarkers. Both lipid sources did impair glucose tolerance, but only when WPH was the protein source, although without altering insulin levels, basal glucose and GLUT-4 translocation. Changes in gut microbiota suggest that this hyperlipidic diet does not invert the *Bacteroidetes:Firmicutes* ratio. We conclude that high intake of *trans* fatty acids did not trigger inflammation, but antagonized the WPH-induced HSPs expression.

Key words: dyslipidemia, lard, vegetable oil, metabiome

1. Introduction

Trans fats are unsaturated fatty acids containing at least one double bond in the *trans* configuration. These fatty acids are mainly present in partially hydrogenated oils (PHO) (Mozaffarian and others 2006). PHO intake has been associated with the development of several diseases, such as type-2 diabetes (Salmerón and others 2001), cardiovascular disease (Mozaffarian and others 2006), infertility (Chavarro and others 2007) and Alzheimer's disease (Morris and others 2003).

The presence of non-natural *trans* fats has been banned from processed foods in some countries since 2016. In spite of that, the ban has not been enacted in many other countries like Brazil, where some industrialized foods containing varying levels of *trans* fatty acids continue to be produced and sold (Brasil 2003; USFDA 2015).

High-fat diets can be harmful to health because they may lead to inflammation (De La Serre and others 2010) and result in obesity (Hamilton and others 2015). Since diet and obesity are intimately associated with each other and with the gut microbiota profile (Ley and others 2006; Turnbaugh and others 2008; Hamilton and others 2015; Rea and others 2016), the microbiota composition becomes a valid criterion to assess the effect of the fat in terms of chronic metabolic disorders in the host. While diets rich in saturated fat have been known to adversely modulate the profile of the gut microbiota (Hildebrandt and others 2009; Murphy 2010), few studies are known about the effect of a hyperlipidic diet containing only vegetable *trans*-fats, rather than saturated pork or animal fat, on the microbiota.

Heat shock proteins (HSPs) are a group of proteins that are endogenously produced as part of the natural defense system in response to several stressing conditions (Lianos and others 2015). It has been reported however, that consumption of high-fat diets reduces HSP production and impair glucose tolerance in both animals and humans (Chung and others 2008).

Studies have also shown that high-fat-diet-induced obesity is associated with the activation of inhibitors of kappa-B kinase (IKK) and c-Jun NH_2-terminal kinase (JNK) phosphorylation, which may promote the inflammation pathways and insulin resistance (Shoelson and others 2006). On the other hand, current evidence also demonstrates that induction of HSP70 and HSP25 is able to inhibit JNK and IKK activation thus improving glucose tolerance in animals that became obese by consuming a high-fat diet (Gupte and others 2009a,b). Consequently, new food components that could induce the expression of HSPs should be explored.

For instance, we have found that consumption of a whey protein hydrolysate (WPH) increases skeletal muscle HSP70 and HSP90 expressions in rats fed a normolipidic diet, but predisposing the animal to potential injuries (De Moura and others 2013; Moura and others 2014). In another study, we also observed that both intact whey protein and WPH can attenuate inflammation and still tend to protect the normal gut microbiota profile in mice fed a high-fat diet with lard as the main lipid source (Monteiro and others 2016).

Few reports were found addressing the effect of hyperlipidic diets containing all-vegetable, partially hydrogenated, oil on HSP expression or about the possibility that whey protein could attenuate the deleterious effects caused by PHO consumption. Apart from this, little is known about how a hyperlipidic, *trans*-fat containing diet, but exempt of pork fat, could impact the inflammation pathways and the gut microbiota.

Thus, the aim of the present study was to investigate the metabolic effect of a hyperlipidic diet containing only-vegetable *trans*-fatty acids on the HSP system, the gut microbiota profile, antioxidant parameters, inflammatory markers, and the possible tissue- and gut-microbiota protective effects of co-ingesting WPH.

2. MATERIALS AND METHODS

2.1 Animals and Ethics

The Ethics Committee on the use of animals of the University of Campinas approved all experimental procedures (CEUA-UNICAMP, protocol 3821-1). Forty male C57BL/6 mice (21 days old, specific-pathogen free) were obtained from the Multidisciplinary Center for Biological Investigation (University of Campinas, SP, Brazil). The animals were maintained under controlled conditions (55% humidity, 22 ± 1°C, inverted 12-hour light/dark cycle) and remained in individual cages with free access to chow (Nuvital, Brazil) and water, for 18 weeks.

2.2 Experimental diets and design

After adaptation of five weeks, the animals (23,68±1,95g) were randomized into five groups (n=8 per group): control (AIN 93-G), high-fat diet containing unhydrogenated oil with casein (OCAS) or whey protein hydrolysate (OWPH), high-fat diet containing partially hydrogenated oil with casein (HOCAS) or whey protein hydrolysate (HOWPH). All diets were based on AIN 93-G (Reeves and others 1993), except that the protein content was 12% and high-fat diets contained 35% of unhydrogenated oil or 35% of partially hydrogenated oil. The whey protein hydrolysate or casein was the only protein source.

The partially hydrogenated fat was obtained from a local food-ingredients industry, and the unhydrogenated oil was prepared in the laboratory by mixing vegetable oils (93% of soy oil and 7% of palm kernel oil). Whey protein hydrolysate was obtained from Hilmar Ingredients (Dalhart, TX, USA) and casein from Synth (São Paulo, SP, Brazil). Diets formulation are shown in Table 1.

Table 1. Diets formulation (g/kg of diet)

Ingredients	**Control**	**OCAS**	**OWPH**	**HOCAS**	**HOWPH**
Corn Starch	489.55	139.55	117.49	139.55	117.49
Dextrinized starch	132.00	132.00	132.00	132.00	132.00
Sucarose	100.00	100.00	100.00	100.00	100.00
WPH	-	-	160.00	-	160.00
Casein	137.93	137.93	-	137.93	-
Soybean oil	40.00	40.00	40.00	40.00	40.00
Hydrogenated oil	-	-	-	350.00	350.00
Unhydrogenated oil	-	350.00	350.00	-	-
Fiber (cellulose)	50.00	50.00	50.00	50.00	50.00
Mineral mixture	35.00	35.00	35.00	35.00	35.00
Vitamin mixture	10.00	10.00	10.00	10.00	10.00
L-Cystine	3.00	3.00	3.00	3.00	3.00
Choline bitartrate	2.50	2.50	2.50	2.50	2.50
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014
Total	1000	1000	1000	1000	1000

Total lipids of the diets were quantified by the method of Bligh and Dyer (Bligh and Dyer 1959). Total protein content, moisture and ash were determined according to the Official Methods of Analysis of the AOAC International (AOAC 2012). Nutrient composition of the diets can be seen in Table 2.

Table 2. Nutrient composition of the diets

	Control	**OCAS**	**OWPH**	**HOCAS**	**HOWPH**
Proteins (%)	12.94±0.12	12.16±0.43	12.74±0.29	13.00±0.08	12.03±0.13
Lipids (%)	3.8±0.22^{b}	39.72±0.13^{a}	39.07±0.16^{a}	39.63±0.7^{a}	39.69±0.28^{a}
Moisture (%)	7.52±0.13^{a}	3.41±0.07^{c}	3.92±0.15^{b}	3.94±0.21^{b}	3.74±0.14^{bc}
Ash (%)	2.39±0.1^{b}	2.19±0.12^{bc}	3.03±0.02^{a}	1.96±0.19^{c}	2.82±0.06^{a}
Carbohydrates (%)	73.35	42.53	41.23	41.47	41.72
Energy	3.79	5.76	5.68	5.75	5.72

Nutrient composition data were expressed as means ± SEM. Different superscript letters indicate statistical differences at $p<0.05$. Values of energy are expressed in kcal/g of diet.

The fatty acid profiles of the unhydrogenated and partially hydrogenated oils (Table 3) were characterized according to the American Oil Chemists' Society (AOCS 2009). The analyses were performed using a capillary gas chromatograph (CGC AGILENT 68650 SERIES GC SYSTEM), under the following conditions: capillary column: DB-23 AGILENT (50% cyanopropyl-methylpolysiloxane), 60 m × 0.25 mm × 0.25 μm; column flow rate: 1.0 mL/min; carrier gas: Helium; volume injected: 1.0 μL. Fatty acids composition of both oils can be seen in Table 3.

Table 3. Fatty acids composition of the lipid fraction of the diets

Fatty acid	Partially Hydrogenated Oil (%m/m)	Unhydrogenated Oil (%m/m)
C8:0	0.18	0.23
C10:0	0.23	0.23
C12:0	3.17	3.1
C14:0	1.34	1.13
C15:0	0.05	0.05
C16:0	13.16	10.9
C16:1	-	0.1
C17:0	0.12	0.1
C18:0	22.2	3.57
C18:1t	26.06	-
C18:1	28.08	22.65
C18:2t	0.23	0.19
C18:2	3.42	50.37
C18:3t	0.32	0.48
C18:3	0.23	5.6
C20:0	0.49	0.36
C20:1	0.08	0.21
C22:0	0.45	0.45
C24:0	0.19	0.17

The animals received the experimental diet for 13 weeks and, after that, they were anesthetized intraperitoneally using a mixture of ketamine (10 mg/kg) and xylazine (150 mg/kg) and then euthanized. All animals were weighted and the naso-anal lengths were measured to calculate the Lee index (Bernardis and Patterson 1968). Adipose tissues were removed and weighted. Liver, kidney, heart, gastrocnemius muscle and cecum fecal samples were dissected and frozen in liquid nitrogen.

2.3 Western blot analysis

The gastrocnemius muscle samples were homogenized in the extracting buffer as previously described (Moura and others 2014). The total protein content of the muscle was determined by the Lowry method (Lowry and others 1951). The extracts were subjected to SDS-PAGE and transferred using a semi-dry system (Bio-Rad, CA, USA) to a nitrocellulose membrane. The membranes were incubated overnight at 4°C with specific primary antibodies

to assess the protein level of: HSP90 (Enzo Life Sciences, Farmingdale, NY, USA; Ref. ADI-SPA 831), HSP70 (Enzo Life Sciences ADI-SPA 810), HSP60 (Enzo Life Sciences ADI-SPA 806), HSP25 (Enzo Life Sciences ADI-SPA 801), p-IKK (Cell Signaling Technology, Beverly, MA, USA; Ref. 9246S), IKK (Cell Signaling Technology, Beverly, MA, USA; Ref. 9242S), p-JNK (Cell Signaling Technology, Beverly, MA, USA; Ref. 4668S), JNK (Cell Signaling Technology, Beverly, MA, USA; Ref. 9252S), TLR4 (Santa Cruz, CA, USA; Ref. sc30002), CD14 (Santa Cruz, CA, USA; Ref. sc9150), MyD88 (Santa Cruz, CA, USA; Ref. sc11356), TNF-α (Santa Cruz, CA, USA; Ref. sc8301), catalase (Santa Cruz, CA, USA; Ref. sc271803), NF-kB (Abcam, Cambridge; Ref. ab7970), SOD (Abcam, Cambridge; Ref. ab51254), GPx (Abcam, Cambridge; Ref. ab22604), GLUT-4 (Abcam, Cambridge; Ref. ab654), GAPDH (Enzo Life Sciences; Ref. ADI-CSA 335-E). For detection, the appropriate secondary antibodies were used. The bands were visualized using a UVITEC Cambridge instrument (model Alliance LD2) and the band intensities were quantified using the digital program ImageJ (v. 1.50 for Windows).

2.4 Biochemical analysis

Blood samples were collected through portal vein puncture and centrifuged at 3000 *x g* (4 °C, 15 min) to obtain the serum. The serum parameters were determined using commercial clinical kits: triacylglycerols (TG), cholesterol, total proteins, high density lipoprotein (HDL), uric acid, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (purchased from Laborclin; Vargem Grande, Paraná, Brazil), albumin (from Laborlab; Guarulhos, São Paulo, Brazil) and glucose (Labtest; Lagoa Santa, Minas Gerais, Brazil). Insulin levels were determined using Elisa kit (EZRMI-13K) from Millipore. Spectrophotometric microplate reader (Biotech Epoch, Winooski, VT) was used to determinate all serum parameters following the manufacturer's instructions.

2.5 Glucose tolerance test (GTT)

The oral glucose tolerance test (GTT) was performed in the twelfth week of the experiment. Glucose (2g/kg) was given orally by gavage and blood samples were collected from the tail vein at 0, 15, 30, 60 and 120 min for the determination of glucose concentrations. Blood glucose levels were measured using an Accu-Chek Active glucometer (Roche Diagnostics, Mannheim, Germany) with appropriate test strips.

2.6 Determination of glycogen

Glycogen content was measured in the heart, liver and kidney using a phenol-sulfuric acid technique as previously described by Lo and others (1970).

2.7 Histological analysis of liver tissue

Liver histology was performed in order to qualitatively assess hepatic steatosis in the animals of all experimental groups. For that, fragments of the hepatic lobe were collected, cut into slices (3mm thick, approximately), fixed in buffered 4% paraformaldehyde, dehydrated in an ascending series of ethanol, diaphanized in xylene, embedded in paraffin, cut in the microtome (5-µm thick) and stained with hematoxylin–eosin. The stained liver slices were microscopically examined under 10× and 20× objectives.

2.8 Identifying the intestinal microbiota

Total DNA was extracted from the cecal contents with the QIAmpDNA Stool Kit. The library construction and sequencing were performed as recommended by the manufacturer (Illumina, San Diego, CA, USA). For each sample, the 16S rRNA gene was amplified using the direct amplifier corresponding to the following sequence: 5´-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***ACTCCTACGGGAGGCAGCAG** -3´.

In this sequence, the portion in italic font corresponds to the adapter Nextera® transposase sequences A, and the sequence in bold, to the initiator widely conserved 338F. The reverse initiator used was 5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***TTACCGCGGCTGCTGGCAC** -3', the portion in italics corresponding to the adapter Nextera® transposase sequences B, and the sequence in bold, to the initiator of ample utilization 533R. The kit "Illumina TruseqDNA Sample Preparation v2" was used for the preparation of the libraries, labeling each sample with a bar code. Sequencing was performed on an Illumina Hiseq2000 equipment at the Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP, SP, Brazil). After sequencing, the identity of sequences was calculated using the megablast technique. The taxonomy was filed seeking the best megablast against GreenGenes. To determine the best BLAST, sequences derived from bacterial VLPs in custom databases were built with the help of GreenGenes database.

2.9 Data analysis

All data were expressed as means ± SEM and were analysed by ANOVA, followed by the Duncan *post-hoc* test, using SPSS (Statistical Package for the Social Sciences, Chicago, USA – software, version 23.0 for Windows). Differences with $p < 0.05$ were considered to be statistically significant. All graphs were performed with GraphPad Prism (San Diego, CA, USA - version 5).

3. RESULTS

3.1 Heat shock proteins, p-JNK and p-IKK expression

Both unhydrogenated and partially hydrogenated oils (PHO) with casein tended to reduce HSP90 expression, but did not differ from the control, while unhydrogenated oil with

WPH (OWPH) increased HSP90 expression compared to the unhydrogenated oil with casein (OCAS) or PHO with either casein (HOCAS) or WPH (HOWPH; Figure 1A). Similarly, WPH in both unhydrogenated and partially hydrogenated-oil diets increased HSP60 expression when compared to casein (OCAS; Figure 1B). In the diet containing the unhydrogenated oil, it was observed that casein dramatically reduced HSP25, while WPH remained its levels close to those of the control group. In the diets containing PHO, neither one of the proteins showed an effect on the expression of HSP25 (Figure 1C). Particularly for HSP70, no statistical difference was observed among the groups (Figure 1D).

While HOCAS inhibited JNK phosphorylation in comparison to OCAS, the unhydrogenated oil only tended to increase JNK phosphorylation irrespective of the protein (Figure 1E). No alteration in IKK phosphorylation was observed (Figure 1F).

3.2 Inflammatory pathway

We assessed the expression of the following inflammatory parameters: Toll-like receptor 4 (TLR4), cluster of differentiation 14 (CD14), myeloid differentiation factor 88 (MyD88), nuclear factor kappa B (NF-κB) and tumor necrosis factor-alpha (TNF-α). However, no significant differences in the expression of such parameters among the groups were found (Figures 1 G, H, I, J and K, respectively).

3.3 Antioxidant system

We observed that the intake of partially hydrogenated oil with casein (HOCAS) reduced glutathione peroxidase (GPx) expression in comparison to the control group (Figure 1L) and WPH increased catalase expression in relation to casein when in the presence of the unhydrogenated oil. However, there was no statistical difference in catalase expression between the protein sources when the animals ingested the partially hydrogenated oil (Figure

1M). Additionally, WPH increased superoxide dismutase (SOD) expression in the HOWPH group compared to OCAS (Figure 1N).

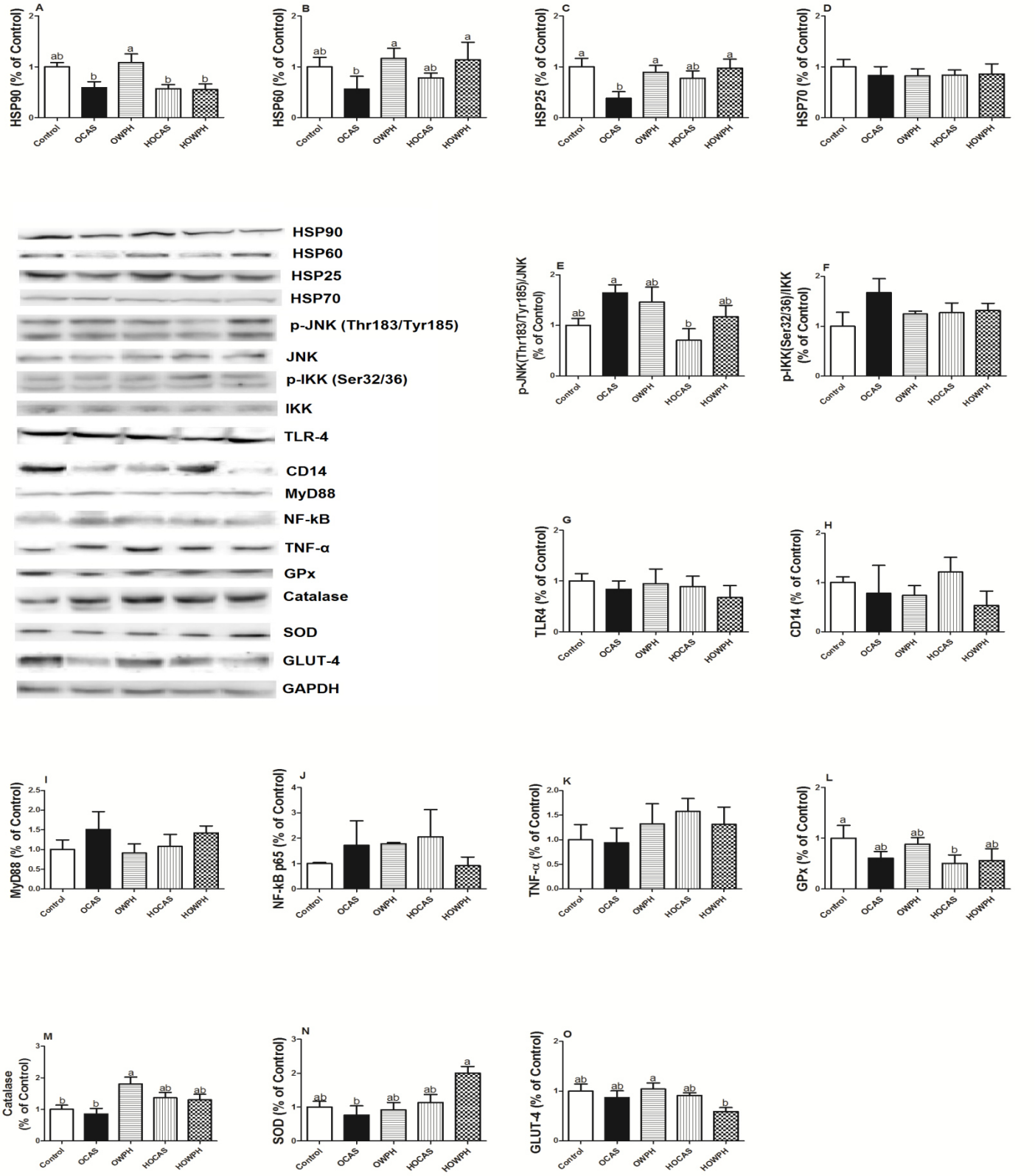

Figure 1. Means and SEM of the Western blot analysis of A) HSP90; B) HSP60; C) HSP25 D) HSP70; E) p-JNK; F) p-IKK; G) TLR4; H) CD14; I) MyD88; J) NF-kB p-65; K) TNF-α; L) GPx; M) Catalase; N) SOD; O) GLUT-4. Western blot determination were done in the gastrocnemius muscle. Diets: CONTROL (AIN 93-G), OCAS (high-fat diet prepared with unhydrogenated oil and casein), OWPH (high-fat diet prepared with unhydrogenated oil and whey protein hydrolysate), HOCAS (high-fat diet prepared with hydrogenated oil and casein), OWPH (high-fat diet prepared with hydrogenated oil and whey protein hydrolysate). Different letters represent significant differences ($p<0.05$).

3.4 Food intake, body weight gain and biochemical parameters

As expected, ingestion of hyperlipidic diets led to depressed food intake. Nevertheless, the depression was greater in the animals that consumed the unhydrogenated oil than in those receiving the PHO (Figure 2A). No effect from either the type of protein or fat source was reflected on the animals' weight (Figure 2C), and neither was total cholesterol (Figure 2D).

The high-density lipoprotein cholesterol (HDL-c) was elevated by both the unhydrogenated and the partially-hydrogenated oil diets when the protein was WPH compared to OCAS (Figure 2E). Meanwhile, the serum triacylglycerols appeared increased in the groups that consumed WPH independent of the fat source (Figure 2F).

WPH increased also the serum levels of uric acid in the group that consumed the partially hydrogenated oil (HOWPH) when compared to OCAS (Figure 2G), whereas the consumption of WPH in both partially hydrogenated and unhydrogenated oils showed impaired glucose tolerance (Figure 2 Q,R). No difference was observed among the groups regarding glucose, albumin, AST, ALT, total proteins, insulin levels (Figure 2 H, I, J, K, L, P, respectively).

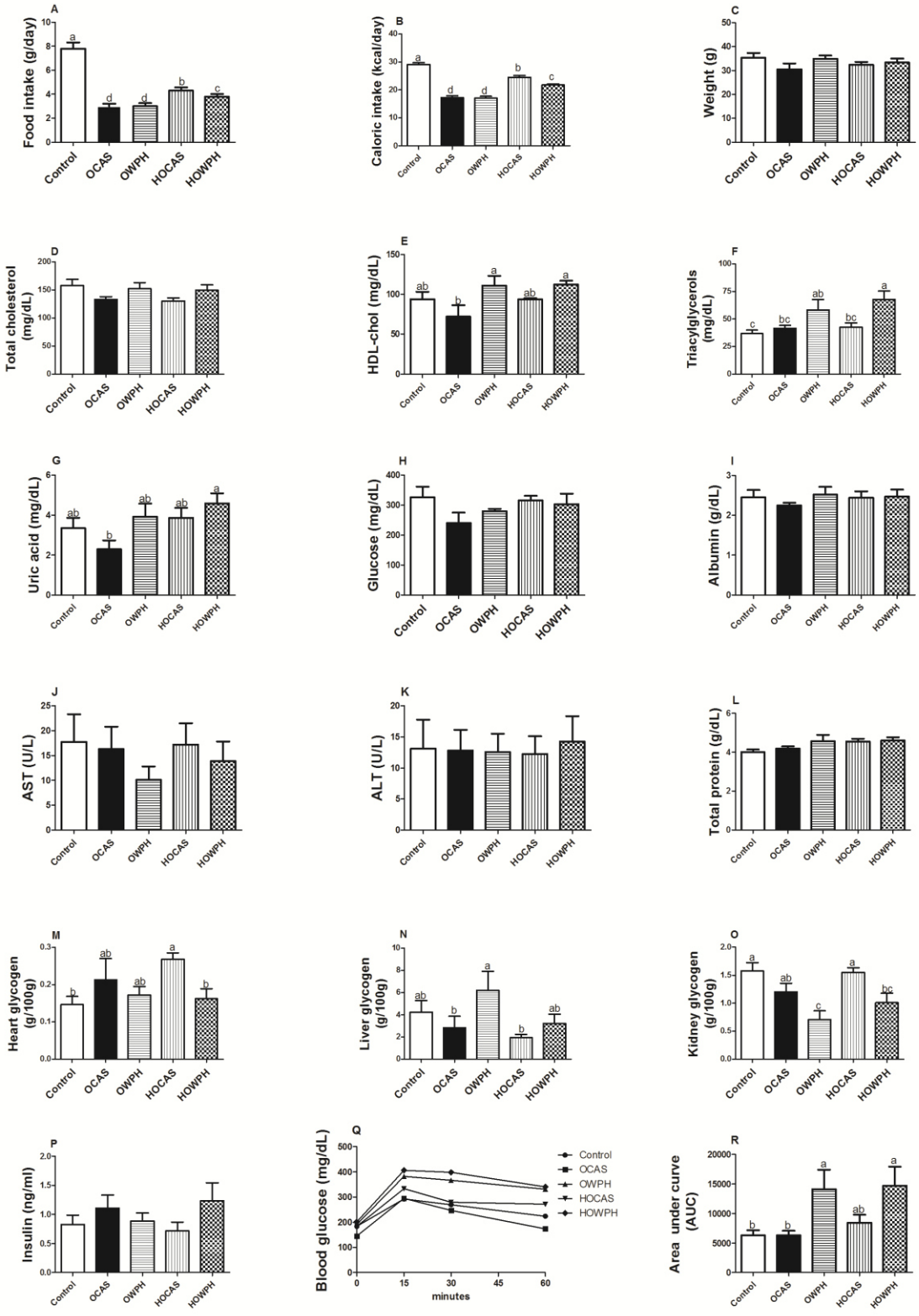

Figure 2. Food intake, caloric intake and body weight evolution curves of the five diet groups (A-C). Effect of whey protein hydrolysate on biochemical parameters: (D) total cholesterol; (E) HDL-chol: high-density lipoproteins; (F) triglycerides; (G) uric acid; (H) glucose; (I) albumin; (J) AST; (K) ALT; (L) total protein. Means and SEM for the (M) heart glycogen concentration; (N) liver glycogen concentration; (O) kidney glycogen concentration; (P) insulin; (Q) area under curve; (R) blood glucose. (Q-R) are results from the glucose tolerance test. Different letters above bars represent significant difference (Duncan test, $p<0.05$).

3.5 Histological examination

Photomicrographs of livers from the experimental groups are shown in Figure 3. Liver from control animals showed normal appearance, with hepatocytes exhibiting normal morphology (Figure 3A,B). It is evident that liver of animals fed with partially hydrogenated oil (Figure 3G-J) showed notable increase of hepatocyte vacuolization typical of fat droplet accumulation (steatosis) compared with those that consumed the unhydrogenated oil (Figure 3C-F) regardless of the protein source. When the diet contained the unhydrogenated oil the WPH worsened the hepatic steatosis (Figure 3C-F).

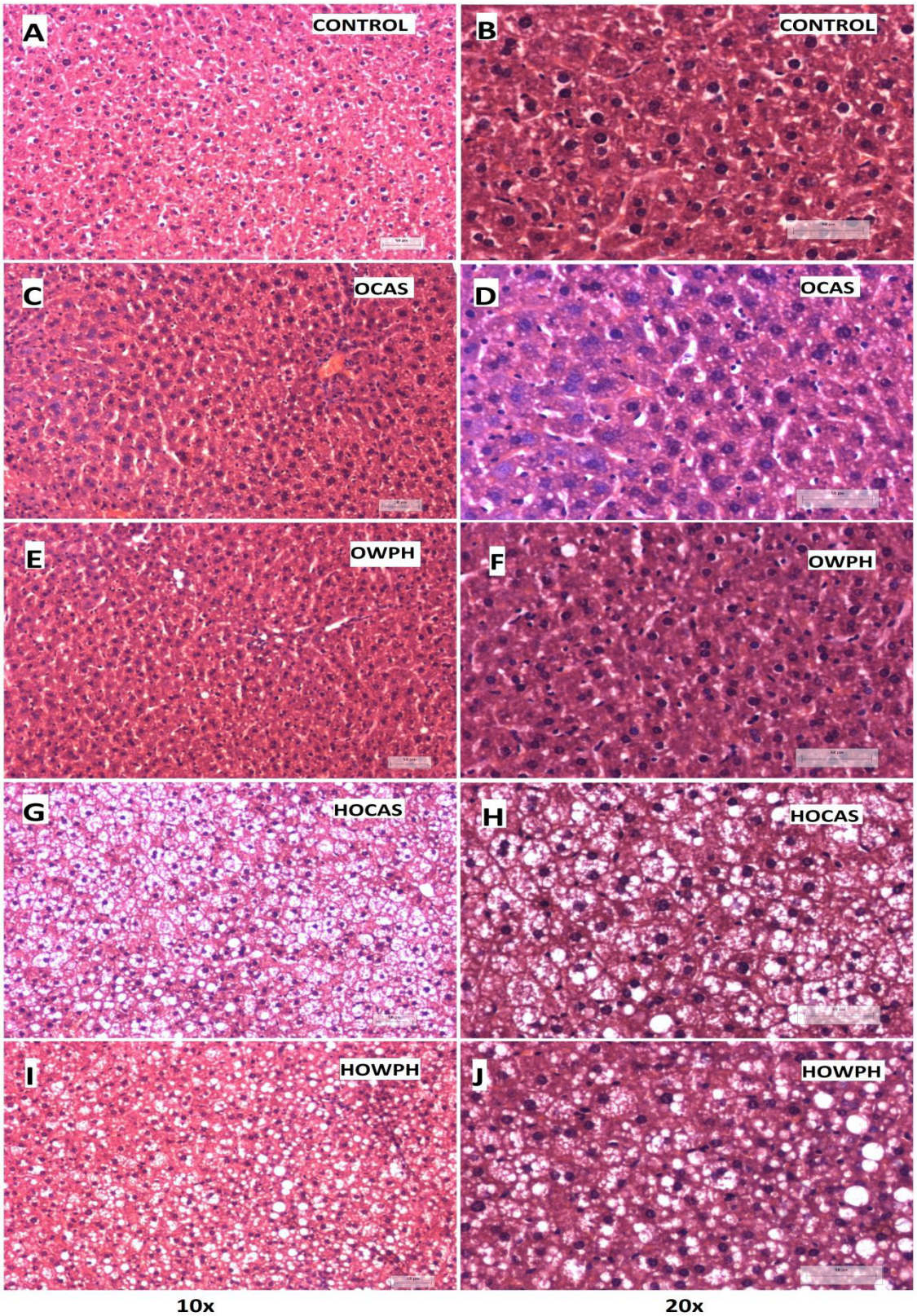

Figure 3. Photomicrographs of liver sections stained with hematoxylin and eosin. Bars (A, B, C, D, E, F, G, H, I, J) = 50 µm. Diets: Control (AIN 93-G), OCAS (high-fat diet prepared with unhydrogenated oil and casein), OWPH (high-fat diet prepared with unhydrogenated oil and whey protein hydrolysate), HOCAS (high-fat diet prepared with hydrogenated oil and casein), HOWPH (high-fat diet prepared with hydrogenated oil and whey protein hydrolysate).

3.6 Organ weights and Lee Index

Although there was a tendency for the excess fat to increase liver weight (~10%), it appeared that the partially hydrogenated oil fostered this increase. Consumption of the unhydrogenated oil with casein (OCAS) brought a reduction of inguinal fat, whereas WPH did not influence the results (Table 4). There was no significant difference in the weight of spleen, kidney, heart and lung, and the Lee indices remained normal for all groups (Table 4).

Table 4. Organ weights and Lee index

	Control		**OCAS**		**OWPH**		**HOCAS**		**HOWPH**	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Spleen (mg/g)	3.04	0.36	3.68	0.74	3.27	0.16	3.08	0.15	3.02	0.23
Kidney (mg/g)	10.93	0.65	11.62	0.77	11.29	0.51	12.35	0.34	12.05	0.61
Heart (mg/g)	5.70	1.09	5.41	0.30	5.33	0.17	4.95	0.19	5.32	0.39
Lung (mg/g)	4.65	0.25	5.18	0.59	4.93	0.14	5.38	0.26	5.33	0.33
Liver (mg/g)	40.27^{c}	1.54	41.01^{bc}	3.38	43.86^{abc}	1.49	46.66^{ab}	2.32	48.07^{a}	1.17
Epididimal fat (mg/g)	31.72^{ab}	3.05	21.30^{b}	4.80	40.10^{a}	3.45	27.30^{ab}	3.86	32.48^{ab}	5.13
Inguinal fat (mg/g)	26.93^{a}	2.34	16.21^{b}	2.50	23.69^{ab}	2.10	19.08^{ab}	2.58	23.31^{ab}	3.66
Lee Index (g/cm³)	0.34	0.004	0.34	0.004	0.34	0.002	0.32	0.005	0.33	0.008

Results were expressed as means ± SEM. Different superscript letters indicate statistical differences at $p<0.05$.

3.7 Glycogen and GLUT-4

Hydrogenation of the oil suppressed GLUT-4 translocation compared to the unhydrogenated oil when in combination with WPH. Other than that, GLUT-4 levels remained comparable to the levels of the control (Figure 1O).

Glycogen levels responded in different ways depending on the organ. Heart glycogen concentrations were higher in the animals that received partially hydrogenated oil plus casein in comparison with the control (Figure 2L), whereas in the liver, the levels increased only in the animals that received the unhydrogenated oil and WPH (OWPH) in comparison with casein (Figure 2M). In the kidney, however, both unhydrogenated and hydrogenated with WPH decreased glycogen levels (Figure 2N).

3.8 Gut microbiota profile

In general, the partially hydrogenated oil did not invert the general dominance of *Bacteroidetes* independent of the protein source, but rather preserved the normal pattern (Figure 4). In contrast, the unhydrogenated oil in combination with WPH brought about a decrease in *Bacteroidetes*. Nevertheless, the *Firmicutes* population was reduced in the experimental diets containing casein (OCAS and HOCAS), contrary to the case when the protein was WPH (OWPH diet). With regard to the other major phyla, the unhydrogenated oil tended to elevate the proportion of *Proteobacteria*, and this increase was more prominent in the OCAS group. Minor phyla *Verrucomicrobia, Actinobacteria, Thermotogae* and others were identified and showed effects of both the high fat and the protein, but only *Verrucomicrobia* responded to some extent (Figure 4). The major genera and species identified in the cecal microbiota are shown in Table 5.

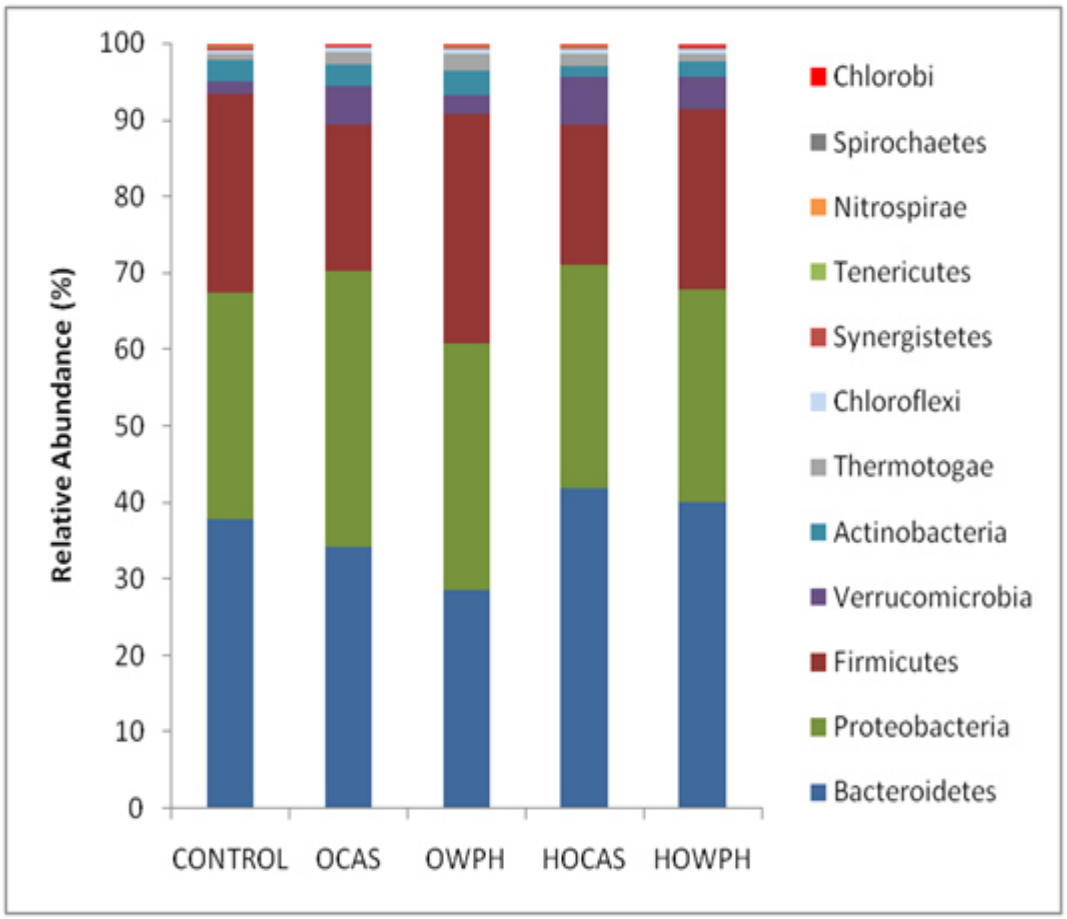

Figure 4. Cecal microbiota composition (% reads basis) of each group according to phyla. Key: Control (AIN 93-G), OCAS (high-fat diet prepared with unhydrogenated oil and casein), OWPH (high-fat diet prepared with unhydrogenated oil and whey protein hydrolysate), HOCAS (high-fat diet prepared with hydrogenated oil and casein), HOWPH (high-fat diet prepared with hydrogenated oil and whey protein hydrolysate).

Table 5. Identification of major genera and species in the cecal microbiota (Mean ± SEM) classified according to the16S rRNA

Genera	**CONTROL**	**OCAS**	**OWPH**	**HOCAS**	**HOWPH**
Helicobacter	22.17 ± 0.02^{ab}	28.38 ± 0.03^{a}	25.99 ± 0.02^{ab}	21.72 ± 0.01^{b}	19.66 ± 0.02^{b}
Bacteroides	16.51 ± 0.02^{a}	18.13 ± 0.01^{a}	11.90 ± 0.01^{b}	20.33 ± 0.01^{a}	19.85 ± 0.02^{a}
Parabacteroides	3.08 ± 0.02^{b}	5.48 ± 0.01^{b}	4.95 ± 0.01^{b}	11.16 ± 0.01^{a}	11.06 ± 0.01^{a}
Oscillospira	8.63 ± 0.01^{ab}	7.34 ± 0.00^{bc}	11.06 ± 0.01^{a}	5.04 ± 0.0^{c}	9.16 ± 0.01^{ab}
Flavobacterium	5.41 ± 0.00	4.02 ± 0.01	4.89 ± 0.00	5.30 ± 0.00	4.20 ± 0.00
Akkermansia	1.32 ± 0.01^{b}	4.55 ± 0.01^{ab}	1.96 ± 0.01^{b}	5.76 ± 0.01^{a}	3.89 ± 0.01^{ab}
Desulfovibrio	2.41 ± 0.00^{ab}	2.60 ± 0.00^{a}	1.74 ± 0.00^{b}	2.72 ± 0.00^{a}	2.83 ± 0.00^{a}
Butyricimonas	8.1 ± 0.01^{a}	3.10 ± 0.00^{b}	3.06 ± 0.00^{b}	3.40 ± 0.00^{b}	2.60 ± 0.01^{b}
Ruminococcus	3.25 ± 0.00^{ab}	1.79 ± 0.00^{c}	3.79 ± 0.01^{a}	1.69 ± 0.00^{c}	2.38 ± 0.00^{bc}
Actinocorallia	2.22 ± 0.00^{a}	2.15 ± 0.00^{a}	1.68 ± 0.00^{ab}	0.49 ± 0.00^{c}	1.03 ± 0.00^{bc}
Natronincola	4.74 ± 0.01^{a}	2.61 ± 0.00^{b}	3.04 ± 0.01^{b}	1.67 ± 0.00^{b}	1.77 ± 0.00^{b}
Clostridium	1.63 ± 0.00^{b}	1.44 ± 0.00^{b}	2.37 ± 0.00^{a}	1.94 ± 0.00^{a}b	2.73 ± 0.00^{a}
Rikenella	0.99 ± 0.00	1.56 ± 0.00	1.82 ± 0.00	1.50 ± 0.00	1.13 ± 0.00
Arcobacter	0.11 ± 0.00^{b}	0.20 ± 0.00^{b}	0.39 ± 0.00^{ab}	0.14 ± 0.00^{b}	0.59 ± 0.00^{a}
Marinitoga	0.79 ± 0.00	1.71 ± 0.01	2.55 ± 0.01	1.82 ± 0.00	1.13 ± 0.00
Species	**CONTROL**	**OCAS**	**OWPH**	**HOCAS**	**HOWPH**
Helicobacter rodentium	20.13 ± 0.02	20.6 ± 0.03	19.14 ± 0.02	14.92 ± 0.01	15.97 ± 0.02
Bacteroides vulgatus	4.52 ± 0.01^{ab}	5.34 ± 0.01^{ab}	3.32 ± 0.01^{b}	8.94 ± 0.02^{ab}	9.83 ± 0.03
Parabacteroides goldsteinii	3.68 ± 0.00^{b}	7.23 ± 0.02^{b}	6.71 ± 0.01^{b}	14.20 ± 0.01^{a}	12.88 ± 0.02^{a}
Akkermansia muciniphila	2.12 ± 0.01^{b}	6.94 ± 0.02^{ab}	3.74 ± 0.01^{ab}	8.22 ± 0.02^{a}	6.075 ± 0.02^{ab}
Actinocorallia cavernae	3.70 ± 0.01^{a}	3.41 ± 0.01^{ab}	2.73 ± 0.01^{ab}	0.70 ± 0.00^{c}	1.79 ± 0.01^{bc}
Butyricimonas virosa	7.89 ± 0.01^{a}	1.98 ± 0.00^{b}	2.57 ± 0.00^{b}	2.94 ± 0.00^{b}	2.56 ± 0.00^{b}
Desulfovibrio piger	1.6 ± 0.00^{b}	2.15 ± 0.00^{ab}	1.41 ± 0.00^{b}	2.27 ± 0.00^{ab}	3.11 ± 0.00^{a}
Bacteroides rodentium	3.48 ± 0.01	4.44 ± 0.00	3.48 ± 0.00	5.05 ± 0.01	3.94 ± 0.01
Rikenella microfusus	1.67 ± 0.00	2.43 ± 0.01	2.85 ± 0.01	2.18 ± 0.00	1.84 ± 0.00
Bacteroides dorei	1.47 ± 0.01	3.53 ± 0.02	0.88 ± 0.00	0.75 ± 0.01	1.67 ± 0.01
Arcobacter marinus	0.16 ± 0.00^{b}	0.31 ± 0.00^{b}	0.65 ± 0.00^{ab}	0.20 ± 0.00^{b}	1.02 ± 0.00^{a}
Bacteroides acidifaciens	4.81 ± 0.01^{ab}	3.48 ± 0.01^{b}	3.94 ± 0.00^{ab}	6.86 ± 0.01^{a}	4.76 ± 0.01^{ab}
Helicobacter ganmani	3.64 ± 0.00	4.48 ± 0.01	4.33 ± 0.00	3.78 ± 0.00	3.07 ± 0.01
Parabacteroides johnsonii	1.07 ± 0.00^{a}	0.59 ± 0.00^{b}	0.83 ± 0.00^{ab}	0.92 ± 0.00^{ab}	1.15 ± 0.00^{a}
Butyricimonas synergistica	5.58 ± 0.01^{a}	2.93 ± 0.00^{b}	2.66 ± 0.00^{bc}	2.01 ± 0.00^{bc}	1.58 ± 0.00^{c}
Marinitoga piezophila	1.09 ± 0.00	2.45 ± 0.02	3.89 ± 0.02	2.49 ± 0.00	1.61 ± 0.01
Oribacterium sinus	1.45 ± 0.00	1.09 ± 0.00	1.83 ± 0.00	0.86 ± 0.00	1.78 ± 0.00
Helicobacter mastomyrinus	1.09 ± 0.00^{b}	1.85 ± 0.00^{a}	1.72 ± 0.00^{a}	1.20 ± 0.00^{b}	1.15 ± 0.00^{b}
Helicobacter mesocricetorum	1.34 ± 0.00^{b}	2.32 ± 0.00^{a}	2.22 ± 0.00^{a}	1.54 ± 0.00^{b}	1.29 ± 0.00^{b}
Porphyromonas canis	0.92 ± 0.00^{a}	0.68 ± 0.00^{ab}	0.75 ± 0.00^{ab}	0.38 ± 0.00^{b}	0.90 ± 0.00^{a}

Data are expressed as % of total bacteria. Different superscript letters indicate statistical differences at $p<0.05$.

By observing Table 5, it is evident that up or down changes in genera and species were induced by both the high unhydrogenated vegetable oil and the PHO as could be seen with genera *Helicobacter, Parabacteroides*, *Akkermansia* and *Clostridium*, or *Butyricimonas*, *Actinocorallia* and *Natronincola*. It was noticed for instance that whereas the high vegetable oil doubled the growth of *Parabacteroides goldsteinii*, it also lowered the populations of the genus *Butyricimonas* (accounted for by the *Butyricimonas virosa* and *Butyricimonas synergistica* species). On the other hand, the large presence of dietary *trans*-fat caused a further two-fold increase of *Parabacteroides goldsteinii* while depressing the *Actinocorallia* and *Natronincola* genera. No protecting effect, however, could be attributed to the WPH.

4. DISCUSSION

Based on previous findings of our group indicating that a whey protein hydrolysate (WPH) was able to elevate the expression of protective HSPs in rats consuming a normolipidic diet (De Moura and others 2013; Moura and others 2014), and partially protect the gut microbiota of mice consuming a lard-containing high-fat diet (Monteiro and others 2016), the main objective of the present study was to determine if such ameliorating effects would also hold in the case of a hyperlipidic diet containing *trans* fatty acids, but free of pork fat. In this respect, our expectations were that the inclusion of WPH in a hyperlipidic diet would at least partly neutralize the adverse effects of an oil-rich diet intake.

The results showed that WPH consumption increased muscle HSP90, HSP60 and HSP25 expressions in the hyperlipidic unhydrogenated-oil diet. However, WPH did not influence HSPs in the diet with partially hydrogenated oil. These data suggest that the presence of the *trans*-fatty acids may have inhibited the protective effect of WPH. Additionally, no effect of the protein or lipid sources was found in the HSP70 muscle expression. Similar results were reported by Gupte and others (2009b), who verified that a high-fat diet was unable to change HSP70 expression in rat muscle.

WPH intake has shown to increase HSP70 and HSP90 expression, but not to influence HSP60 and HSP25 in muscle of rats receiving a normolipidic diet (De Moura and others 2013; Moura and others 2014). In contrast, the present results are showing that a hyperlipidic diet exempt of pork fat leads to a different effect of the WPH regarding HSPs expression. Despite WPH having induced some HSPs, our results indicate that WPH did not protect the animals from impaired glucose tolerance in the GTT, although the insulin levels, basal glucose and GLUT-4 expression were not affected.

Although it has been demonstrated that HSP70 and HSP25 induction is able to inhibit JNK and IKK activation, and thus improve glucose tolerance in high-fat-diet induced obesity (Gupte and others 2009a,b), our results did not find a correlation between either the HSPs induction, inhibition of JNK and IKK or with glucose tolerance.

In spite of chronic pathologies such as inflammation, obesity and Type-2 diabetes mellitus, among others, have been associated to the consumption of a high-fat diet with lard (Buettner and others 2007), our results show that the hyperlipidic diets containing either unhydrogenated or hydrogenated oils failed to induce the inflammatory cascade. However, this could be attributed to either the duration of the treatment or the absence of lard in the diets. Kubant and others (2015) showed that rats fed a high-fat diet containing only vegetable partially hydrogenated oil as main lipid source for 14 weeks did not develop obesity nor had insulin impaired, unlike animals fed a high-fat diet containing lard, and the authors suggested that these might happened due the duration of the experiment or the fatty acids composition of the diets. Here, the results showed only impaired glucose tolerance. Thus, the lack of a strong adverse response from such large intake of *trans*-fat could come from the absence of lard in the diet. Therefore, the metabolic stress caused by a hyperlipidic diet containing *trans*-fatty acids seems to be surprisingly less harmful than expected if it were a lard-containing high-fat diet (Kubant and others 2015).

Considering that the expression of such enzymes as GPx, catalase and SOD can act as free-radical scavengers under conditions of various types of stress, including dietary stress, their lack of expression can worsen the metabolic toll imposed by the dietary insult (Blokhina and others 2002). A study by Feillet-Coudray and others (2009) reported that SOD and GPx were not influenced by a high-fat diet in the gastrocnemius muscle of rats. Another study (Marineli and others 2015), however, showed that a high-fat diet may decrease SOD and GPx expression in the soleus of rats. Here we see that only animals of the HOCAS-group showed lower GPx expression in comparison to the control-group and SOD expression was not influenced by the high-fat diet, independent of the type. Additionally, a high-fat diet was demonstrated to increase catalase in several tissues (Meng and others 2011; Rindler and others 2013), except in the gastrocnemius (Feillet-Coudray and others 2009). In agreement with the study of Ebaid and others (2012) reporting that whey protein diminishes oxygen radicals and therefore improve the levels of the oxidative markers, we find that catalase expression was increased only in the OWPH group.

The lower food intake shown by the animals consuming the high-fat could be due to the fat-induced secretion of satiety peptides (Duca and others, 2013).

Previous studies have shown that oral administration of whey proteins exerts a positive effect by reducing fat infiltration and lower fat deposition in the liver of rats (Hamad and others 2011). Moreover, data from Monteiro and others (2016) have suggested that the whey proteins could have a protective effect against non-alcoholic steatosis. In contrast with these findings, the histological analysis of our experiment indicated that WPH had no effect against the fatty infiltration caused by a high-oil diet. Moreover, our histological data show that *trans*-fatty acids increased fatty infiltration in the liver independent of the protein consumed.

A high-fat diet with lard has been shown to alter the gut microbiota composition,

inverting the normal *Bacteroidetes:Firmicutes* ratio (Hildebrandt and others 2009; Kim and others 2012; Hamilton and others 2015). Changes in the intestinal microbiota composition can lead to increased body fat content and insulin resistance (Bäckhed and others 2004; Ley and others 2005). Schwiertz and others (2009) reported that the proportion of *Bacteroidetes* increases in overweight and obese subjects thus diminishing the *Bacteroidetes*:*Firmicutes* ratio. In turn, we have reported that mice fed a high-fat diet associated to whey protein hydrolysate partly preserved the gut microbiota (Monteiro and others 2016). In the present study, however and contrary to our expectation, the hydrogenated lipid did not adversely affect the *Bacteroidetes*:*Firmicutes* ratio, but rather appeared to be conservative in contrast to the unhydrogenated oil.

Nothing has been reported in the literature regarding the sizeable increase of *Parabacteroides goldsteinii* as induced by *trans*-fats. Nevertheless, this pathogenic species that has been associated with appendicitis, peritonitis, intra-abdominal infections and bacteremia in humans (Awadiel-Kariem and others 2010), was promoted in our experiments not only by the high lipid intake, but also substantially by the *trans*-fat.

The role of genus *Akkermansia* on health has been rather controversial. While patients with multiple sclerosis have shown gut microbiome alterations that include increases in *Akkermansia*, coupled with decrements of beneficial *Butyricimonas* (Jangi and others 2016), ingestion of *A. muciniphila* has shown to attenuate atherosclerotic lesions by restoring the gut barrier and improving the metabolic endotoxemia induced by inflammation in mice (Li and others 2016). In our study, we have observed both an increase of *Akkermansia muciniphila* and a depression of *Butyricimonas virosa* and *B. synergistica*; both alterations being more related to the high lipid intake than to the *trans*-fat intake (Table 5). The population increments seen in mucin-thriving bacteria of our experiments appear to have been beneficial based on the nearly absence of inflammation. Decrements in essential butyric acid-

producing bacteria have also been reported to occur in insulin-resistant, obese patients (Moreno-Indias and others 2016) and, therefore, leave the mixed results difficult to interpret at this time.

In conclusion, the data obtained indicate that WPH increases HSP90, HSP60 and HSP25 expressions, but without influencing HSP70, in a group of mice consuming a hyperlipidic diet with unhydrogenated oil. However, when the diet contained high amounts of *trans*-fatty acids, the WPH was not able to raise HSPs expression. Thus, these results suggest that the intake of partially hydrogenated fat inhibited the potentially tissue-protective, anti-stress effect of WPH. Also, the main alterations expected to occur in the gut microbiota profile, as a result of the high fat intake, here were not observed – most probably due to the lack of pork fat in the diet. Considering that the high intake of either unhydrogenated or hydrogenated oil did not affect the inflammatory parameters, and that other health parameters also did not undergo great alterations, it is suggested that the extensive adverse health effects commonly attributed to the consumption of *trans*-fats are the result of some kind of synergism with the lard.

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DISCUSSÃO GERAL

4. DISCUSSÃO GERAL

Evidências demonstram que o consumo da proteína do soro do leite hidrolisada (PSLH) aumenta a expressão de HSP70 e HSP90, mas não influencia a expressão das HSP60 e HSP25 em músculo de ratos alimentados com uma dieta normocalórica (DE MOURA et al., 2013; MOURA et al., 2014). Diferentemente, neste estudo, o consumo de PSLH elevou a expressão das HSP90, HSP60 e HSP25 no músculo de animais alimentados com uma dieta hiperlipídica contendo óleo não hidrogenado. Todavia, a PSLH não influenciou a expressão das HSPs na dieta contendo óleo parcialmente hidrogenado. Adicionalmente, as fontes proteica e lipídica não foram capazes de influenciar a expressão da HSP70. Resultados similares foram relatados por Gupte et al. (2009a), que verificaram que uma dieta hiperlipídica não alterou a expressão da HSP70 em músculo de ratos. Apesar da PSLH ter induzido a expressão de HSPs, os resultados indicam que esta fonte proteica não protegeu os animais da intolerância à glicose após o teste de tolerância à glicose (GTT). Entretanto, não se notou qualquer alteração no nível de insulina, glicose basal e translocação de GLUT-4.

Tem sido relatado que a indução de HSP70 e HSP25 é capaz de inibir a ativação de JNK e IKK e, assim, melhorar a tolerância à glicose em animais obesos induzidos por uma dieta rica em gordura (GUPTE et al., 2009a,b). Contudo, na presente pesquisa não foi observada correlação entre a indução de HSPs, inibição de JNK e IKK com a tolerância à glicose.

Uma dieta com alto teor de lipídios pode prejudicar a saúde do consumidor e levar a patologias graves, como inflamação, obesidade e diabetes mellitus tipo 2 (BUETTNER; SCHÖLMERICH; BOLLHEIMER, 2007). Os resultados aqui mostraram que a dieta hiperlipídica não foi capaz de desenvolver um quadro característico de obesidade. Tal fato pode ser atribuído ao tempo de duração do experimento ou até mesmo à ausência de gordura animal na composição da dieta, uma vez que já se foi evidenciado que quando há

essencialmente banha de porco como fonte lipídica na composição de uma dieta hiperlipídica, os animais tendem a apresentar maior ganho de peso e/ou mostrar co-morbidades associadas à obesidade (MURPHY et al., 2010; KAWASAKI et al., 2012; BATISTA et al., 2014; HAMILTON et al., 2015; KUBANT et al., 2015).

A redução das atividades das enzimas antioxidantes, como a GPx, a catalase e a SOD, pode piorar o estresse oxidativo, uma vez que essas enzimas atuam como "sequestradores" de radicais livres em condições desse tipo de estresse (BLOKHINA; VIROLAINEN; FAGERSSTEDT, 2002). A proteína do soro de leite já mostrou reduzir os radicais de oxigênio e melhorar os níveis dos marcadores oxidativos e, conseqüentemente, melhorar o sistema antioxidante (EBAID; BADR; METWALLI, 2012). No presente estudo, notou-se que o único parâmetro antioxidante que a PSLH influenciou foi a expressão de catalase, compreendendo apenas o grupo que consumiu óleo não hidrogenado e WPH, onde sua expressão foi aumentada.

As proteínas do soro de leite mostraram exercer um efeito positivo na histologia do fígado de ratos, uma vez que a administração oral de proteínas de soro de leite diminuiu a infiltração de gordura (HAMAD et al., 2011). Monteiro et al. (2016) sugeriram que as proteínas do soro de leite podem mostrar um efeito protetor contra a esteatose hepática não alcoólica. Em contraste com esses resultados, a análise histológica deste experimento indicou que a PSLH não apresentou qualquer efeito protetor contra a infiltração de gordura causada pela dieta hiperlipídica (principalmente com óleo parcialmente hidrogenado).

A literatura evidencia que uma dieta com alto teor de lipídio pode alterar a composição da microbiota intestinal, como, por exemplo, aumentar a relação *Firmicutes*:*Bacteroidetes* (KIM et al., 2012; HAMILTON et al., 2015). As mudanças na composição da microbiota intestinal podem levar a um aumento do teor de gordura corporal e da resistência à insulina (BÄCKHED et al., 2004; LEY et al., 2005). Monteiro et al. (2016)

relataram que camundongos alimentados com uma dieta *hiperlipídica* suplementada com proteína de soro de leite intacta apresentaram maior proporção de *Bacteroidetes* à *Firmicutes*. Entretanto, na presente pesquisa, nem a fonte lipídica nem a fonte proteica promoveram a inversão da relação *Bacteroidetes*:*Firmicutes*. De modo semelhante, Schwiertz et al. (2009) reportaram que a proporção de *Bacteroidetes* aumentou em indivíduos com sobrepeso e obesos, enquanto houve uma menor proporção de *Firmicutes*.

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CONCLUSÃO GERAL

5. CONCLUSÃO GERAL

Apesar de os animais terem ingerido uma dieta hiperlipídica, não foi notado o desenvolvimento de um quadro característico de obesidade.

Embora pesquisas do nosso laboratório tenham mostrado que as proteínas do soro do leite foram capazes de elevar a expressão de algumas HSPs (*heat shock proteins*) em ratos alimentados com uma dieta normocalórica (DE MOURA et al., 2013; MOURA et al., 2014) e também amenizar os efeitos adversos na microbiota intestinal de camundongos causados por uma dieta hiperlipídica com banha de porco (MONTEIRO et al., 2016), tais efeitos não foram completamente reproduzidos neste estudo.

No presente estudo, foi observado que a proteína do soro do leite hidrolisada (PSLH) eleva a expressão de algumas HSPs (HSP90, HSP60 e HSP25) apenas na dieta contendo óleo não hidrogenado, não influenciando a expressão da HSP70. Assim, os resultados sugerem que a ingestão de gordura parcialmente hidrogenada possa ter inibido o possível efeito protetor do consumo da PSLH.

Além disso, as alterações no perfil da microbiota intestinal que eram esperadas após o consumo de uma dieta hipercalórica não foram observadas.

Portanto, sugere-se que a ausência de todos os efeitos adversos conhecidos na saúde associados com os ácidos graxos *trans* seja devido, principalmente, à ausência de banha de porco ou gordura animal na composição da dieta hiperlipídica.

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ANEXO

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CERTIFICADO

Certificamos que o projeto intitulado **"EFEITO DO CONSUMO DA PROTEÍNA DO SORO DO LEITE HIDROLISADA NA MICROBIOTA INTESTINAL E SISTEMA HEAT SHOCK PROTEINS EM CAMUNDONGOS ALIMENTADOS POR UMA DIETA RICA EM GORDURAS TRANS"**, protocolo nº **3821-1**, sob a responsabilidade de **Prof. Dr. Jaime Amaya-Farfán / Gessika Cristina Borges Castro**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica ou ensino, encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais e do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal - CONCEA**, e foi aprovado pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em reunião de **08 de junho de 2015**.

Vigência do projeto: 08/2015-12/2015
Espécie/Linhagem: Camundongo isogênico / C57BL/6/Junib
No. de animais: 40
Peso/Idade: 04 semanas / 12gr
Sexo: machos
Origem: CEMIB/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

Campinas, **08 de junho de 2015**.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva