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NARCIZA MARIA DE OLIVEIRA ARCANJO

EFEITO PROTETOR DO VINHO TINTO CONTRA OXIDAÇÃO EM CARNE MARINADA E CAPACIDADE REDOX ATIVA DO RESVERATROL EM SISTEMAS MODELO

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Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Centro de Tecnologia, Universidade Federal da Paraíba em cumprimento dos requisitos para obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientadora: Dra. Marta Suely Madruga

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BANCA EXAMINADORA

Marta Suely Madruga

Profa. Dra. Marta Suely Madruga – DEA / CT / UFPB
Orientador/Coordenador da Banca Examinadora

Prof. Dr. Mario Estévez Garcia – IPROCAR / UNEX
Coorientador

Prof. Dr. Fábio Anderson Pereira da Silva –DEA / CT / UFPB
Examinador Interno

Prof. Dra Marciane Magnani – DEA / CT / UFPB
Examinador Interno

Janeeyre Ferreira Maciel

Prof Dra Janeeyre Ferreira Maciel – DEA / CT / UFPB
Examinador Externo

Mônica Tejo Cavalcanti

Prof Dra Mônica Tejo Cavalcanti – CCTA / UFCG
Examinador Externo

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RESUMO

Efeito Protetor do Vinho Tinto Contra Oxidação em Carne Marinada e Capacidade Redox Ativa do Resveratrol em Sistemas Modelo

O objetivo desta tese foi de avaliar e elucidar a ação dos compostos fenólicos do vinho tinto nas reações de oxidação em carne marinada e a capacidade redox ativa do resveratrol em sistemas modelos envolvendo proteína do soro humano e bactéria ácido láctica. No primeiro estudo, avaliou-se o impacto da marinação com vinhos tintos produzidos a partir de diferentes uvas na estabilidade oxidativa e na qualidade global de carne bovina (*Longissimus lumborum)* durante 7 dias de armazenamento refrigerado a 4 ºC e também na carne assada. Para tanto, quatro sistemas de marinação foram conduzidos: Carbernet (CAB), Tempranillo (TEM) e Isabel (ISA), incluindo um controle, utilizando água. Nos bifes armazenados sob refrigeração, observou-se que os componentes fenólicos específicos presentes no vinho foram os responsáveis por diversas bioatividades. Os componentes do vinho ISA, protegeram de forma mais eficaz as proteínas contra a oxidação, além de melhorar a maciez dos bifes quando comparado aos outros tratamentos. Entretanto, variedades de vinho CAB e TEM, ricas em procianidinas, foram mais efetivas contra a oxidação lipídica. A composição fenólica e o conteúdo em ácidos orgânicos dos vinhos ISA podem explicar seus efeitos antimicrobianos contra enterobactérias, enquanto os açúcares podem ter promovido o crescimento de bactérias lácticas nos tratamentos CAB e TEM. Nos bifes marinados e submetidos ao processo de cocção verificou-se que o ISA também demonstrou maior capacidade de proteção contra a oxidação das proteínas, também foi eficaz no controle da formação de compostos voláteis derivados de lipídios, além disso contribuições positivas foram verificadas nos atributos sensoriais dos bifes. Em geral, o processo de marinação com vinho trouxe benefícios aos bifes bovinos, e em particular vinhos produzidos com uvas Isabel mesmo sendo sensorialmente considerado uma vinho de qualidade inferior, quando comparado aos vinhos produzidos com uvas *Vitis vinifera* podem ser utilizados como ingrediente funcional em produtos cárneos. O segundo experimento avaliou a capacidade do um componente específico do vinho, o resveratrol (RES), em neutralizar o dano oxidativo causado por concentrações patológicas de metilglioxal (MGO) e glioxal (GO) em proteína do soro humano. O resveratrol neutralizou ambos os α-dicarbonílos formando aductos e a ação antioxidante acarretou em uma redução significativa de AGEs (Produtos finais de glicalização avançada). No entanto, ação pró-oxidante do resveratrol também foi observada, pois conjugados resveratrol-α-dicarbonil oxidaram Cys34 (Tiol) e lisina, arginina e / ou prolina por um ataque nucleofílico aos grupos SH (grupo sulfidrila) e ε-NH (grupo amino) em HSA (albumina do soro humano). Estudos futuros ainda precisam ser desenvolvidos para esclarecer melhor o papel pró-oxidativo dos conjugados RES-α-dicarbonil bem como, os mecanismos de sinalização redox e / ou reflexo de dano oxidativo e doença também deve ser consideradas em estudos futuros. O terceiro estudo forneceu uma visão original sobre a base genética e molecular das respostas de *Lactobacillus reuteri* PL503 contra o estresse oxidativo induzido por peróxido de hidrogênio (H_2O_2). Verificou-se que o resveratrol (100 µM) protegeu a *L. reuteri* PL503 contra a carbonilação proteica de forma plausível através de vários mecanismos, incluindo eliminação direta de espécies reactivas de oxigênio (ROS), regulação positiva do gene dhaT e promoção da síntese de compostos contendo enxofre. A carbonilação de proteínas como reflexo do dano oxidativo às bactérias e suas conseqüências, bem como o papel das proteínas carbonilas como moléculas sinalizadoras implicadas nas respostas das bactérias ao estresse oxidativo, precisam ser mais investigadas.

Palavras chave: processo de oxidação, vinhos, compostos bioativos, carne, carbonílicos de proteínas, bactéria ácido lática.

ABSTRACT

Protective effect of red wine against marinated meat oxidation and redox activity of resveratrol in model systems.

The objective of this thesis was to evaluate and elucidate the action of red wine phenolic compounds on the oxidation reactions in marinated meat and the active redox capacity of resveratrol in model systems involving human serum protein and lactic acid bacteria. In the first study, the impact of marination with red wines produced from different grapes on oxidative stability and on the overall quality of beef (*Longissimus lumborum*) was evaluated during 7 days of refrigerated storage at 4 ºC and also in roasted beef. Four marking systems were conducted: Carbernet (CAB), Tempranillo (TEM) and Isabel (ISA), including a control, using water. In the steaks stored under refrigeration, it was observed that the specific phenolic components present in the wine were responsible for several bioativities. The ISA wine components protected proteins more effectively against oxidation, as well as improving the tenderness of steaks when compared to other treatments. However, CAB and TEM varieties, rich in procyanidins, were more effective against lipid oxidation. The phenolic composition and organic acid content of ISA wines may explain its antimicrobial effects against enterobacteria, while sugars may have promoted the growth of lactic acid bacteria in CAB and TEM treatments. In the steaks marinated and submitted to the cooking process it was verified that the ISA also demonstrated a greater protection capacity against the oxidation of the proteins, it was also effective in controlling the formation of volatile compounds derived from lipids, besides positive contributions were verified in the sensorial attributes two steaks. In general, the marination process with wine has brought benefits to bovine steaks, and in particular wines produced with Isabel grapes even being sensorially considered a wine of inferior quality, when compared to the wines produced with Vitis vinifera grapes can be used as functional ingredient in products meat. The second experiment evaluated the ability of a specific wine component, resveratrol (RES), to neutralize the oxidative damage caused by pathological concentrations of methylglyoxal (MGO) and glyoxal (GO) in human serum protein. Resveratrol neutralized both α-dicarbonyls by forming adducts and the antioxidant action resulted in a significant reduction of AGEs (Advanced glycation end products). However, pro-oxidant action of resveratrol was also observed, since resveratrol-α-dicarbonyl conjugates oxidized Cys34 (Tiol) and lysine, arginine and / or proline by a nucleophilic attack to SH (sulfhydryl group) and ε-NH amino) in HSA (human serum albumin). Future studies still need to be developed to further elucidate the pro-oxidative role of the RES-α-dicarbonyl conjugates as well as mechanisms of redox signaling and / or oxidative damage reflex and disease should also be considered in future studies. The third study provided an original insight into the genetic and molecular basis of *Lactobacillus reuteri* PL503 responses against oxidative stress induced by hydrogen peroxide (H_2O_2). Resveratrol (100 μM) has been shown to protect *L. reuteri* PL503 against plausible protein carbonylation through various mechanisms, including direct elimination of reactive oxygen species (ROS), up-regulation of the dhaT gene and promotion of the synthesis of compounds containing sulfur. Carbonylation of proteins as a reflection of oxidative damage to bacteria and their consequences, as well as the role of carbonyl proteins as signaling molecules implicated in bacterial responses to oxidative stress, need to be further investigated.

Key words: oxidation process, wines, bioactive compounds, meat, protein carbonyls, lactic acid bacteria.

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LISTA DE ABREVIATURAS E SIGLAS

AAS	Semialdeído σ-aminoadípico
ADQ	Análise Descritiva Quantitativa
AGEs	Produtos Finas da glicalização avançada
ANOVA	Análise de Variância
BSA	Albumina do Soro bovino
DM	Diabetes metillus
GGS	Semialdeído α-glutâmico
GO	Glioxal
HSA	Albumina do Soro humano
MDA	Malonaldeido
MGO	Metilglioxal
PCR	Reação em Cadeia da Polimerase
ROS	*Reactive Oxygen Species*
SPME	*Solid Phase Micro Extraction*
TBARs	Substâncias Reativas ao Ácido Tiobarbitúrico
TEP	Tetraetoxipropano
WBSF	*Warner Bratzler Shear Force*

SUMÁRIO

1 INTRODUÇÃO

As reações de oxidação têm sido consideradas uma das principais causas da deterioração da qualidade de carnes e seus derivados, além de desempanhar um papel essencial na patogênese de doenças degenerativas relevantes (LUND et al., 2011). A oxidação de proteínas alimentares abrange uma rede complexa de reações químicas, tendo em vista que os mecanismos e a natureza dos produtos formados são dependentes dos sistemas envolvidos e de como as reações são iniciadas (DAVIES, 2005; SOLADOYE et al., 2015).

Similarmente ao que ocorre na oxidação lipídica, a oxidação proteica é descrita como um processo de reações em cadeia mediadas por radicais livres, envolvendo uma iniciação, propagação e terminação. Entretanto, a principal diferença está baseada na alta complexidade das vias e na grande variedade de compostos que pode ser formada no processo de oxidação das proteínas (LUND et al., 2007a, b).

A indústria de produtos cárneos tem usado estratégias variadas para minimizar a degradação da qualidade de seus produtos como resultado das reações oxidativas. A aplicação de antioxidantes tem sido reconhecida como uma estratégia contra a deterioração oxidativa. No entanto, a preocupação toxicológica do uso de antioxidantes sintéticos tem levado a um interesse crescente pelo uso de produtos naturais com propriedades antioxidantes (SHAHIDI, 2000).

Preservar a carne contra a deterioração oxidativa, a descoloração e a deterioração microbiana é a principal preocupação dos mercados varejistas, visto que essas são as vias que afetam a vida útil dos produtos cárneos, em particular a carne bovina (BEKHIT et al., 2013; HES; GRAMZA-MICHAŁOWSKA, 2017). A aplicação de extratos vegetais obtidos de frutas, especiarias, ervas, oleaginosas e plantas vêm se destacando dada a demanda do consumidor nos "aditivos naturais", definidos como sendo aqueles naturalmente presentes no reino vegetal e aos quais efeitos biológicos já foram atribuídos (BALASUNDRAM et al. , 2006; KUMAR et al.,2015; FERNANDES et al., 2016; RIAZI et al., 2016; FERREIRA et al., 2017;).

O vinho tem sido sugerido como componente em processos tecnólogicos, pois é considerado uma bebida complexa, devido aos aspectos qualitativos e quantitativos, além de ser fonte de compostos fenólicos, que são considerados compostos redox ativos(RIZZON; MIELE, 2006; KEKELIDZE et al., 2018). A composição e o teor em compostos fenólicos no vinho variam de acordo com as condições de vinificação, métodos e tecnologias empregadas. O tipo de uva utilizada é considerada um importante fator que influencia diretamente na qualidade fenólica de vinhos (MENG et al., 2012).

A uva 'Isabel' é uma cultivar pertencente à espécie *Vitis labrusca*, uma uva rústica, de aroma e sabor marcante, de maturação tardia e amplamente difundida no Brasil devido à sua fácil adaptação a diferentes condições climáticas e alta produtividade (ASSIS et al., 2011). "Isabel" juntamente com outras uvas *V. labrusca* são amplamente utilizadas na produção de vinhos e sucos. Vinhos provenientes de uvas dessa espécie apresenta uma participação de 80% da produção brasileira (DEBASTIANI et al., 2015; ARAÚJO et al., 2016). Segundo Arcanjo et al. (2017), Nixford e Hermosín-Gutiérrez (2010), vinhos produzidos a partir de uvas 'Isabel' exibem composições fenólicas e antioxidantes consideravelmente diferentes do que vinhos produzidos com uvas Vitis vinifera.

Na Espanha, que tem tradição na produção de vinho, a variedade Tempranillo tem se destacado dentre as uvas viníferas, por fornecer a estrutura de alguns dos melhores vinhos tintos da Espanha, tendo-se espalhado para outros países/regiões, Portugal, Argentina, Austrália, Califórnia (RAMOS et al.,2015). De acordo com Camargo, Tonietto e Hoffmann (2011), no Brasil esta variedade de uva vem sendo produzida recentemente na região Sul e a sua produção encontra-se em expansão.

A marinação da carne utilizando vinhos trata-se de prática usada há décadas para melhorar as propriedades sensoriais de cortes bovinos de baixo valor comercial, e para diversificar a oferta de produtos cárneos processados. Porém o conhecimento científico sobre o efeito do processo de marinação na qualidade da carne é, no entanto, escasso. Os mecanismos envolvidos permanecem desconhecidos, bem como o efeito da marinação com vinho no processo oxidativo das proteínas, de como os danos da oxidação proteica trazem consequências relevantes sobre a textura, o valor nutricional aos produtos cárneos marinados e seus efeitos na saúde dos consumidores (SOLADOYE et al., 2015; ESTÉVEZ e LUNA, 2017).

Os compostos fenólicos presentes no vinho não têm apenas uma influência direta nas características sensoriais, mas também são referenciados como agentes que protegem contra doenças cardiovasculares, obesidade, câncer e envelhecimento (BAGUL et al., 2015; TUNG et al., 2015; ANDRÉ et al., 2016; XANTHOPOULOU et al., 2016). Eles também foram mencionados como compostos prebióticos, pois podem ser capazes de exercer benefícios para a população bacteriana do cólon (BIASI et al., 2014; CUEVA et al. 2017).

O resveratrol (3,5,4′-trihidroxi-trans-estilbeneno) é um polifenol abundante em vinhos com benéficios à saúde que incluem proteção cardiovascular (GRESELE et al., 2011), efeitos sobre a obesidade (ANDRÉ et al., 2016; CALMASINI et al., 2018), antienvelhecimento (LI et al., 2017), anticancerígeno (BAGUL et al., 2015; DHAR et al., 2015), anti-inflamatório (TOMÉ-CARNEIRO et al., 2013; TUNG et al., 2015); antidiabético (FERREIRA et al., 2018).

Por se tratar de um composto de natureza óxido-redutiva (redox), o resveratrol atua como um potente eliminador de radicais livres. No entanto, suas propriedades pró-oxidantes também tem sido bem documentadas, com ênfase nos efeitos e implicações clínicas (DE LA LASTRA, 2007; RAUF et al., 2015)

Além das suas propriedades redox, o resveratrol também pode estar envolvido na modulação de processos fisiológicos através da regulação da expressão gênica e em estudos de sua implicação em vias de sinalização direcionadas (RAUF et al., 2015). Vários estudos comprovaram que a albumina sérica humana (HSA) se liga ao resveratrol e a outros estilbenos (NAIR et al., 2015; CAO et al., 2016). No entanto, as consequências redox de tais interações são praticamente desconhecidas.

Considerando o exposto, esta tese teve por objetivo avaliar e elucidar a ação dos compostos fenólicos do vinho tinto provenientes de três uvas distintas (Cabernet Sauvignon, Tempranillo e Isabel) no processo de oxidação de bifes mariandos, bem como avaliar o potencial redox ativo do resveratrol em sistemas modelos contendo proteína do soro humano condições diabéticas e bactéria láctica (*Lactobacillus reuteri* PL503) submetida ao estresse oxidativo.

2. REVISÃO DA LITERATURA

2.1 PROCESSO OXIDATIVO EM CARNES

A carne é um alimento de qualidade, sendo fundamental na dieta humana, seja *in natura* ou processada, por ser fonte de proteínas biodisponíveis, lipídeos, vitaminas e minerais (LORENZO; PATEIRO, 2013; LORENZO et al., 2018). Por sua propriedade química, a carne se deteriora facilmente (MAYSONNAVE et al., 2014; MOREIRA et al., 2017), fazendo-se necessário a sua preservação contra a deterioração oxidativa, descoloração e deterioração microbiana, os quais afetam sua vida útil (HES;GRAMZA-MICHAŁOWSKA, 2017). Dentre as deteriorações que ocorrem na carne, a deterioração química é alvo de muito estudos, principalmente os estudos de oxidação lipídica e protéica (KARAKAYA et al., 2011; CHENG et al., 2017).

As reações de oxidação ocorrem naturalmente nos tecidos vivos durante o metabolismo celular para manter o bom funcionamento do organismo. Quando há um equilíbrio entre os fatores pró-oxidantes e antioxidantes no meio celular, o processo de oxidação é benéfico, pois garante uma maior eficiência na produção de energia para o funcionamento celular. Após o abate dos animais e durante os eventos *post mortem*, o equilíbrio entre os componentes pro-oxidantes e antioxidantes é desbalanceado devido as mudanças bioquímicas que ocorrem durante a conversão do músculo em carne, fazendo com que se iniciem os processos de oxidação dos componentes da carne, sobretudo proteínas e lipídeos (LEÃO et al., 2017).

A diminuição de vida de prateleira, perda de nutrientes e água, formação de compostos tóxicos, juntamente com as alterações nas características sensoriais de cor, sabor e odor, constituem indicativos da deterioração oxidativa em carnes ou produtos cárneos. Quando os produtos cárneos são expostos ao oxigênio ou a formas reativas de oxigênio ocorre a perda de pelo menos um elétron dos seus constituintes (ácidos graxos e/ou aminoácidos) levando aos processos de oxidação lipídica e/ou proteica, tendo em vista que estas moléculas, são facilmente suscetíveis a danos oxidativos em decorrência da rápida depleção de antioxidantes endógenos após o abate (KARAKAYA et al., 2011; FALOWO et al., 2014).

2.1.1 Oxidação Lipídica

Os lipídeos são de grande importância em carnes, dependendo da sua natureza e forma, conferem características desejáveis de suculência, sabor, aroma e valor nutricional. São encontrados nos produtos cárneos em diferentes formas: triacilglicerol entre as fibras,

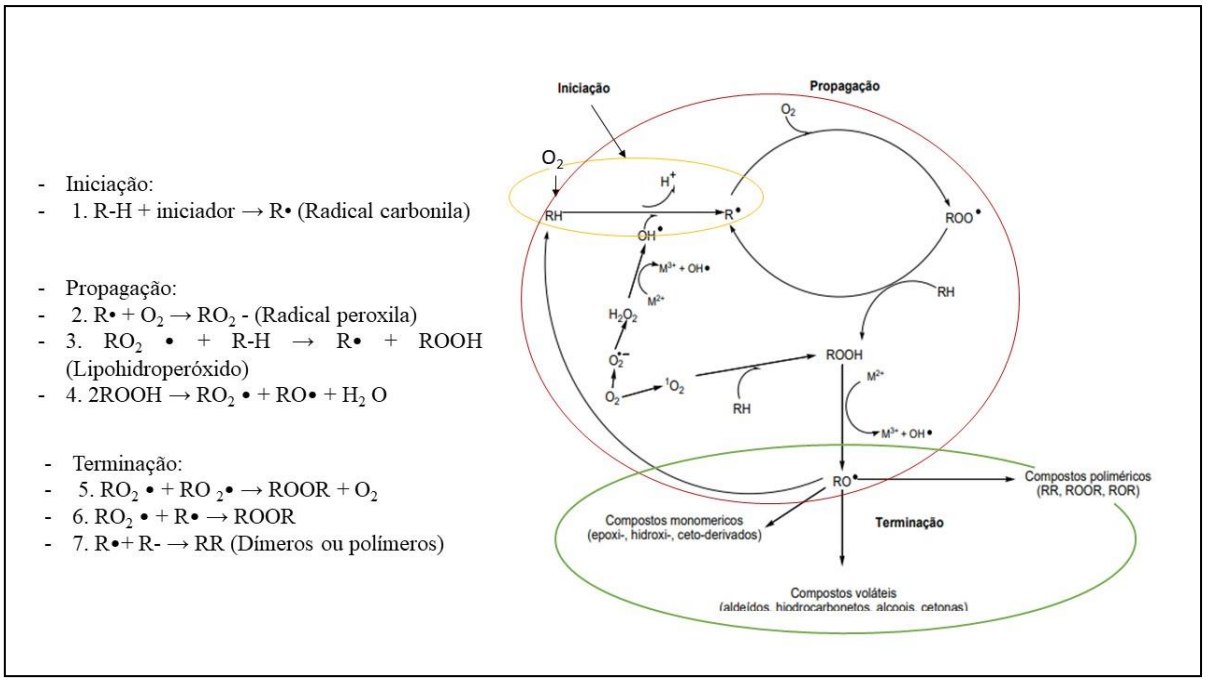
componentes de membranas, tecido adiposo e hormônios esteróides. Por sua estrutura química, alguns lipideos são facilmente oxidáveis, levando à rancificação dos produtos cárneos com perdas sensoriais e nutricionais (BERASATEGI et al., 2014; KUMAR et al., 2015).

A oxidação lipídica é descrita como uma deterioração dos ácidos insaturados presentes na carne, envolvendo a molécula de oxigênio (MAPIYE et al., 2012; FALOWO et al., 2014). Os ácidos graxos que compõem os lipídeos são oxidados de forma principal por um processo de auto-oxidação, que é um mecanismo autocatalítico dos radicais livres, o qual consiste em três fases descritas como: reação de iniciação, propagação e terminação (Figura 1).

- A reação de iniciação: ocorre devido à ação de um iniciador (calor, radiação, UV, metais de transição, espécies reativas de oxigêncio-ROS ou espécies reativas de nitrogênio-RNS) promovendo a abstração de um átomo de hidrogênio no carbono adjacente a uma ligação dupla do ácido graxo insaturado, o que requer menor perda de energia do hidrogênio, formando radicais livres (R•). Dessa forma, quanto maior o número de ligações duplas na molécula de ácido graxo, mais susceptível a oxidação é este ácido graxo (KIRK, 1984; FERRARI,1998) .
- Já a propagação: esta etapa caracteriza-se pela reação do radical livre com o oxigênio molecular formando um radical peroxila (ROO•), esse radical é altamente reativo e abstrai com facilidade um hidrogênio de outro ácido graxo insaturado, gerando hidroperóxidos (ROOH) e novo radical livre (R•). Os hidroperóxidos, gerados na presença de metais ou calor, podem decompor-se em radicais alcoxila e peroxila, realimentando a cadeia oxidativa e aumentando a velocidade das reações de propagação. Porém, em certo momento com o esgotamento de subtratos ocorre uma diminuição das reações de propagação (GRAY, 1978; WANG et al., 1995; SAMPAIO et al., 2012).
- A fase de terminação: ocorre a formação de espécies não radicalares por meio da combinação de dois radicais. Os principais produtos finais da oxidação lipídica são derivados da decomposição dos hidroperóxidos como álcoois, aldeídos, cetonas, ésteres e hidrocarbonetos que apresentam baixo peso molecular e alta volatilidade (KUBOW, 1992).

Além dos iniciadores da reação de oxidação lipídica em carnes, outros fatores também interferem na velocidade e modo que o processo de oxidação ocorre, dentre estes citam-se: pH, atividade de água, composição e tipo de ácidos graxos, área de exposição dos produtos ao ar, (SOUZA et al., 2007).

Figura 1: Etapas da oxidação lipídica.



Fonte: Arcanjo, 2018

Os fatores mencionados interferem diretamente na velocidade com que inicia-se e propaga-se as reações de oxidação dos lipideos, onde o tipo de ácido graxo e o grau de insaturação presente, têm influência significativa na oxidação, ou seja, quanto maior o grau de insaturação na cadeia dos ácidos graxos, maior a susceptibilidade a oxidação (os ácidos linoléico e linolênico oxidam-se 64 e 100 vezes mais rápido do que o ácido oléico, respectivamente). A temperatura de armazenamento, influencia na velocidade da reação de acordo com seu aumento, onde a cada acréscimo de 10 °C na temperatura, a reação do oxigênio com a gordura insaturada duplica. Outro fator que influencia a oxidação lipídica é a atividade de água (aw) dos alimentos, sendo que a presença de água livre aumenta a atividade catalítica dos metais e a quantidade de O_2 dissolvido, portanto, o risco de oxidação aumenta a medida que atividade de água aumenta. Porém, em valores de atividade de água baixa a reação também ocorre com facilidade devido à um maior contato entre os ácidos graxos e os agentes pró-oxidantes.

Volden et al. (2011) citaram que os compostos voláteis que podem ser formados no processo de oxidação lipídica das carnes ou em produtos cárneos dependerão principalmente dos ácidos graxos presentes em sua composição. Por exemplo, a degradação dos ácidos graxos da família n-9 pode produzir voláteis como: hexanal, heptenol, decanal, octanal, heptanal, nonanal; os ácidos graxos da família n-3 darão origem aos voláteis: 1-penten-3-ol e propanal;

e dos ácidos graxos da família n-6 formarão:hexanal, pentanal, pentilfurano, pentanol, hexanol, 1-octenol e 2-octenol.

Os compostos formados no processo de oxidação lipídica, como aldeídos, cetonas e outros compostos voláteis, além dos malonaldeídos (MDA) e óxidos de colesterol conferem odores desagradáveis às carnes e aos produtos cárneos, especialmente em carnes pré-cozidas que, após dias de armazenagem, apresentam aroma/sabor de requentado (UTRERA et al., 2014). Além de alterações sensoriais de sabor e aroma nas carnes e nos produtos cárneos, os compostos da oxidação promovem a modificação da cor da carne pela transformação do pigmento oximioglobina, de coloração vermelho brilhante em metamioglobina, tornando a carne marrom-acinzentada, aspecto que o consumidor rejeita. Observa-se ainda alteração na textura da carne, ocasionando formação de complexos com outros componentes, a exemplo das proteínas (HALLENSTVEDT et al., 2012).

A ingestão de alimentos oxidados pode acarretar em implicações adversas ao organismo, gerando riscos de alterações no estado antioxidante dos tecidos e células levando a riscos patológico-toxicológicos (UTRERA et al., 2014). Esse fato, tem levado a indústria de alimentos a buscar alternativas para reduzir, inibir, controlar o processo de oxidação lipídica.

O uso de antioxidantes e, principalmente a utilização de antioxidantes naturais tem se destacado nesta busca por alternativas mais saudáveis. Estudos estão sendo desenvolvidos focando na aplicação de antioxidantes naturais em carnes e produtos cárneos, utilizando por exemplo, antioxidantes na formulação de produtos cárneos (CANDO et al., 2014; UTRERA, et al., 2014; HERNÁNDEZ-LOPEZ et al., 2016; FERREIRA et al., 2017), e também utilizando como estratégias dietéticas, ou seja, processando um produto em que ocorra, a diminuição ou substituição parcial de gordura ou sal (RODRÍGUEZ-CARPENA, et al., 2012).

Segundo Armenteros et al. (2016), a aplicação de antioxidantes naturais, mistura de alho, canela, cravo e óleo essencial de alecrim, e também um extrato de *Rosa Canina* L. através da injeção por salmoura em presuntos ibéricos cozidos constitui-se uma boa estratégia para aumentar a estabilidade oxidativa do produto sem modificar as propriedades físico-químicas do produto final. Hernandéz-López et al. (2016) verificaram que por meio de suplementação alimentar com abacate foi possível promover uma melhor estabilidade oxidativa dos lipídeos em costelas de porco processadas.

2.1.2 Oxidação Proteica

As proteínas são componentes principais do tecido muscular e desempenham papel importante em carnes e produtos cárneos, nos aspectos sensoriais, nutrionais e tecnólogicos da carne (ESTÉVEZ, 2011). As proteínas da carne são, essencialmente, similares em todos os animais de abate; sendo as miofibrilares representadas pela actina, miosina, tropomiosina, troponina, actininas, proteínas C e M e que formam os miofilamentos grossos e finos que constituem a miofibrila. Representam 52 a 56% das proteínas musculares, sendo a miosina em maior proporção, seguida da actina (PIRES et al., 2002).

De acordo com Li e Wick (2001), as propriedades funcionais das proteínas miofibrilares influenciam na qualidade final de produtos desenvolvidos a base de carne. Isso ocorre porque após o abate dos animais, parte dos mecanismos antioxidantes presentes entra em colapso e os músculos ficam expostos à oxidação lipídica e protéica durante o manuseamento, armazenagem e processamento industrial. Esse processo faz com que o estado físico-quimico e integridade das proteínas afetem sua funcionalidade e, por conseguinte, a qualidade e valor dos produtos da carne processada (UTRERA et al., 2012).

A percepção de que as proteínas musculares seriam vulneráveis as reações oxidativas e que os produtos destas reações poderiam afetar negativamente a qualidade da carne proporcionou um maior interesse das pesquisas envolvendo o impacto da oxidação proteica na qualidade de alimentos cárneos. Vários componentes do tecido muscular são apontados como precursores que levam a formação de ROS e radicais livres, como os lipídeos, heme pigmentos, íons metálicos e enzimas antioxidantes, e tais componentes promovem as reações de oxidação de proteínas (XIONG, 2000; SOLADOYE et al., 2015).

A oxidação proteica consiste em uma reação em cadeia muito complexa, onde a natureza e os mecanismos dos produtos formados são dependentes dos sistemas que estão envolvidos e como as reações são iniciadas. A reação ocorre em três etapas (iniciação, propagação e terminação), sendo mediada pela presença de radicais livres, que diferente de como ocorre na oxidação lipídica, muitas são as vias e muitos são os produtos derivados do processo de oxidação das proteínas (LUND et al., 2007 a, b).

Aminoácidos tanto da cadeia principal quanto da cadeia lateral das proteínas são susceptíveis a oxidação, onde a reação em cadeia é iniciada quando espécies reativas de oxigênio (ROS) abstraem um átomo de hidrogénio da molécula de proteína para produzir um radical protéico (PO•), que se converte em radical peroxila (POO •), na presença de oxigênio. A reação subsequente com Fe^{2+} ou abstração de um átomo de hidrogênio, a partir de outra

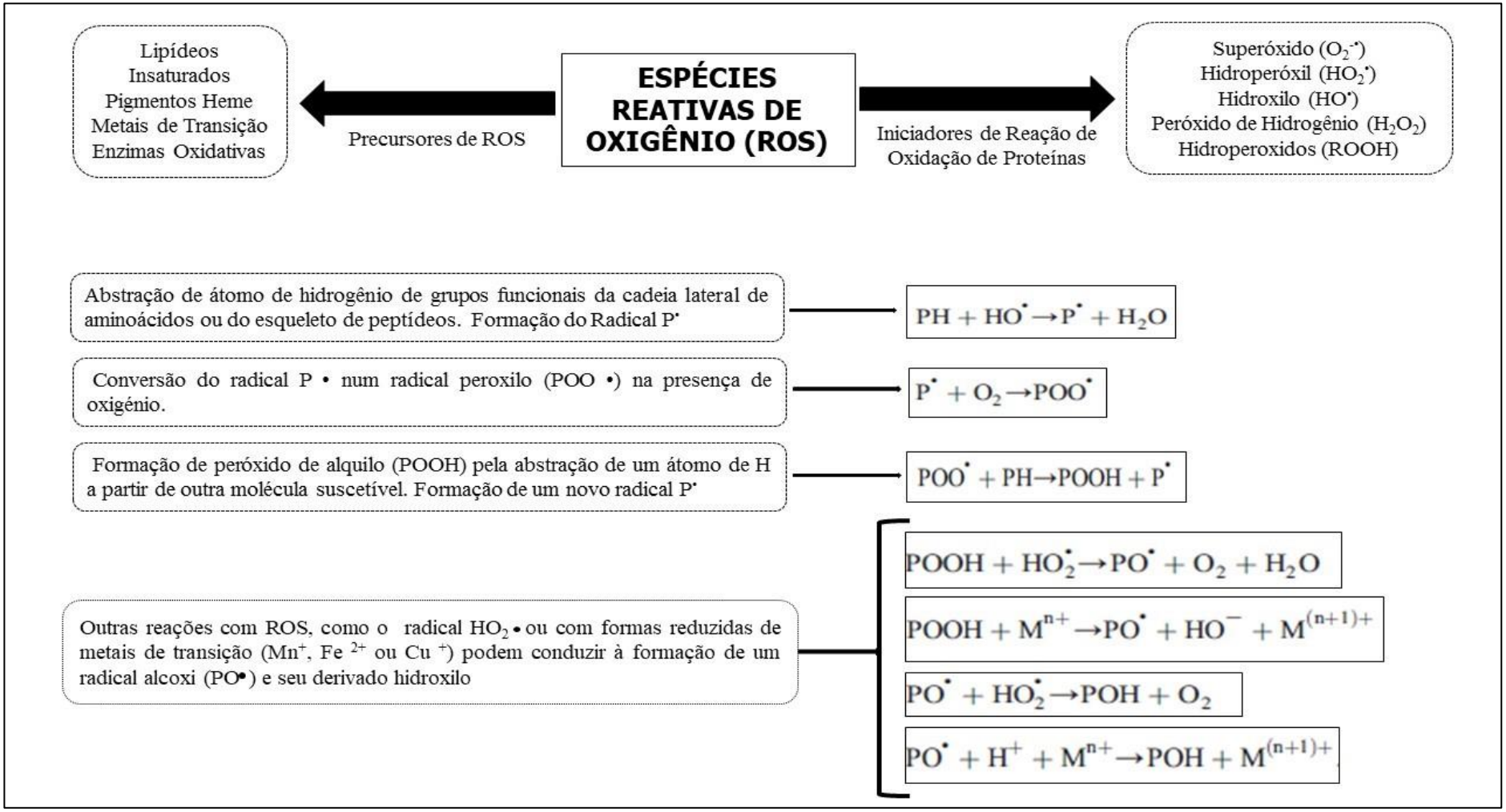
molécula suscetível, podem facilmente produzir radical peróxido de alquila (POOH), com geração de mais radical proteíco. Outras reações com ROS, como o radical hidroxil ou com formas reduzidas de metais de transição, pode levar a formação de PO• e seu derivado hidroxila (POH) (STADTMAN;LEVINE 2003; LUND et al., 2011). Dependendo do alvo e do agente oxidante, a oxidação de proteínas se propaga e termina de acordo com vários mecanismos e as consequências incluem a perda de grupos sulfidrilo, a formação de compostos carbonílicos, a formação de ligações cruzadas, e a modificação dos aminoácidos aromáticos, entre outros (SOLADOYE et al., 2015). Para uma melhor compreensão, o mecanismo geral de oxidação de proteínas é mostrado na Figura 2.

A oxidação de alguns aminoácidos, como arginina, lisina, prolina e treonina leva a formação de compostos carbonílicos por meio de reações de desaminação (XIONG, 2000). A formação de compostos carbonílicos, em relação as demais mudanças citadas nas proteínas após o processo de oxidação, vem tendo destaque como uma das modificações mais proeminentes nas proteínas oxidadas (LEVINE et al., 1990; STADTMAN; LEVINE, 2003). Os compostos carbonílicos geralmente são quantificados usando a técnica de dinitrofenilhidrazina (DNPH) (ESTÉVEZ, 2011) que é provavelmente o método mais frequente para avaliar a oxidação de proteínas (Protox) em carnes e sistemas biológicos (ESTÉVEZ et al., 2008a; ESTÉVEZ, 2011; FERREIRA et al., 2018; HERNÁNDEZ-LÓPEZ et al., 2016).

Recentemente, compostos carbonílicos específicos, os semialdeídos α-aminoadípico e γ-glutâmico (AAS e GGS, respectivamente) foram identificados em proteínas miofibrilares oxidadas, onde a oxidação, catalisada por metais, das cadeias laterais dos aminoácidos básicos (lisina, arginina e prolina) têm sido apontada como a principal rota de carbonilação de proteínas e a fonte mais potente e de maior ataque oxidativo direto às proteínas (SOLAYEDE et al., 2015). Como uma consequência de oxidação catalisada por metais, a lisina é convertida em semialdeídos α-aminoadípico (AAS), e arginina e prolina em semialdeído γ-glutâmico (GGS) (VILLAVERDE; ESTEVEZ, 2013).

Os semialdeídos podem ser formados a partir de grupos ô-amino de aminoácidos alcalinos via reação Maillard induzida pelos α-dicarbonilos, tais como glioxal (GO) e metilglioxal (MGO) (AKAGAWA et al., 2002a). Esses semialdeídos tornaram-se marcadores de oxidação e são determinados por um procedimento de derivatização descrito por Akagawa et al. (2006) com posterior identificação por análise de cromatografia líquida acoplada por espectro de massa com ionização por electrospray (LC-ESI-MS) (ESTÉVEZ et al., 2009).

Figura 2: Mecanismo Geral de Oxidação de Proteínas.



Fonte: Adaptado de Estevéz (2011).

Além da formação dos semialdeídos AAS e GGS, outra modificação que pode ser detectada como um marcador de oxidação de proteínas é a perda de grupos sulfidrilo dos aminoácidos cisteína, cistina e metionina, os quais são sensíveis a quase todas as ROS, e a sua perda em sistemas cárneos pode ser um reflexo de um dano oxidativo específico para as proteínas da carne, pois leva a formação de ligações cruzadas e produção de compostos derivados contendo enxofre (SOLADOYE et al., 2015).

A ligação cruzada de proteínas é amplamente avaliada por determinação de ligações dissulfeto ou fluorescência do triptofano (ESTÉVEZ et al., 2008b). A formação de ligações de dissulfeto, em particular, tem sido associada com a ação de mioglobina hipervalente, por este mecanismo ser diferente do mecanismo catalisado por metais envolvidos na carbonilação de proteínas (LUND et al., 2011). A análise da depleção de triptofano por espectrofluorometria também tem sido explorada como uma expressão de oxidação proteíca em isolados de proteínas miofibrilares, emulsões à base de carne e alimentos musculares processados (UTRERA et al., 2012).

As proteínas do músculo cárneo, que exercem função expressiva na qualidade da carne, quando oxidadas resulta em perda de qualidade nutricional que envolvem a perda de aminoácidos essenciais, a diminuição da digetibilidade e da solubidade das proteínas e funcionalidade protéica (LUND et al., 2011; SOLADOYE et al ., 2015) (Figura 3).

Soladoye et al. (2015) relataram que o decaimento do valor nutricional das proteínas pode estar diretamente relacionado à perda de sua digestibilidade, assim dificultando a absorção intestinal subsequente de pequenos peptídeos e aminoácidos. Já Xiong (2000) descreveu que a oxidação proteica leva a alterações na estrutura terciária de proteínas cárneas, isso faz com que ocorra em desdobramento, aumento da hidrofobicidade, formação de agregados e desnaturação irreversível das proteínas (Figura 3).

No que diz respeito aos aspectos sensoriais da carne, com relação a dureza de produtos cárneos, sabe-se que a formação de ligações cruzadas contribui para a estabilização de agregados proteicos, ocasionando o encurtamento de miofibrilas e uma constrição mecânica contra o intumescimento, aumentando a dureza dos produtos de carne (LIU; XIONG, 2000; LUND et al ., 2007 b; WANG et al ., 2017). Além das ligações cruzadas, a inativação de enzimas proteolíticas também estão envolvidas no aspecto de dureza da carne (LUND et al., 2007 b; KIM et al., 2010). Com relação ao aspecto de sabor e aroma da carne, Estévez (2011) evidenciou a associação entre compostos carbonílicos específicos da oxidação de proteínas (AAS e GGS) e a degradação de aminoácidos via reação de Strecker na formação de compostos voláteis responsáveis pelo aroma desejável em produtos cárneos curados.

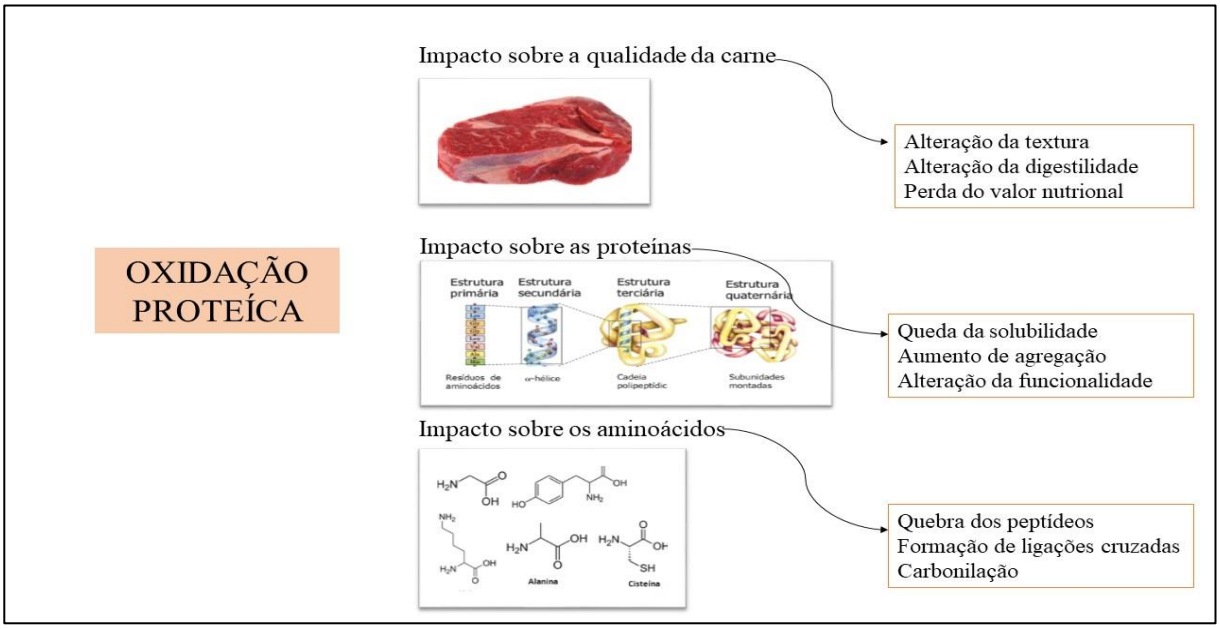
No que refere aos aspectos nutricionais, uma revisão recente (ESTÉVEZ e LUNA, 2017) e vários artigos enfatizaram o papel de produtos variados de oxidação protéica em vários distúrbios patológicos, incluindo proteínas carbonilas e produtos de oxidação da tirosina e triptofano (STOCKER et al., 2015; ROMBOUTS et al., 2017). Os produtos da oxidação das proteínas apresentam ação tóxica, e enfatiza-se que alguns desses compostos são formados durante a digestão e, a combinação, a natureza e o potencial de toxicidade desses compostos gerados durante a digestão são expostos à mucosa do cólon (SANTARELLI et al., 2010; BOLDO et al., 2018).

Indica-se que o consumo de carnes, principalmente de carne vermelha está associada ao câncer e outras patologias (FOGELHOLM et al., 2015; ELMAFDA et al., 2017; BOLDO et al., 2018; HEMERYCK et al., 2018; GIVENS, 2018). No entanto, indica-se que o ferro heme possui potencial na promoção da oxidação lipídica / protéica e na formação de compostos nitrosos (BASTIDE et al., 2011; HEMERYCK et al., 2018).

É sabido que a utilização de temperaturas acima de 60 ºC na carne e/ou em produtos cárneos pode desenvolver a clivagem oxidativa do anel porfirínico, desencadeando a liberação do ferro heme, o que pode aumentar a intensidade da oxidação lipídica e proteica. Após o cozimento da carne, a quantidade de ferro heme é reduzida e o teor de ferro não-heme aumenta (PURCHAS; BUSBOOM; WILKINSON, 2006), o que é crucial para a iniciação da oxidação proteica via reação de Fenton (ESTÉVEZ, 2011). Ainda é importante considerar que a extensão da oxidação proteica da carne também depende do método e do tempo de cocção (SOLADOYE et al., 2015).

Ferreira et al. (2018) descreveram no estudo sobre o papel da oxidação de proteínas na perda nutricional e mudanças de textura em hamburgueres de frango prontos para consumo por diferentes técnicas de cocção (fervura, assamento e grelhamento), as quais influenciaram no desenvolvimento de oxidação de proteínas, que dentre as técnicas aplicadas o processo de fervura parece ser a mais prejudicial nas proteínas de frango.

Figura 3 : Consequências da oxidação sobre os aminoácidos, proteínas e carne



Fonte: Arcanjo (2018) e https://pt.dreamstime.com/imagem-de-stock-carne-crua-image17630391; https://sites.google.com/site/1ctcproteinaseacucares/proteinas/c-estrutura; https://brainly.com.br/tarefa/937922.

A percepção do consumidor sobre a qualidade da carne: sensorial, segurança, influência na saúde e os efeitos da oxidação de proteínas em produtos cárneos têm desafiado tecnólogos a desenvolver estratégias antioxidantes. A maioria dos estudos confirmam o crescente interesse em antioxidantes naturais, principalmente de plantas que contêm altos níveis de compostos fenólicos, como inibidores de oxidação de proteínas nos alimentos musculares (ARMENTEROS et al., 2016; CANDO et al., 2014).

2.2 COMPOSTOS FENÓLICOS

2.2.1 Vinhos Tintos e Compostos Fenólicos

O vinho é o produto da fermentação alcoólica completa ou parcial do mosto de uvas sãs e maduras. Diversas variedades de uvas são empregadas na produção de vinhos e, pela legislação brasileira, o vinho pode ser elaborado a partir de uvas viníferas e uvas não viníferas, onde “vinho de mesa de viníferas” é o vinho elaborado exclusivamente com uvas das variedades *Vitis vinífera* (Cabernet Sauvignon, Syrah, Merlot, Tempranillo, Cabernet Franc, entre outras). O“vinho de mesa de americanas” é definido como o vinho elaborado com uvas do grupo das uvas americanas (*Vitis labrusca* ou *Vitis bourquina*) e/ou híbridas (Isabel, Bordô, Niagara, e

outras), podendo conter em sua composição, vinhos de variedades viníferas, sendo também denominado de “vinho comum” (BRASIL, 2004).

A produção global de vinho, é estimada entre 240 e 250 milhões de hectolitros, com quase oito milhões de hectares plantados para fins vitivinícolas (Organización Internacional de la Viña y el Vino - OIV, 2017). Os principais produtores mundiais de vinho são Itália, França e Espanha, dentre outros países incluindo EUA, Argentina e China. O Brasil ocupa a 15 ª posição no ranking mundial de produção de vinhos, de acordo com a OIV no ano de 2015 (OIV, 2017; FERRARI et al., 2018).

Vinhos tintos de mesa são destaques na produção e consumo da população brasileira. A produção de vinho de mesa comum foi de aproximadamente 210 milhões de litros, representando um percentual de 85% do vinho consumido nacionalmente em 2015 (UVIBRA, 2015).

O elevado consumo de vinho comum, deve-se ao fato de que as uvas americanas e híbridas representarem mais de 80% do volume de uvas processadas no Brasil, com aproximadamente 400 mil toneladas/ano. A cultivar Isabel, também denominada “uva rústica” ou “uva comum”, “Isabella”, “Brasileira”, “Nacional”, é representante de cerca de 50% desse volume, constituindo-se matéria-prima básica para a elaboração de vinho de mesa (NIXFORD; HERMOSÍN-GUTIÉRREZ, 2010). A uva Isabel é bastante cultivada na país, pois oferece fácil adaptação à variabilidade das condições edafoclimáticas, com elevada produtividade, longevidade e relativa rusticidade (ROMBALDI, 2014).

A qualidade de um vinho está diretamente ligada à presença de constituintes químicos e a principal classe desses constituintes são os compostos fenólicos, capazes de influenciar diretamente sobre suas características sensoriais, como a cor, sabor, amargor e adstringência (GARRIDO; BORGES, 2011). Além de conferir propriedades sensoriais ao vinho, os fenólicos têm atraído muito interesse devido às suas propriedades antioxidantes e seus efeitos potencialmente benéficos à saúde (GRIS et al., 2011a; GRIS et al., 2011b; GRIS et al. 2013).

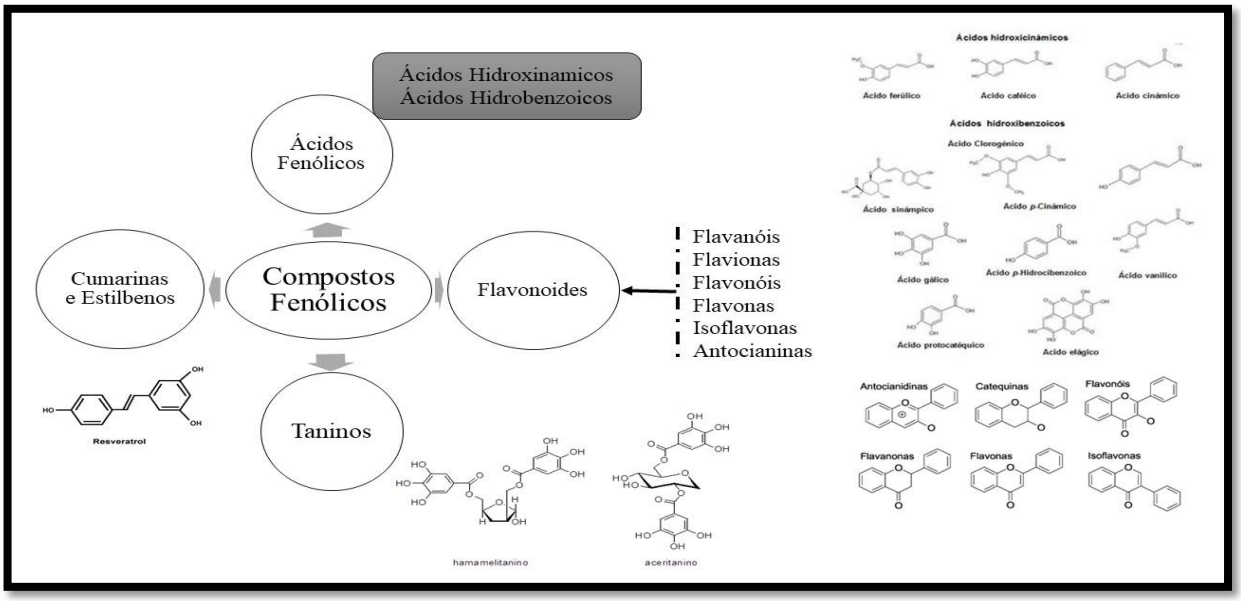
A composição fenólica dos vinhos apresenta uma grande variação de acordo com as condições ambientais e climáticas, tipo de solo, variedade de uva, grau de maturação e, ainda nos vinhos, as práticas enológicas empregadas, como a duração do processo de maceração, controle da fermentação e armazenamento são de grande influência (GRANATO et al., 2010; MARACAJÁ et al. , 2017;).

Os compostos fenólicos são originalmente sintetizados nos tecidos da pele e sementes de uvas e posteriormente transferidos para o vinho durante o processo de vinificação. Os

microrganismos e o barril de carvalho, onde o envelhecimento ocorre, também contribuem para a composição fenólica dos vinhos (LORRAIN et al., 2013; FERNANDEZ et al., 2017).

Eles são agrupados em classes onde os ácidos fenólicos englobam os ácidos hidroxicinâmicos e hidrobenzóicos; os flavonoides estão subdivididos em flavanois, flavionas, flavonóis, flavonas, isoflavonas e antocianinas; têm-se também os taninos, cumarinas e estilbenos (Figura 4).

Figura 4: Representação das diferentes classes de compostos fenólicos e suas estruturas química



Fonte: Arcanjo, 2018 e
https://www.google.com.br/search?q=estruturas+quimicas+de+compostos+fen%C3%B3licos&source

Os ácidos fenólicos são os compostos fenólicos mais comuns e amplamente distribuídos entre os metabólitos fenólicos, possuem estrutura simples e a partir deles são obtidos fenólicos mais complexos. Dividem-se em dois tipos principais - o ácido benzóico e derivados de ácido cinâmico que possuem um anel aromático e uma cadeia lateral de três carbonos (TSAO, 2010; GRANATO et al., 2016; PADILHA et al., 2017).

Lima et al. (2015) verificaram que em uvas *V. labrusca* o ácido caféico (valores 15,3-17,9 mg/ L), seguido por gálico (2,2 a 3,4 mg/ L), cinâmico (0,5 a 2,0 mg/ L), clorogênico (1,8 a 3,2 mg/ L) e *p*-cumárico (1,1 a 1,7 mg/ L) foram os principais. Castilhos et al. (2017) fizeram uma abordagem da composição fenólica e sensorial em vinhos tintos produzidos por uvas Isabel, utilizando dois processos, a pré-secagem da uva e da produção de vinho submerso e, verificaram que entre os ácidos identificados, os ácidos caftárico, *trans*-coutárico e *p*-

coumárico, 27,94 mg/L, 16,47 mg/L e 24,66 mg/L, respectivamente foram os ácidos que apresentaram maiores concentrações.

Em uvas e vinhos também podem ser encontrados os seguintes fenólicos: ácidos benzoicos (p-hidroxibenzóico, protocatequímico, vanílico, siríngico, salicílico e gentísico); ácidos cinâmicos (p-cumárico, ferrúlico) e o ácido gálico sintetizado também através do ácido chiquímico, dentre os outros ácidos fenólicos (MURELO et al., 2015).

Os flavonóides são compostos fenólicos que englobam uma larga faixa de substâncias coloridas e cujas estruturas provêm de duas rotas bioquímicas diferentes: a rota do ácido chiquímico via fenilalanina e a rota do ácido malônico. Derivados de bagas de uva, antocianinas e flavonóides em particular são os principais responsáveis pelas propriedades sensoriais do vinho, como cor e sabor (LIANG et al., 2012). Os flavonóides mais encontrados em uvas e vinhos são catequinas, epicatequina (flavanóis), antocianinas (vinhos tintos), flavonóis como quercetina, campferol e miricetina (LIANG et al, 2014; GARAGUSO; NARDIN, 2015).

As antocianinas são a principal razão para o desenvolvimento e mudança na cor durante o amadurecimento da baga. A biossíntese de antocianina na uva é, normalmente, influenciada por uma série de fatores ambientais, tais como a exposição à luz solar, irradiação ultravioleta, temperatura do ar e precipitação, assim como a variedade de uva e as práticas vitícolas utilizadas. Estes fatores podem modificar significativamente a composição das antocianinas da baga da uva e o seu teor final no vinho (ZHANG et al. 2017).

Os taninos condensados, também conhecidos como as proantocianidinas das uvas e dos vinhos, são oligômeros e polímeros de catequina e epicatequina. Estes compostos são formados durante o processo de envelhecimento dos vinhos, sendo responsáveis pela cor e adstringência (JACKSON, 2008). Efeitos benéficos à saúde têm sido atribuídos aos flavan-3-ols monômeros e as proantocianidinas na prevenção da peroxidação lipídica (GRIS et al., 2011a).

Os estilbenos compreendem junto com os ácidos fenólicos formm a classe dos compostos não flavonoides presentes em uvas e vinhos. O *trans*-resveratrol é o estilbeno mais abundante em uvas, enquanto que o isômero *cis* é formado durante o processo de vinificação. O teor de *trans*-resveratrol do vinho é proveniente das uvas, principalmente de suas cascas e sementes, podendo conter de 1,5 a 5,0 mg/L de vinho. Maiores concentrações destes compostos são encontrados em vinhos tintos de variedades de *Vitis vinífera* (FERNÁNDEZ-MAR et al., 2012; GAMBINI et al., 2013).

O resveratrol, assim como a maioria dos compostos fenólicos, é sintetizado quando as uvas são expostas ao estresse, ou seja, estes compostos são produzidos como uma estratégia de defesa da planta contra danos ou doenças. A síntese do resveratrol ocorre por via enzimática a

partir do aminoácido fenilalanina, onde três enzimas chave estão envolvidas: liase amónio fenilalanina, coenzima-A ligase e estilbeno sintase (FERNÁNDEZ-MAR et al., 2012; BAVARESCO et al., 2016).

O resveratrol, juntamente com os compostos fenólicos, tem sido alvo de pesquisas em decorrência de sua atividade antioxidante. Os vinhos são considerados excelentes fontes de antioxidantes, como resultado da presença dos compostos fenólicos, que sofrem influência das diferentes regiões de cultivo, da variedade da uva, do estágio de maturação da uva, das condições climáticas, das práticas enológicas e do tempo de envelhecimento (BURIN et al., 2011; STOCKHAM et al., 2013; COLETTA et al., 2014).

2.2.2 Atividade Antioxidante dos Compostos Fenólicos do Vinho Tinto

O vinho tinto é a bebida mais citada quanto aos benefícios promovidos pela sua ação antioxidante de seus compostos fenólicos, que apresentam a capacidade de inibir o desenvolvimento de radicais livres, tanto em matrizes alimentares, quanto em radicais gerados no corpo humano, contribuindo para prevenção de neoplasias e vários tipos de câncer (YANG et al., 2013; ZAROMA-ROS et al., 2013), distúrbios cardiovasculares (MEDINA-REMÓN et al., 2015), diabetes (BABU et al., 2013), dentre outras doenças (GUILDFORD; PAUSED, 2011; YAN et al., 2014).

De acordo com pesquisas que vêm sendo realizadas, quando a abordagem é a relação vinho e saúde, pode-se afirmar que incluir vinho na dieta de forma regular obtém-se um aumento na capacidade antioxidante total no plasma, lipoproteínas HDL, atividade fibrinolítica e antitrombina, além de redução do dano oxidativo e agregação de plaquetas (BASLI et al., 2012). Estudos realizados em diferentes partes do mundo, com diferentes grupos da população, sugeriram que o consumo moderado (1-2 copos por dia) de vinho reduz o risco de doenças cardiovasculares (BASLI et al., 2012; HIGGINS; LLANOS, 2015; FRAGOPOULOU et al., 2018).

Investigações com consumidores de vinhos dos Estados Unidos e de alguns países europeus exploraram as percepções sobre os possíveis efeitos à saúde do que eles consideram ser um consumo moderado de vinho. Os entrevistados percebem o vinho como um produto bastante saudável se consumido moderadamente (CHANG et al., 2016; VECCHIO et al., 2017).

Em matrizes alimentares, principalmente em carnes e produtos cárneos, que são considerados alimentos perecíveis, susceptíveis a alterações químicas e microbianas; os

compostos fenólicos podem interagir com as proteínas e lipídeos, retardando ou inibindo reações de degradação (WEISS et al., 2015).

A indústria de alimentos faz uso de compostos fenólicos sintéticos, como o BHT (Butil hidroxitolueno) e BHA (Butil hidroxianisol), porém já vem sendo relatado que estes antioxidantes podem atuar como um iniciador de tumor ou quando consumidos em altas doses podem causar danos ao DNA e promover o desenvolvimento de câncer no estômago (OKUBOA et al., 2003; DOLATABADI; KASHANIAN, 2010). Portanto, muitas pesquisas com a utilização de antioxidantes naturais estão sendo desenvolvidas, principalmente, utilizando extratos naturais provenientes de resíduos oriundos do processamento agroindustrial, como por exemplo, do processamento das uvas e vinhos (ROCKENBACH et al., 2011).

A utilização de extrato de uva em salsichas de carne bovina, nas concentrações de 100, 300 e 500 ppm, mantiveram o odor fresco e sabor da carne cozida por mais tempo do que o controle segundo estudo de Kulkarni et al. (2011). Além disso, não houve alteração na concentração de substâncias reativas ao ácido tiobarbitúrico (TBARS) durante o período de armazenamento (-18°C por 4 meses), indicando uma forte proteção contra reação de oxidação de lipídica nas salsichas. Pereira (2015) desenvolveu um microencapsulado de extratos hidroalcoólicos de coprodutos de vinho e suco de uva das variedades Bordô e Niágara para aplicação em patê cremoso de carne de frango; Casagrande (2015) também utilizou extratos de coprodutos de vinho e suco de uva, visando aplicar em linguiça de frango. Ambos os estudos descreveram que obtiveram resultados satisfatórios com relação ao efeito antioxidante dos extratos de compostos fenólicos.

Maqsood et al. (2015) investigaram o impacto de distintos compostos fenólicos (ácido tânico, catequina, ácido cafeico e ácido gálico) em concentração de 200 ppm na retenção de qualidade de carne de camelo sob armazenamento refrigerado por 9 dias, verificando que com a adição desses compostos à carne, os valores de peróxido e substâncias reativas ao ácido tiobarbitúrico, juntamente com as contagens microbianas foram retardadas especialmente, em amostras adicionadas com ácido tânico e catequina em comparação com amostras controle ($p < 0,05$).

O resveratrol, principal composto fenólico do vinho, possui uma capacidade antioxidante intrínseca que pode estar relacionada com os seus efeitos quimiopreventivos. Estudos em ratos, suínos e seres humanos parecem indicar que o resveratrol pode suprimir aumentos patológicos da peroxidação de lípideos e outras macromoléculas, *in vivo* (SILVA et al., 2015). Em estudo desenvolvido por Bagul et al. (2015) demonstrou que o resveratrol atenuando, assim, o estresse oxidativo cardíaco e as complicações

do diabetes em ratos, através da desacetilação de NFkB-p65 (fator de transcrição e histona 3 (proteína que participa da regulação de genes).

Fernandes et al. (2017) no estudo de revisão sobre os flavonóides do vinho na saúde e prevenção de doenças, relataram que através de estudos *in vitro* os compostos do vinho exerce atividades inibitórias das enzimas, α-glicosidase e a α-amilase, as quais tem a função de controlar o nível de insulina no sangue de pacientes diabéticos.

Os compostos fenólicos também promovem efeitos benéficos sobre os microrganismos, pois eles são extensivamente metabolizados pela microbiota e os metabólitos formados são mais ativos, fazendo com que os microrganismos mantenham sua capacidade de funcionalidade e sobrevivência. Além de que os metabólitos de compostos fenólicos formados por microrganismos intestinais, incluindo probióticos, exercem uma variedade de benefícios na saúde do hospedeiro (FERNANDES et al., 2017; ATTRI; GOEL,2018).

Assim, a utilização de compostos fenólicos em alimentos, bem como sua ingestão, traz efeitos benéficos à saude do consumidor, e ainda pode prolongar o tempo de vida útil dos produtos e melhorar suas características sensoriais (KULKARNI et al. 2011; BASLI et al., 2012; FERREIRA et al., 2018).

2.2.3 Interação dos Compostos Fenólicos com Proteínas em Sistema Alimentar

Compostos fenólicos são substâncias bioativas de interesse crescente devido à relação direta destas substâncias naturais em produtos alimentares impedindo reações de oxidação e, em circunstâncias especiais, atuarem em efeitos pró-oxidantes em sistemas alimentares (ESTÉVEZ; HEINONEN, 2010; GANHÃO et al., 2010).

A função dos compostos fenólicos de proteger proteínas miofibrilares contra oxidação tem sido pouco estudada. Estévez et al. (2008a) relataram o efeito dos compostos fenólicos selecionados na oxidação de proteínas avaliadas por meio de espectroscopia de fluorescência. Considerando que ambos os efeitos, antioxidantes e pró-oxidantes foram relatados nesse documento, os mecanismos químicos específicos de interação entre os compostos fenólicos, proteínas miofibrilares, e outros componentes das reações oxidativas (isto é, metais) permanecem desconhecidas.

Estevéz e Heinonen (2010) postulam que, muitos estudos têm-se centrado sobre a ocorrência e as consequências da oxidação de proteínas em carnes e produtos derivados (LUND et al., 2007 a; VENTANAS et al., 2007). No entanto, mais estudos sobre esta questão são

necessários, pois os mecanismos envolvidos na degradação por oxidação de proteínas musculares e o destino de aminoácidos particulares durante o manuseio, processamento e armazenamento de alimentos musculares são pouco compreendidos. A compreensão desses mecanismos complexos em alimentos musculares é essencial para entender completamente a química básica de oxidação de proteínas e empregar metodologias altamente sensíveis e específicas (OZDAL et al., 2013).

A interação fenólico-proteína pode influenciar na eficiência dos compostos fenólicos e é afetada por vários fatores, incluindo a quantidade e a estrutura química dos compostos fenólicos, o tamanho, a conformação e a carga das moléculas de proteína. É necessário que se avalie os compostos fenólicos de plantas como inibidores da oxidação de proteínas miofibrilares para que as consequências de suas interações sejam entendidas (OZDAL et al., 2013).

Assim como os produtos de oxidação, a interação fenólico-proteína, pode levar à alterações nas propriedades físico-químicas das proteínas, tais como: a solubilidade, a estabilidade térmica e digestibilidade (Figura 5) (RAWEL et al., 2001; LABUCKAS et al., 2008). Sendo influenciadas por parâmetros como temperatura, pH, os tipos de proteínas, a concentração de proteína, os tipos e estruturas de compostos fenólicos, a concentração de sal e a adição de reagentes (OZDAL et al., 2013).

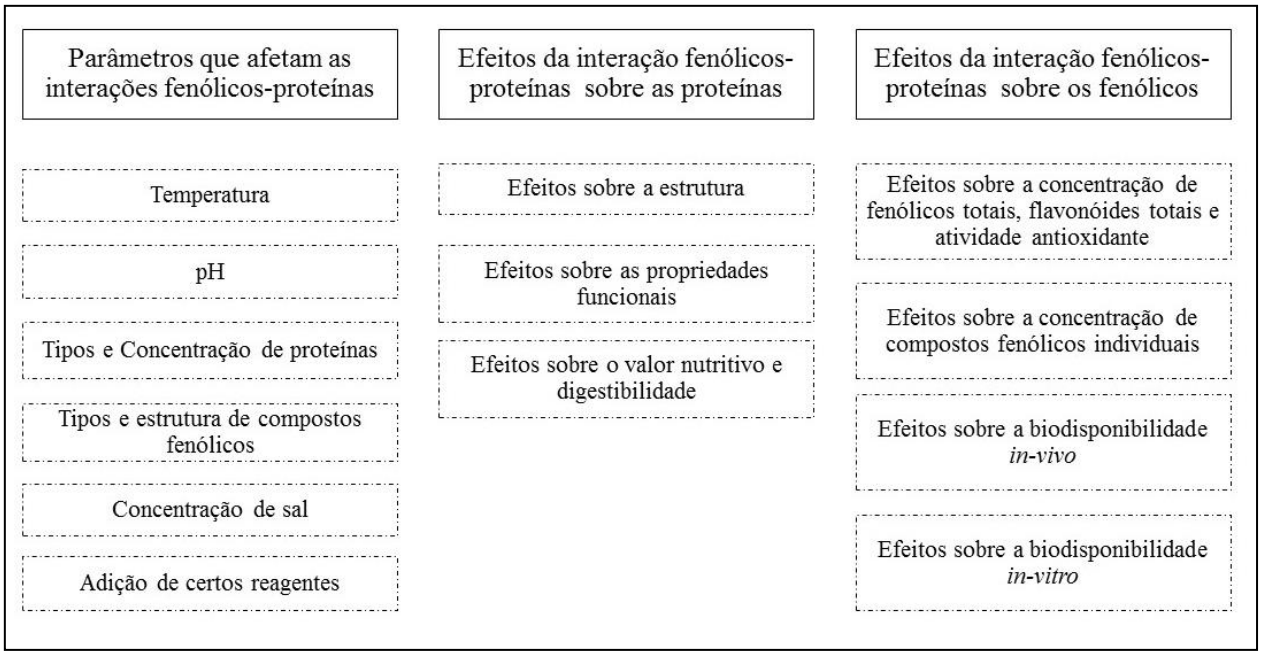
Além disso, as propriedades nutricionais de proteínas podem ser afetadas devido à modificação dos aminoácidos essenciais e por meio da inibição de proteases (KROLL et al., 2003). Os polifenóis podem interagir com proteínas de forma reversível e irreversível. Em interações reversíveis, geralmente atuam forças não covalentes, tais como ligações de hidrogénio, ligação hidrofóbica e forças de van der Waals são envolvidas (PRIGENT et al., 2003; PONCET-LEGRANDETAI, 2006; RICHARD et al., 2006), ao passo que em interações irreversíveis, ligações covalentes formam-se entre o polifenóis e proteínas (OZDAL et al., 2013).

Os estudos de Ozdal et al. (2013) de revisão sobre a interação entre compostos fenólicos e proteínas e suas alterações, destacam a forma de como ocorre a interação entre estes componentes e apresentam resultados contraditórios, como por exemplo, o efeito da temperatura sobre a afinidade entre as proteínas e polifenóis, os quais indicaram em um estudo que a afinidade diminui com o aumento da temperatura, em contrapartida outro estudo relata que aconteceu o oposto (Figura 5) (PRIGENT et al.,2003; TSAI; SHE, 2006;).

Assim o desenvolvimento de trabalhos sobre o tema interação proteína-fenólico é necessário para esclarecer os resultados controversos obtidos até agora pelos estudos

desenvolvidos e para compreender melhor os mecanismos subjacentes das interações proteína-fenólicos, bem como, os fatores que afetam o grau dessa interação.

Figura 5: Resumo dos parâmetros e efeitos que afetam as proteínas e os compostos fenólicos quando submetidos a um sistema alimentar.



Fonte: Arcanjo, 2018.

2.2.4 Compostos Fenólicos e Proteína do Soro Humano na Presença de Metabólitos do Diabetes

Compreender a base molecular da doença é crucial para entender os mecanismos patológicos subjacentes e para projetar estratégias profiláticas e terapêuticas específicas. Nesta linha, o estudo da bioquímica de proteínas tornou-se um tema de enorme interesse, dado seus papéis variados como moléculas de sinalização, moduladores da expressão gênica e executores da maioria das funções biológicas (DEAN et al., 1997).

As proteínas são alvos para mudanças pós-tradicionais atípicas, incluindo oxidação, glicação e nitrosação, com algumas dessas modificações levando a funcionalidades prejudicadas. De forma particular, o processo de oxidação de proteínas gera receios devida a formação e o acúmulo no organismo de produtos de oxidação de proteínas acarretando em desenvolvimento de doenças relacionadas à idade, por exemplo, a diabetes tipo II (DEAN et al., 1997).

A Diabetes Mellitus (DM) é “uma doença crônica comum, caracterizada por uma hiperglicemia crônica com distúrbios no metabolismo dos hidratos de carbono, lípidos e proteínas, resultantes da deficiência na secreção ou ação da insulina, ou de ambas" (American Diabetes Association, 2010) e que afeta atualmente 8,3% (371 milhões de pessoas) da população mundial (International Diabetes Federation, 2014).

Esta doença metabólica é classificada em três tipos principais: o diabetes mellitus tipo 1 é especificamente definido como uma doença autoimune caracterizada pela destruição de células β, levando à falta absoluta de insulina; diabetes mellitus tipo 2 é devido a um defeito secretor de insulina secretora no contexto de resistência à insulina; e o diabetes mellitus gestacional é geralmente diagnosticado no segundo ou terceiro trimestre da gravidez (American Diabetes Association, 2015).

Produtos de glicação avançada ou conhecidos também por AGEs são considerados os mais importantes para tentar explicar como a hiperglicemia crônica conduz aos danos celulares e teciduais observados nessa doença, pois efeitos patológicos dos AGEs estão relacionados à capacidade destes compostos de modificar as propriedades químicas e funcionais das mais diversas estruturas biológicas (PEPPA et al., 2003; BARBOSA et al., 2008).

Alguns mecanismos que explicam a formação dos AGEs são relatados na literatura : i) via Reação de Maillard, ii) via do “estresse carbonílico”, iii) por envolviemnto com neutrofilos, monócitos e macrófagos (BARBOSA et al., 2008). Contudo, pela via do “estresse carbonílico”, na qual ocorre a oxidação de lipídeos ou de açúcares acarreta na formação compostos dicarbonílicos intermediários altamente reativos, a autoxidação de glicose, por exemplo, produzem metilglioxal (MGO) e glioxal (GO), que interagem com aminoácidos para formar AGEs. Estes compostos dicarbonílicos chegam a ser 20 mil vezes mais reativos do que a glicose e são os principais intermediários da formação de AGEs (HUEBSCHMANN et al., 2006).

De acordo com Wang e Ho (2012) o metilglioxal (MGO) e o glioxal (GO), ambos líquidos de cor amarela, conhecidos como espécies de carbonilo reativo, podem ser gerados de forma endógena e exógena (corpo humano e sistema alimentar) (RABBANI; THORNALLEY, 2015). O MGO e GO podem ser gerados de forma exogéna a partir da auto-oxidação de açúcar, da reação de Maillard, bem como, a degradação da fermentação lipídica e microbiana. Na autoxidação de açúcar, a MGO é formada pela fragmentação do açúcar por condensação de retro-aldol, na qual o oxigênio desempenha um papel importante. Este processo ocorre principalmente em alimentos que contêm uma grande quantidade de carboidratos, especialmente monossacarídeos (SAREMI et al., 2017).

A oxidação de proteínas é manifestada como mudanças químicas variadas, no entanto, a formação de carbonílicos de proteínas foi identificada como a modificação mais severa induzida em proteínas por espécies de oxigênio reativo (ROS) (STADTMAN; LEVINE, 2003). Trnková et al. (2015) analisaram as interconexões e os produtos comuns entre a oxidação da proteína mediada por radicais e a reação de Maillard, enfatizando as consequências biológicas relevantes do dano protéico.

O plasma humano contém uma variedade de proteínas, tais como albumina de soro humano (HSA), que desempenha um papel fundamental no transporte e distribuição de ligandos endógenos e exógenos presentes no sangue (GONDIM et al., 2018). A interação de proteínas com carbonilos reativas pode resultar em inativação e modificação de proteínas celulares essenciais que podem potencialmente levar à citotoxicidade (YANG et al., 2011).

A formação de espécies reativas de carbonila e processos de glicação protéica (estresse de α-dicarbonílicos) são responsáveis pelos efeitos colaterais crônicos em pacientes diabéticos com hiperglicemia persistente (RABBANI; THORNALLEY, 2015). Os dicarbonílicos acima mencionados, GO e MGO são encontrados em níveis elevados em pacientes diabéticos e sua hepatotoxicidade, nefrotoxicidade e neurotoxicidade estão bem documentadas (YANG et al., 2011; ALLAMAN et al., 2015).

Os α-dicarbonilos dietéticos prejudicam a digestibilidade e o valor nutricional das proteínas alimentares e, posteriormente, após a absorção, podem contribuir para a circulação de α -dicarbonilos e induzem toxicidade em células humanas (AMOROSO et al., 2013). Embora a patogênese de α-dicarbonilos reativos seja geralmente atribuída à sua capacidade de reagir com lisina e arginina ligadas a proteínas para formar produtos finais de glicação avançada, também está bem documentado que AAS e GGS também são formados em resíduos de proteína na presença de GO e MGO (AKAGAWA et al., 2002 a, b).

Akagawa et al. (2002a) descreveram originalmente o mecanismo mediado por α-dicarbonilo pelo qual AAS e GGS foram formados a partir de aminoácidos alcalinos em proteínas plasmáticas de ratos diabéticos. Os mesmos autores relataram concentrações 2-3 vezes maiores de AAS em ratos diabéticos em comparação com as homólogas de controle e consideradas como um biomarcador precoce e confiável de estresse oxidativo ligado ao diabetes (AKAGAWA et al., 2002b). A acumulação de AAS em proteínas plasmáticas foi proposta para induzir mudanças no ponto isoelétrico das proteínas, mudanças conformacionais e, eventualmente, inativação (AKAGAWA et al., 2002b).

Além disso, os resíduos de AAS podem estar implicados na formação de ligações cruzadas de proteína (isto é, AGEs) através da condensação de aldol ou com outro resíduo de

lisina via formação de base de Schiff. Estas condensações podem contribuir para aumentar a resistência à remoção por meios proteolíticos, bem como impedir a sua função (AKAGAWA et al., 2002b). A nível molecular, o AAS e seu produto final de oxidação (ácido α-aminoadípico, AAA) podem estar implicados no desenvolvimento de complicações diabéticas, como insuficiência renal (SELL et al., 2008), cataratogênese (FAN et al. , 2008) e aterosclerose (SAREMI et al., 2017).

No combate a uma formação desses compostos, muitos estudos têm focado na utilização de componentes biologicamente ativos do reino vegetal por possuírem capacidade dietética e / ou farmacológica, grande atenção tem focado em flavonóides de plantas alimentares como antioxidantes eficazes (TRNKOVÁ et al., 2015). Atualmente, os flavonóides (genisteína, quercetina, epicatequina, luteolina, resorinol e floroglucinol) e ácidos graxos fenólicos (ácidos cinâmico, isoferulico, elágico e clorogênico) de plantas têm sido investigados como inibidores naturais contra a glicação. Dentre todos os fenólicos, o resveratrol (3,5,4-tri-hidroxiestilbeno) é um estilbeno pertencente a uma subclasse de fitoalexinas e sintetizada em resposta a patógenos e estresse abiótico em plantas (SILVA et al., 2015).

O efeito antioxidante, antiinflamatório, anticancerígeno e fitoestrógeno do resveratrol, bem como, a inibição da agregação plaquetária tem sido amplamente relatada (BAGUL et al., 2015; CALMASINI et al., 2018). No entanto, a atividade potencial do resveratrol contra a formação de produtos de oxidação de proteínas (AAS e GGS) na presença de compostos dicarbônicos não foi documentada ainda (SHEN et al., 2017).

Shen et al. (2017) constataram que o resveratrol apresentou uma capacidade significativa de inibição contra a formação de AGES em sistemas modelos com albumina do soro bovino (BSA) indicando que atráves de uma reação de conjunção o resveratrol pode prender o metilglioxal, provocando a inibição da reação de glicação. Outros estudos relatam a capacidade potencial do resveratrol de protegar células, inibir a formação de AGEs quando na presença de Metioglioxal e glioxal, e verificaram que é agente contra complicações diabéticas a longo prazo (LIU et al., 2013; CIDDI; DODDA, 2014; SEO et al., 2014; NAIR et al., 2015). Porém, como já mencionado, mecanismos moleculares subjacentes não são bem compreendidos e, mais especificamente, o efeito do resveratrol contra a formação de carbonilos protéicos específicos em proteínas humanas, sob condições hiperglicêmicas, é desconhecido.

2.3 PROCESSO DE MARINAÇÃO E SEU EFEITO NA QUALIDADE DA CARNE

Os consumidores estão cada vez mais conscientes dos fatores que influenciam a qualidade e a segurança da carne (MAYSONNAVE et al., 2014; MOREIRA et al., 2017). Em consequência disto, tendem a consumir carnes provenientes de sistemas pecuários sustentáveis, além de rejeitarem qualquer intervenção envolvendo aditivos sintéticos ou ingredientes potencialmente prejudiciais mesmo que adicionados em concentrações estabelecidas pela legislação (CHENG et al., 2017). Preservar a carne de deteriorações oxidativa, microbiana vem se apresentando como um grande desafio para a indústria de alimentos; uma vez que esses processos são os principais agentes que afetam a vida útil da carne (RYSMAN et al., 2016; HES; GRAMZA-MICHAŁOWSKA, 2017; CHENG et al; 2017).

Estrátegias ou técnicas são aplicadas à carne *in natura* com a finalidade de ampliar sua conservação e manter suas qualidades sensoriais. Dentre as técnicas de conservação, a marinação apresenta grande potencial no comércio é um processo que vem se desenvolvendo desde os anos 80, em países como os Estados Unidos, Reino Unido, Noruega, Suécia e Finlândia, que possuem regulamentação e mercado estabelecido, com boa aceitação dos consumidores por conferir melhoria no sabor e textura das carnes (DAGUER et al., 2010; FREITAS; OLIVEIRA-FILHO, 2016).

A expressão "marinação" se origina de línguas latinas, que se refere à técnica de embeber carnes em salmouras. Embora ocorra bastante variação entre os diversos países em que é aplicada, tem-se por marinação a adição de sal, fosfatos e condimentos à carne em emio aquoso (BJÖRKROTH, 2005). A marinação é uma técnica simples que permite uma condimentação antecipada da carne através de um meio líquido, ligeiramente ácido, onde deve-se equilibrar o pH da solução marinada com a carne escolhida (OLIVO, 2006).

Industrialmente, a marinação é aplicada de forma estática ou dinâmica. A forma estática é feita por imersão da carne na salmoura, quando os ingredientes penetram gradativamente por difusão, sem aplicação de força externa. No processo dinâmico, empregam-se a injeção e o massageamento da carne. A imersão da carne em salmoura talvez seja o método de marinação mais antigo, onde a migração dos ingredientes para o interior das miofibrilas depende da concentração de sólidos da salmoura e do tempo de imersão da carne, sendo que o tecido conjuntivo apresenta-se como uma barreira para a introdução da salmoura (XARGAIÓ et al., 2007).

De acordo com Offer, Trinick (1983) e Ertbjerg et al. (1999), a marinação pode afetar a maciez da carne por ação do pH na indução do inchaço das fibras musculares e/ou tecido

conectivo, e pelo aceleramento da proteólise, favorecendo o enfraquecimento da estrutura muscular através de um aumento da solubilização do cólageno no cozimento.

Para que se alcance uma maciez nas carnes, o desenvolvimento do marinado deve estar diretamente relacionado ao tipo de ingrediente e aditivos alimentares, uma vez que estes agem diretamente nas características intrínsecas do alimento e o modificam quimicamente, garantindo qualidade, segurança e singularidade aos produtos. Os tipos de ingredientes que compõe um marinado são bastante variados, tais como; vinagre, suco de limão, vinho, molho de soja, salmoura, óleos essenciais (EOs), sais, amaciantes, ervas, especiarias e ácidos orgânicos (PATHANIA et al., 2010).

Os ingredientes adicionados no marinado tem como finalidade principal o aumento de retenção de água pela carne, garantindo a fixação de sabores e aromas, e melhorando a suculência e a textura da carne (XARGAYÓ et al., 2007). Sendo assim, um marinado pode levar características e sabores regionais para os consumidores de outras regiões com diferentes influências alimentares, promovendo a diversidade de culturas alimentares.

A marinação utilizando vinhos é uma forma de melhorar as propriedades sensoriais (textura, sabor e cor) de carnes,e pode influenciar na estabilidade química e formação de compostos potencialmente tóxicos, mas tanto tem sido pouco estudado. Os potenciais efeitos da marinação do vinho na qualidade e segurança da carne bovina seriam determinados pela sua composição em compostos bioativos e isto é, por sua vez, dependente da variedade da uva (BALASUNDRAM et al., 2006). Acredita-se que os ácidos orgânicos, valor de pH e o álcool do vinho, alteram as propriedades estruturais do colágeno, agindo nas miofibrilas durante a marinação, causando um aumento de volume, pois esses componentes do vinho na carne diminuem o pH e alteram o ponto isoelétrico das proteínas. Assim, pode ser que ocorra aumento da capacidade de retenção de água, e a estrutura muscular pode ser deformada pela umidade adquirida e tornando a carne mais macia. Porém, o conhecimento científico, mecanismos de ação, isto é, o efeito da marinação do utilizando apenas o vinho como único componente da marinação, na qualidade da carne, no entanto, não é conhecido (ISTRATI et al., 2012).

Kargiotou et al. (2011) investigaram o efeito da marinação em molho de soja e vinho tinto sobre a deterioração microbiana da carne bovina crua e outros parâmetros de qualidade, incluindo oxidação e textura. Os autores perceberamm que as marinadas testadas foram efetivas contra a proliferação de microorganismos de deterioração da carne e também resultaram em redução da oxidação lipídica.

Istrati et al. (2015) estudaram o efeito combinado de marinação e especiarias à base de vinho, na degradação de proteínas miofibrilares e na estabilidade da cor da carne no músculo

bovino. Esses estudos e outros (NISIOTOU et al., 2013; RHOADES et al., 2013) concluíram que a marinação à base de vinho com temperos variados aumentou a maciez da carne sem efeitos negativos sobre a cor, apresentando efeitos antimicrobianos e com diminuição oxidação lipídica.

Os mecanismos subjacentes de tais efeitos permanecem desconhecidos, bem como, o efeito do vinho-marinação na oxidação de proteínas, assumindo consequências relevantes sobre a textura, o valor nutricional e até mesmo os efeitos na saúde dos alimentos musculares (SOLADOYE et al., 2015; ESTÉVEZ; LUNA, 2017).

De particular interesse são as possíveis interações moleculares entre os componentes do vinho (polifenóis) e os componentes proteícos, dados os adutos bem conhecidos formados entre os fenólicos da uva e os resíduos protéicos. Portanto, novas investigações são necessárias para estabelecer sólidos conhecimentos envolvendo os diversos efeitos que a marinação do vinho pode ter sobre a oxidação lipídica / protéica, cor, textura e deterioração microbiana na carne bovina.

2.4 BACTÉRIAS LÁTICAS *VERSUS* COMPOSTOS FENÓLICOS

O papel protetor dos microrganismos em processos envolvidos no desenvolvimento do sistema imunológico e regulação da resposta a patógenos, entre outros está ganhando interesse crescente devido à sua capacidade de neutralizar o estresse oxidativo no cólon em humanos e animais (SPYROPOULOS et al., 2011; STÜMER et al., 2012).

Microrganismos que proporcionam benefícios à saúde quando presentes no trato gastrointesntinal ou quando consumidos em quantidades adequadas são considerados de microrganismos probióticos, ou seja, são aqueles capazes de beneficiar o hospedeiro por meio de diferentes mecanismos de ação (WEDRYCHOWICZ et al , 2016). Dentre os benéficios do consumo de bactérias probióticas destaca-se que são uma barreira protetora natural entre os potenciais efeitos nocivos dos componentes da dieta e da mucosa intestinal, ou seja, eles podem atuar como restauradores da homeostase entérica fisiológica (STÜMER et al., 2012; WEDRYCHOWICZ et al., 2016).

Bactécias do gênero *Lactobacillus* são habitantes do trato gastrointestinal de humanos e animais e têm sido intensamente pesquisados durante as últimas décadas por seus efeitos sobre o próprio trato gastrointestinal (HOU et al., 2015). São constituintes do grupo de bactérias ácido láticas, pois produzem através da fermentação de carboidratos (especialmente glicose) ácido lático como produto principal (MENEZES et al., 2018).

A espécie *L. reuteri* representa o grupo de lactobacilos heterofermentativos, ou seja, além de produzir ácido lático, são capazes de produzir também etanol, ácido acético e dióxido de carbono (CARR et al., 2002) e, cepas de *L. reuteri* têm sido sugeridas como tendo atividades de promoção da saúde devido à sua ocorrência no intestino delgado ou devido a ingestão (LEE et al., 2008; ZHAO; GANZLE, 2018).

A administração oral de *L. reuteri* reduz os distúrbios e infecções gastrointestinais e contribui para uma microbiota equilibrada (SHORNIKOVA et al., 1997). De acordo com os mecanismos relacionados aos seus efeitos probióticos, incluindo tolerância à enzima digestiva, alta adesão a células, exclusão competitiva contra invasões de patógenos (HOU et al., 2014; WANG et al., 2012), efeito hipocolesterolêmico comprovado (TARANTO et al., 2000), também já foi relatado que L. reuteri protege contra o estresse oxidativo e inibe a acreção de produtos de oxidação no lúmen (AMARETTI et al., 2013).

Evidências mostram que os efeitos probióticos são específicos de uma determinada cepa (SZAJEWSKA et al., 2014). *L. reuteri* DSM 17938 foi estudada na diarreia aguda em crianças e descobriu-se serem eficaz, seguro e bem tolerado pelas crianças (DINLEYICI et al., 2015), *L. reuteri* ATCC PTA 6475 foi relacionada com a apoptose de células derivadas de leucemia mielóide humana (IYER et al., 2008), *L. reuteri* ATCC PTA 4659 (origem humana) e *L. reuteri* R2LC (origem aminal) melhoraram os sinais clínicos de colite ulcerativa em estudo modelo com ratos (AHL et al., 2016).

Enquanto os benefícios de *L. reuteri* contra o estresse oxidativo e distúrbios intestinais, como colite ulcerativa são documentados (PETRELLA, 2016), os mecanismos moleculares implicados nas respostas dessas bactérias probióticas sob condições pró-oxidantes específicas (como aquelas induzidas por um sistema gerador de radical hidroxila) são ainda não definido.

Sabe-se que o cólon é particularmente suscetível ao estresse oxidativo devido a uma intensa geração de espécies reativas de oxigênio (ROS) que freqüentemente excedem as capacidades antioxidantes (SANDERS et al., 2004). A carne vermelha foi identificada como uma fonte de espécies pró-oxidantes (SOLADOYE et al., 2015) e ainda está relacionada ao aparecimento do câncer colorretal (CCR) pelo IARC (INTERNATIONAL AGENCY FOR RESEARCH ON CANCER, 2015). Uma lesão química duradoura no cólon causado pelas espécies pró-oxidantes leva a condições patológicas nas quais o estresse oxidativo é conhecido por desempenhar um papel relevante, incluindo doença inflamatória intestinal (DII), colite ulcerativa e o CRC supracitado, como também implicam ou levam ao estresse oxidativo dessas bactérias (GACKOWSKI et al., 2002; ZHU; LI, 2012; WANG et al., 2017).

Algumas enzimas, tais como as oxidases superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa redutase (GR) são responsáveis pela interação direta com ROS, constituindo um mecanismo de defesa enzimáticas. Também existe defesas antioxidantes não enzimáticas (glutationa, tiorredoxina, vitamina C, vitamina E) e sistemas de reparo para proteger os micorganismos contra o estresse oxidativo. No entanto, esses sistemas antioxidantes próprios, geralmente, não são suficientes para impedir que os organismos vivos sofram danos oxidativos. Portanto, a ingestão de alimentos que contenham compostos antioxidantes, têm sido amplamente estudados no que diz respeito a sua proteção contra estresse oxidadivo de células viáveis (WANG et al., 2017).

Nesse sentido, estudos recentes relatam que os principais constituintes dos alimentos, como fibra alimentar e polifenóis, influenciam positivamente a composição e a funcionalidade da microbiota intestinal (DUDA-CHODAK et al., 2015; MARCHESI et al., 2016; MENDIS et al., 2016). Além disso, algumas pesquisas foram focadas no isolamento e identificação de bactérias intestinais humanas capazes de metabolizar os polifenóis da dieta (CUEVA et al., 2016).

Os polifenóis provenientes da dieta são pouco absorvidos no intestino delgado, ou seja, podem não estar em sua forma biodisponível (OZDAL et al., 2015). Os polifenóis quando na presença dos microrganismos presentes no colón, sofrem reações metabólicas, resultando no aumento da sua biodisponibilidade. Consequentemente os polifenóis modulam a composição dos microrganismos do intestino através da inibição de bactérias patogênicas e da estimulação de bactérias benéficas (MUÑOZ-GONZÁLEZ et al., 2013; OZDAL et al., 2015).

Assim, torna-se evidente que os efeitos benéficos atribuídos ao consumo de polifenóis são conferidos principalmente a estes metabólitos fenólicos microbianos (biodisponíveis) e seus outros conjugados (BOTO-ORDÓÑEZ et al., 2011; SELMA et al., 2009).

Dolara et al. (2005) avaliaram a influencia dos polifenóis do vinho tinto (antocianinas, flavonóis, ácidos fenólicos, catequina, epicatequina e proantocianidinas) na carcinogênese, microflora intestinal, no dano oxidativo e nos perfis de expressão gênica da mucosa em ratos, concluíram que os polifenóis do vinho tinto reduz o processo de carcinogênese química do cólon em roedores, modificam a ecologia microbiana, reduzem a oxidação do DNA da mucosa e têm efeitos complicados na regulação gênica.

Entretando, pouca informação está disponível sobre as respostas biológicas de bactérias probióticas a espécies oxidativas específicas e, em particular, na presença de compostos bioativos, como o resveratrol. A análise da expressão gênica é uma ferramenta valiosa e altamente específica para entender como os fatores externos influenciam precisamente

determinadas funções biológicas e rotas metabólicas. Por exemplo, ferramentas genômicas avançadas têm sido cruciais para revelar a base molecular dos efeitos benéficos do resveratrol em células do cólon tumorigênico e também para compreender os efeitos antiproliferativos da *L. reuteri* em células derivadas da leucemia mielóide humana (IYER et al., 2008). As respostas genéticas e moleculares de *L. reuteri* na presença de resveratrol são, até onde sabemos, desconhecidas.

Assim, faz-se necessário o desenvolvimento de pesquisas que tenham enfoque na avaliação dos possíveis benefícios de compostos fenólicos na manutenção da funcionalidade celular de bactérias benéficas, particularmente quando expostas ao estresse oxidativo.

3. MATERIAL E MÉTODOS

3.1 DELINEAMENTO EXPERIMENTAL

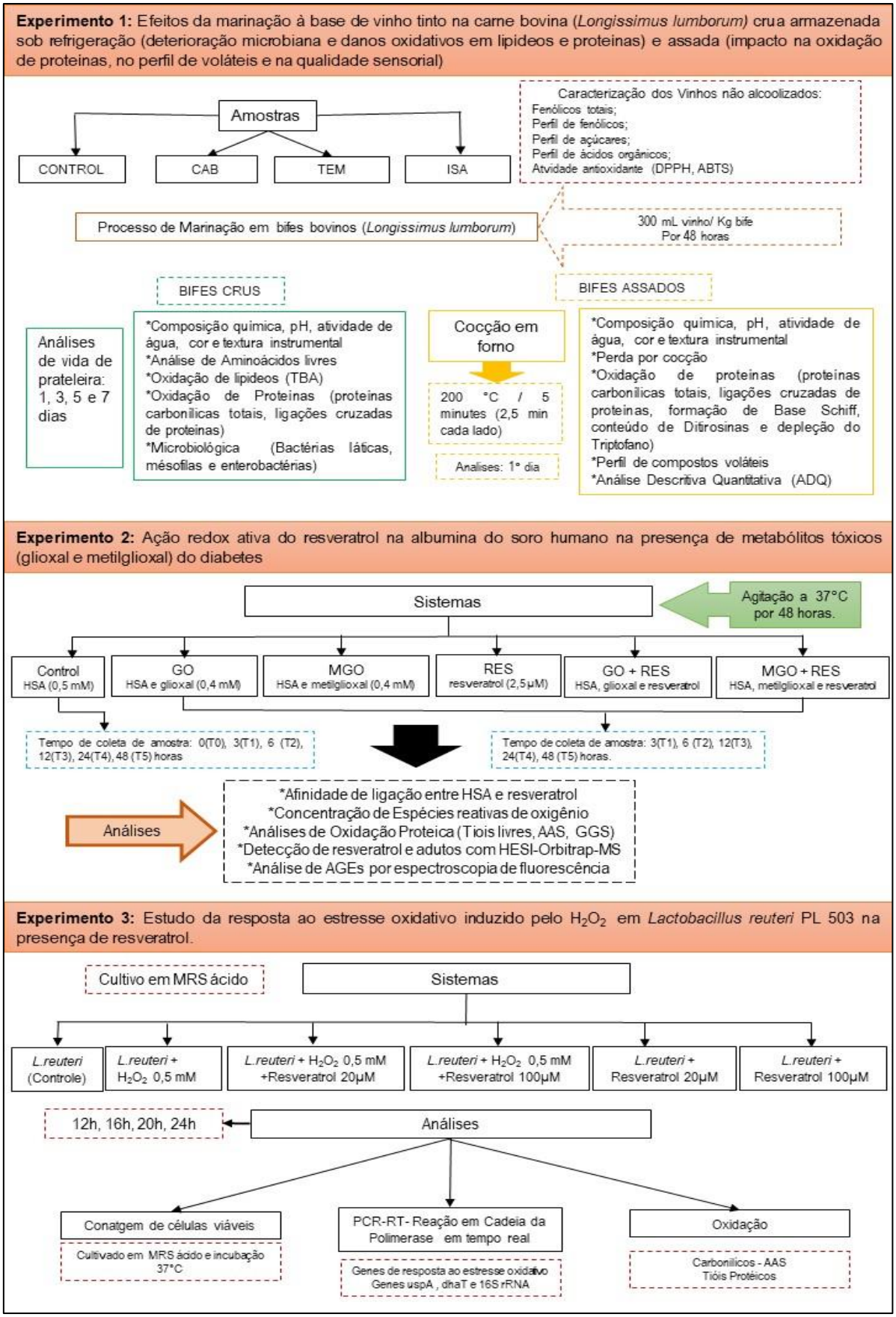
Os estudos envolvidos na tese foram realizados na Universidade de Extremadura (UNEX, Cáceres/Espanha), nos Laboratórios do Instituto de Investigação "Carne y Productos Cárnicos" através do Programa de Doutorado Sanduíche no Exterior (PDSE/CAPES), processo Nº 88881.135018/2016-01, no período de seis meses - abril a outubro de 2017. Análises complementares foram realizadas na Universidade Federal da Paraíba (UFPB, Brasil), nos laboratórios do Departamento de Engenharia de Alimentos, Centro de Tecnologia, Campus I. A pesquisa envolveu o desenvolvimento de três experimentos distintos os quais resultaram na produção de quatro artigos científicos (Figura 6).

O primeiro estudo envolveu o entendimento da ação dos compostos fenólicos presente no vinho tinto, produzido com uvas das espécies *V. labrusca* ('Isabel') e *V. vinifera* ('Tempranillo' e 'Cabernet Sauvignon'), em bifes bovinos (*Longissimus lumborum*) marinados sob armazenamento refrigerado (carne crua), frente à deterioração microbiana, deteriorações oxidativas e de cor e, também em bifes assados sobre a oxidação de proteínas, perfil volátil e propriedades sensoriais.

Tomando-se por base que os conhecimentos científicos do efeito do resveratrol frente a formação de carbonilas proteicas a partir de proteínas humanas em condições de hiperglicêmia são escassos, um segundo estudo foi realizado objetivando o conhecimento da eficácia do resveratrol como inibidor das alterações químicas induzidas em sistemas modelos formados por aldeídos carbonilados - glioxal (GO) e metilglioxal (MGO) – e albumina do soro humano (HSA). Este estudo abrangeu estudos sobre a formação de carbonilas proteicas precoces, de semi-aldeídos ácido α-aminoadipico (AAS) e γ-glutamico (GGS), o acúmulo de produtos finais da glicalização avançada (AGEs) e a depleção de outros grupos funcionais e aminoácidos em proteínas, como resíduos de triptofano e tióis.

No terceiro experimento foram avaliados os efeitos (protetor) do resveratrol na resposta ao estresse oxidativo induzido pelo peróxido de hidrogênio (H_2O_2) em bactéria ácido lática (*Lactobacillus reuteri* PL503*).*

Figura 6: Fluxograma geral dos experimentos realizados



Fonte: Arcanjo, 2018

3.2 EXPERIMENTO 1: EFEITO DA MARINAÇÃO À BASE DE VINHO TINTO NA CARNE BOVINA (*Longissimus lumborum)* CRUA ARMAZENADA SOB REFRIGERAÇÃO (DETERIORAÇÃO MICROBIANA E DANOS OXIDATIVOS EM LIPÍDEOS E PROTEÍNAS) E ASSADA (IMPACTO NA OXIDAÇÃO DE PROTEÍNAS, NO PERFIL DE VOLÁTEIS E NA QUALIDADE SENSORIAL)

3.2.1 Material

Foram utilizados três vinhos tintos seco produzidos com uvas distintas, onde dois vinhos (Bronte, vinho de Tierra Castilla, 2016, 13% vol. Álcool, Vinícola Féliz Soles SL, Ciudad Real, Espanha) produzidos com uvas Cabernet Sauvignon*(Vitis vinífera)* e Tempranillo *(Vitis vinífera)*, foram obtidos no supermercado cidade de Cáceres da Espanha. E terceiro vinho (Quinta do Morgado da Serra Gaúcha, 2016, 10,5% vol. de álcool, Vinícola Fante, Flores da Cunha, Brasil) produzido com uva Isabel *(Vitis labrusca)*, foi obtido em João Pessoa, Brasil. Todos os vinhos foram produzidos no mesmo ano, considerados vinhos jovens e nenhum teve maturação em barril de carvalho. Os vinhos permaneceram na garrafa 12 meses antes de serem processados para análise e subsequente marinação (início de 2017).

Três cortes bovinos (*longissimus lumborium)*, foram adquiridos em Frigorífico local da cidade de Cáceres (Espanha), e transportados sobre refrigeração (< 10 °C) para o Laboratório de Tecnologia de Alimentos – TECAL, e processados imediatamente, segundo descrição no item 3.2.3.

3.2.2 Caracterização dos Vinhos

Inicialmente os vinhos foram submetidos a um processo de remoção do álcool, evitando-se a interferência do etanol na ação dos compostos fenólicos frente aos processos oxidativos da carne bovina. O processo consistiu em evaporar 20% do volume do vinho em Rota evaporador (Meidolph, Laborota 4000) a uma temperatura de 50 °C, com bomba a vácuo (Buchi, Vacuum Pump V-700 e Vacuum controller V-800) operando a uma pressão de 124 mBar. Os vinhos desalcoolizados foram acondicionados em frascos ambar e armazenados sob congelamento (– 20 °C) até o dia das análises, observando-se um período não superior a 60 dias. Em seguida, os extratos de vinho foram caracterizados quanto ao teor de fenólicos totais, perfil de compostos fenólicos, perfil de ácidos orgânicos e açúcares, e atividade antioxidante.

3.2.2.1 Conteúdo de Fenólicos totais

O teor fenólico total (TPC) nos vinhos tintos foi determinado pelo método de Folin-Ciocalteau proposto por Singleton e Rossi (1965) com adaptação. Alíquotas de 100 µL de extrato etanólico de vinho foram misturados com 500 µL de solução aquosa de Folin-Ciocalteau a 10%, após 2 min., com 400 µL de carbonato de sódio a 7,5%. Após a mistura ter sido incubada durante 15 min. num banho de água a 50 ° C para a formação da cor, a absorvância foi medida num espectrofotómetro Hitachi U-3900 (Hitachi, Ltd, Tóquio, Japão) a 760 nm contra um reagente em branco (Folin-Ciocalteau a 10% e carbonato de sódio a 7,5%). Para quantificação dos fenóis totais do vinho, uma curva padrão (10 a 90 µg / mL) preparada com ácido gálico (Sigma-Aldrich, Brasil) foi elaborada e plotada em amostras para expressar resultados como mg de equivalentes de ácido gálico (GAE) por litro da amostra.

3.2.2.2 Atividade antioxidante in vitro

O teste DPPH (redução do radical 2,2-difenil-1-picrilidrazilo) foi realizado de acordo com Brand-Wiliams et al. (1995) com algumas modificações. Uma solução de DPPH • (0,004% p / v) foi preparada com valores de absorbância entre 0,7 a 0,8 determinada em espectrofotômetro UV-Visible (Ultravioleta Microproce 0798U, Quimis, São Paulo, Brasil), a 515 nm a partir da qual a solução foi preparada diariamente e armazenada sob refrigeração até o momento da utilização. Uma alíquota de 350 µL do extrato de vinho foi transferida para tubo de vidro e adicionada com 3,15 mL de solução de DPPH, agitada e, após 30 min., a absorbância da mistura foi registrada no espectrômetro UV-Visible Quimis. Foi construída a curva de calibração Trolox (2,5 -15 µM) e a mesma foi plotada como a porcentagem (%) de inibição versus concentração de Trolox.

A capacidade antioxidante equivalente de Trolox (TEAC) - a captura de radicais de 2,2-azino-bis (3-etilbenzotiazolina-6-sulfónico) (ABTS • +) foi determinada de acordo com o método descrito por Re et al. (1999). A amostra foi preparada em água destilada e através do teste com ABTS • +, obtida por reacção de 5 mL de ABTS (7 mM) com 88 µl de persulfato de potássio 140mM (concentração final de 2.45 mM), onde o sistema foi mantido em repouso à temperatura ambiente por um período de 12 a 16 horas na ausência de luz. Posteriormente, a absorvância da amostra foi lida a 734 nm em espectrofotômetro UV-Visible (Ultravioleta Microproce 0798U, Quimis, São Paulo, Brasil), após a mistura de 50 µL de amostra e 950 µL de solução ABTS • +, deixando descansar por 6 min. Foi preparado um branco e a leitura foi

realizada de acordo com o procedimento descrito, sem a adição da amostra. Foi construída a curva de calibração Trolox (2,5 -15 μM) e a mesma foi plotada como a porcentagem (%) de inibição versus concentração de Trolox.

3.2.2.3 Perfil de fenólicos

A análise dos fenólicos monoméricos foi realizada utilizando o método descrito por Ibern-Gómez et al. (2002) com modificações. A separação analítica dos compostos fenólicos foi realizada em aparelho de HPLC Shimadzu "Prominence" (Shimadzu Corp., Kyoto, Japão) equipado com um sistema de solvente quaternário (LC-20AD), um desgaseificador online DGU-20AS, um amostrador automático SIL-20A , um detector de fluorescência RF-10A XL e um detector de matriz de diodo SPD-M20A. Utilizou-se uma coluna Agilent Poroshell 120 SB-C18 de fase reversa (150 × 4,6 mm, tamanho de partícula de 2,7 μm) e uma coluna de guarda (10 × 4,6 mm) preenchida com o mesmo material. (A) Ácido trifluoroacético aquoso a 0,2 % e (B) acetonitrila com ácido trifluoroacético a 0,2 % foram utilizados como eluentes. Foi utilizado uma programação de gradiente, variando-se a concentração de eluente B 7 % 0-5 min.; 5-25 min., 7-18 % de B; 25-46 min., 18-34 % de B; 46-60 min., 34-65 % B. O fluxo foi mantido a 0,5 mL / min., e a temperatura da coluna foi mantida constante (30 °C). Antes da análise, 8 μL dos vinhos não alcoolizados foram filtrados usando-se um filtro PVDF de 0,45 μm (Agilent). Com base na identificação espectral, os fenólicos monoméricos foram quantificados em cinco subclasses: ácidos hidroxibenzóicos (como equivalentes de ácido gálico, 280 nm), catequinas (expressos como equivalentes de catequina; detecção de fluorescência λex = 280 nm, λem = 322 nm), ácidos hidroxicinâmicos (como equivalentes de ácido cafeico, 320 nm), flavonóis (como equivalentes a quercetina, 365 nm) e antocianinas (como equivalentes de cloreto de delfinidina, 520 nm).

O teor total de procianidina foi quantificado pelo método descrito por Ollé et al. (2010) com modificações. As procianidinas oligoméricas e poliméricas foram despolimerizadas na presença de um agente nucleofílico (floroglucinol) em meio ácido. Aliquotas de 0,5 mL de cada vinho não alcoolizado foi evaporado até secura sob vácuo num SpeedVac a 35 ° C. O sedimento seco foi dissolvido em 1 mL de acetato de etilo, transferido para um frasco de vidro de 4 mL e re-seco sob azoto. O reagente de floroglucinólise foi preparado como se segue: inicialmente foi preparada uma solução metanólica de HC1 0,1 M (solução A). A solução B foi então preparada dissolvendo 500 mg de floroglucinol em 10 mL de solução A. Finalmente, a solução C foi preparada dissolvendo 100 mg de ácido ascórbico em 10 mL de solução B. Uma vez preparada

as soluções, deu-se inicio a reação pela adição de 1 mL da solução C aos frascos contendo o sedimento de vinho. Eles foram agitados no vórtice para se dissolverem completamente, e em seguidas foram homogeneizados em agitador magnético por 30 min. a 50 ºC. A reação foi interrompida colocando-se as amostras em banho de gelo, e diluindo-se o meio reacional com 1,5 mL de uma solução de acetato de amónio 40 mM. A solução final foi então filtrada através de um filtro PVDF de 0,45 μm (Agilent) antes da análise no sistema de HPLC. O mesmo sistema cromatográfico e coluna utilizados para a identificação dos compostos fenólicos monoméricos também foram utilizados para a identificação dos produtos de floroglucinólise. (A) 0,1% de ácido fórmico aquoso (v / v) e (B) acetonitrilo com 0,1 % de ácido fórmico foram usados como eluentes. Foi utilizado uma programação de gradiente, variando-se a concentração do eluente B de 0-5 min., 7 % de B ; 5-24 min., 7-18 % de B; 24-45 min., 18-34 % de B; 45-55 min., 34-65 %. O fluxo foi mantido a 0,5 mL / min e a temperatura da coluna foi mantida constante a 30 °C. Os produtos de floroglucinólise foram caracterizados por seus espectros UV-Vis, tempos de retenção relativos aos padrões externos. Um detector de fluorescência (λex = 280 nm, λem = 322 nm) foi usado simultaneamente para melhorar o procedimento de identificação. Procianidinas totais foram determinadas como a soma das subunidades quantificadas.

3.2.2.4 Perfil de açúcares e ácidos orgânicos

Um extrato aquoso dos vinhos foi preparado e utilizado na determinação do perfil de açúcar e ácidos orgânicos. Cerca de 0,25 g de vinho foram diluídos em 5 mL de água Mili-Q, seguindo homogeneização e extração de acordo com método de Zeppa et al. (2001) com modificação. Alíquotas de 20 μL de extrato aquoso de cada filtrado foram usadas para determinar o perfil de açúcar por meio de injeção manual em Cromatógrafo Líquido de Alta Eficiência (356 LC, Varian), uma válvula Rheodyne com loop de 20 μL; acoplado a uma coluna Agilent Hi-Plex Ca (7,7 x 300 mm, 8 μ), a uma temperatura de 85 °C, detector de índice de refração (VARIAN 356), sistema de bombeamento com configuração isocrática de alta pressão e software de processamento GALAXIE Chromatography Data System. A fase móvel utilizada foi a água, com vazão de 0,6 mL / min. A duração da corrida foi de 30 min. A quantificação dos açúcares foi realizada pela injeção de curvas padrão de glicose, frutose e sacarose nas mesmas condições cromatográficas.

Para a quantificação de ácidos orgânicos no vinho tinto, 20 μL do extrato aquoso da amostra filtrada foram injetados em Cromatógrafo Líquido de Alta Eficiência (Shimadzu,

Japão), equipado com sistema de solventes binários, válvula Rheodyne com cabo de 20 μL; acoplado a um Lichrospher 100 RP18 (250 x 4 mm, 5 mm), a uma temperatura de 25 ° C, detector por arranjo de diodos (SPD-M20A), em comprimentos de onda de 210 nm, bombeando com configuração de gradiente de alta pressão (LC-20AT) e o software de processamento LCsolution versão 1.22 SP1. A fase móvel utilizada foi 0,009 M de ácido sulfúrico a um fluxo de 0,7 mL / min. A duração da corrida foi de 30 minutos. A quantificação dos ácidos orgânicos foi realizada pela injeção de curvas padrão de ácidos tartárico, málico, succínico, láctico e acético nas mesmas condições cromatográficas.

3.2.3 Marinação dos Bifes

O processo de marinação envolveu quatro tratamentos distintos, nomeadamente 'Carbernet' (CAB), 'Tempranillo' (TEM) e 'Isabel' (ISA), e um grupo CONTROL de bifes tratadas com água destilada, utilizando mesma relação (volume de vinho/ carne).

Os cortes bovinos foram inicialmente submetidos a toalete com remoção de gordura externa, ligamentos e tendões. Em seguida foram cortados em bifes de 100 g (aprox. 2,5 cm de espessura) e distribuídos aleatoriamente em bandejas de poliestireno (11 × 16 × 4 cm) em grupos de quatro unidades. Os bifes foram marinados com 300 mL de vinho desalcoolizado por kg de carne bovina, sob agitação vigorosa para garantir a distribuição uniforme do vinho, seguido de embalagem com filmes de PVC (permeabilidade ao oxigênio: ~ 17 cm^3 / m^2/ dia. atm; permeabilidade à umidade: <5 g / m^2/ dia (Tecnodur SL, Valência, Espanha) e mantidos sob refrigeração (4 ± 1 °C) por 48 horas. Após 24 horas do início do processo, os bifes foram virados para uma melhor incorporação do líquido de mariação. Finalmente, ao atingir-se as 48 horas de marinação, os bifes foram removidos das bandejas e o excesso de líquido foi drenado em papel de filtro. Os bifes foram pesados em balança analítica e colocados em recipientes plásticos limpos e refrigerados (4 ± 2 °C) por 7 dias sob luz fluorescente simulando exibição de varejo (1620 lux, 12 horas de luz / 12 horas de escuridão). Os bifes marinados crus foram analisados nos intervalos de 1, 3, 5 e 7 dias após o processo de marinação. Todo o processamento de marinação foi replicado três vezes em lotes de produção independentes correspondentes. Assim, nove fatias de três diferentes lotes de produção foram obtidas por tratamento. Todos os bifes crus foram analisados em duplicata.

Um segundo lote de bifes marinados foi submetido ao processo de cocção logo após o término da marinação (48 horas), sendo os bifes marinados assados em forno (Unox®, Mod. GN2.1, Cadonegue, Itália) até uma temperatura interna de 200 °C por um período de 5 min.

(2,5 min. para cada lado dos bifes). Finalmente, os bifes marinados e assados, foram resfriados a temperatura ambiente (25 °C) e analisados em triplicata.

Detalhadamente, nos bifes marinados crus foram analisados em relação aos parâmetros de composição química, pH, atividade de água, cor e textura instrumental, aminoácidos livres (FAA), reação ao ácido tiobarbitúrico (TBARs), proteínas carbonílicas totais, ligações cruzadas de proteínas (formação de Base de Schiff e ligações de dissulfeto) e análises microbiológicas. Para os bifes assados os procedimentos analíticos foram: composição química, pH, atividade de água, cor e textura instrumental, perda por cocção, oxidação de proteínas (proteínas carbonílicas totais, ligações cruzadas de proteínas, formação de Base Schiff, conteúdo de Ditirosinas e depleção do Triptofano), compostos voláteis e análise sensorial (Análise Quantitativa Descrita - ADQ). Todos os procedimentos experimentais realizados nos bifes crus e assados estão descritos nas sessões a seguir e quando necessário incluso as particularidades para cada tipo de procedimento.

3.2.4 Análise dos Parametros Físico-Químicos

3.2.4.1 Composição química, pH, atividade de água e cor instrumental

Os conteúdos de umidade e proteína foram determinados usando métodos oficiais (AOAC, 2000). O método de Folch, Lees e Sloane Stanley (1957) foi usado para determinar o teor de gordura na carne bovina marinada. A atividade de água foi medida em um medidor de atividade de água Lab Master (Novasina AG, Neuheimstrasse, Suíça) onde as amostras foram colocadas em cápsulas circulares de polietileno e inseridas no equipamento, sendo a leitura realizada automaticamente após alguns min. de toda a amostra de rastreamento . O pH foi determinado usando um medidor de pH portátil (TESTO 205, Lenzkirch, Alemanha). O pH intramuscular foi avaliado na carne bovina à temperatura ambiente e o equipamento foi calibrado com soluções tampão a pH 7,0 e 4,0.

As medidas de cor da superfície da carne foram realizadas usando um colorímetro Minolta Chromameter CR-300. As medidas dos parâmetros de cor (L * - luminosidade, a * - cor vermelha, b * - cor amarela) foram realizadas em triplicatas em pontos aleatórios na superfície das fatias da carne. As medições de cor foram feitas à temperatura ambiente com o iluminante D65 e um ângulo de zero grau. Uma diferença numérica total da cor (ΔE) entre as estacas de carne no dia 1 e no dia 7 de armazenamento foi calculada como: ΔE1-7 = [(L7-L1) 2+ (a7-a1) 2+ (b7-b1) 2)] ½ e utlizada apenas nas amostras de carne crua.

3.2.4.2 Determinação da força de cisalhamento

A avaliação da força de cisalhamento (WBSF) foi determinada utilizando-se Texturometro TA XT-2i (Stable Microsystems, Godalming, Surrey, Reino Unido), equipado com a lâmina triangular de Warner-Bratzler (WB), conforme metodologia de Hiudobro et al. (2005) com modificações. Os bifes crus (dia 7) e assados (dia 1) foram cortados em fatias com dimensões de 20 mm x 30 mm x 15 mm (espessura x comprimento x largura). Nas análises, as amostras foram cortadas com uma lâmina de Warner-Bratzler em uma direção perpendicular às fibras musculares. O texturometro foi operado a uma velocidade de 1,0 mm/segundo e distância de 10 mm e com força de 5g. As análises foram realizadas em triplicado em cada lote de processamento.

3.2.4.3 Perda por cocção

As amostras foram pesadas antes e depois do cozimento, e o percentual de perda de cozimento (CL) foi calculado de acordo com a Eq. (1), como segue:

$$CL = (\text{Peso inicial} - \text{Peso final}) / \text{Peso inicial} \times 100$$

3.2.4.4 Análise de Aminoácidos livres

Os bifes marinados (10 g) foram homogeneizados duas vezes com 50 mL de água desionizada e centrifugados a 5000 g a 4 °C durante 10 min. Sobrenadantes combinados, contendo nitrogênio solúvel em água, foram filtrados através de papel de filtro Whatman No. 1. Alíquotas de 25 mL deste filtrado foram misturados com 25 mL de ácido tricloroacético a 20% (TCA), sedimentados à temperatura ambiente durante 30 min, centrifugados a 5000 g a 4 ° C durante 10 min e filtrados através de papel de filtro Whatman n ° 4. Uma aliquota do filtrado, contendo nitrogênio não proteico, foi tratada com 3 mL de reagente o- ftalaldeído. Aliquotas de 40 mg de o-ftalaldeído foram dissolvidos em 5 mL de etanol, 25 mL de tetraborato de sódio 0,1 M, 0,1 mL de p-mercaptoetanol e diluidos ao volume de 50 mL com água desionizada. A absorvância foi lida a 340 nm contra o reagente o-ftalaldeído. O teor de aminoácidos livres foi calculado usando tirosina como padrão e expresso como g Aminoacidos livres / 100 g de carne (HAGEN et al., 1989).

3.2.4.5 Substâncias reativas ao ácido tiobarbitúrico

A oxidação lipídica foi determinada pelo método das substâncias reativas ao ácido tiobarbitúrico (TBARS), relatado por Ganhão et al. (2011). Resumidamente, 2,5 g de amostras foram homogeneizadas com 7,5 mL de ácido perclórico 3,86% e 0,25 mL de BHT 4,2%. O homogeneizado misturado foi centrifugado (3500 rpm durante 3 min) e filtrado através de Whatman n° 54 papel de filtro em frasco volumétrico e lavado com ácido perclórico. O filtrado foi ajustado para 10 mL por adição de ácido perclórico (3,86%). Em seguida, 2,0 mL deste filtrado com 2,0 mL de TBA 0,02 M foi levado ao banho de água a 90 °C durante 30 min. para desenvolver a reação de cor. Foi necessário realizar um procedimento em branco para cada amostra analisada, considerando-se que a utilização de vinho no processo de marinação conferiu uma coloração rosa aos bifes marinados. Este procedimento consistiu em submeter os bifes marinados as mesmas condições analíticas, excetuando-se a adição dos 2,0 mL do reagente TBA. As leituras das absorvâncias foram realizadas a 532 nm em espectrofotômetro UV-VIS (UV-1800, Shimadizu, Tóquio, Japão). Os resultados foram plotados contra uma curva padrão preparada com concentrações conhecidas de tetraetoxipropano (TEP) (5,67x10-^{8} a 11,34 x10-^{7} g TEP/ mL), e expressos em miligramas de malonodialdeído (MDA) por quilo de carne.

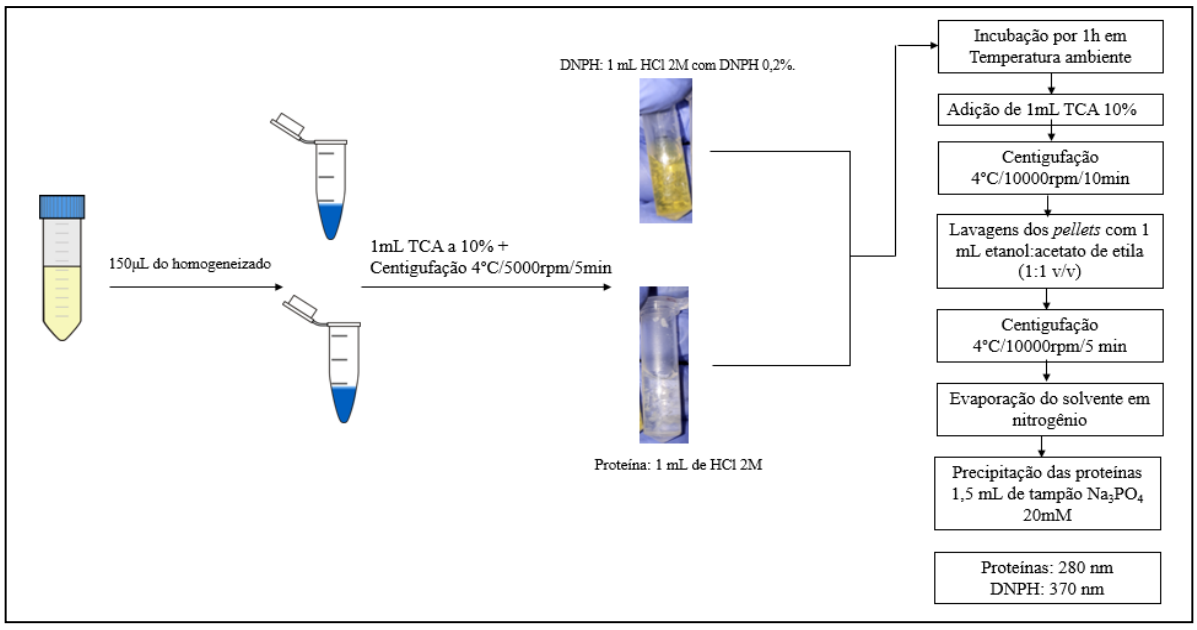
3.2.5 Análises de Oxidação Proteíca

3.2.5.1 Quantificação dos compostos carbonílicos totais

As carbonilas totais foram determinadas por meio do método dinitrofenilhidrazina (DNPH) descrito por Ganhão et al. (2010) com algumas modificações. Aliquota de 1,0 g de amostra foi homogeneizado com 9,0 mL de tampão Na3PO4 20 mM e NaCl 0,6 M pH 6,5 (1:10). A partir deste homogeneizado, duas alíquotas de 150 µL de cada amostra foram colocadas em tubos Eppendorf de 2 mL correspondentes: uma foi tratada para a quantificação de proteínas e a outra para a quantificação de carbonilas. As proteínas foram então precipitadas pela adição de 1 mL de ácido tricloroacético a 10% (TCA) frio, seguido de centrifugação a 4 ° C a 600 g durante 5 min. e os sobrenadantes foram rejeitados. Para a determinação dos compostos carbonilas, adicionou-se 1 mL da solução de HCl 2M com 0,2% de DNPH aos sedimentos. Para a determinação de proteínas, adicionou-se 1 mL de HCl 2M às pastilhas. Após incubação à temperatura ambiente por 1 h, as proteínas foram novamente precipitadas com 1 mL de TCA a 10% frio, seguido de centrifugação a 4 ° C, 1200 g por 10 min e lavadas duas

vezes com 1 mL de etanol/acetato de etila (1:1 v / v). Os sedimentos foram dissolvidos em 1,5 mL de tampão Na3PO4 20 mM, pH 6,5, adicionado com hidrocloreto de guanidina 6 M (Figura 7). A concentração de proteína foi calculada a partir de leituras de absorbancia a 280 nm utilizando uma curva padrão de albumina de soro bovino (BSA). A quantidade de carbonilas para os bifes foi expressa em nmoles de carbonilas por mg de proteína usando um coeficiente de extinção molar de hidrazonas (21,0 nM -1 cm -1) com leituras de absorbância a 370 nm. Para os bifes assados os resultados foram expressos como percentual de inibição contra a formação de proteínas carbonilas nos bifes marinados em relação ao tratamento controle, sendo calculada como: % de inibição = [100- (T x 100) / C)], onde C é a concentração de carbonilas de proteína nas amostras de CONTROLE e T é a concentração de proteína carbonilas em cada uma das contrapartes tratadas.

Figura 7: Esquema geral da determinação de compostos carbonílicos totais



Fonte: Arcanjo, 2018.

3.2.5.2 Ligações cruzadas de proteínas

As ligações cruzadas das proteínas foram avaliadas através da quantificação das ligações dissulfeto (bifes crus) e determinação da fluorescência da base de Schiff (bifes crus e assados).

As ligações de dissulfeto foram analisadas seguindo a metodologia de Rysman et al. (2014). Frações de 1,0 g de cada bife foi homogeneizado com o auxilio de ultra-turrax com 25

mL de 100 mM de tampão Tris pH 8,0 adicionado 6 M de Cloridrato de Guanidina (GuHCl). Os homogenatos foram centrifugados (20 min. a 1500 g e 4 ° C), e os sobrenadantes filtrados (papel de filtro qualitativo, 11 µm de retenção de partículas). Três mL dos filtrados foram submetidos à redução de dissulfeto pela adição de 50 µL de 1-octanol e 100 µL de boro-hidreto de sódio a 30% (p / v), recentemente preparado em NaOH 1M. Após incubação a 50 ° C durante 30 min, adicionou-se uma alíquota de 1,35 mL de HCl 6 M, seguida de agitaçãoo durante 10 min. Os tióis totais foram determinados com 4,4'-ditiodipiridina (4-DPS) nos filtrados reduzidos. Alíquotas de 250 mL do filtrado foram misturadas com 1,25 mL de tampão de ácido cítrico 1M e pH 4,5, adicionado de 6M GuHCl e 250 mL de solução 4-DPS (4 mM DPS-4 em 12 mM HCl). A absorbância foi medida a 324 nm contra o tampão de ácido cítrico 1M pH 4,5 adicionado de GuHCl 6M antes da adição de 4-DPS (A_{pre}) e após 30 minutos de reação com 4-DPS no abrigo da luz e a temperatura ambiente (A_{pos}). Uma mistura de 1,25 mL de tampão de ácido cítrico 1 M pH 4,5 adicionado de GuHCl 6 M e 250 µL da solução 4-DPS foi preparada como um branco (A_{branco}). A absorbância correspondente a concentração de tiol foi calculada subtraindo A_{pre} e A_{branco} de $A_{pos.}$ (Equação 1). A concentração de tiol foi calculada com base em uma curva padrão de cinco pontos variando de 2,5 a 500 µM de cisteína em 6M GuHCl em tampão de ácido cítrico 1 M (pH 4,5). O teor de dissulfeto foi calculado como metade da diferença entre tióis totais e livres divididos por dois.

Equação 1: $ABS(Tióis\ livres) = A\ pos - (Apre + A\ branco)$

Onde, A_{branco} é a absorbância apenas do tampão ácido cítrico com solução 4-DPS ; $A_{pré}$ é a absorbância após a reação da amostra com o tampão ácido citrico e solução de 4-DPS; $A_{pós}$ é a absorbância após 30 min de reação com 4-DPS.

A análise das bases de Schiff fluorescentes foi realizada usando espectroscopia de fluorescência, como descrito por Estévez et al. (2008b). Homogeneizados de bifes (1:10 p / v) em tampão fosfato de sódio pH 6,0 com ureia 8 M foram obtidos usando-se um ultraturrax. Após a diluição (1:20 v / v), as amostras foram transferidas para uma cubeta de quartzo de 4 mL com quatro paredes planas (101-QS 10 × 10 mm, Hellma Analytics, Müllheim, Alemanha). O espectro de emissão para a base de Schiff foi registado entre 400 nm e 500 nm de comprimento de onda com excitação estabelecida a 350 nm (espectrómetro de Luminescência Perkin-Elmer LS 55, Beaconsfield, UK). As larguras da fenda de excitação e emissão foram fixadas em 10 nm e os dados foram coletados a 500 nm por min.. Os resultados para os bifes

crus foram expressos como unidades de intensidade de fluorescência emitidas por estruturas de base de Schiff a 450 nm. Estes valores foram corrigidos de acordo com a concentração de proteína de cada amostra, aplicando um fator de correção (Cf = Pt / Pp) onde Pt é a média total da quantidade de proteína de todas as amostras e Pp é o conteúdo de proteína em cada tipo de amostra. Para os bifes assados os resultados foram expressos como percentual de inibição contra a formação de bases de Schiff nos bifes marinados em relação ao tratamento controle, sendo calculada como: % de inibição = [100- (T x 100) / C)], onde C é a fluorescência emitida pelas amostras CONTROL e T é a fluorescência emitida por cada das contrapartes tratadas.

3.2.5.3 Ditirosinas

O teor de ditirosina foi medido pelo método relatado por Davies et al. (1987) modificado por Zhang et al. (2017). Um homogenato de carne (1:10 w / v) foi produzido com tampão fosfato de sódio pH 6,0 com ureia a 8 M, e subsequentemente filtrado para remover materiais insolúveis. As amostras foram transferidas para uma cubeta de quartzo de 4 mL com quatro paredes planas (101-QS 10 × 10 mm, Hellma Analytics, Müllheim, Alemanha) e a intensidade de fluorescência foi medida num espectrómetro de Luminescência Perkin-Elmer LS 55 (Beaconsfield, UK) com excitação ajustado em 325 nm e conjunto de emissão em 420 nm e uma largura de 10 nm para excitação e emissão. Os resultados foram expressos como unidades de intensidade de fluorescência emitidas por ditirosinas a 420 nm. Estes valores foram corrigidos de acordo com a concentração de proteína de cada amostra, aplicando um fator de correção (Cf = Pt / Pp) onde Pt é a média total da quantidade de proteína de todas as amostras e Pp é o conteúdo de proteína em cada tipo de amostra. Para os bifes assados os resultados foram expressos como percentual de inibição contra a formação de ditirosinas nos bifes marinados em relação ao tratamento controle, sendo calculada como: % de inibição = [100- (T x 100) / C)], onde C é a fluorescência emitida pelas amostras CONTROL e T é a fluorescência emitida por cada um dos contrapartes tratadas.

3.2.5.4 Determinação da fluorescência de triptofano

A fluorescência natural do triptofano foi avaliada por espectroscopia de fluorescência (ESTÉVEZ et al., 2008b). Os bifes foram moídos e homogeneizados de acordo com o processo descrito por Utrera et al. (2012). Uma alíquota de 1 mL dos homogenatos foi redissolvida em 20 mL do tampão fosfato de sódio 20 mM e depois dispensada em uma célula

espectrofluorométrica de quartzo de 4 mL. Os espectros de emissão de triptofano foram registrados de 300 a 400 nm com o comprimento de onda de excitação estabelecido em 283 nm (espectrômetro de luminescência LS 55 Perkin-Elmer, MA, EUA). As larguras de fenda de excitação e de emissão foram ajustadas a 10 nm e os dados foram recolhidos a 500 nm por min. Os resultados foram corrigidos de acordo com a concentração protéica de cada amostra, aplicando-se um fator de correção previamente reportado e eventualmente expresso em unidades de intensidade de fluorescência. A percentagem de inibição contra a depleção de triptofano pelas marinadas foi calculada como % de inibição = [100- (C x 100) / T)], onde C é a fluorescência emitida pelas amostras CONTROL e T é a fluorescência emitida por cada um dos tratados homólogos.

3.2.6 Análise do Perfil de Voláteis

Um grama de bife marinado, assado e triturado foi dispensado em um frasco de vidro (4 mL) SPME e incubado a 37 °C para o desprendimento e coleta dos voláteis. A amostragem do Headspace (HS) foi realizada seguindo método previamente descrito por Estévez et al. (2003). A fibra de SPME, revestida com um filme de divinilbenzeno-carboxeno-poli(dimetilxilosano) (DVB / CAR / PDMS) de 50/30 µm, foi previamente condicionada a 220 °C durante 45 min; a seguir a fibra de SPME foi exposta durante 30 min ao “headspace” do frasco de SPME, sendo este mantido a uma temperatura de 37 ° C para a captura dos compostos volateis.

Para a separação e identificação dos compostos voláteis a fibra SPME foi transferida para um injetor de um cromatógrafo a gás HP5890GC série II (Hewlett-Packard, EUA) acoplado a um detector seletivo de massa (modelo Agilent 5973). Os voláteis foram separados utilizando uma coluna de 5% de fenil-95% de dimetil-polissiloxano (Restek, EUA) (30 m x 0,25 mm., espessura do filme de 1,0 mm). Hélio foi utilizado como gás de arraste a uma pressão de 18,5 psi, resultando em um fluxo de 1,6 mL / min. A fibra de SPME foi dessorvida e mantida na porta de injecão a 220 °C durante toda a corrida cromatográfica. O programa de temperatura foi isotérmico durante 10 min a 40 °C e depois elevado a uma taxa de 7 °C / min a 250 °C e mantido durante 5 min. A temperatura da linha de transferência de GC / MS foi 270 °C. O espectrômetro de massa operou no modo de impacto de elétrons com uma energia eletrônica de 70 eV, uma tensão multiplicadora de 1650 V e coleta de dados a uma taxa de 1 scan / s em uma faixa de m / z 40–300. Todos os voláteis (exceto ésteres) detectados nos cromatogramas foram identificados positivamente, comparando seus espectros e índices de retenção linear (LRI) com os dos compostos padrão (Sigma-Aldrich, Steinheim, Alemanha). Os ésteres foram

tentativamente identificados comparando seus espectros e índices de retenção linear (LRI) com os da biblioteca Wiley. Áreas cromatográficas de MS são fornecidas como unidades de área (AU).

3.2.7 Análise Sensorial

Os bifes marinados e assados foram avaliadas sensorialmente utilizando-se metodologia de análise descritiva quantitativa (ADQ), por um painel treinado composto por 10 julgadores (funcionários e alunos da Faculdade de Medicina Veterinária de Cáceres, Espanha) que participam frequentemente de avaliações sensoriais de carnes e derivados. Uma ficha, contendo definições e referências de doze atributos agrupados em quatro parâmetros (aparência, odor, sabor e textura), foi utilizada pelos julgadores para validação dos termos (Figura 8). Os bifes marinados foram assados em forno a 200 °C por 5 min (Unox®, Mod. GN2.1, Cadonegue, Itália), resfriados e cortados em retângulos, e avaliados separadamente por tratamento. Três sessões foram realizadas em uma sala de painéis sensoriais com cabines equipadas com luz fluorescente branca. A ordem da amostra foi randomizada seguindo o desenho de quadrado latino de Williams (*Williams Latin Square*). Os bifes assados foram mantidos à temperatura ambiente, posteriormente servidos em placas de vidro, juntos com um copo de água (150 mL) e biscoito sem sal (seguindo o protocolo de enxágue entre as amostras). Os julgadores usaram uma escala quantitativa linear não estruturada, tendo nos extremos os valores de 0 cm com "pouco" a 10 cm "muito", para todos os atributos. Para o atributo de cor foi usada a nomenclatura "claro" para "escuro". O software FIZZ versão 2.20 C - Análise Sensorial e Gerenciamento de Teste de Computador (v. 2.20: Biosystemes, Couternon, França) foi usado para coletar os dados.

Figura 8: Lista de validação dos termos descritores sensoriais para bife bovino marinado em vinho e assado.

Análisis descriptivo cuantitativo en Carne de Ternera Marinada

Estudiante: Narciza Arcanjo

Tabla de definiciones y referencias para atributos na análisis descriptivo cuantitativo en carne de ternera marinada en vino tinto.

	ATRIBUTO	DEFINICIÓN	EXTREMOS/ REFERENCIAS
APARENCIA	Intensidad de Color	Intensidad de color marrón caracteristica de carne de ternera asada, con variacion de el marrón claro al marrón oscuro	Clara: carne de ternera asada a 75°C Oscura: Carne de ternera asada a 200 °C y marinada en vino
	Brillo	cantidad de luz reflejada por el cuerpo en comparación con la cantidad de luz que incide sobre el mismo	Poco: Carne de ternera asada Mucho: Carne de ternera asada con aceite
AROMA/OLOR	Olor carne de ternera assada	Intensidad de aroma característico de la carne bovina asada	Poco: Carne de ternera en agua por 1 hora y asada a 75°C Mucho: Carne de ternera a la plancha con (KNOR)
	Olor a vino	Intensidad de aroma característico de vino tinto.	Poco: 10 mL de vino tinto sin alcohol diluido en 40 mL de agua. Mucho: Vino tinto sin alcohol y calentado en baño (100°C por 20 minutos)
SABOR Y FLAVOR	Sabor acido	Percepcion de la intensidad de sabor acido	Poco: Sumo de limon diluido 1:100 Mucho: Sumo de limon diluido 1:10
	Flavor a carne de ternera asada	Intensidad de sabor característico de la carne asada	Poco: Carne de ternera cocida por 1 hora y asada a 75°C Mucho: Carne de ternera asada con (avecrem KNOR)
	Flavor a vino	Intensidad de sabor característico de vino en la carne asada	Poco: Carne asada Mucho: Carne marinada en vino y asada
	Flavor a rancio	Percepción de alteración de sabor producida por el contacto del aire en las sustancias grasas	Poco: Cacahuetes Mucho: Cacahuetes posto al aire por 3 dias.
TEXTURA EN BOCA	Terneza/dureza	Propiedad de textura que ofrece poca resistencia a la masticación, variando de duro hasta suave	Poco: queso Mucho: Carne de ternera asada 200°C por 10 minutos.
	Jugosidad	Humedad dada por la presencia de jugos en la carne	Poco: Carne de ternera asada 200°C por 10 minutos Mucho: Carne de ternera asada a 200°C por 4 minutos
	Fibrosidad	Percepción debida a la presencia de fibras en los dientes	Poco: Salsicha Mucho: Esparragos blancos
	Masticabilidad	energia requerida para masticar el alimento	Poco:Yogurt Mucho: Pulpo en conserva

Fonte: Arcanjo, 2018.

3.2.8 Análises Microbiológicas

Pesou-se 10 g de cada bife foram homogeneizadas com 90 mL de água de peptona a 1% durante 1 min. com o auxilio de um *Stomacher*. Diluições decimais seriadas foram feitas e inoculadas em meios de cultura adequados para cada microrganismo avaliado. Todas as pesquisas utilizou a técnica de plaqueamento por superfície. 0,1mL de cada amostra diluídas e foram espalhadas com alças de Drigalski em placas de petri contendo ágar deMan, Rogosa e Sharpe (MRS) acidificado para contagem de bactérias láticas. As placas foram incubadas em estufa a 30 °C por 24 h em microaerofilia. Para as bactérias mesófilas o meio de cultura utilizado foi o Ágar Padrão (PCA), onde 0,1 mL das amostras foram espalhadas na superfície da placa e incubadas a 30 °C por 24 horas. O meio Ágar violet red bile glucose **(**VRBG) foi utilizado para a pesquisa de enterobactérias, após um volume de 0,1 mL de amostra ser plaqueada, e incubada a 37 °C por 24 horas. As placas contendo entre 10 e 300 colônias foram contadas e os resultados foram expressos em $\log_{10}$ ufc/g.

3.2.9 Análise Estatística

Os dados obtidos de todas as análises realizadas nos bifes crus e assados foram avaliados por meio de Análise de Variância unidirecional (ANOVA) para avaliar o efeito do tipo de vinho utilizado no processo de marinação. O teste de Tukey foi realizado quando ANOVA revelou diferenças significativas ($P < 0,05$) entre os tratamentos. O nível de significância foi estabelecido em $P < 0,05$. O software SPSS (v. 18.0) foi usado para realizar o teste estatístico. Na análise sensorial teste de normalidade de Shapiro-Wilk foi aplicado para avaliar se dados apresentaram uma distribuição normal.

Para os bifes crus o Modelo Linear Geral com medidas repetidas foi utilizado para analisar os dados de oxidação lipídica (TBARS), oxidação de proteínas (concentração de proteínas carbonilas), reticulação de proteínas, estabilidade da cor da carne e contagens microbianas ao longo do tempo de armazenamento refrigerado. No modelo estatístico misto, a fatia individual de carne bovina foi considerada como efeito aleatório, enquanto o tipo de uva, o tempo de armazenamento e a interação Tratamento × Tempo foram considerados como efeitos fixos.

3.3 EXPERIMENTO 2: AÇÃO REDOX ATIVA DO RESVERATROL NAS PROTEINAS SÉRICAS HUMANAS NA PRESENÇA DE METABÓLITOS TÓXICOS (GLIOXAL E METILGLIOXAL) DO DIABETES

3.3.1 Material

Resveratrol (RES) (3,4 ′, 5-tri-hidroxi-trans-estilbeno), glioxal (GO), metilglioxal (MGO), albumina de soro humano (HSA), cianoboro-hidreto (NaCNBH3), ácido dietilenotriaminopentacético (DTPA), dodecil sulfato de sódio (SDS), ácido 4-aminobenzóico (ABA) e Ácido β-(N-Morfolino) etanosulfônico (MES) monohidratado foram adquiridos da Sigma-Aldrich Co. Ltd. (Steinheim, Alemanha). Di-hidrogenofosfato de sódio (NaH2PO4), hidrogenofosfato dissódico (Na2HPO4), ácido tricloroacético (TCA), acetato de sódio anidro, metanol, acetonitrila, éter dietílico, etanol e ácido clorídrico foram obtidos de Scharlau Labs S.L. (Barcelona, Espanha). A água utilizada foi purificada por passagem atravrés de um sistema Milli-Q (Millipore Corp., Bedford, MA). Todas as soluções foram preparadas no mesmo dia da análise.

3.3.2 Sistema Modelo da Interação Proteína-Resveratrol-AGEs

O estudo envolveu seis sistemas de interação os quais estão descritos na Tabela 1: i) CONTROL incluiu apenas HSA (0,5 mM) como amostras de controle negativo; ii) GO incluiu HSA e glioxal (0,4 mM); iii) MGO incluiu HSA e metilglioxal (0,4 mM); iv) RES incluiu apenas o resveratrol (2,5 µM); v) GO + RES incluiu HSA, glioxal (0,4 mM) e resveratrol (2,5 µM) e, finalmente, vi) MGO + RES incluiu HSA, metilglioxal (0,4 mM) e resveratrol (2,5 µM) Todas as concentrações finais nas suspensões de proteínas e os níveis de reagentes (GO, MGO e RES) simulam a concentração de α-dicarbonil no plasma de pacientes diabéticos (LAPOLLA et al., 2003) e a concentração de RES no plasma humano após consumo de vinho tinto (SMOLIGA et al., 2014). Todas as unidades de reação foram preparadas em triplicata (6 x 3 = 30 no total) e incubadas a 37 ° C por 48 h com agitação constante. As amostras foram coletadas em horários fixos (3, 6, 12 24 e 48h) e posteriormente analisadas quanto à concentração de AAS e GGS, tióis livres, AGEs e perda da fluorescência de triptofano. Todos os sistemas foram armazenados em freezer a -80 °C até o dia das análises.

Tabela 1: Descrição dos sistemas de reação para avaliar a interação resveratrol com proteinas séricas humanas na presença de AGEs

Sistemas	**HSA (mL)**	**Resveratrol (µL)**	**Glioxal (µL)**	**MetilGlioxal (µL)**	**Água (µL)**
Control	4	-	-	-	702
GO	4	-	1,85	-	700
MGO	4	-	-	1,4	700,5
RES	4	700	-	-	1,85
GO+RES	4	700	1,85	-	-
MGO+RES	4	700	-	1,4	0,45

3.3.3 Análise da Afinidade de Ligação entre HSA e Resveratrol

A saturação da fluorescência intrínseca de proteína (triptofano) por resveratrol foi analisada para avaliar a afinidade de ligação do resveratrol de acordo com o procedimento descrito por N 'soukpoé-Kossi et al. (2006). HSA (0,5 mM) foi dissolvida em tampão de fosfato de sódio (pH 7,0; 10 mM). O resveratrol foi dissolvido em água destilada de modo a produzir soluções estoque de 52 µM. Os espectros de fluorescência foram registrados em um espectrômetro de luminescência Perkin Elmer LS 55 (Perkin Elmer, Cambridge, Reino Unido) usando uma cubeta de fluorescência Suprasil de quartzo de 10 mm (Hellma, Alemanha). A fim de quantificar a interação potencial entre resveratrol e HSA, estes últimos foram titulados em cubeta por adições sucessivas de resveratrol (0-45 mM concentrações finais). Os espectros de emissão de fluorescência foram registrados de 300 a 400 nm com excitação a 280 nm. As fendas de excitação e emissão foram ajustadas para 10 nm e a velocidade de varredura foi de 500 nm / min. Todas as experiências foram realizadas a 22 ± 1 °C. A intensidade de fluorescência foi lida com o máximo de emissão de proteína a 337 nm. A constante de taxa de extinção bimolecular (Kq) foi calculada usando a equação 2 Stern-Volmer:

Equação 2: $F0 / F = 1 + Kq\tau0 [Q]$

onde F0 e F são as intensidades de fluorescência das soluções proteicas na ausência e presença do extintor, respectivamente; [Q] é a concentração do extintor, e $\tau0$ ($\approx 5 \times 109$ s) é a vida útil d fluoróforo na ausência do extintor.

3.3.4 Análise da Concentração de ROS (Espécies Reativas de Oxigênio)

Para verificar a potencial formação de ROS nas unidades experimentais, o superóxido foi avaliado em unidades experimentais seguindo o método descrito por Susanto et al. (2006) como se segue. Para cada unidade experimental, foram preparadas três misturas de reacção i) uma com SOD (100 U / mL), ii) outra com DETCA (3 mM) e, finalmente, com NADH (100 mM) e DETCA (3 mM). Os tubos de ensaio foram incubados durante 1 hora a 37º C com agitação regular. O sobrenadante foi misturado com o citocromo c (20 μM) e a absorvância foi imediatamente lida a 550 nm usando um espectrofotômetro Hitachi U-2000. As diferenças de absorvância entre a amostra com ou sem SOD foram utilizadas para calcular a liberação do radical superóxido utilizando o coeficiente de extinção molecular do citocromo c de 21 $mM^{-1}cm^{-1}$.

3.3.5 Análises de Oxidação Proteica

3.3.5.1 Determinação de tiois livres (Grupo Sulfidrila)

A quantificação dos grupos sulfidrila livres (tióis) foi realizada de acordo com o método proposto por Rysman et al. (2014). A absorbância foi medida a 324 nm logo após a reação contra um tampão de ácido cítrico 1,0 M (pH 4,5) adicionado de cloridrato de guanidina 6,0 M e 250 μL da solução de 4-DPS (Apre) e também após 30 min. de reação no escuro com 4-DPS (Apos). Para o branco foi preparado uma mistura de 1,25 mL de tampão de ácido cítrico 1,0 M (pH 4,5) adicionado de cloridrato de guanidina 6,0 M e 250 μL da solução de 4-DPS foi utilizada como branco (Abranco). A absorbância correspondente a concentração de tióis livres foi calculada pela subtração de Apre e Abranco de Apos (Equação 1). A concentração de tiol foi calculada com base numa curva padrão de cinco pontos variando de 2,5 a 500 μM de L-cisteína em GuHCl 6 M em tampão de ácido cítrico 1 M (pH 4,5). O teor de tiol foi expresso como μM.

3.3.5.2 Determinação dos semialdeídos α-aminoadípico (AAS) e γ-glutâmico (GGS)

A determinação do AAS e GGS foi realizada de acordo com o método descrito por Utrera et al. (2011) com modificações. Aliquotas de 500 µL de cada sistema foram misturados com 1,5 mL de ácido tricloroacético (TCA) 10% em tubo eppendorf seguido de agitação. A mistura foi centrifugada (4,0 ºC) durante 5 min. a 5.000 rpm e o sobrenadante foi descartado. Em seguida, foram adicionados aos tubos 0,5 mL de uma solução tampão de ácido 2-(N-Morfolino) etanosulfônico (MES) 0,25 M (pH 6,0), ureia 8,0 M e ácido dietilenotriamina penta-acético (DTPA) 1,0 mM,; 0,5 mL de tampão MES 0,25 M (pH 6,0) + 50 mM de ácido para-aminobenzóico (ABA) e 0,25 mL de tampão MES 0,25 M (pH 6,0) + 0,1 M de cianoborohidreto de sódio. A mistura foi agitada em vortex até completa dissolução dos *pellets*. Após homogeneização, os tubos foram levados para banho-maria a 37 ºC por 90 min. Em seguida, adicionou-se 0,5 mL de TCA 50 % e após 5 min a mistura foi centrifugada a 10.000 rpm por 10 min a 4 ºC e o sobrenadante foi removido. Na fase seguinte, os *pellets*. foram lavadas duas vezes com 1 mL de TCA 10% e 1 mL da solução de etanol:éter etílico (1:1 v/v). Entre cada lavagem, os tubos foram centrifugados a 7000 rpm por 7 min a 4 ºC. Ao final da etapa de lavagem, o solvente foi evaporado com auxílio de gás nitrogênio a baixa pressão. Para a etapa de hidrólise, adicionou-se 1 mL de ácido clorídrico (HCl) 6 N aos tubos, os quais foram vedados e armazenados durante 20 h em estufa a 110 ºC. Em seguida, o conteúdo hidrolisado contido nos eppendorfs foram secos a vácuo em *SpeedVac*TM 47 e reconstituídas com 200 µL de água Mili-Q e filtradas em filtro com membrana de celulose (porosidade 0,45 µm, Pall Corp., New York, USA) antes da injeção no cromatógrafo.

As amostras (1,0 µL) foram injetadas em Cromatografo líquido UFLC ‘Shimadzu Prominence (Shimadzu Corp., Kyoto, Japão) equipado com detector de fluorescência, RF-10A XL . Tampão acetato de sódio (50 mM, pH 5,4) e acetonitrila pura foram utilizados como eluentes, foi utilizado um programa de gradiente, variando a concentração de eluente B de 0 % (min. 0) a 8 % (min. 20). A separação cromatográfica ocorreu a fluxo constante (1 mL/min) e a temperatura da coluna foi mantida constante a 30 °C. Os tempos de onda de excitação e emissão foram estabelecidos em 283 e 350 nm, respectivamente. A identificação dos semialdeídos derivatizados foi realizada por comparação dos tempos de retenção das amostras com um padrão injetado nas mesmas condições anteriormente descritas. Os picos correspondentes a AAS-ABA e GGS-ABA foram integrados manualmente a partir de cromatogramas FLD e as áreas resultantes foram traçadas contra uma curva padrão ABA com

concentrações conhecidas que variaram de 0,1 a 0,5 mM. Os resultados foram expressos como nmol de carbonila por mg de proteína.

3.4.6 Detecção de Resveratrol e Aductos com HESI-Orbitrap-MS

As amostras (48 h de incubação nas condições previamente mencionadas) foram preparadas por diluição (1:40) das suspensões com acetonitrila de grau cromatográfico e metanol (1: 1). Uma infusão direta das amostras foi realizada em um instrumento Orbitrap quadripolar Q-Exativo (Thermo Fisher Scientific, Bremen, Alemanha) usando uma bomba de seringa (11 Plus, Harvard Apparatus, Holliston, MA, EUA) e uma seringa de 500 μL (Hamilton , Reno, NV, EUA) a um fluxo de 5 μL. min^{-1}. As análises foram realizadas usando a fonte Ion Max da Thermo Fisher Scientific operando no modo de detecção HESI de íons negativos e aplicando os seguintes parâmetros: fluxo de gás da bainha 5 unidades arbitrárias e temperatura capilar de 320 °C. O alvo AGC foi definido como $1e^6$ e o tempo máximo de injeção para 50 ms; a tensão de pulverização foi operada a 3,5 kV, e a fragmentação na fonte a 40,0 eV. O nível de RF da lente S foi ajustado para 50,0 e o alcance da varredura para m / z 50-1000 no modo de polaridade iónica negativa. A análise dos dados MS foi realizada utilizando o software Trace Finder da Thermo Fisher Scientific.

3.4.7 Análise de AGEs por Espectroscopia de Fluorescência

O AGEs foram analisados usando um espectrômetro de fluorescência Perkin-Elmer LS-55 (Perkin-Elmer, Beaconsfield, U.K.). Antes da análise, as soluções de proteínas humanas foram diluídas com tampão de fosfato de sódio 100 mM, pH 7,4. Os AGEs foram excitados a 350 nm, e a fluorescência emitida foi registrada de 400 a 500 nm. As fendas de excitação e emissão foram ajustadas para 10 nm e a velocidade de varredura foi de 500 nm / min. Os resultados foram expressos como intensidade de fluorescência (unidades de área).

3.4.8 Análise Estatística

Todas as unidades experimentais foram preparadas em triplicado e cada amostra individual em cada tempo de amostragem foi analisada três vezes para cada medida. O efeito da adição dos reagentes sobre a concentração de tióis livres, proteinas carboniladas e AGEs foi

analisado por uma Análise de Variância (ANOVA). O efeito do tempo de incubação foi avaliado por uma ANOVA de medidas repetidas. O teste de Tukey foi usado para comparações múltiplas dos meios. O nível de significância foi definido em $p < 0,05$.

3.4 EXPERIMENTO 3: **:** ESTUDO DA RESPOSTA AO ESTRESSE OXIDATIVO INDUZIDO PELO H_2O_2 EM *Lactobacillus reuteri* PL503 NA PRESENÇA DE RESVERATROL

3.4.1 Material

Todos os produtos químicos e reagentes utilizados neste estudo foram de grau analítico ACS e adquiridos a Sigma Chemicals (Sigma-Aldrich, Alemanha), Scharlab S.L. (Espanha), Pronadisa (Laboratório Conda, Espanha), Applied Biosystems (EUA), Epicenter (EUA) e Acros Organics (Espanha). O *L. reuteri* PL503 foi isolado a partir de fezes de porco e identificado por 16S rRNA por Ruiz-Moyano et al. (2008).

3.4.2 Delienamento Experimental

A cepa utilizada neste estudo, *L. reuteri* PL503, foi estocada sob congelamento em glicerol a -80 °C. Para preparar culturas de trabalho, esta estirpe foi cultivada consecutivamente duas vezes em caldo MRS (Conda Laboratory) contendo 0,5% de ácido acético a 10% (v / v) a 37 °C durante 24 h. Seis grupos experimentais foram considerados dependendo da adição e concentração de H_2O_2 e resveratrol : 1. CONTROLE (L. reuteri em caldo MRS); 2. H2O2 (L. reuteri em caldo MRS + 0,5 mM H2O2); 3. LRES (L. reuteri em caldo MRS + 20 µM de resveratrol); 4. HRES (L. reuteri em caldo MRS + 100 µM de resveratrol); 5. H2O2-LRES (L. reuteri em caldo MRS + H2O2 0,5 mM + resveratrol 20 µM); 6. H2O2-HRES (L. reuteri em caldo MRS + 0,5 mM de H2O2 + 100 de resveratrol) .

Três repetições foram realizadas para cada tratamento. Tubos experimentais foram inoculados com 100 µl da cultura de *L. reuteri* PL503 em caldo MRS e incubados a 37 ° C por até 24 h em condições microaerofílicas. Amostras das culturas foram coletadas em quatro tempos (12, 16, 20 e 24 h) da inoculação. Para contagem de células viáveis, 100 µL *L. reuteri* PL503 foram espalhados em ágar Man Rogosa Sharpe (MRS) ao mesmo tempo e condições dos tubos experimentais.

Tabela 2: Descrição dos tratamentos com bactérias *L. reuteri* PL503 avaliados

Grupos Experimentais	Volume MRS (µL)	H_2O_2 (µL)	Resveratrol (µL)
Controle (*L. reuteri*)	4900	2,5	0
H_2O_2 (*L. reuteri* + 0,5 mM H2O2)	4900	2,5	0
LRES (*L. reuteri* + 20 µM de resveratrol)	4890	2,5	10
HRES (*L. reuteri* + 100 µM de resveratrol)	4850	2,5	50
H2O2-LRES (*L. reuteri* + H2O2 0,5 mM + resveratrol 20 µM)	4890	2,5	10
H2O2-HRES (*L. reuteri* + 0,5 mM de H2O2 + 100 de resveratrol)	4850	2,5	50

3.4.3 Análise de Expressão Genética

- *Extração do RNA*

A extração do RNA ocorreu utilizando-se Kit MasterPure™ RNA Purification (Epicentre na Illumina Company, Madison, Wisconsin). Para rompimento da célula 1,0 mL de cada amostra foram agitadas e em seguida levadas para centrifugação por 10 min. a 4 °C e 10.000 rpm. Após descarte do sobrenadante adicionou-se 300 µL da solução Tissue and Cell Lysis Solution e Proteinase K (para o preparo da solução diluente utilizou-se para cada amostra a relação de 1,0 µl de Proteinase K a 50 µg/µl em 300 µL de Tissue and Cell Lysis Solution). Em seguida procedeu-se com a incubação em banho a seco a 65 °C por 15 min., seguido de agitação a cada 5 min. A seguir procedeu-se com etapa de precipitação dos ácidos nucleicos totais. Para tanto, adicionou-se 175 µL de MPC Protein – reagente de precipitação, seguida de agitação e centrifugação por 10 min., 4 °C e 10.000 rpm. O sobrenadante foi transferido para um novo eppendorf, ao qual foi adicionado 500 µL de isopropanol com agitação cuidadosa dos eppendorf, por 20-30 vezes, seguido de repouso por, 10 min. à temperatura de -20 °C, e finalmente, centrifugação por 10 min. a 4 °C e 10.000 rpm.

Para remover o DNA contaminante descartou-se o sobrenadante e tratou-se os *pellets* formados com 200 µL de uma solução Dnase e Rnase-Free (Thermo Fisher Scientific, EUA). Os *pellets* foram colocados em banho-maria a 37 °C por 30 min., adicionados de 200 µL de 2XT e homogeneizados em vortex. Alíquotas de 200 µL de MPC Protein Precipitation foi adicionada aos *pellets*, seguida de agitação por 10 segundos, deixando-se os tubos em banho de gelo por 5 min., seguido de centrifugação por 10 min. a 4 °C e 10.000 rpm. O sobrenadante foi

transferido para um novo eppendorf, ao qual foi adicionado 500 µL de isopropanol, seguido de repouso por 10 min. a -20 °C. Uma nova centrifugação por 10 min. a 4 °C e 10.000 rpm foi realizada, tendo-se descartado o sobrenadante. Os *pellets* foram então lavados duplamente com 200 µL de etanol a 70 %, e resuspendidos em 35 µL de TE Buffer. Após descartar o sobrenadante uma lavagem dos *pellets* foi realizada com 200 µL de etanol a 70 %. Após repetir o procedimento da lavagem os *pellets* foram resuspendidos em 35 µL de TE Buffer (10 mM Tris-HCl [pH 7,5], 1 mM EDTA) e mantidos a -80 ° C até realização das análises posteriores. A concentração de RNA (ng / µL) e a pureza (relação A260 / A280) foram determinadas espectrofotometricamente usando uma alíquota de 1,5 µL no Nanodrop 2000 (Thermo Scientific, EUA).

- *Transcrição reversa (DNA_c)*

A síntese de cDNA foi realizada com 500 ng de ARN total e seguindo as instruções do fabricante do kit de Reagente PrimeScript ™ RT (Takara Bio Inc., Japão). O DNAc foi armazenado a -20 °C até novo uso em reações de PCR. Para tanto um volume de , adicionou-se 5 µl do Mix (preparado com as soluções do Kit) e 5 µl da amostra do RNA previamente extraído, com posterior aquecimento a 37 ºC por 15 min. e 85 °C por 5 segundos, em termociclador. Ao final, as amostras foram homogeneizadas e armazenadas em temperatura de -20 ºC até uso posterior em reações de qPCR.

- *PCR em tempo real (RT-PCR)*

As reações de qPCR foram realizadas em um sistema de tempo real ViiA ™ 7 da Applied Biosystems (Applied Biosystems) em placas de reação de 96 poços ópticos MicroAmp, seladas com tampas adesivas ópticas (Applied Biosystems). A tecnologia SYBR Green foi usada. Os sistemas de reação obteve um volume final de 12,5 µL e consistia em 2,5 µl de molde de DNA_c, 6,25 mL de SYBR® Premix Ex Taq ™ (Takara Bio Inc.), 0,625 mL de ROX ™ Referência tintura (Takara Bio Inc.), 2,37 uL de água ultrapura estéril e 300 mM de cada par de iniciadores. Os métodos qPCR para amplificação dos genes UspA, dhaT e 16S rRNA foram conduzidos com as seguintes condições de ciclos térmicos: 1 ciclo 8 min a 95 °C, 40 ciclos a 95 °C por 10 s, 53 °C por 10 s e 72 durante 25 s. Após o ciclo qPCR final, as curvas de fusão foram realizadas aquecendo os produtos a 60-99 °C e os valores das temperaturas de fusão

foram verificados para assegurar a veracidade dos resultados e especificidade dos pares de primers. Os valores do ciclo do limiar (Ct) representam o ciclo de PCR em que ocorreu um aumento da fluorescência, ao longo de um limiar definido, em cada gráfico de amplificação. Todas as amostras foram analisadas em triplicata, incluindo amostra de controle consistindo em adicionar água ultrapura estéril em vez de DNA molde, e as reações de qPCR foram repetidas pelo menos duas vezes. A razão de expressão foi calculada usando o método $2^{-\Delta\Delta}$CT relatado por Livak e Schmittgen (2001). O gene 16S rRNA foi utilizado como controle endógeno para a quantificação relativa da expressão dos genes UspA e dhaT. O grupo experimental controle foi utilizado como calibrador em cada tempo de amostragem.

3.4.4 Análise de Oxidação Proteica

Os estudos da oxidação proteica envolveu a determinação Tióis livres e semialdeídos α-aminoadípico (AAS). Antes de seguir para as determinaçãoes foi necessário realizar um tratamento prévio nos grupos experimentais, com a finalidade de extrair o meio de cultivo, uma vez que o mesmo contém em sua composição extratos de carne. Uma alíquota de 500 µL das amostras foi centrifugada a 10.000 rpm a 4 °C por 10 min. Em seguida, lavou-se duplamente o *pellet* formado com BPS (difosfato de sódio, fosfato dissódico e cloreto de sódio), e com etanol: éter dietílico (1:1 v/v). Entre uma lavagem e outra o sobrenadante foi descartado após centrifugações. Para a análise de tióis o *pellet* foi dissolvido em 1,25 mL do tampão de ácido cítrico ácido cítrico 1,0 M (pH 4,5) adicionado de cloridrato de guanidina 6,0 M.

3.4.4.1 Quantificação do semialdeído α-aminoadípico (AAS)

Previamente foi realizada a síntese do composto padrão do AAS. O N-acetil-L-AAS foi sintetizado a partir da N-acetil-L-lisina utilizando a atividade da lisil oxidase da membrana da casca do ovo seguindo o procedimento descrito por Akagawa et al. (2002a). Resumidamente, 10 mM N-acetil-L-lisina foi incubada com agitação constante com 5 g de membrana da casca de ovo em 50 ml de tampão fosfato de sódio 20 mM, pH 9,0 a 37 °C durante 24 h. A membrana da casca de ovo foi então removida por centrifugação e o pH da solução foi ajustado para 6,0 usando HCl 1M. Os aldeídos resultantes foram ativados redutivamente com 3 mmol de ABA (ácido 4- aminobenzoico) na presença de 4,5 mmol de cianoboro-hidreto de sódio (NaBH3CN) a 37 °C durante 2 h com agitação. Em seguida, os derivados ABA foram hidrolisados em 50

mL de HCl 12 M a 110 °C durante 10 h. Os hidrolisados foram evaporados a 40 °C até secarem. O AAS-ABA resultante foi purificado usando cromatografia em coluna de sílica gel e usando acetato de etilo / ácido acético / água (20: 2: 1, v / v / v) como solvente de eluição. A pureza da solução resultante e a autenticidade dos compostos padrão obtidos após os procedimentos acima mencionados foram verificados usando MS e ^{1}H NMR [18,19].

Para a quantificação do AAS, alíquotas de 500 µL de cada grupo experimental foi misturado com 1,5 mL de ácido tricloroacético (TCA) 10% em tubo eppendorf seguido de agitação. O procedimento foi realizado como descrito no item 3.3.5.2. Padrões (0,1 µL) foram executados e analisados nas mesmas condições. A identificação do semialdeído derivado nos cromatogramas FLD foi realizada comparando o seu tempo de retenção com o do composto padrão. O pico correspondente ao AAS-ABA foi integrado manualmente a partir de cromatogramas FLD e as áreas resultantes foram traçadas contra uma curva padrão ABA com concentrações conhecidas que variaram de 0,1 a 0,5 mM. Os resultados foram expressos como nmol do composto carbonilo por mg de proteína.

3.4.4.2 Análise de tióis proteicos

A quantificação dos grupos sulfidrila livres (tióis) das amostras foi realizada de acordo com o método proposto por Rysman et al. (2014). Todo o procedimento de determinação foi realizado de acordo com item 3.3.5.1. Os resultados foram expressos como µmol de grupos tiol livres por mg de proteína.

3.4.5 Análise Estatística

Os dados da análise (n = 3) foram coletados e submetidos a análises estatísticas. Para avaliar o efeito de diferentes concentrações de resveratrol na presença peróxido de hidrogênio na espressão genética e química da bactéria foi aplicada uma análise de variância (ANOVA) (SPSS v. 15.5). O teste de Tukey foi aplicado quando a ANOVA encontrou diferenças significativas entre os tratamentos. A significância estatística foi estabelecida em $p \leq 0,05$.

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4 RESULTADOS

A apresentação dos resultados obtidos nos três experimentos foi realizada em forma de artigos, de acordo com as normas estabelecidas pelo Programa de Pós Graduação em Ciência e Tecnologia de Alimentos da UFPB (Norma Complementar nº 03/2011).

ARTIGO I: Bioactivities of wine components on marinated beef during aging
Submetido: Food Research International; Fator de Impacto: 3.520

ARTIGO II: Benefits of wine-based marination of strip steaks prior to roasting: inhibition of protein oxidation and impact on sensory properties
Aceito para publicação: Journal of the Science of Food and Agriculture; Fator de Imapcto: 2.379

ARTIGO III: Antioxidant and Pro-Oxidant Actions of Resveratrol on Human Serum Albumin in the Presence of Toxic Diabetes Metabolites: Glyoxal and Methyl-Glyoxal
Publicado: Biochimica et Biophysica Acta - BBA - General Subjects v. 1862, p. 1938-1947, 2018. Fator de Impacto: 4.702

ARTIGO IV: Effects of resveratrol on hydrogen peroxide (H_2O_2) - induced oxidative stress in Lactobacillus reuteri PL503

Bioactivities of wine components on marinated beef during aging

N.M.O. Arcanjo[1], D. Morcuende[2], M.J. Andrade[3], P. Padilla[2,3] , M.S. Madruga[1], M. Estévez[2]♣

[1] Department of Food Engineering, Federal University of Paraiba, Joao Pessoa, CEP 58051-900, PB, Brazil.

[2] IPROCAR Research Institute, TECAL Research Group, University of Extremadura, 10003, Cáceres. Spain.

[3] IPROCAR Research Institute, HISEALI Research Group, University of Extremadura, 10003, Cáceres. Spain.

♣ Corresponding author:
Mario Estévez (mariovet@unex.es)
Tel. +34927257100 (Ext. 51390)
Fax. +34927257110
Department of Animal Production and Food Science, Food Technology,
University of Extremadura, 10003, Spain.

Abstract

The objective of this study was to identify particular wine components as responsible for the bioactivities observed in marinated bovine muscle *Longissimus lumborum* during 7 days of refrigerated storage at 4 ºC. Depending on the grape variety, four marinades were elaborated (300 mL dealcoholized wine/ kg meat): Carbernet (CAB), Tempranillo (TEM) and Isabel (ISA), including a CONTROL group of samples. CAB and TEM, rich in procyanidins, were more effective against lipid oxidation while ISA, rich in hydroxycinnamic acids, protected proteins against oxidation more efficiently. The lower Warner Bratzler shear force values in beef stakes marinated with ISA could be explained by the inhibition of protein cross-linking. Caftaric acid, the most abundant hydroxycinnamic acid in ISA, was tentatively identified as responsible for this relevant bioactivity. The particular phenolic composition of ISA wine and its high content in organic acids, may explain its effects against *Enterobacteriaceae* while sugars may have promoted the growth of lactic-acid bacteria in beef marinated with CAB and TEM.

Keywords: Red wine; tenderization; lipid oxidation; protein oxidation; microbial spoilage

1. INTRODUCTION

Beef is a pricey and high-quality food item. Consumers are increasingly aware of the factors having an influence on the quality and safety of beef.[1] New consumers demand beef from sustainable livestock systems and reject any intervention involving synthetic additives or any non-meat ingredient observed as potentially hazardous while added at legal concentrations.[2] Preserving meat against oxidative deterioration, discoloration and microbial spoilage is the main concern of retailers given that those are the main reasons affecting shelf life of beef.[3,4] The application of extracts from fruits, herbs and plants is of increasing interest given the consumer's demand in so called "natural additives": those naturally present in plant kingdom and to which numerous effects have been ascribed.[2]

These natural antioxidants typically consist of phenolic compounds, with several species of these compounds such as hydroxycinnamic acids (*p*-coumaric acid, caffeic acid and ferulic acid), hydroxybenzoic acids (gallic acid, salicylic acid and gentistic acid), catechins, flavonoids (myricetin, quercetin and malvidin-3-glucoside), and hydroxylated trans-stilbenes (resveratrol), being present in wines. Phenolic compounds are originally synthesized in the skin tissues and seeds of grapes and subsequently transferred to the wine during vinification process. Microorganisms and the oak barrel where aging occurs also contribute to the phenolic composition of wines. [2, 5, 6] Wine phenolics do not only have a direct influence on the sensorial characteristics of wine: certain phenolics naturally present in red wine have been found to protect against cardiovascular diseases, obesity, cancer and aging .[7, 8] In addition, recent scientific evidences suggest that wine polyphenols perform their beneficial effects through interactions with the intestinal microbiota, since they appear to modulate such microorganisms and, at the same time, are metabolized by intestinal bacteria into specific bioavailable metabolites. [6]

Wine marination is a traditional practice made to improve the sensory properties of particular low-value meat cuts and diversify the offer of processed muscle foods. The scientific knowledge on the effect of wine marination on meat quality is, however, scarce. Kargiotou et al. [9] investigated the effect of marination in soy sauce and red wine on the microbial spoilage of raw meat and other quality parameters, including oxidation and texture. Istrati et al. [10] studied the combined effect of wine-

based marination and spices, on the degradation of myofibrillar proteins and meat color stability on bovine muscle. These studies and others [11, 12] concluded that wine-based marination with assorted spices increased meat tenderness without negative effects on the color of the meat, antimicrobial effects and lipid oxidation. The underlying mechanisms of such effects remain unknown as well as the effect of wine-marination on the oxidation of proteins, with this damage having relevant consequences on the texture, the nutritional value and even the health effects of muscle foods.[13,14] Of particular interest are the possible molecular interactions between wine components (polyphenols) and protein components given the well-known adducts formed between grape phenolics and protein residues. Other wine components such as reducing sugars and organic acids may also have an influence on microbial spoilage, oxidative status and sensory properties of wine-marinated beef. Hence, further investigations are needed to settle solid fundamental knowledge on the assorted effects of particular wine bioactive compounds on lipid/protein oxidation, color, texture and microbial spoilage in retailed beef.

The 'Isabel' grape is a cultivar belonging to the species *Vitis labrusca*, a very rustic and highly fertile grape, widespread in Brazil due to its easy adaptation to different climatic conditions and high productivity.[15] 'Isabel' along with other *V. labrusca* grapes are widely used in wine production. According to Arcanjo et al. [16] and Nixford and Hermosín-Gutiérrez [17], wines produced from 'Isabel' grapes display considerably different phenolic composition and antioxidant activities than wines produced with *Vitis vinifera* grapes. It is estimated that, for example, of the total Brazilian wine production 80% was intended for the production of wines with *V. labrusca* grapes. However, the quality of wines from 'Isabel' grape is low and the production of these wines is not allowed in the European Union.[18] Hence, it is of technological interest to study this grape and the corresponding wine as a source of bioactive compounds to enhance the quality of muscle foods.

The objective of this work is to evaluate the effectiveness of red wine produced with 'Isabel' grapes compared to wines made from *V. vinifera* grapes ('Tempranillo' and 'Cabernet Sauvignon'), to protect bovine muscles (*Longissimus lumborum*) against microbial deterioration and oxidation reactions under refrigerated storage. The potential mechanisms of red-wine marination on meat texture are also discussed.

2. MATERIAL AND METHODS

2. 1. Chemicals and raw material

All chemical and reagents used for the work were purchased from Merck KGaA (Darmstadt, Germany). The water used in HPLC analysis was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA, USA).

Beef samples (3 muscles *L. lumborum* from 3 different animals) were acquired from a local slaughterhouse (Cáceres, Spain), transported under refrigeration (< 10 ºC) to the Food Technology Laboratory, of the Faculty of Veterinary, University of Extremadura, Spain, and immediately processed. Three red wines were used for marination purposes. Two monovarietal wines, 'Cabernet Sauvignon' and 'Tempranillo', with Protected Geographical Indication (PGI Tierra de Castilla Wines, "Bronte" from Felix Solis S.L. Winery, Ciudad Real, Spain) and produced by standard wine making technology (13.0 % vol. alcohol), were purchased from a supermarket in Cáceres (Spain). Another monovarietal wine (Quinta do Morgado from Serra Gaúcha, 2016, 10.5 % vol. alcohol, Fante Winery, Flores da Cunha, Brazil) produced from 'Isabel' grape, was obtained from João Pessoa, Brazil. All wines were produced the same year (2016; young wines) and none had maturation in oak barrel. The wines remained in the bottle 12 months before being processed for analysis and subsequent marination (early 2017).

2. 2. Characterization of red wines

2. 2. 1. Total phenolic content

The total phenolic content (TPC) in the red wines was determined by the Folin-Ciocalteau method proposed by Singleton and Rossi [19] with adaptation. A volume of 100 µL of wine extract was mixed with 500 µL of aqueous solution of 10% of Folin-Ciocalteau, and after 2 minutes, with 400 µL of 7.5% sodium carbonate. The mixture was then incubated for 15 minutes in a water bath at 50 °C for the color formation and its absorbance was measured in a U-3900 Hitachi spectrophotometer (Hitachi, Ltd, Tokyo, Japan) at 760 nm against a blank reagent. For quantification of the wine's total phenols, a standard curve (10 to 90 µg/mL) prepared with gallic acid (Sigma-Aldrich, Brazil) was elaborated

and plotted against samples to express results as mg of gallic acid equivalents (GAE) per litre of sample.

2. 2. 2. In vitro antioxidant activity

The DPPH tests (2,2-diphenyl-1-picrylhydrazyl radical reduction) were performed according to Brand-Wiliams et al. [20] with some modifications. Preparation of a solution of DPPH • (0.004% w/v) with a range of between 0.7 and 0.8 absorbance at 515 nm and from which the solution was prepared daily and stored in a refrigerator until the moment of use. An aliquot of 350 µL of the wine extract was transferred to a tube and added with 3150 µL of DPPH solution, stirred, and after 30 minutes the amount of DPPH was recorded in UV-Visible spectrometer (Ultravioleta Microproce 0798U, Quimis, São Paulo, Brazil). The results were expressed as percent inhibition of DPPH radical.

The Trolox equivalent antioxidant capacity (TEAC) -2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical capture (ABTS•+) was determined according to the method described by Re et al. [21]. The sample was prepared in distilled water and reacted with 5 mL of ABTS (7 mM) and 88 µL potassium persulfate 140 mM (final concentration of 2.45 mM), and the mixture was kept to stand at ambient temperature (~22 ºC) for a period of 6 hours in absence of light. Subsequently, the sample absorbance was read at 734 nm. Reading the prepared blank was performed according to the procedure described, without the addition of the sample. A Trolox calibration curve (concentrations ranging from 2.5 to 15 µM) was prepared and plotted against the samples to calculate the percentage (%) of inhibition versus concentration of Trolox.

2. 2. 3. Phenolics profile

Analysis of monomeric phenolics was performed using an HPLC method described by Ibern-Gómez et al. [22] with some modifications. Analytical separation of phenolic compounds was carried out on a Shimadzu “Prominence” HPLC apparatus (Shimadzu Corp., Kyoto, Japan) equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS online degasser, an SIL-20A autosampler, a RF-10A XL fluorescence detector, and a SPD-M20A Diode Array Detector. The column consisted of a reversed-phase Agilent Poroshell 120 SB-C18 column (150×4.6 mm, 2.7 µm particle size) and a guard column (10 × 4.6 mm) filled with the same material. (A) 0.2% aqueous

trifluoroacetic acid and (B) acetonitrile with 0.2 % trifluoroacetic acid were used as eluents. A gradient program was used, varying eluent B concentration from 7 % (min 0) to 7 % (min 5); 5-25 min, 7-18 % B; 25-46 min, 18-34 % B; 46-60 min, 34-65 % B. The flow rate was kept at 0.5 mL/min, and the temperature of the column was maintained constant at 30 °C. Prior to analysis, de-alcoholized wines were filtered using a PVDF 0.45 µm filter (Agilent) and injection volume was 8 µL. On the basis of spectral identification, monomeric phenolics were quantified in five subclasses: hydroxybenzoic acids (as gallic acid equivalents, 280 nm), catechins (expressed as catechin equivalents; fluorescence detection λ_{ex}= 280 nm, λ_{em}= 322 nm), hydroxycinnamic acids (as caffeic acid equivalents, 320 nm), flavonols (as quercetin equivalents, 365 nm) and anthocyanins (as delphinidin chloride equivalents, 520 nm).

Total procyanidin content was quantified by an analytical HPLC method described by Ollé et al. [23] with some modifications. The oligomeric and polymeric procyanidins were depolymerized in the presence of a nucleophilic agent (phloroglucinol) in an acid medium. A volume of 0.5 mL of each de-alcoholized wine was evaporated to dryness under vacuum in a SpeedVac at 35 ºC. The dried pellet was dissolved in 1 mL ethyl acetate, transferred to a 4 mL glass flask and re-dried under nitrogen. The phloroglucinolysis reagent was prepared as follows: a 0.1 M HCl methanol solution was prepared (solution A). Solution B was then prepared by dissolving 500 mg of phloroglucinol in 10 mL of solution A. Finally, solution C was prepared dissolving 100 mg of ascorbic acid in 10 mL of solution B. The reaction started adding 1 mL of solution C to the flasks containing wine pellet. They were vortexed to completely dissolve and stirred in a magnetic stirrer for 30 minutes at 50 ºC. The reaction was stopped by placing the samples in an icebath and by diluting the reaction medium with 1.5 mL of a 40 mM ammonium acetate solution. The final solution was then filtered through a PVDF 0.45µm filter (Agilent) before HPLC analysis. The same chromatographic system and column employed for the identification of monomeric phenolic compounds were used for the identification of phloroglucinolysis products. (A) 0.1% aqueous formic acid (v/v) and (B) acetonitrile with 0.1 % formic acid were used as eluents. A gradient program was used, varying eluent B concentration from 7% (min 0) to 7 % (min 5); 5-24 min, 7-18 % B; 24-45 min, 18-34 % B; 45-55 min, 34-65 %. The flow rate was kept at 0.5 mL/min, and the temperature of the column was maintained constant at 30 °C.

Phloroglucinolysis products were characterized by their UV-vis spectra and retention time relative to external standards. A fluorescence detector (λ_{ex}= 280 nm, λ_{em}= 322 nm) was used simultaneously to improve the identification procedure. Total procyanidins was determined as the sum of the quantified subunits.

2. 2. 4. Sugar and organic acids profile

An aqueous extract of the wines was prepared and used in the determination of sugar and organic acids profile. Twenty-five grams of wine were diluted in 5 mL Mili-Q water, following homogenization and extraction according to Zeppa, Conterno and Gerbi [24] with modification. Aliquots of 20 µL of aqueous extract of each filtrate were used to determine the sugar profile by means of manual injection in a High Performance Liquid Chromatograph (356 LC, Varian), a Rheodyne valve with a 20 µL loop, coupled with an Agilent Hi-Plex Ca column (7.7 x 300 mm, 8 µm), at a temperature of 85 °C, refractive index detector (VARIAN 356), pumping system with high pressure isocratic configuration and processing software GALAXIE Chromatography Data System. The mobile phase used was water, at a flow rate of 0.6 mL/min. The duration of the run was 30 minutes. The quantification of sugars was performed by injecting standard curves of glucose, fructose and sucrose under the same chromatographic conditions.

For the quantification of organic acids in the red wine, 20 µL of the aqueous extract of the filtered sample were injected in a High Performance Liquid Chromatograph (Shimadzu, Japan), equipped with binary solvent system, Rheodyne valve with handle of 20 µL; coupled with an Lichrospher 100 RP18 (250 x 4 mm, 5 mm), at a temperature of 25 ° C, detector by diode array (SPD-M20A), at wavelengths from 210 nm, pumping with high pressure gradient configuration (LC-20AT) and LCsolution versão 1.22 SP1 processing software. The mobile phase used was 0.009M sulfuric acid at a flow rate of 0.7 mL / min. The duration of the run was 30 minutes. The quantification of organic acids was performed by injecting standard curves of acids under the same chromatographic conditions.

192 **2. 3. Preparation of red wines extracts for marination**

193 Ethanol was removed from wines. The process consisted in evaporating 20 % of the wine volume in
194 a Rotary Evaporator (Meidolph, Laborota 4000) at a temperature of 50 °C, using a vacuum pump
195 (Buchi, Vacuum Pump V-700 and Vacuum controller V-800) operating at a pressure of 124 mBar.
196 The de-alcoholized wines were packed in amber bottles and stored under freezing at - 20 °C until
197 the day of marination, for a period not exceeding 60 days.

198 **2. 4. Experimental setting**

199 Initially, beef muscles were freed from external fat, ligaments and tendons. The three muscles were
200 then sliced in 100 g pieces (aprox. 2.5 cm thickness) and randomly distributed into in polystyrene
201 trays (11 × 16 × 4 cm) in groups of four slices. Slices were marinated with 300 mL of de-alcoholized
202 wine per kg of beef, vigorously agitated to guarantee homogenized distribution of marinade and
203 subsequently wrapped with PVC films (oxygen permeability: ~17 cm^3/m^2 day atm; moisture
204 permeability: <5 g/m^2 day; Tecnodur S.L., Valencia, Spain) and kept under refrigeration (4 ± 1 °C)
205 for 48 hours. After 24 hours from the beginning of the marination process, the muscles were turned
206 upside down and allowed to be marinated for additional 24 h. Following marination, the meat
207 samples were removed from the trays and the excess liquid was allowed to drain off on laboratory
208 filter paper. Beef slices were weighed on analytical balance, and placed in similar clean plastic
209 containers and refrigerated (4 ± 2 ° C) for 7 days under fluorescent light simulating retail display
210 (1620 lux, 12 hours light/12 hours darkness). Depending on the type of wine, 4 different group of
211 samples, namely ‘Carbernet’ (CAB), ‘Tempranillo’ (TEM) and ‘Isabel’ (ISA) were elaborated including
212 a CONTROL group of samples treated with distilled water using the same liquid/meat ratio and
213 following the procedure previously described for wine marination. The raw marinated beef samples
214 were analyzed at days 1, 3, 5 and 7 after the marination process. The whole processing was
215 replicated three times in corresponding independent production batches. Hence 9 slices from three
216 different production batches were obtained per treatment. All slices were analyzed in duplicate for
217 all experimental procedures described as follows.
218

2.5. Analysis of marinated beef

2.5.1. Chemical composition, pH, water activity and instrumental color and texture

Moisture and protein contents were determined using official methods.[25] The method of Folch, Lees and Sloane Stanley [26] was used for determining fat content in marinated beef. The water activity was measured on a Lab Master water activity meter (Novasina AG, Neuheimstrasse, Switzerland) where the samples were placed in polyethylene circular capsules and inserted into the equipment, with the reading being performed automatically after a few minutes of the entire tracking sample. pH was determined using a portable pH meter (TESTO 205, Lenzkirch, Germany). The intramuscular pH was evaluated in the bovine muscles at room temperature and the equipment was calibrated with buffer solutions at pH 7.0 and 4.0. Surface color measurements of raw beef muscles were accomplished using a Minolta Chromameter CR-300. The measurements of the color parameters (L * - luminosity, a * - red color, b * - yellow color) were performed in triplicates at random points on the surface of the beef slices. Color measurements were made at room temperature with illuminant D65 and a 0 angle. A numerical total color difference (ΔE) between beef stakes at day 1 and day 7 of storage was calculated as: $\Delta E_{1\text{-}7} = [(L_7\text{-}L_1)^2+(a_7\text{-}a_1)^2+(b_7\text{-}b_1)^2)]^{1/2}$. Warner-Bratzler shear force (WBSF) assessment was performed in a TA XT-2i texture-meter (Stable Microsystems, Godalming, Surrey, UK). Samples (day 7) were prepared in slices of dimensions 2 mm × 30 mm × 15 mm (thickness × length× width). In the analyses, samples were cut with a Warner-Bratzler blade in a direction perpendicular to the muscle fibres. Analyses were performed in triplicate in each processing batch.

2.5.2. Free amino acids (FAA) analysis

Meat (10 g) was homogenized twice with 50 mL deionized water and centrifuged at 5000 g, 4 °C for 10 minutes. Combined supernatants, containing water-soluble nitrogen, were filtered through Whatman No. 1 filter paper. Twenty-five mL of this filtrate were mixed with 25 mL of 20 % trichloroacetic acid (TCA), settled at room temperature for 30 minutes, centrifuged at 5000 g at 4 °C for 10 minutes and filtered through Whatman No. 4 filter paper. An aliquot from the filtrate, containing non-protein nitrogen, was treated with 3 mL of o-phthalaldehyde reagent. Forty mg of o-phthalaldehyde were dissolved in 5 mL ethanol, 25 mL of 0.1 M sodium tetraborate, 0.1 mL of β-

mercaptoethanol and brought to volume (50 mL) with deionized water. The absorbance was read at 340 nm against the o-phthalaldehyde reagent. The FAA content was calculated using tyrosine as standard and expressed as g FAA/100 g muscle. [27]

2.5.3. Thiobarbituric Acid Reactive Substances

The lipid oxidation was determined by the thiobarbituric acid reactive substances (TBARS) method reported by Ganhão, Estévez and Morcuende [28]. Briefly, 2.5 g of samples was homogenized with 7.5 mL of 3.86% perchloric acid and 0.25 mL of 4.2% BHT. The homogenate blended was centrifuged (3500 rpm for 3 minutes) and filtered through Whatman no. 54 filter paper into volumetric flask and washed with perchloric acid. The filtrate was adjusted to 10 mL by adding perchloric acid (3.86%). Next, 2.0 mL of this filtered with 2.0 mL of 0.02 M TBA was brought to the water bath at 90 °C for 30 minutes to develop the color reaction. It was necessary to perform a blank for each sample because of the pigmentation of the wine conferring pink staining to the muscles. The blank procedure for each sample consisted of subjecting the sample to water with the same conditions in the absence of the reactive TBA. Absorbance readings at 532 nm in UV-VIS spectrophotometer (UV-1800, Shimadizu, Tokyo, Japan) and the results from the samples were plotted against a standard curve prepared with known concentrations of tetraethoxypropane (TEP). The results were expressed as milligrams of malonodialdehyde (MDA) per kilogram of meat.

2.5.4. Protein carbonyls

Total protein carbonyls were determined by means of the dinitrophenylhydrazine (DNPH) method described by Ganhão, Morcuende and Estévez [29] with some modifications. 1.0 g of sample was homogenized with 9.0 mL of 20 mM Na_3PO_4 buffer and 0.6 M NaCl pH 6.5 (1:10). From this homogenate, two 150µL aliquots from each sample were placed in corresponding 2 mL Eppendorf tubes: one is treated for the quantification of proteins and the counterpart for the quantification of carbonyls. The proteins were then precipitated by the addition of 1 mL of cold 10% trichloroacetic acid (TCA), followed by centrifugation at 4°C at 600 g for 5 minutes and the supernatants were discarded. For the determination of the carbonyl compounds 1 mL of the 2M HCl solution with 0.2% DNPH was added to the pellets. For determination of proteins 1 mL of 2M HCl was added to the

pellets. After incubation at room temperature for 1 hour, proteins were precipitated again with 1 mL of cold 10 % TCA, followed by centrifugation at 4°C, 1200 g for 10 minutes and washed twice with 1 mL of ethanol/ethyl acetate (1:1 v/v). The pellets were dissolved in 1.5 mL of 20 mM Na_3PO_4 buffer pH 6.5 added with 6 M guanidine hydrochloride. Protein concentration was calculated from absorbance readings at 280 nm using a standard curve of bovine serum albumin (BSA). The amount of carbonyls was expressed in nmoles of carbonyls per mg of protein using a molar extinction coefficient of hydrazones (21.0 nM^{-1} cm^{-1}) with absorbance readings at 370 nm.

2.5.5. Protein cross-linking

Protein cross-linking was assessed by means of determination of Schiff bases fluorescence and quantification of disulphide bonds. The analysis of fluorescent Schiff bases was performed using fluorescence spectroscopy as described by Estévez et al. [30]. Sample homogenates (1:10 w/v) in sodium phosphate buffer pH 6.0 with 8 M urea were obtained using an ultraturrax. After dilution (1:20 v/v), samples were transferred to a 4 mL quartz cuvette with four flat walls (101-QS 10 × 10 mm, Hellma Analytics, Müllheim, Germany). The emission spectrum for the Schiff base was recorded between 400 nm and 500 nm wavelength with excitation set at 350 nm (Perkin-Elmer LS 55 Luminescence spectrometer, Beaconsfield, UK). The excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per minute. The results were expressed as units of fluorescence intensity emitted by Schiff base structures at 450 nm. These values were corrected according to the protein concentration of each sample by applying a correction factor (Cf = Pt/Pp) where Pt is the total average of the amount of protein from all samples and Pp is the content of protein in each type of sample.

Disulphide bonds were analyzed following the methodology of Rysman et al. [31]. 1.0 g of each sample was homogenized with the aid of ultra-turrax with 25 mL of 100 mM tris buffer pH 8.0 added 6M Guanidine Hydrochloride (GuHCl). Homogenates were followed for centrifugation (20 minutes at 1500 g and 4 °C), and the supernatants were filtered (qualitative filter paper, 11 µm particle retention). Three mL of filtrate were subjected to disulphide reduction by addition of 50 µL of 1-octanol and 100 µL of freshly prepared 30 % (w/v) sodium borohydride in 1 M NaOH. After incubation at 50 °C for 30 minutes, an aliquot of 1.35 mL of 6M HCl was added, followed by stirring for 10

minutes. Total thiols were determined with 4,4'-dithiodipyridine (4-DPS) in the reduced filtrates. A volume of 250 µL of the filtrate was mixed with 1.25 mL of 1 M citric acid buffer and pH 4.5 added 6 M GuHCl and 250 µL of 4-DPS solution (4 mM DPS-4 in 12 mM HCl). The absorbance was measured at 324 nm against 1 M citric acid buffer pH 4.5 added with 6 M GuHCl prior to the addition of 4-DPS (A_{pre}) and after 30 minutes of reaction with 4-DPS in the light coat and at room temperature (A_{pos}). A mixture of 1.25 mL of 1 M citric acid buffer pH 4.5 added of 6 M GuHCl and 250 µL of the 4-DPS solution was prepared as a white (A_{blank}). The absorbance corresponding to the thiol concentration was calculated by subtracting A_{pre} and A_{blank} to A_{pos}. The thiol concentration was calculated based on a standard five points curve ranging from 2.5 to 500 µM cysteine in 6M GuHCl in 1 M citric acid buffer (pH 4.5). The disulphide content was calculated as half of the difference between total and free thiols divided by two.

2.5.6. Microbiological analysis

On each sampling day, total viable count (TVC), *Enterobacteriaceae* and lactic acid bacteria (LAB) counts were determined. Ten g of each sample were taken aseptically, transferred to sterile bags and homogenized for 1 minute with 90 mL of 1% peptone water. Serial decimal dilutions were made using the same solution.

For the TVC, 0.1 mL of the appropriate dilutions was poured and spread on petri dishes containing plate count agar (PCA), which were incubated at 30 °C for 24 hours. For *Enterobacteriaceae* and LAB counts, the same procedure was followed using violet red bile glucose agar (VRBG) and Man, Rogosa and Sharpe agar (MRS), respectively. VRBG plates were incubated at 37 °C for 24 hours. For LAB, the inocula were overlaid with about 10 mL of MRS and incubated at 30 °C for 24 hours.

After incubation, typical colonies for each microbial group were enumerated when the number of colonies per plate ranged between 10 and 300 colonies. The obtained counts were then transformed into logarithms of the number of colony forming units (log cfu/g).

2.6. Statistical analysis

The wine-based marination with three different types of grapes (main variable under study) was performed to a three pack of beef slices and the whole processing was made three times (9 beef slices per treatment). Data obtained from analyses at each sampling point were evaluated by one-way Analysis of Variance (ANOVA) to evaluate the effect of grape. Tukey's test was performed when ANOVA revealed significant ($P < 0.05$) differences between treatments. The General Linear Model with repeated measures was used to analyze the data of lipid oxidation (TBARS), protein oxidation (protein carbonyls concentration), protein cross-linking, meat color stability and microbial counts over time of chilled storage. In the mixed statistical model, the individual beef slice was considered as a random effect, while the type of grape, the storage time and the Treatment × Time interaction were considered as fixed effects. The Tukey's test was used for multiple comparisons of the means. The significance level was set at $P < 0.05$. SPSS (v. 18.0) software was used to carry out the statistic test.

3. RESULTS AND DISCUSSION

3.1. Characterization of wines from three different grapes

In order to assess their potential as bioactive components in the preparation of marinades, wines elaborated from three different cultivars ('CAB', 'TEM' and 'ISA') were analyzed for their composition in total phenolics and phenolic, organic acids and sugars profiles (Table 1). The antiradical activities of the wines were also assessed. In order to evaluate the impact of grape variety and minimize the influence of other external factors, grapes and wines harvested and produced the same year and having a similar aging in bottle (< 1 year) were selected for the present experiment. The TPC was higher in 'CAB' and 'TEM' than in the 'ISA' counterpart. The analysis of the phenolic profile revealed that the most remarkable differences were found for specific groups such as procyanidins as the wines made from *V. vinifera* grapes ('TEM' and 'CAB') had three-fold times more concentration of such phenolics than wines elaborated from 'ISA' grapes. 'CAB' had significantly higher amounts of catechins than the other two cultivars. Conversely, the concentration of hydroxycinnamic acids was significantly higher in 'ISA' wine than in the other two types of wine, with caftaric acid being one of

the most abundant. The concentration of anthocyanins, responsible for the bright red color of young wines, was abnormally low in 'TEM' which could be due to a poor preservation of the wine during maturation. Regardless of the assorted number of factors having an impact on the chemical composition of grapes and wines, such as the variety of grapes used, conditions under which they were grown, wine making techniques, maturity, and processing parameters, the samples under study had chemical profiles compatible with those from wines studied in previous works. [2] The composition of wines in phenolic compounds was consistent with the *in vitro* antiradical activity, following the decreasing order: 'TEM' > 'CAB' > 'ISA'. Among wines, 'ISA' appeared to have a higher concentration of organic acids with lactic, succinic and tartaric being the most abundant. Remarkable differences were also found between wines for the sugar profile with 'CAB' showing the highest concentration of the monosaccharides glucose and fructose. The potential impact of these wine components on particular quality traits of marinated beef will be discussed in the corresponding section.

3.2. Chemical composition of marinated beef

Marination in red wine extracts led to a liquid uptake of around 1% on average and no significant differences on this regard were found between treatments. The impact of this treatment on the proximate composition of the muscles was negligible and all of them had statistically similar amounts of moisture (77.5%), total lipids (2.9%), proteins (16.5%) and ashes (1.1%). Wine marination lowered the pH of beef stakes from 5.50 in CONTROL samples to 5.48, 5.32 and 5.13 in 'CAB', 'TEM' and 'ISA', respectively. The lower pH in 'ISA' may have been caused to the uptake of organic acids from this wine variety. The water activity remained unchanged by marination and no significant differences were found between beef samples (water activity=0.98).

3.3. Wine marination against lipid and protein carbonylation

Oxidation is a major threat to the sensory quality and safety of muscle foods and effects to both lipids and proteins.[14, 32] The extent of lipid and protein oxidation was evaluated in the marinated beef muscles by using the TBARS and DNPH methods, respectively. The effect of marination in red wines on TBARS values in raw bovine muscles stored for 7 days at 4 °C is shown in Figure 1A. The

increasing concentration of TBARS in CONTROL samples over storage time reflects intense lipid oxidative degradation likely promoted by the release of pro-oxidants and the collapse of endogenous antioxidant defenses in postmortem muscles and the exposure to fluorescent light during the simulated retail display (photo-oxidation).[3] A strong inhibitory effect against lipid oxidation was observed in marinated beef compared to CONTROL. Compared to the latter, marinated beef samples had significantly lower TBARS values throughout the storage assay. On the seventh day of storage, beef stakes marinated with 'CAB' and 'TEM' presented lower TBARS values than those treated with 'ISA' wine. Taking into consideration that the raw CONTROL samples surpassed at day 7 the TBARS threshold limit for displaying rancid taste, set at 1 mg MDA/kg by Ergezer and Serdaroğlu [33], it is reasonable to consider that rancidity may be perceived in such samples upon culinary treatment. Malondialdehyde and other TBARS are known to display toxicity upon oral intake [32] and hence, the efficient antioxidant effect of wine marination may be regarded as beneficial in terms of both sensory and health aspects. The inhibitory effect on oxidation can be plausibly attributed to the large diversity of phenolic compounds with antioxidant potential detected in the red wines under evaluation. Balasundrama, Sundram and Samman [2] reviewed the antioxidant potential of assorted agricultural products and highlighted wines as a remarkable source of phenolic compounds. Kargiotou et al. [9] treated fresh meat slices by immersion in marinades based on red wine and found that marinated meats showed significantly lower TBARS values than CONTROL samples and meats marinated with soy sauce, attributing these results to the phenolic components of the wine. These effects, in line with our own results prove that an uptake of such bioactive compounds took place within the meat tissues during marination with these phenolics being able to control lipid oxidation likely neutralizing reactive oxygen species (ROS) and binding pro-oxidant meat elements like iron.[2]

Wine marination had also a positive impact on protein carbonylation, yet, these effects were not so remarkable than those previously reported for lipid oxidation (Figure 1A vs Figure 1B). The formation of carbonylic proteins responds to the oxidative deamination of alkaline amino acids (mainly lysine, arginine and proline) in the presence of ROS and transition metals.[34] Recent studies also report the significant contribution of a Maillard-mediated mechanism to the carbonylation of

409 food proteins.[35] This carbonylation pathway may even take place at the concentration of reducing
410 sugars found in postmortem muscle (< 20 mM) [36] and hence, such mechanisms may have been
411 taken place in our samples given that wines contributed considerable amounts of
412 monosaccharides. Regardless of the underlying mechanisms involved, the significant
413 accumulation of protein carbonyls occurred at day 5 and therefore, more slow than lipid oxidation,
414 which is in agreement with previous reports describing protein carbonylation as a late event in the
415 oxidation of muscle foods.[37, 38] In line with TBARS results, wine marination also led to a significant
416 decrease in protein carbonylation as a likely consequence of the presence of phenolic compounds,
417 which has the capacity to protect the proteins against carbonylation. [30, 39] The modest antioxidant
418 protection of wine marinades against protein oxidation as compared to lipid oxidation has been
419 observed in many previous [40-42] and may respond to the complex interaction mechanisms
420 governing the redox consequences of the actions of phenolics on muscle proteins. Likewise, 'ISA'
421 seemed to be less efficient against lipid oxidation at day 7 while displayed the most intense effect
422 against protein oxidation at day 5, which reflects that diverse bioactive compounds display different
423 actions on lipids and proteins. However, all wine marinades display intense antioxidant protection
424 on proteins and this antioxidant effect may also be ascribed as a positive outcome of beef
425 marination since protein carbonyls are linked to the impairment of protein functionality [43], loss of
426 protein digestibility and nutritional value [44, 45] and health risks to consumer upon intake.[14] This is,
427 to our knowledge, the first study to report the protection of wine marinades against meat protein
428 oxidation. Applying antioxidant solutions to intact meat pieces is a technological challenge that
429 may compromise the effectiveness given the limited diffusion of bioactive compounds into the meat
430 core, as suggested by Perricone et al.[46] and Lahmar et al.[42]. Given that oxidative stress may
431 primarily take place on the meat surface, strongly stimulated by molecular oxygen and the pro-
432 oxidant effect of light (~ photooxidation), we may then hypothesize that the antioxidant effects of
433 the wine components on the surface was appropriate to display significant effects in the meat
434 sample.

435

436

3.4. Wine marination against beef discoloration

Lipid oxidation and more recently, protein oxidation, have been reported to occur simultaneously with myoglobin oxidation with the oxidation of heme iron being influenced by the oxidation of other muscle components. [47] Hence, lipid and myoglobin oxidation are commonly studied together and identified as responsible for the discoloration of beef during aging and retail display. Such discoloration took place during the refrigeration of the beef muscles from the present study and manifested as a gradual decrease of L*, a* and b* over the storage of the marinated beef stakes (Figure 2). Wine-based marination not only affected the color displayed by beef samples at day 1, it also influenced the evolution of color parameters during storage. After marination, treated samples tended to be less bright and red than CONTROL samples which can be directly attributed to the wine pigments, namely anthocyanins. The parameter a* (Figure 2B) is the most important for the evaluation of meat color quality, since reduction of red intensity is considered as indicative of oxidation of myoglobin. The calculated total color difference undergone by the beef stakes during cold storage indicate that CONTROL samples ($\Delta E_{1\text{-}7}$= 14.50) suffered the most intense color deterioration followed by beef samples marinated with 'CAB' ($\Delta E_{1\text{-}7}$= 11.82), 'ISA' ($\Delta E_{1\text{-}7}$= 9.76), and 'TEM' ($\Delta E_{1\text{-}7}$= 7.44) ($p < 0.05$). Again, these results are in good agreement with lipid and proteins oxidation measurements and 'TEM', with the most intense *in vitro* antioxidant activity, seemed to better protect beef against discoloration. Hence, myoglobin may have also been benefited from the antioxidant effects of bioactive phenolics from wine extracts. To similar conclusions came Istrati et al. [10] who reported a similar color trend during 14 days of beef aging and confirmed that wine marination inhibited the formation of metmyoglobin and even facilitated the reducing conditions required to convert metmyoglobin into oxymyoglobin during the retail display of beef muscles. Color is considered the most important sensorial characteristic for the consumer at the time of purchase, being the basic criterion for their choice, since it reflects the chemical status and myoglobin content in the muscle.[47] Though the protection of wine phenolics against beef discoloration may have a reflection on the visual appearance of meat, in the absence of a consumer assessment, the impact of red wine marination on consumer's purchasing decision is unknown and may be covered in future studies.

3.5. Wine marination against protein cross-linking

The oxidative reactions occurred during beef maturation includes the formation of protein cross-links through a number of mechanisms.[34, 48] In the present study, we assessed protein aggregation through the analyses of two chemical structures: Schiff bases (Figure 3A) and disulfide bonds (Figure 3B). The fluorescence emitted by Schiff base structures increased regularly over storage, only the 'ISA' treatment at day 5 and both 'ISA' and 'TEM' at day 7 seemed to be able to significantly decrease the formation of Schiff bases as compared to the CONTROL. During the storage period, increases in the concentration of disulphide bonds were also observed, with the marinated beef samples having at the end of the assay significantly lower amounts of such cross-links than the CONTROL counterpart. It is worth noting the correspondence in the evolution of both cross-links during beef storage, although their chemical nature and formation pathways are different and the corresponding analytical methods are based on two completely different approaches (spectrophotometry versus fluorescence spectroscopy). Since both Schiff bases and disulphide bonds are formed in proteins as a result of oxidative stress, it is reasonable to consider that antioxidant components of wine played a role in inhibiting the formation of these cross-links in aged beef. Schiff bases are formed as a result of the reaction between proteins carbonyls and free amino groups from alkaline amino acids (i.e. lysine).[34] Therefore, the protection against protein carbonylation may also have an effect on the subsequent formation of Schiff bases. This makes sense when assessing the particular effectiveness of 'ISA' marination against the formation of protein carbonyls and Schiff bases. This wine variety seemed to be also particularly effective at inhibiting the formation of disulphide bonds in aging beef. 'ISA' samples had the lowest concentration of such cross-links at days 5 and 7, with 'TEM' samples matching the same quantities the last day of storage. Disulphide bonds are the oxidation product of two cysteine residues located at the same polypeptide chain (~intramolecular cross-link) or at different proteins (~intermolecular cross-link). [49] Given the complex composition of wine extracts in bioactive compounds, it seems complicated to identify which components of 'ISA' extract, were responsible for its particular effectiveness against protein cross-linking. We may, however, hypothesized whether hydroxycinnamic acids (i.e. caftaric acid) played a role given the abundance of such

493 phenolics in this grape. In previous studies we have observed other hydroxycinnamic acids such
494 as chlorogenic acid being highly effective against meat protein oxidation [39, 50] but we lack
495 information on the bioactivity of most abundant hydroxycinnamic acid in 'ISA' wine: caftaric acid.
496 A detailed examination of the potential bioactivities of this phenolic compound with special
497 attention to the molecular interactions with food proteins is well-deserved. Protein cross-linking
498 has been associated with the formation of protein aggregates, increased particle size, increased
499 meat toughness and impaired texture properties in muscle foods. This issue will be covered in
500 detailed in the following section.

501 ***3.6. Wine marination and beef tenderness***

502 The marination of meat is a traditional practice to which several effects are alleged, including meat
503 softening and improvement of taste and succulence.[9, 10] Some of these assumptions have been tried
504 to be backed up by scientific studies but contradictory results were found. In particular, Istrati et al.
505 [51] found a tenderization effect of wine-based marinades on beef loin stakes aged for 14 days. These
506 authors also found a more intense proteolytic activity in marinated samples compared to the
507 CONTROL counterparts which may explain the higher softness in treated beef. The authors,
508 however, confessed difficulties to explain the underlying mechanism and considered an effect of the
509 organic acids naturally present in wine. They reported that organic acids are involved in the muscle
510 structure decay, improvement of the cathepsines activity and increase of collagen conversion to
511 gelatin at low pH during cooking. The same authors in a subsequent study [10] found similar effects of
512 wine-based marinades on beef tenderness but the straightforward connection with proteolytic activity
513 was unclear as a qualitative electrophoretic analysis precluded establishing a clear causality
514 connection. To opposite conclusions came Kargiotou et al. [9] who observed increased toughness in
515 beef cuts treated with wine-based marinades. Results from the present study contribute to support
516 the idea of the tenderization effect of wine-based marination of beef stakes but the underlying
517 mechanisms here discussed are different to the aforementioned studies (Figure 4). Only beef
518 samples marinated with 'ISA' wine extract showed a significantly lower WB shear force than
519 CONTROL samples while samples from the other two treatments took intermediate positions. Unlike
520 results obtained by Istrati et al. [51], the present ones cannot be linked to the extent of proteolysis as

the concentration of FAA in the beef samples increased during aging (from 0.9 to 2.1 mg/g sample, in average) and no significant differences were found between group of samples. While differences in protein degradation may have explained differences in tenderization, differences in protein aggregation would explain differences in meat toughness and those were certainly found in the present study. The inhibition of protein cross-linking in beef stakes marinated with 'ISA' wine extracts may have a manifestation in the lower WB shear force values.While the meat tenderization mechanism with acid marinades as proposed by Istrati et al. [51] is not completely known, this may not be ruled out in the present study as 'ISA' wine extracts were also the richest in such organic acids. Previous studies reported that the formation of Schiff bases and disulfide bonds impair meat texture, compromising meat tenderness and increasing hardness in processed muscle foods. [13, 29]

3.7. Wine marination and microbial counts

The behavior of TVC, *Enterobacteriaceae* and LAB in the marinated beef during storage is shown in Figure 5. TVC increased during the whole storage for all treatments. All the marinades were efficient in inhibiting TVC growth on beef ($p < 0.05$). The most positive effect of the marination process with wine on TVC was observed at day 5, when a significant reduction of up to 1.4 log cfu/g was detected in the marinated batches in regard to the CONTROL one. Nevertheless, all samples, especially the CONTROL ones, should be considered microbiologically unacceptable after 5 days because of TVC higher than 7 log cfu/g. [52] These TVC levels could be due to the high TVC initial level in all groups of samples. As expected, the growth of *Enterobacteriaceae* and LAB was lower than that of TVC. *Enterobacteriaceae*, a hygiene indicator and also part of the meat microbial population (Figure 5B), showed initial levels higher than 3.0 log cfu/g, indicating that the steaks could have been processed under inadequate hygiene conditions.[53] It is remarkable that a relevant reduction of *Enterobacteriaceae* levels was observed in all treatments after 3 days of storage. At days 3 and 5, *Enterobacteriaceae* counts were significantly lower in the 'ISA' treatment compared to the other group of samples. Despite the fact that LAB counts showed an increase during refrigeration, the behavior of this microbial group diverged from that of the other analyzed microorganisms at day 7. In particular, higher LAB counts were found in the 'CAB' and 'TEM' samples than in the 'ISA' and CONTROL groups at the end of the refrigeration, indicating that such marinated procedures were

549 more favorable to the growth of LAB. The highest concentration of sugars in the *V. vinifera* wines
550 (particularly in 'CAB' samples) could have stimulated the growth of LAB. Furthermore, it has to be
551 taken into account that LAB are the most resistant bacteria within the group of Gram-positive to the
552 action of antimicrobial compounds.[54] This could be related to their ability to tolerate conditions of
553 osmotic stress and to respond effectively to the outflow of K + caused by many antimicrobial
554 compounds. [55]

555 Some authors have reported the effectiveness of wine marinades as inhibitors of microbial growth
556 in refrigerated beef. [9,10] While the mechanism of action of phytochemicals as antimicrobial needs
557 further clarification, profuse scientific literature has reported the antimicrobial activity of phenolic
558 compounds.[55, 56] In this study 'ISA' wine extract was the most effective against microbial spoilage,
559 which may be attributed to the specific phenolic components in this wine extract or to the higher
560 concentration of organic acids in these samples that may have lowered the pH (Table 1).
561 Hydroxycinnamic acids, particularly abundant in 'ISA' wine extracts (Table 1), have long been known
562 as effective antimicrobial compounds.[57] Caftaric acid, already identified as a discriminating bioactive
563 compound between wine extracts, has recently been identified as a relevant contributor to the
564 antibacterial activity of Argentinian red wines. [58] The high microbial counts at the beginning of
565 marination could have had an important influence on the slight antimicrobial effect detected during
566 cold storage. To similar conclusions came Fernandes et al. [59] after evaluating the antimicrobial
567 effects of oregano extract on sheep burgers during refrigerated storage.

568 **CONCLUSIONS**

569 The data presented in this study showed diverse bioactivities of wine marinades on aging meat,
570 including improved tenderization, protection against oxidative damage and discoloration and
571 inhibition of bacterial growth. An attempt to identify particular wine components responsible for each
572 of these effects was made and caftaric acid, an abundant hydroxycinnamic acid in Isabel wine,
573 deserves particular attention as its molecular interaction with meat proteins is hypothesized to play
574 a role in its bioactivity. From a practical point of view, the present data support the technological
575 benefit of using wine extracts to marinade beef cuts. In particular, Isabel grapes and its low-quality
576 wine may be used with success as a functional ingredient in muscle foods.

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FIGURE CAPTIONS

FIGURE 1. Concentration of TBARS (A) and protein carbonyls (B) (means ± standard deviation) in bovine *m. Longissimus lumborum* muscle during refrigerated storage (4 ºC/7 days).

Different letters at the same sampling time denote significant differences ($p < 0.05$) between treatments. Ns: no significant differences.

FIGURE 2. Evolution of lightness (A), redness (B), and yellowness (C) (means ± standard deviation) in bovine *m. Longissimus lumborum* muscle during refrigerated storage (4 ºC/7 days).

Different letters at the same sampling time denote significant differences ($p < 0.05$) between treatments. Ns: no significant differences.

FIGURE 3. Formation of cross-links measured as fluorescent Schiff Bases (A) and disulphide bonds (B) (means ± standard deviation) in bovine *m. Longissimus lumborum* muscle during refrigerated storage (4 ºC/7 days).

Different letters at the same sampling time denote significant differences ($p < 0.05$) between treatments. Ns: no significant differences.

FIGURE 4. Warner-Bratzler shear force measured in bovine *Longissimus lumborum* muscle after refrigerated storage (4 ºC/7 days).

Different letters denote significant differences ($p < 0.05$) between treatments.

FIGURE 5. Evolution of microbial counts during storage (4 ºC/7 days) of marinated bovine *Longissimus lumborum* muscle. (A) Total Viable Count (B) *Enterobacteriaceae (C) Lactic Acid Bacteria*

Different letters at the same sampling time denote significant differences ($p < 0.05$) between treatments. Ns: no significant differences. *: Counts above the enumeration limit of the plating method.

TABLES

TABLE 1. Chemical characterization of red wines used for marination.

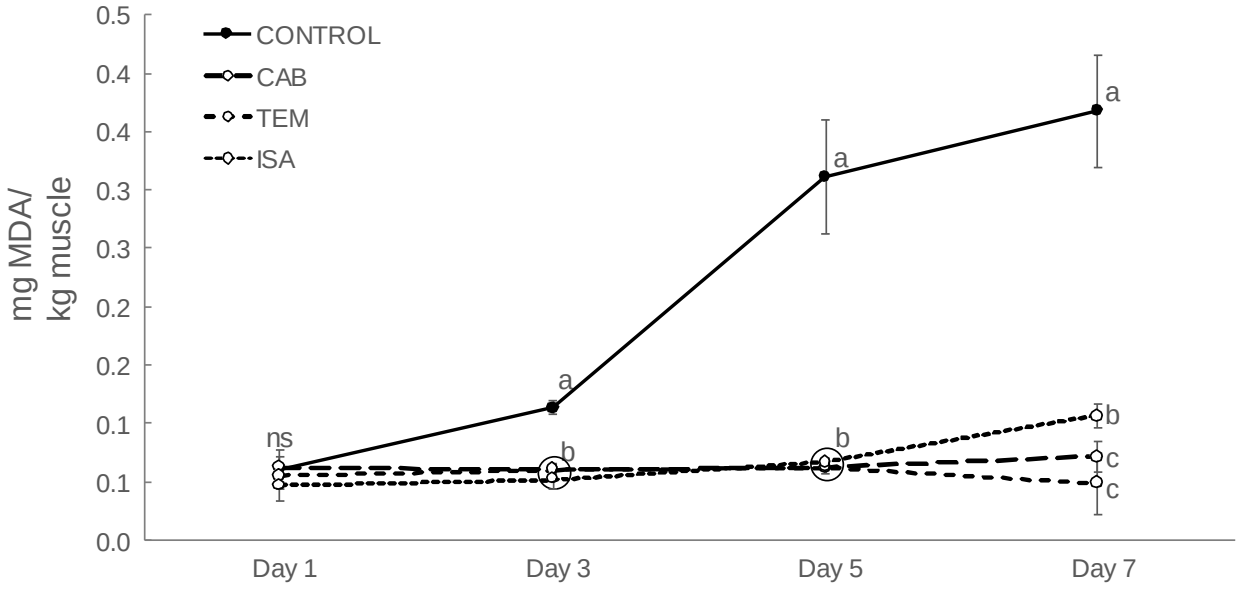
	Cabernet	**Tempranillo**	**Isabel**
Total phenolics mg GAE/100 mL	453.14^{a} ± 2.03	479.15^{a} ± 10.35	307.86^{b} ± 9.02
Antioxidant Activity %Inhibition			
ABTS	54.01^{b} ± 0.92	67.91^{a} ± 1.71	46.14^{c} ± 0.95
DPPH	57.17^{b} ± 0.98	68.99^{a} ± 1.23	46.98^{c} ± 1.20
Phenolics profile mg/L			
Hydroxycinnamic acids	48.62^{c} ± 4.17	54.70^{b} ± 0.74	94.51^{a} ± 0.20
Hydroxybenzoic acids	15.48^{b} ± 0.44	18.99^{a} ± 0.82	18.04^{a} ± 0.04
Catechins	50.95^{a} ± 0.34	21.67^{c} ± 0.23	26.34^{b} ± 0.07
Flavonols	23.84^{a} ± 0.57	12.16^{b} ± 2.31	1.65^{c} ± 0.07
Procyanidins	435.66^{a} ± 7.21	474.64^{a} ± 6.89	137.94^{b} ± 13.97
Anthocyanins	82.66^{a} ± 0.26	10.70^{c} ± 0.11	44.96^{b} ± 0.12
Organic acid profile mg/mL			
Tartaric acid	52.89^{a} ± 1.30	38.30^{b} ± 2.14	59.02^{a} ± 2.73
Lactic acid	116.17^{b} ± 2.65	39.33^{c} ± 5.42	137.69^{a} ± 2.37
Acetic acid	94.67 ± 5.39	93.45 ± 2.67	ND*
Citric acid	4.72^{c} ± 0.29	7.97^{b} ± 0.42	17.77^{a} ± 0.75
Succinic acid	22.46^{b} ± 0.65	4.0^{c} ± 0.39	75.88^{a} ± 2,04
Propionic acid	ND	46.77^{a} ± 0.43	44.93^{b} ± 0.55
Sugar Profile g/100 mL			
Sucrose	1.13^{b} ±0.03	2.51^{a}± 0.15	2.51^{a} ± 0.06
Glucose	5.55^{a} ± 0.36	2.73^{b} ± 0.24	2.63^{b} ± 0.23
Fructose	5.63^{a} ± 0.66	2.55^{b} ± 0.12	3.02^{b} ± 0.09

* Compound not detected.

Mean ± standard deviation followed by the same letters in the same rows do not differ at 5% according to Tukey's test.

FIGURE 1

(A)


CONTROL
CAB
TEM
ISA
mg MDA/ kg muscle
0.5
0.4
0.4
0.3
0.3
0.2
0.2
0.1
0.1
0.0
Day 1
Day 3
Day 5
Day 7
ns
a
b
a
b
a
b
c
c

(B)

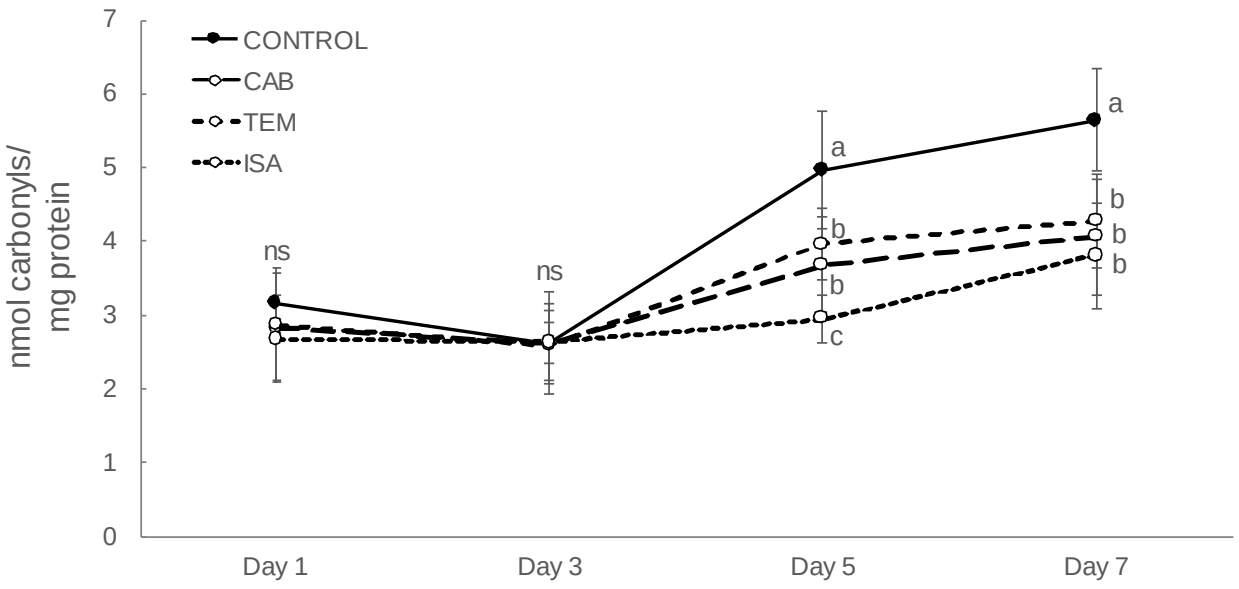

CONTROL
CAB
TEM
ISA
nmol carbonyls/ mg protein
7
6
5
4
3
2
1
0
Day 1
Day 3
Day 5
Day 7
ns
ns
a
b
b
b
a
b
b
c

FIGURE 2

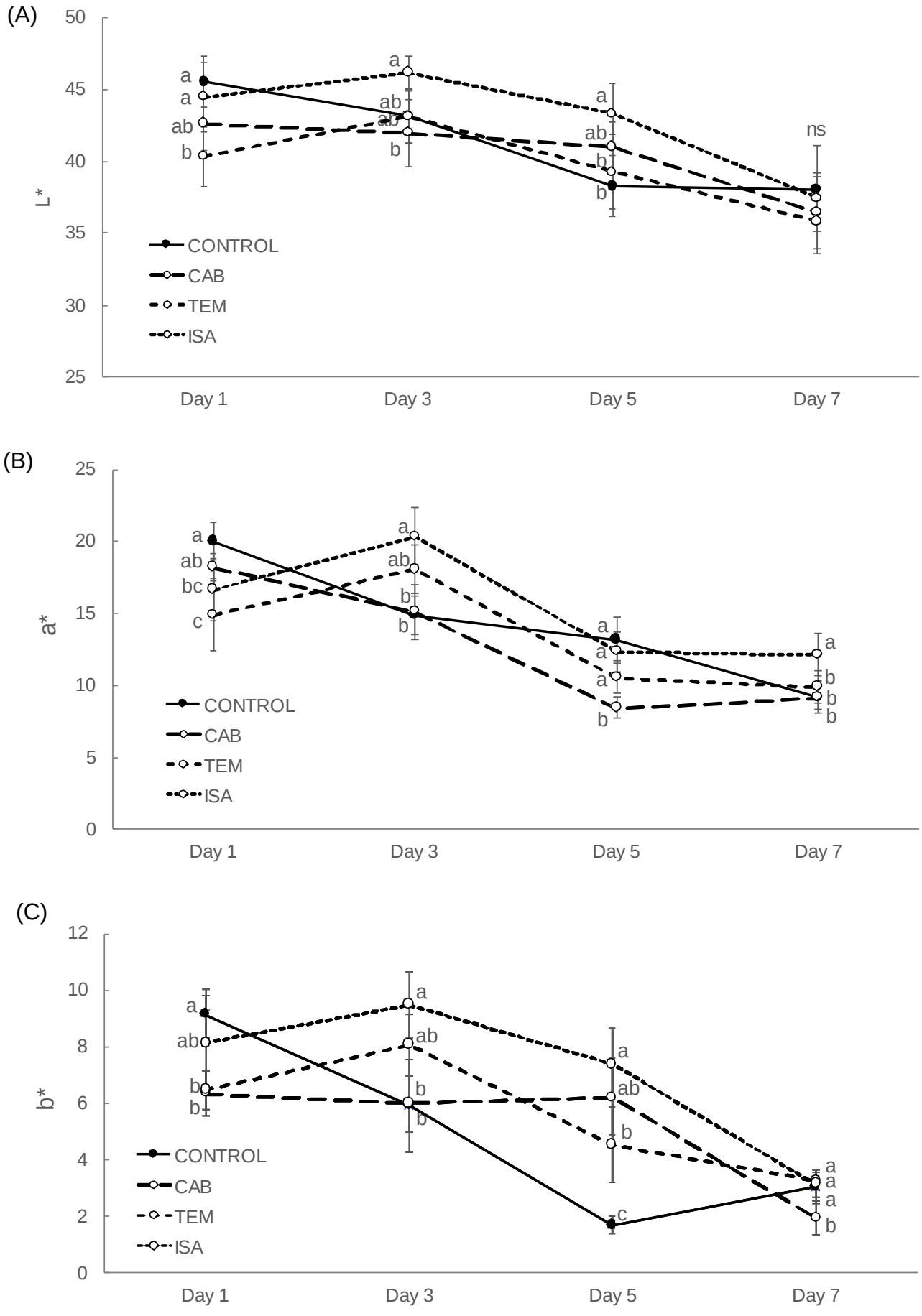

(A)
L*
50
45
40
35
30
25
Day 1
Day 3
Day 5
Day 7
CONTROL
CAB
TEM
ISA
ns
(B)
a*
25
20
15
10
5
0
(C)
b*
12
10
8
6
4
2
0

FIGURE 3

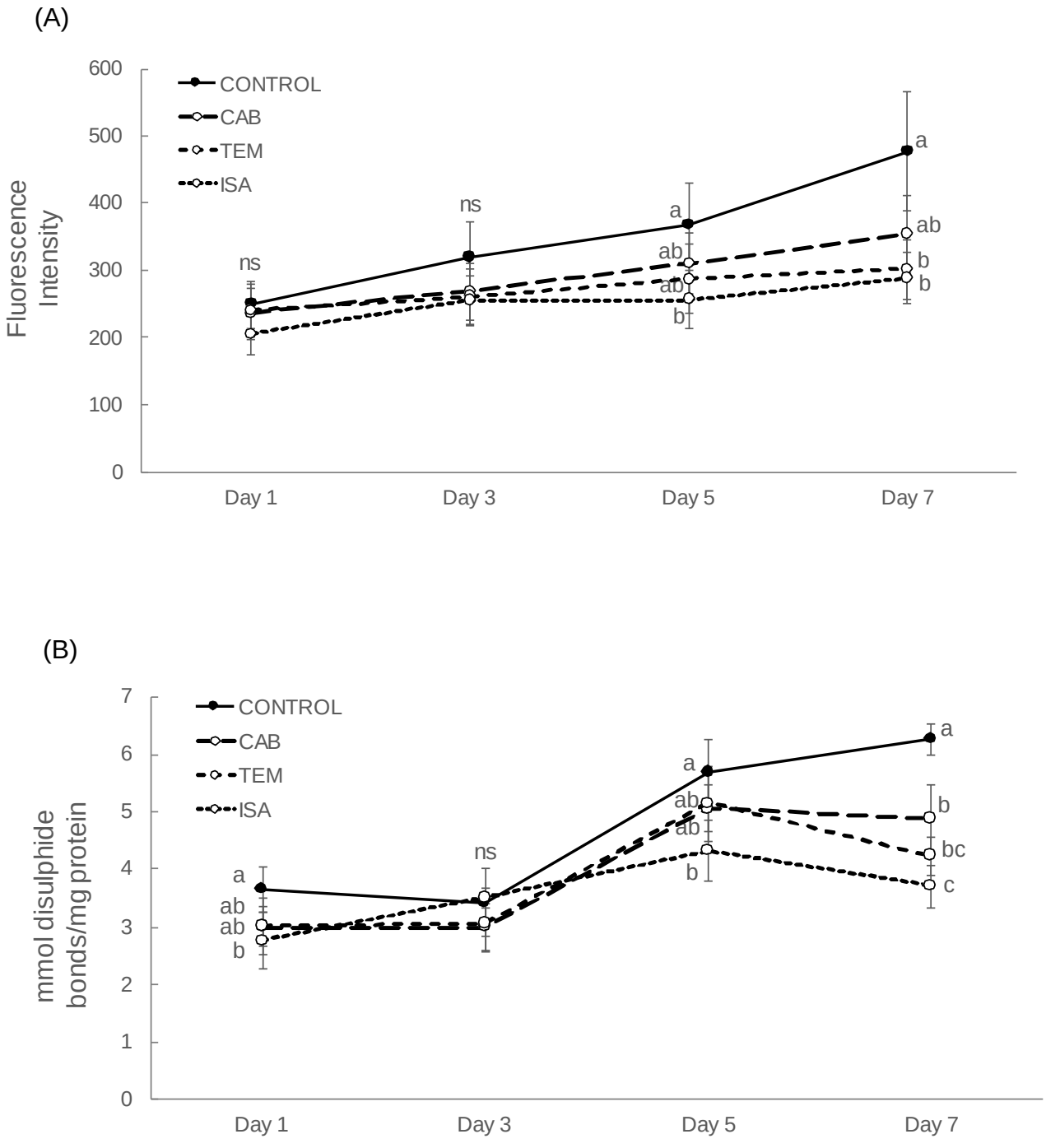

(A)
Fluorescence Intensity
600
500
400
300
200
100
0
CONTROL
CAB
TEM
ISA
ns
ns
a
ab
ab
b
a
ab
b
b
Day 1
Day 3
Day 5
Day 7
(B)
mmol disulphide bonds/mg protein
7
6
5
4
3
2
1
0
CONTROL
CAB
TEM
ISA
a
ab
ab
b
ns
a
ab
ab
b
a
b
bc
c
Day 1
Day 3
Day 5
Day 7

FIGURE 4

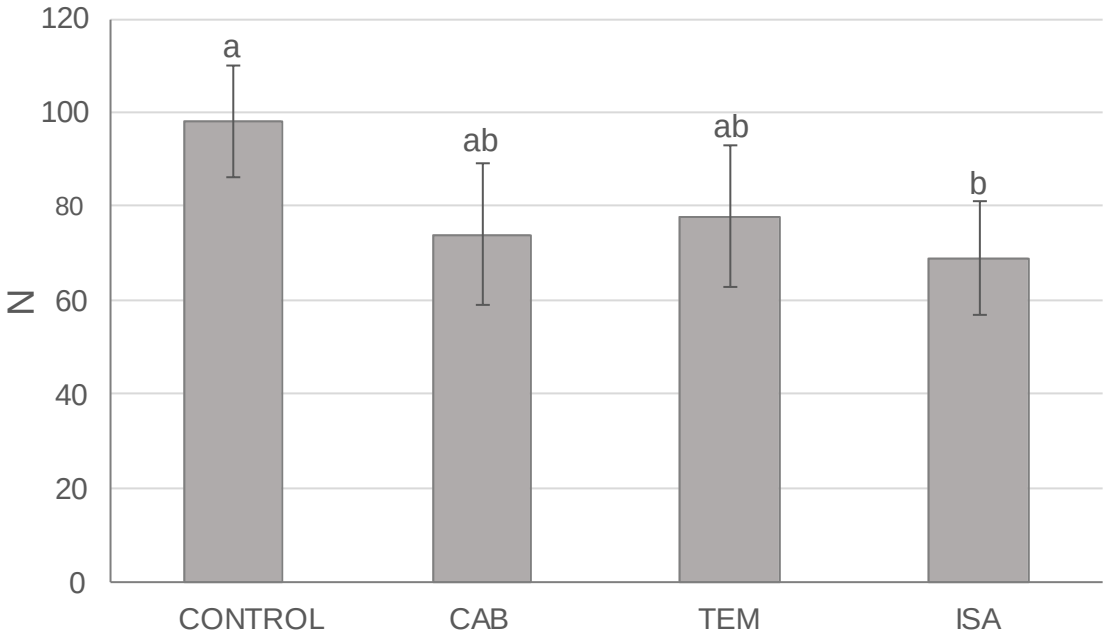
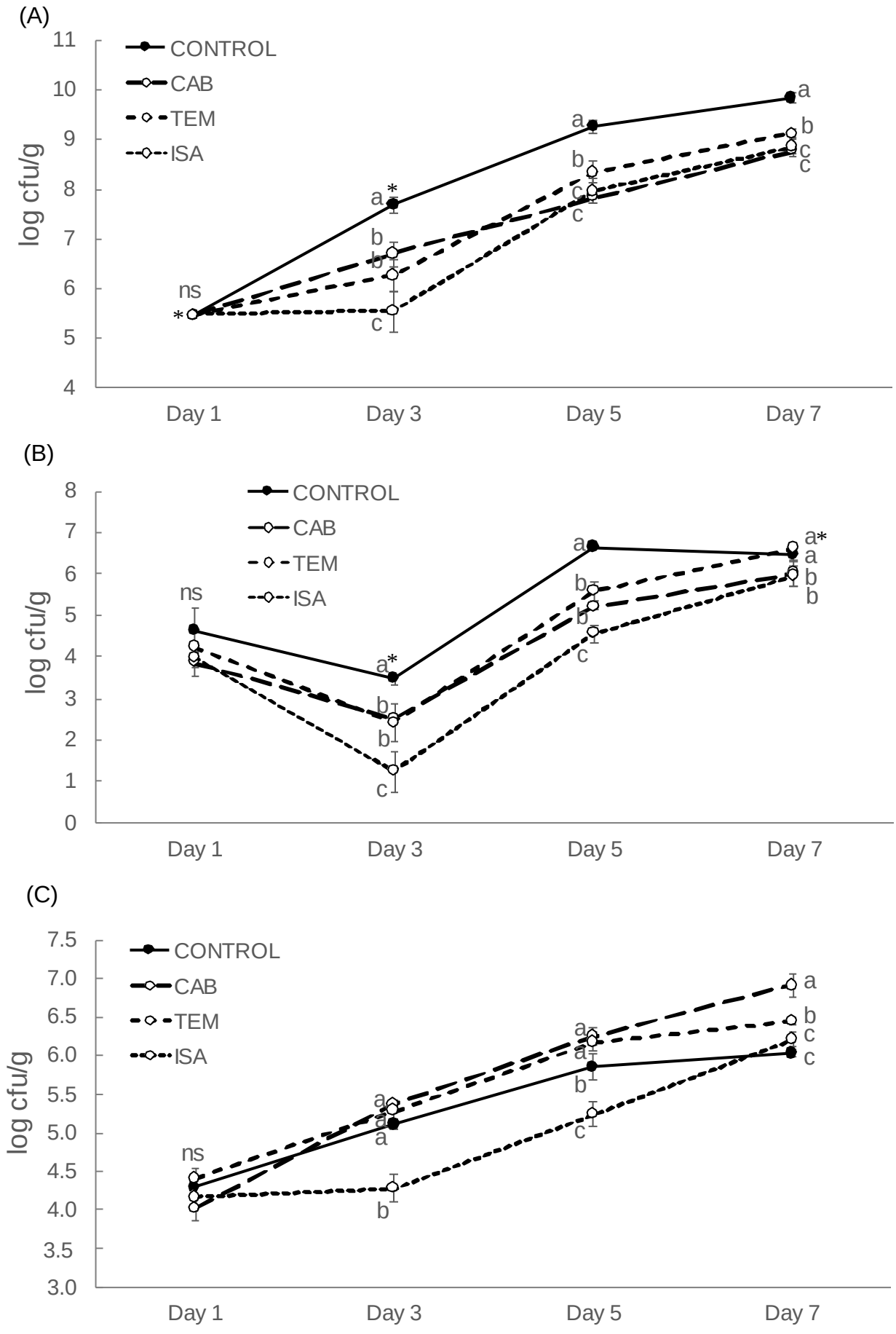


FIGURE 5


(A)
log cfu/g
CONTROL
CAB
TEM
ISA
Day 1
Day 3
Day 5
Day 7
(B)
log cfu/g
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CAB
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ISA
Day 1
Day 3
Day 5
Day 7
(C)
log cfu/g
CONTROL
CAB
TEM
ISA
Day 1
Day 3
Day 5
Day 7

Benefits of wine-based marination of strip steaks prior to roasting: inhibition of protein oxidation and impact on sensory properties

N.M.O. Arcanjo[1], S. Ventanas[2], A. González-Mohíno[2], M.S. Madruga[1], M. Estévez[2]

[1] *Department of Food Engineering, Federal University of Paraiba, Joao Pessoa, CEP 58051-900, PB, Brazil.*

[2] *IPROCAR Research Institute, TECAL Research Group, University of Extremadura, 10003, Cáceres. Spain.*

Corresponding author:
Mario Estévez (mariovet@unex.es)
Tel. +34927257100 (Ext. 51390)
Fax. +34927257110
Department of Animal Production and Food Science, Food Technology, University of Extremadura, 10003, Spain.

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Abstract

The objective of this study was to evaluate the impact of red wine-based marination on the oxidative stability and overall quality of roasted beef strip steaks. Four treatments were considered, according to the type of wine (300 mL dealcoholized wine/ kg meat): 'Cabernet sauvignon' (CAB), 'Tempranillo' (TEM) and 'Isabel' (ISA) including a CONTROL. The formation of potentially harmful protein oxidation products during roasting, including protein carbonyls and dityrosines, was inhibited by bioactive components of the wine. 'ISA' marinades were particularly resistant to protein oxidation which could be due the particular composition of this wine in phenolic compounds. Wine-based marination was also effective in controlling the formation of lipid-derived volatile compounds such as hexanal, octane-2,5dione and heptan-2-one which led to a reduced perception of rancidity by panelists. Additionally, wines contributed to spicing roasted beef with wine-derived flavors from esters, alcohols and lactones. Hence, marination may be a feasible means to alleviate the potential negative effects that oxidative reactions cause to meat proteins, improve beef quality and diversify beef cuts into a variety of safer and more flavored meat products. Among wines, 'ISA' appeared to be most promising in terms of antioxidant protection while the limited consumer's acceptance of steaks treated with this wine may be regarded as a drawback to be sorted out in future studies.

Keywords: Red wine; beef quality; protein oxidation; volatile compounds; sensory evaluation

1. INTRODUCTION

Nowadays, consumer perception on beef quality is not only dependent on sensory attributes: safety and health concerns have recently become critical issues. [1] In regards to sensory attributes, texture and flavor are traditionally regarded as essential quality traits of beef. [2] Lately, however, consumers of red meat are particularly concerned on the potential impact of beef consumption on their health. This concern is supported by the reports from the WHO linking red meat consumption with cancer and the scientific evidences which provide strength to the alarm from health authorities.[3-5] While epidemiological studies establish this relationship, the chemical threat is not identified: the potential carcinogenic compounds naturally present in red meat are indefinite. There are some suspicions on heme iron and its ability to promote lipid and protein oxidation and formation of nitroso compounds which may eventually induce noxious DNA mutations.[5-7] It is worth noting that some of these compounds are formed during digestion and the combination of foods in the diet influence the pool, nature and potential toxicity of species generated during digestion, and ultimately exposed to the colonic mucosa.[3, 8] A relatively new study [9] identified certain tyrosine and tryptophan oxidation products as discriminating metabolites during digestion of red meat *vs* white meat, suggesting that these species may play a still unclear role in the pathogenesis of these disorders. A recent review and several original articles emphasized the role of assorted protein oxidation products, including protein carbonyls, oxidized tryptophan and dityrosines, on various health disorders. [8, 10-11]

While the intake of pro-oxidant species entails harmful effects, the administration of dietary phytochemicals with antioxidant activity (including wine) have been found to reduce the generation of potentially toxic compounds in gastrointestinal models.[12] For this reason, among others, food scientists propose formulating muscle foods with plant phytochemicals and the examples in literature are countless. [13-16] Wine marination may have an influence on sensory properties (texture, flavor and colour) [17, 18] but also on the chemical stability and formation of potentially toxic compounds, but both and particularly the latter has been scarcely studied. The potential effects of wine marination on beef quality and safety would be determined by on their composition in bioactive compounds and that is, in turn, dependent on the grape variety .[19] 'Isabel' along with other *Vitis labrusca*, grapes are widely used in wine production. [20] Only in Brazil, 80% of the wine production is made from *V. labrusca* grapes. However, the quality of wines from this variety is low and the production of 'Isabel' wines is not allowed in the European Union. [21] Hence, it is of technological interest and economically viable to study this grape and the corresponding wine as a source of bioactive compounds to enhance the quality of muscle foods. This study was designed to evaluate the impact of marination with wines made from three different varieties (Cabernet sauvignon, Tempranillo and Isabel) on the extent of lipid and protein oxidation, volatiles profile and sensory properties of roasted beef strip steaks. Special attention was paid to modifications in meat proteins that may lead to loss of nutritional value and potential harmful effects on the consumer: protein carbonyls, tryptophan oxidation and dityrosines.

2. MATERIAL AND METHODS

2.1 Chemicals and raw material

All chemical and reagents used for the work were purchased from Merck (Darmstadt, Germany) or Sigma Chemicals (Sigma–Aldrich, Steinheim, Germany). The water used in HPLC and UPLC analysis was purified by passage through a Milli-Q system (Millipore Corp., Bedford,MA, USA). Beef samples (3 muscles *Longissimus lumborum* from 3 different animals) were acquired from a local slaughterhouse (Cáceres, Spain), transported under refrigeration (< 7 ºC) to the Food
Technology Laboratory of Institute of Meat and Meat Products (IPROCAR) at the University of Extremadura (Spain) and immediately processed. Three red wines were used for marination purposes. Two wines (Bronte, wine from Tierra Castilla, 2016,13.0% vol. alcohol, Féliz Soles S.L. Winery, Ciudad Real, Spain), produced with 'Cabernet Sauvignon' and 'Tempranillo' grapes, were purchased from a supermarket in Cáceres (Spain). Another wine (Quinta do Morgado from Serra Gaúcha, 2016, 10.5% vol. alcohol, Fante Winery, Flores da Cunha, Brazil) produced from 'Isabel' grape, was obtained from João Pessoa, Brazil. All wines were produced the same year (2016; young wines). The wines remained in the bottle 12 months before being processed for analysis and subsequent marination (early 2017).

2.2 Preparation and characterization of red wines extracts

Ethanol was removed from wines. The process consisted in evaporating 20 % of the wine volume in a Rotary Evaporator (Meidolph, Laborota 4000) at a temperature of 50°C, using a vacuum pump (Buchi, Vacuum Pump V-700 and Vacuum controller V-800) operating at a pressure of 124 mBar. The de-alcoholized wines were packed in amber bottles and stored under freezing at - 20°C until the

day of marination (less than 2 weeks). The total phenolic content (TPC) in the red wines was determined by the Folin-Ciocalteau method proposed by Singleton and Rossi. [22] The DPPH tests (2,2-diphenyl-1-picrylhydrazyl radical reduction) were performed according to Brand-Wiliams et al.[23] The Trolox equivalent antioxidant capacity (TEAC) - 2,2-azino-bis(3-ethylbenzothiazoline-6sulfonic acid) radical capture ($ABTS^{\bullet+}$) was determined according to the method described by Re et al.[24]

The quantification of organic acids and sugars was analyzed by High Performance Liquid Chromatography (HPLC). An aqueous extract of the wines was prepared and used in the determination of sugar and organic acids profile. Twenty-five grams of wine were diluted in 5 mL Mili-Q water, following homogenization. Aliquots of 20 µL of aqueous extract of each filtrate were used to determine the sugar profile by means of manual injection in a High Performance Liquid Chromatograph (356 LC, Varian), a Rheodyne valve with a 20 µL loop, coupled with an Agilent HiPlex Ca column (7.7 x 300 mm, 8 µm), at a temperature of 85 °C, refractive index detector (VARIAN 356), pumping system with high pressure isocratic configuration and processing software GALAXIE Chromatography Data System. The mobile phase used was water, at a flow rate of 0.6 mL/min. The duration of the run was 30 minutes. The quantification of sugars was performed by injecting standard curves of glucose, fructose and sucrose under the same chromatographic conditions. For the quantification of organic acids in the red wine, 20 µL of the aqueous extract of the filtered sample were injected in a High Performance Liquid Chromatograph (VARIAN, Waters 2690, California, USA), equipped with binary solvent system, Rheodyne valve with handle of 20 µL; coupled with an Agilent Hi-Plex H column (7.7 x 300 mm, 8 µm), at a temperature of 65 °C, detector by diode array (VARIAN 330), at wavelengths from 220 to 275 nm, pumping with high pressure gradient configuration (VARIAN 230) and GALAXIE Chromatography Data System processing software. The mobile phase used was 0.009M sulfuric acid at a flow rate of 0.7 mL/min. The duration of the run was 30 minutes. The quantification of organic acids was performed by injecting standard curves of tartaric, malic, succinic, lactic and acetic acids under the same chromatographic conditions.

2.3 Experimental setting

Beef muscles were freed from external fat and connective tissue, sliced in 100 g pieces (aprox. 2.5 cm thickness) and randomly distributed into in polystyrene trays (11 × 16 × 4 cm) in groups of four slices. Beef slices were marinated with 300 mL of de-alcoholized wine per kg of beef, vigorously agitated to guarantee homogenized distribution of marinade, subsequently wrapped with PVC films (oxygen permeability: ~17 cm^3/m^2 day atm; moisture permeability: <5 g/m^2 day; Tecnodur S.L., Valencia, Spain) and kept under refrigeration (4 ± 1 °C) for 48 hours. After 24 hours from the beginning of the marination process, the muscles were turned upside down and allowed to be marinated for additional 24 hours. Following marination, the meat samples were removed from the trays and the excess liquid was allowed to drain off on laboratory filter paper. Depending on the type of wine, 4 different group of samples, namely ‘Carbernet sauvignon’ (CAB), ‘Tempranillo’ (TEM) and ‘Isabel’ (ISA) were elaborated including a CONTROL group of samples treated with distilled water using the same liquid/meat ratio and following the procedure previously described for wine marination. All samples were roasted in an oven (Unox®, Mod. GN2.1, Cadonegue, Italy) until an internal temperature of 200 °C was reached for a period of 5 minutes (2.5 minutes for each side of

the steaks). Roasted samples were allowed to cool down at room temperature (22°C) and immediately analyzed. The whole processing was made three times and all experimental procedures were carried out in triplicate, totalizing nine beef slices per treatment.

2.4 Analysis of roasted marinated beef

2.4.1. Chemical composition, pH, water activity, instrumental colour and texture

Moisture and protein contents were determined using official methods. [26] The method of Folch, Lees and Sloane Stanley [27] was used for determining fat content in marinated beef. The water activity (a_w) was measured on a Lab Master water activity meter (Novasina AG, Neuheimstrasse, Switzerland). The intramuscular pH was determined using a portable pH meter (TESTO 205, Lenzkirch, Germany) at room temperature (22°C). Surface colour measurements were performed in triplicates at random points on the surface of the roasted beef slices using a Minolta Chromameter CR-300 at room temperature (22°C) with illuminant D65 and a 0 angle. Warner– Bratzler shear force (WBSF) assessment was performed in a TA XT-2i texture-meter (Stable Microsystems, Godalming, Surrey, UK). Samples were prepared in slices of dimensions 2 mm × 30 mm × 15 mm (thickness×length×width). In the analyses, samples were cut with a Warner–Bratzler blade in a direction perpendicular to the muscle fibres. Analyses were performed in triplicate in each processing batch.

2.4.2. Cooking loss

Samples were weighed before and after cooking, and the cooking loss (CL) percentage was calculated as follows:

CL = (Initial weight−final weigh)/Initial weight x 100.

2.4.3. Tryptophan depletion

The natural fluorescence of tryptophan was assessed by using fluorescence spectroscopy. [28] Meat samples were ground and homogenized according to the process described by Utrera, et al. [29] One mL aliquot of the homogenates was diluted (1:20) in 20 mL of 100 mM Na_3PO_4 buffer pH 6.0 with 8 M urea and then dispensed in a 4 mL quartz spectrofluorometer cell. Emission spectra of tryptophan were recorded from 300 to 400 nm with the excitation wavelength established at 283 nm (LS 55 Perkin-Elmer luminescence spectrometer, MA, USA). Excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per minute. These values were corrected according to the protein concentration of each sample by applying a correction factor (Cf = Pt/Pp) where Pt is the total average of the amount of protein from all samples and Pp is the content of protein in each type of sample. The percent inhibition against the tryptophan depletion by each of the marinades was calculated as % inhibition = [100-(C x 100)/T)], where C is the tryptophan in the CONTROL samples and T is the tryptophan emitted in each of the treated counterparts.

2.4.4 Protein carbonyls

Total protein carbonyls were determined by means of the dinitrophenylhydrazine (DNPH) method described by Ganhão, Morcuende, and Estévez, [30] with some modifications. A homogenate with 1.0 g of sample was pre-capped with 9.0 mL of 20 mM Na_3PO_4 buffer and 0.6 M NaCl pH 6.5 (1:10). From this homogenate, 150µL aliquots from each sample were precipitated by the addition of 1 mL of cold 10 % trichloroacetic acid (TCA), followed by centrifugation at 4 °C at 600 g for 5 minutes and the supernatants were discarded. For the determination of the carbonyl compounds 1 mL of the 2 M HCl solution with 0.2 % DNPH was added to the pellets. For determination of proteins, 1 mL of 2 M HCl was added to the pellets. After incubation at room temperature for 1 h, proteins were precipitated again with 1 mL of cold 10% TCA, followed by centrifugation at 4 °C, 1200 g for 10 min and washed twice with 1 mL of ethanol/ethyl acetate (1: 1 v/v). The pellets were dissolved in 1.5 mL of 20 mM Na_3PO_4 buffer pH 6.5 added with 6M guanidine hydrochloride. Protein concentration was calculated from absorbance readings at 280 nm using a standard curve of bovine serum albumin (BSA). The amount of carbonyls was expressed in nmoles of carbonyls per mg of protein using a molar extinction coefficient of hydrazones (21.0 nM^{-1} cm^{-1}) with absorbance readings at 370 nm. The percent inhibition against the gain of protein carbonyls by each of the marinades was calculated as % inhibition = [100-(T x 100)/C)], where C is the concentration of protein carbonyls in the CONTROL samples and T is the concentration of protein carbonyls in each of the treated counterparts.

2.4.5. Dityrosines

The dityrosine content was measured by the method reported by Davies, Delsignore and Lin [31] as modified by Zhang et al. [32] A meat homogenate (1:10 w/v) was produced with 100 mM Na_3PO_4 buffer pH 6.0 with 8 M urea, and subsequently filtered through gauze to remove insoluble materials. Samples were transferred to a 4 mL quartz cuvette with four flat walls (101-QS 10×10 mm, Hellma Analytics, Müllheim, Germany) and fluorescent intensity was measured on a Perkin-Elmer LS 55 Luminescence spectrometer (Beaconsfield, UK) with excitation set at 325 nm and emission set at 420 nm and a slit width of 10 nm for both excitation and emission. The results were expressed as units of fluorescence intensity emitted by dityrosines at 420 nm. The results were corrected according to the protein concentration of each sample and the percent inhibition against the formation of dityrosines by each of the marinades was calculated as previously reported for carbonyls.

2.4.6. Protein cross-links

Protein cross-linking was assessed by means of determination of Schiff bases fluorescence. The analysis of fluorescent Schiff bases was performed using fluorescence spectroscopy as described by Estévez et al.[28] Sample homogenates (1:10 w/v) in 100 mM Na_3PO_4 buffer pH 6.0 with 8 M urea were obtained using an ultraturrax. After dilution, (1:20 v/v) samples were transferred to a 4 mL quartz cuvette with four flat walls (101-QS 10 × 10 mm, Hellma Analytics, Müllheim, Germany). The emission spectrum for the Schiff base was recorded between 400 nm and 500 nm wavelength with excitation set at 350 nm (Perkin-Elmer LS 55 Luminescence spectrometer, Beaconsfield, UK). The excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per

minute. The results were expressed as units of fluorescence intensity emitted by Schiff base structures at 450 nm. The results were corrected according to the protein concentration of each sample and the percent inhibition against the formation of dityrosines by each of the marinades was calculated as previously reported for carbonyls and dityrosines.

2.4.7. Volatiles profile

One gram of minced sample was dispensed in a 4 mL SPME glass vial. The Headspace (HS) sampling was performed following a method previously described. [33] The SPME fiber, coated with divinylbenzene-carboxen-poly (dimethylxilosane) (DVB/CAR/PDMS) 50/30 µm, was preconditioned prior analysis at 220 °C during 45 min. The SPME fiber was exposed during 30 min to the headspace of the vial while incubated in an oven at 37°C. The SPME was then transferred to a HP5890GC series II gas chromatograph (Hewlett–Packard, USA) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m 0.25 mm id., 1.0 mm film thickness). GC-MS conditiond were published elsewhere.[33] All volatiles (except esters) detected in the chromatograms were positively identified by comparing their spectra and linear retention indexes (LRI) with those from standard compounds (Sigma–Aldrich, Steinheim, Germany). Esters were tentatively identified by comparing their spectra and linear retention indexes (LRI) with those from Wiley library. Chromatographic areas from MS are provided as area units (AU).

a

2.4.8 Sensory profile

Roasted beef samples were evaluated using a quantitative-descriptive analysis (QDA) method by a trained panel composed of 10 people (staff and students of the Faculty of Veterinary Medicine of Cáceres, Spain, who frequently participate in sensorial evaluations of meat and meat products). Twelve attributes grouped in appearance, odor, flavor and texture were evaluated. Upon roasting and letting the samples stand at room temperature for 1 min, beef strip steaks were cut into rectangles (1 cm thickness) and each sample was evaluated separately. A total of 3 sessions were performed in a sensory panel room with booths equipped with white fluorescent light. The order of the sample was randomized following the William Latin Square design. The steaks were served on glass plates along with a glass of water (150 mL) and unsalted biscuit to follow the protocol of rinsing between the samples. The evaluators used a 10 cm linear unstructured quantitative scale with "little" to "much" extremes for all attributes, except for color attribute ("light" to "dark"; in that case). The FIZZ software 2.20 C version- Sensory Analysis and Computer Test Management (v. 2.20: Biosystemes, Couternon, France) was used to collect the data.

2.5 Statistical analysis

The wine-based marination with three different types of grapes (main variable under study) was performed to a three pack of beef slices and the whole processing was made three times (9 beef slices per treatment). The normality of the sensory analysis data were verified by the Shapiro-Wilk. Data obtained from analyses at each sampling point were evaluated by one-way Analysis of Variance (ANOVA), to evaluate the effect of grape. Tukey's test was performed when ANOVA revealed significant ($p< 0.05$) differences between treatments. The significance level was set at $p< 0.05$. SPSS (v. 18.0) software was used to carry out the statistic test.

3. RESULTS AND DISCUSSION

3.1 Chemical composition and antiradical properties of wines

Wines under study ('CAB', 'TEM' and 'ISA') were analyzed for their composition in phenolics, organic acids and sugars and antiradical properties (Table 1). The total phenolic content was higher in 'CAB' and 'TEM' than in the 'ISA' counterpart. The analysis of the phenolic profile revealed that the most remarkable differences were found for specific groups such as procyanidins (more abundant in 'TEM' and 'CAB' than in 'ISA' grapes), catechins ('CAB' had significantly higher amounts of than the other two cultivars) and hydroxycinnamic acids (significantly higher in 'ISA' wine than in the other two cultivars). The composition of wines in phenolic compounds was consistent with the *in vitro* antiradical activity, following the decreasing order: 'TEM' > 'CAB' > 'ISA'. Among wines, 'ISA' appeared to have a higher concentration of organic acids with lactic, succinic and tartaric being the most abundant. Remarkable differences were also found between wines for the sugar profile with 'CAB' showing the highest concentration of the monosaccharides glucose and fructose. Regardless of the assorted number of factors having an impact on the chemical composition of grapes and wines, such as the variety of grapes used, conditions under which they were grown, wine making techniques, maturity, and processing parameters, the samples under study had chemical profiles compatible with those from wines studied in previous works.[25] The potential impact of these wine components on particular quality traits of marinated beef will be discussed in the corresponding section.

3.2 Physico-chemical properties of roasted strip steak as affected by marination

The wine-based marination process did not affect the chemical composition or the water activity of the steaks (Table 2). Only significant differences were observed in the color values (L*, a* and b*) and the pH. The treatments 'TEM' and 'ISA' led to steaks with a significantly lower pH value compared to the 'CAB' and CONTROL counterparts. The analysis of the organic acids of the wines revealed that 'ISA' had the highest concentrations of lactic, citric and succinic acids and that the wine 'TEM' had the highest concentration of acetic and propionic acids (data not shown). While the pH of the roasted steaks was influenced by the contribution of organic acids by the corresponding wines, their color was clearly affected by the wine pigments, anthocyanins.[34] The results from this study show that marinated steaks were darker and less yellow than the CONTROL steaks. Only steaks marinated with 'TEM' had a less intense red color than the other three types of samples. Previous studies evaluated the impact of the wine marination on the color displayed by beef muscles during retail display. [17,18] However, those studies did not consider an eventual cooking procedure so the present results are unprecedented in this regard. Besides the color, the texture of cooked beef is an essential quality trait for consumers of red meat.[1] Previous studies observed a

tenderization effect of wine marination on aging beef. [17, 18] This tenderization effect was not found in the cooked samples from the present study as roasted steaks from the four groups presented similar WBSF values. It is hence, plausible that the mechanisms implicated in such tenderization are not relevant in cooked samples. The aforementioned studies attributed the tenderization effect of wine marination to: i) an enhancement of the proteolysis in aged meat by organic acids from wines and ii) an inhibition of the strengthening caused by oxidation-mediated protein cross-linking by wine polyphenols. Apparently, the impact of high temperatures on meat, namely, the dehydration process and the denaturation and coagulation of meat proteins, neutralized the potential tenderization effects of wine components and equalized all steaks in terms of toughness. The cooking loss in the steaks varied from 36.24 - 37.15%, with these values being in the range of that reported in the study by Yancey, Wharton and Apple. [35] The impact of wine marination on cooking loss was negligible which partly explains the similar water content and texture properties between treatments.

3.3 Protein oxidation in roasted strip steaks as affected by marination

The oxidation of proteins in cooked meat has become a topic of scientific and technological interest owing to its influence on food quality and consumer's safety.[36, 37] The influence of wine marination of lipid oxidation has already been studied and expected antioxidant effects were found.[18] Conversely, the present study investigated the effect of wine-based marination on protein oxidation. Diverse expressions of the oxidative damage to meat proteins were measured including tryptophan depletion and the formation of protein carbonyls, dityrosines and protein crosslinks (Figure 1).

Tryptophan is an essential amino acid with relevant biological functions and its oxidation leads to the formation of kynurenine derivatives.[38] Tryptophan is highly sensitive to oxidation and several studies agree that between 30 and 60% of the tryptophan concentration in raw meat can be oxidized as affected by the cooking method.[29, 39] Wine marination inhibited tryptophan depletion in roasted strip steaks. The percent inhibition depended on the type of wine and followed the decreasing order 'ISA'> 'TEM'> 'CAB'. 'ISA' wine, in particular, protected almost 70% of the tryptophan oxidized in the CONTROL samples. This antioxidant effect may be attributed to the elevated concentration of phenolic acids and polyphenols in the wines under study. Utrera and Estévez[40] showed the effectiveness of assorted phytochemicals as inhibitors of tryptophan oxidation *in vitro*. The protection of tryptophan may not only be regarded as a beneficial effect in terms of nutritional value. The oxidation products of tryptophan (kynurenines, not quantified in the present study) have been found to be implicated in impaired physiological functions in target tissues such as brain and the gut. [8, 41]

Alkaline amino acids (lysine, arginine and proline) are also very susceptible to radical species. [42] The formation of carbonyl compounds has been described as one of the utmost salient alterations in oxidized proteins.[43] These compounds are formed in cooked meats as a result of the oxidative deamination of the aforementioned amino acids in the presence of transition metals such as iron.[36,42]

A significant effect of the wine-based marinades was observed against the formation of protein carbonyls in roasted strip steaks (p <0.05). Once again, this antioxidant effect could be ascribed to the radical scavenging activity of wine phenolics. In this particular case, 'ISA' appeared as the most effective inhibitor of protein carbonylation. The phenolic composition of this wine, which is under current examination, may be responsible for this effective protection on meat proteins. It is worth noting that the ability of wine components to protect proteins against carbonylation is more limited than that displayed by the same wines on the other expressions of protein oxidation. Though limited, this protective effect is relevant given that the formation of protein carbonyls involves the loss of essential amino acids and a potential health threat in accordance to the review by Estévez and Luna.[8] In this regard, the formation of dityrosines is of particular interest given the profuse recent scientific evidences of the pathological conditions induced by this protein oxidation product in experimental animals upon oral administration. According to Yang et al. [11] the intake of dityrosines impairs assorted systemic metabolic processes, facilitates fat accumulation in liver, induces hepatic and renal injury, affects the gut microbiota function and increase the risk factor for cardiovascular diseases. Ding et al. [44] observed that dietary dityrosines disrupt pancreas functionality leading to glucose metabolism disorder. Furthermore, as commented in the introduction, dityrosines and kynurenines were identified as candidates to explain the implication of red meat consumption on colorectal cancer at a molecular level. Wine marination effectively reduced the formation of dityrosines in roasted beef with 'ISA' being more effective than 'TEM' which was, in turn, more effective than 'CAB'. Taking into account the above mentioned arguments, wine marination seems to be a feasible way to decrease the formation of dityrosines in cooked beef up to a 50% as compared to conventional (non-marinated) beef.

The formation of cross-links seemed to be inhibited by wine marination (around 30%) but, unlike the other measurements of protein oxidation, no significant differences were found between types of wine. The fluorescent structures assessed in the present study can be attributed to the formation of Schiff Bases [45] which consist of the reaction between protein carbonyls and amino groups. However, cross-links may be formed in proteins by other mechanisms, via the oxidation of two cysteines leading to the formation of disulfide bonds and the above mentioned dityrosines.[38] The benefits of the inhibition of Schiff base formation may also be emphasized given the role of these structures in the functionality and digestibility of meat proteins.[37, 45-46] Considering the fact that this is the first study involving the analysis of protein oxidation in a wine-based meat marination system with thermal application, the mechanisms of how the interaction between meat proteins and specific phenolic compounds occurs need to be further clarified.

3.4 Volatiles profile of roasted strip steak as affected by marination

Among the total volatile compounds detected in the headspace of the roasted samples, 24 volatiles were identified and analyzed for their potential influence on beef flavor (Table 3).
Meat lipids are known to influence the production of odour-active compounds in cooked beef, due to the occurrence of hydrolytic and oxidative reactions.[47] In this study, hexanal was the most abundant aldehyde, particularly in the CONTROL samples, which had seven times higher concentration of these compounds than the marinated counterparts. Nieto et al. [48] identified hexanal as the most abundant aldehyde in cooked lamb with this volatile being generated from diverse unsaturated fatty acids. Hexanal and other saturated aldehydes may have an impact on the taste of roasted beef, where octanal is described as fruity and green, and heptanal is associated with a "penetrating" odor.[48] Pentanal, hexanal, heptanal, octanal and nonanal showed significant differences ($p < 0.05$) between the CONTROL and the other treatments, suggesting more intense lipid oxidation processes and a more intense onset of rancid flavors in the CONTROL samples. Bioactive compounds from the wine likely contributed to this positive effect against lipid oxidation in the marinated steaks, which agrees with the aforementioned results on protein oxidation. The Maillard reaction is another relevant source of aroma and flavor compounds in meat.[33] Strecker degradation of amino acids is a pathway of the Maillard reaction that produces characteristic volatile compounds in roasted beef, such as benzaldehyde.[49,50] This compound was not detected in the samples treated with 'TEM' and lower values were found in the beef marinated with 'ISA' ($p < 0.05$).

Seven alcohols were found in marinated steaks and among these, three (3-methyl-butan-1-ol, 2methyl-butan-2-ol and phenyl-ethyl alcohol) were only detected in the treated samples and another (pentan-1-ol) was only present in the CONTROL beef. Lorenzo and Domínguez [51] reported the occurrence of 1-octen-3-ol in cooked steaks, with this alcohol being described as an important volatile component of cooked beef providing a characteristic 'fungic' odor.[52] On the other hand, 1pentanol, which is commonly found at low concentrations in meat and imparts fruity and balsamic odors [53], was found only in the CONTROL. The volatiles 3-methyl-butan-1-ol and 2-methyl-butan2-ol are superior alcohols, indicators of positive quality of wines.[54] Considering that they were not detected in the CONTROL sample, these alcohols, together with phenylethyl alcohol and the esters, probably originated from the wine.

Esters are relevant odor-active compounds due to their low detection thresholds, transmitting fruity notes. Propanoic acid, 2-hydroxy, ethyl ester was identified in larger area units, with emphasis on 'ISA' group as compared to the other treatments (p <0.05). According to Lorenzo & Domínguez [51] in their study on the effect of cooking processes on cooking loss, lipid oxidation and formation of volatile compounds in pork, the heat treatment can lead to degradation of the esters mainly in baked steaks. The abundance of esters in our samples compared to previous studies[52] reflects the considerable contribution of wine marination to volatile esters with striking potential flavor activity. Two ketones were identified only in the CONTROL samples with these compounds being typically linked to cooked meats submitted to severe technological conditions and hence, with elevated lipid oxidation rates.[33, 55] This finding in a general agreement with other findings on lipid-derived aldehydes and alcohols showing the higher oxidative instability of CONTROL samples compared to the marinated counterparts. Butyrolactone was identified in the marinated steaks, with higher counts in 'CAB' treatment. According to Perestelo et al.[56,57] lactones impart a sweet and buttery aroma to the wine, and consequently they may confer such aromatic notes to the marinated meat.

3.5 Sensory profile of roasted strip steak as affected by marination

The panelists detected significant differences ($p < 0.05$) for color (intensity and acceptability), odor (meat, wine and acid), and flavor attributes (wine and rancidity) and juiciness between treatments (Table 4). However, brightness, fibrousness, hardness and meat flavor were similar among the four group of samples. Marinated steaks had higher intensity of color, odor and wine flavor, acid odor, with lower perception for rancidity compared to CONTROL steaks. When evaluating the appearance of marinated steaks, the panelists detected a difference in color intensity, observing a behavior similar to that already described by instrumental evaluation, where the coordinates L *, a *, b * also presented variation ($p < 0.05$) between the treatments. Among treatments, 'ISA' was identified as the group of samples with the least appealing appearance. The perception of meaty odor was more intense in the CONTROL samples while the wine odor was easily perceived in the marinated steaks ('CAB', 'TEM' and 'ISA'), a result that was already expected. Possibly wine odor resulted from the presence of volatile components present in wines, such as alcohols (3-methyl-1butanol and 2-methyl-1-butanol, phenylethyl alcohol) and mainly esters and lactones, which were not detected in CONTROL samples. Higher values for acid odor detected in steaks 'TEM' and 'ISA', probably resulted from the presence of some organic acids, which also affected the pH values in these samples.

The panelists perceived a more intense rancidity in the CONTROL samples than in the marinated steaks. This result can be explained by the more abundant concentration of lipid-derived aldehydes such as hexanal in the CONTROL samples compared to the marinated counterparts. Mezgebo et al. and Hunt et al. [55, 59] found positive correlations between hexanal and meat flavor scores, indicating that the results obtained in this study are consistent. The meat texture is a parameter that implies meat acceptance and succulence depends on intramuscular fat and water loss during cooking. In this study the perception of greater succulence in the CONTROL and 'ISA' steaks are difficult to explain as no other physico-chemical parameter seem to support this difference. The lack of significant differences for the other texture parameters as well as the absence of differences in the instrumental texture measurement indicates that probably the detected differences in juiciness are more likely to be caused by interaction with the session.

4. CONCLUSIONS

The results obtained in this study demonstrate the potential ability of the wine-based marinades to inhibit the oxidation of proteins during culinary preparation, limiting the potential adverse effects of protein carbonyls, dityrosines and tryptophan oxidation products on consumer's health. 'ISA' wine appeared as particularly effective against protein oxidation, which can be attributed to its particular profile in bioactive phenolic compounds. Overall, wine-based marination influenced positively the sensory attributes of roasted beef, reducing lipid oxidation and rancidity and contributing particular aromatic odors from specific esters, alcohols and lactones. As a consequence, marination may be a feasible means to alleviate the potential negative effects that oxidative damage causes on meat proteins, improving beef quality and diversifying muscle foods into a variety of safer and more flavored beef products.

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FIGURE CAPTIONS

FIGURE 1. Effect of wine-based marination on the formation of protein carbonyls, dityrosine, protein cross-links and on the depletion of tryptophan.

[A] Results expressed as percent inhibition against each of the protein oxidation measurements as described in Material and Methods.

[A]

TABLE 1. Chemical composition and antiradical activity of the wines under study (mean ± standard deviation).

	Cabernet	Tempranillo	Isabel
Total phenolics (mg EGA/100g)	453.14^{a} ± 2.03	479.15^{a} ± 10.35	307.86^{b} ± 9.02
Antioxidant Activity (% Inhibition)			
ABTS	54.01^{b} ± 0.92	67.91^{a} ± 1.71	46.14^{c} ± 0.95
DPPH	57.17^{b} ± 0.98	68.99^{a} ± 1.23	46.98^{c} ± 1.20
Total organic acid profile (mg/g)	290.91 ± 7.16	229.82 ± 7.64	335.29 ± 1.62
Total sugar profile (g/100g)	12.31 ± 1.05	7.70 ± 0.35	8.16 ± 0.35

Mean ± standard deviation followed by the same letters in the same rows do not differ at 5% according to Tukey's test.

TABLE 2. Chemical composition, pH, water activity, instrumental colour, texture and cooking loss of roasted strip loin as affected by wine-marination (mean ± standard deviation).

	CONTROL	CAB	TEM	ISA	p[A]
Moisture[B]	53.23±1.25	54.43±1.32	54.69±0.98	55.36±1.18	ns
Protein[B]	36.41±0.69	35.28±0.98	36.95±0.88	36.68±1.01	ns
Lipid[B]	6.75±0.87	6.62±0.74	6.35±0.98	6.62±0.67	ns
Ash[B]	1.45±0.23	1.19±0.17	1.32±0.28	1.46±0.19	ns
pH	5.25^{a}±0.03	5.25^{a}±0.02	5.10^{b}±0.02	5.10^{b}±0.01	**
a_W	0.97±0.01	0.96±0.01	0.97±0.01	0.97±0.01	ns
L*	55.35^{a}±0.88	43.79^{c}±1.07	46.34^{b}±1.25	47.33^{b}±1.07	***
a*	8.58^{a}±0.75	8.05^{ab}±0.66	7.72^{b}±0.45	8.76^{a}±0.98	*
b*	11.22^{a}±0.56	7.31^{d}±0.96	8.52^{c}±0.52	9.96^{b}±0.83	***
WBSF[C]	6.45±0.34	6.66±0.84	6.55±0.78	6.67±0.64	ns
CL[D]	37.15±1.84	36.56±1.98	36.95±2.27	36.24±2.25	ns

[A]Different letters on the same row denote significant differences between means.

Statistical significance: *: p<0.05; **: p<0.01; ***: p<0.001; ns: no significant.

[B]Data expressed as g/100 g sample.

[C]Warner-Bratzler Shear Force; data expressed as Newtons.

[D]Cooking loss; data expressed as percentage of water loss.

TABLE 3. Volatiles profile (area units x 10^6) of roasted strip loin as affected by wine-marination (means ± standard deviation).

	CONTROL	CAB	TEM	ISA	p[A]
Aldehydes					
Pentanal[B]	11.29[a]±3.80	0.58[d]±0.05	0.91[c]±0.21	1.70[b]±0.42	***
Hexanal[B]	263.90[a]±58.84	33.64[b]±3.72	35.01[b]±6.99	40.58[b]±6.87	***
Heptanal[B]	7.07[a]±1.74	1.47[b]±0.42	1.50[b]±0.21	1.66[b]±0.16	***
Benzaldehyde[B]	1.03[a]±0.31	nd[c]	0.80[a]±0.23	0.63[b]±0.09	*
Octanal[B]	8.03[a]±1.82	1.98[b]±0.51	1.86[b]±0.36	2.13[b]±0.51	***
Nonanal[B]	8.83[a]±2.05	3.18[b]±0.45	3.27[b]±0.77	4.30[b]±0.97	***
Total aldehydes	300.15[a]±45.65	40.85[b]±12.56	43.35[b]±12.50	51.00[b]±18.99	***
Alcohols					
3-methyl-butan-1-ol[B]	nd[d]	39.23[c]±3.17	94.51[a]±5.08	49.84[b]±1.49	***
2-methyl-butan-1-ol[B]	nd[d]	4.13[c]±0.62	12.91[a]±1.47	8.99[b]±0.54	***
Pentan-1-ol[B]	5.01[a]±0.80	nd[b]	nd[b]	nd[b]	***
Butane-2,3-diol[B]	5.27[b]±0.91	7.06[a]±1.51	5.96[b]±1.41	5.19[b]±1.61	*
Oct-1-en-3-ol[B]	1.77[a]±0.48	0.50[c]±0.18	0.90[b]±0.08	0.57[c]±0.19	**
2-Ethyl-hexan-1-ol[B]	6.22[a]±1.32	1.97[b]±0.50	6.99[a]±1.73	5.65[a]±1.60	**
Phenethyl alcohol[B]	nd[c]	75.91[a]±13.29	75.63[a]±11.12	56.88[b]±8.69	***
Total alcohols	18.27[c]±6.54	128.8[b]±26.36	196.9[a]±24.78	127.12[b]±28.45	***
Esters					

Butanoic acid, ethyl ester[C]	nd[c]	0.83[b]±0.17	2.22[a]±0.59	1.54[a]±0.57	**
Propanoic acid, 2-hydroxy, ethyl ester[C]	nd[d]	69.25[c]±12.64	101.15[b]±21.19	241.98[a]±16.13	***
Pentanoic acid , ethyl ester[C]	nd[c]	nd[c]	1.25[a]±0.38	0.79[b]±0.40	**
Hexanoid acid, ethyl ester[C]	nd[c]	3.05[b]±0.78	6.43[a]±1.47	2.77[b]±0.25	*
Butanedioic acid , diethyl ester[C]	nd[c]	5.95[b]±2.09	7.42[a]±2.55	3.79[b]±0.73	*
Octanoic acid, ethyl ester[C]	nd[c]	1.37[b]±0.05	5.25[a]±0.95	1.10[b]±0.36	*
Butanoic acid, butyl ester[C]	5.58[b]±1.58	5.79[b]±0.92	7.06[a]±1.92	7.65[a]±1.27	*
Total esters	5.58[d]±1.25	86.24[c]±10.11	130.78[b]±23.11	259.62[a]±32.22	***
Ketones					
Octane-2,5-dione[B]	7.11[a]±1.41	nd[b]	nd[b]	nd[b]	***
Heptan-2-one[B]	0.52[a]±0.09	nd[b]	nd[b]	nd[b]	***
Total ketones	7.63±1.58	-	-	-	
Others					
Butyrolactone[B]	nd[c]	3.12[a]±0.42	2.15[b]±0.38	1.73[b]±0.37	**
Nonanoic acid[B]	0.92[ab]±0.15	1.03[a]±0.40	1.03[a]±0.33	0.76[b]±0.20	*

Different letters on the same row denote significant differences between means.

[A] Statistical significance: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: no significant.

[B] Positive identification of volatile compounds by comparing MS with that of standard compound.

[C] Tentative identification of volatile compounds by comparing linear retention index (LRI) with Wiley library.

TABLE 4. Sensory profile of roasted strip loin as affected by wine-marination.

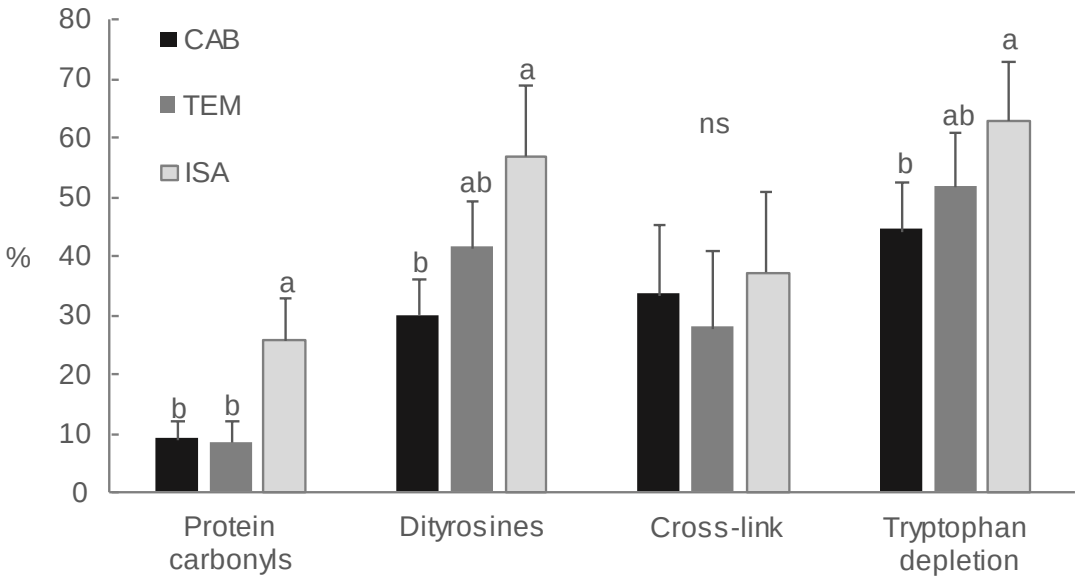
	CONTROL	CAB	TEM	ISA	p^A	p^B
Appearance						
Colour Acceptability	5.78^a ±0.37	5.47^a ± 0.59	5.18^a ± 0.67	4.25^b ±0.31	**	ns
Colour Intensity	2.07± 0.43^c	6.31 ± 0.60^a	6.92 ±0.85^a	5.83 ± 0.59^b	***	ns
Brightness	3.37 ±1.07	3.77 ± 0.98	3.38 ± 0.80	3.76 ± 0.99	ns	ns
Odour						
Meaty odour	6.36 ± 0.58^a	5.07± 0.64^b	3.22 ± 0.43^c	3.88 ± 0.63^c	***	ns
Wine odour	0.73 ± 0.31^b	6.07 ± 0.70^a	6.41± 0.66^a	5.61 ± 0.82^a	***	ns
Sourness	0.91 ± 0.29^c	1.27 ± 0.31^{bc}	1.81 ± 0.35^{ab}	2.08 ± 0.30^a	***	ns
Flavour						
Meaty flavour	6.99 ± 0.62	6.68 ± 0.60	6.27 ±0.62	6.32 ± 0.67	ns	ns
Wine flavour	0.40 ± 0.18^b	6.09 ± 0.60^a	5.62 ± 0.99^a	5.87 ± 2.32^a	***	ns
Rancidity	0.80 ± 0.26^a	0.37 ± 0.12^b	0.31 ± 0.10^b	0.43 ± 0.12^b	**	*
Texture						
Juiciness	5.47 ± 0.41^a	4.71 ± 0.53^b	4.41 ± 0.61^b	5.59 ± 0.38^a	***	**
Fibrousness	5.31 ± 0.47	5.50 ± 0.59	5.81 ± 0.45	5.32 ± 0.41	ns	ns
Hardness	5.26 ± 0.45	5.33 ± 0.39	5.98 ± 0.45	5.64 ± 0.360	ns	*

Different letters on the same row denote significant differences between means.

[A] Statistical significance: *: p<0.05; **: p<0.01; ***: p<0.001; ns: no significant.

[B] Statistical significance to “session effect” *: p<0.05; **: p<0.01; ***: p<0.001; ns: no significant

FIGURE 1



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Antioxidant and pro-oxidant actions of resveratrol on human serum albumin in the presence of toxic diabetes metabolites: Glyoxal and methyl-glyoxal

N.M.O. Arcanjo[a], C. Luna[b], M.S. Madruga[a], M. Estévez[c,*]

[a] Department of Food Engineering, Federal University of Paraiba, Joao Pessoa CEP 58051-900, PB, Brazil [b] Servicio Extremeño de Salud, SES, Gobierno de Extremadura, Cáceres, Spain

[c] IPROCAR Research Institute, Food Technology, University of Extremadura, 10003 Cáceres. Spain

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ABSTRACT

Methylglyoxal (MGO) and glyoxal (GO) are attracting considerable attention because of their role in the onset of diabetes symptoms. Therefore, to comprehend the molecular fundamentals of their pathological actions is of the utmost importance. In this study, the molecular interactions between resveratrol (RES) and human serum albumin (HSA) and the ability of the stilbene to counteract the oxidative damage caused by pathological concentrations of MGO and GO to the human plasma protein, was assessed. The oxidation of Cys34 in HSA as well as the formation of specific protein semialdehydes AAS (α-aminoadipic), GGS (γ-glutamic) and the accumulation of Advanced Glycation End-products (AGEs) was investigated. Resveratrol was found to neutralize both α-dicarbonyls by forming adducts detected by HESI-Orbitrap-MS. This antioxidant action was manifested in a significant reduction of AGEs. However, RES-α-dicarbonyl conjugates oxidized Cys34 and lysine, arginine and/or proline by a nucleophilic attack on SH and ε-NH groups in HSA. The formation of specific semialdehydes in HSA after incubation with GO and MGO at pathological concentrations was reported for the first time in this study, and may be used as early and specific biomarkers of the oxidative stress undergone by diabetic patients. The prooxidative role of the RES-α-dicarbonyl conjugates should be further investigated to clarify whether this action leads to positive or harmful clinical consequences. The biological relevance of human protein carbonylation as a redox signaling mechanism and/or as a reflection of oxidative damage and disease should also be studied in future works.

1. Introduction

Understanding the molecular basis of disease is crucial to comprehend the underlying pathological mechanisms and to design targeted prophylactic and therapeutic strategies. On this line, study of protein biochemistry has become a topic of enormous interest given their assorted roles as signaling molecules, modulators of gene expression, and executers of most biological functions [1]. Proteins are targets for atypical posttranslational changes including oxidation, glycation and nitrosation, with some these modifications leading to impaired functionality. The oxidation of proteins is, in particular, a growing concern given the occurrence and accumulation of protein oxidation products in aging and age-related diseases such as Alzheimer, Parkinson, cataractogenesis, inflammatory bowel diseases, and type II diabetes [1]. Protein oxidation is manifested as assorted chemical changes, yet, the formation of protein carbonyls has been identified as the most severe modification induced in proteins by Reactive Oxygen Species (ROS) [2]. Likewise, protein carbonyls are typically used as indicators of protein oxidation and markers of disease [3]. While most scientific studies use the dinitrophenylhydrazine (DNPH) method to qualify protein carbonyls in biological systems, the analysis of specific protein carbonyls such as α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) provides further molecular insight of specific redox pathways and mechanisms [4]. Interestingly, these carbonyls can be formed from δ-amino groups of alkaline amino acids by two different pathways: i) the 'Stadtman' pathway that involves a metal-catalyzed radical-mediated mechanism [2] and ii) the 'Suyama' pathway that implies a Maillard-mediated pathway induced by reactive α-dicarbonyls such as glyoxal (GO) and methylglyoxal (MGO) [5]. Trnková et al. [6] reviewed the interconnections and common products between radical-mediated protein oxidation and the Maillard reaction, emphasizing the relevant biological consequences of the protein damage.

The formation of reactive carbonyl species and protein glycation processes (α-dicarbonyls stress) is responsible for the chronic side effects in diabetic patients with enduring hyperglycemia [7]. The aforementioned carbonyls, GO and MGO are found at elevated levels in diabetic patients and their hepatotoxicity, nephrotoxicity and neurotoxicity are well documented [8, 9]. The diet is also an external source of these α-dicarbonyls, as they are formed in foods during processing and in the intestinal lumen during digestion [7]. Dietary α-dicarbonyls impair digestibility and nutritional value of food proteins and subsequently, upon absorption, may contribute to circulating α-dicarbonyls and induce toxicity in human cells [10]. While the pathogenesis of reactive α-dicarbonyls is usually attributed to their ability to react with protein-bound lysine and arginine to form advanced glycation endproducts, it is also well documented that AAS and GGS are also formed in protein residues in the presence of GO and MGO. Akagawa et al. [5] originally described the α-dicarbonyl-mediated mechanism by which AAS and GGS were formed from alkaline amino acids in plasma proteins from diabetic rats. The same authors reported 2–3 fold-times higher concentration of AAS in diabetic rats compared to the control counterparts and regarded as an early and reliable biomarker of oxidative stress linked to diabetes [11]. The accumulation was AAS in plasma proteins was proposed to induce changes in the isoelectric point of the proteins, conformational changes and eventually, inactivation [11]. Furthermore, AAS residues may be implicated in the formation of protein cross-links (i.e. AGEs) via aldol condensation or with another lysine residue via Schiff base formation. These condensations may contribute to increasing

* Corresponding author at: IPROCAR Research Institute, TECAL Research Group, University of Extremadura, 10003 Spain. E-mail address: mariovet@unex.es (M. Estévez).

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resistance to removal by proteolytic means as well as impeding function [11]. At a molecular level, AAS and its oxidation end-product (α-aminoadipic acid, AAA) may be implicated in the development of diabetic complications such as renal failure [12], cataractogenesis [13] and atherosclerosis [14].

The dietetic and/or pharmacological usage of biologically active compounds from plant kingdom is of increasing interest owing to their assorted biological effects and well acceptance by consumers. Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a polyphenolic compound naturally occurring in several fruits and vegetables such as grapes, peanuts, and various types of berries [15]. The numerous and assorted benefits alleged to resveratrol, including anti-aging, anticancer, anti-inflammatory, and antidiabetic, has concentrated great attention among physicians, dieticians, food chemists, and consumers [15]. As a redox-active compound, resveratrol is a potent scavenger of free radicals. However, its pro-oxidant properties have also been welldocumented, with these effects having relevant clinical implications [16]. Beyond its redox properties, resveratrol has also been found to modulate multiple physiological processes through gene expression regulation and implication in targeted signaling pathways [15]. The biochemical basis of its biological effects, which may reasonably involve molecular interactions with proteins, is much less understood. Several studies have proven that human serum albumin (HSA) bind resveratrol and other stilbenes [17, 18]. Nevertheless, the redox consequences of such interactions are mostly unknown. As regards to the antidiabetic effects of resveratrol, this compound has been found to exert antiglycation effects in proteins, inhibiting the formation of AGEs [19]. The underlying molecular mechanisms are not well understood and more specifically, the effect of resveratrol against the formation of specific protein carbonyls in human proteins under hyperglycemic conditions is unknown. This study aims to assess the effectiveness of resveratrol as inhibitor of the chemical changes induced by GO and MGO in HSA, covering the formation of early protein carbonyls, AAS and GGS, the accumulation of AGEs and the depletion of other functional groups and aminoacids in proteins such as tryptophan residues and thiols.

2. Material and methods

2.1. *Chemicals*

Resveratrol (3,4′,5-trihydroxy-trans-stilbene), glyoxal, methylglyoxal, albumin from human serum (HSA), cyanoborohydride ($NaCNBH_3$), diethylenetriaminepentaacetic acid (DTPA), sodium dodecyl sulfate (SDS), 4-Aminobenzoic acid (ABA) and 2-(N-morpholino) ethanesulfonic acid (MES) monohydrate were acquired from Sigma-Aldrich Co. Ltd. (Steinheim, Germany). Sodium dihydrogen phosphate (NaH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), trichloroacetic acid (TCA), sodium acetate anhydrous, methanol, acetonitrile, diethylether, ethanol and hydrochloric acid were obtained from Scharlau Labs S.L. (Barcelona, Spain). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA). Solutions were freshly prepared prior to use.

2.2. *Synthesis of AAS-ABA and GGS-ABA*

N-Acetyl-L-AAS and N-acetyl-L-GGS were synthesized from Nαacetyl-L-lysine and Nα-acetyl-L-ornithine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al. (5). Briefly, 10 mM Nα-acetyl-L-lysine and 10 mM Nαacetyl-L-ornithine were independently incubated with constant stirring with 5 g egg shell membrane in 50 mL of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane was then removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mmol ABA in the presence of 4.5 mmol sodium cyanoborohydride ($NaCNBH_3$) at 37 °C for 2 h with stirring. Then, ABA derivatives were hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C in

vacuo to dryness. The resulting AAS-ABA and GGS-ABA were purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20,2:1, v/v/v) as elution solvent. The purity of the solutions and the identification of the synthesized compounds were confirmed by using Fast Atom Bombardment coupled to mass spectrometry (FAB-MS). The spectra and fragmentation patters of the compounds were published elsewhere [20]. The structures of the authentic compounds as synthesized following the aforementioned procedures have been analyzed using ^{1}H NMR techniques and reported by Akagawa et al. [5].

2.3. *Analysis of binding affinity between HSA and resveratrol*

The quenching of protein intrinsic (tryptophan) fluorescence by resveratrol was analyzed to assess the binding affinity of resveratrol following the procedure described by N′ soukpoé-Kossi et al. [21]. HSA (0.5 mM) were dissolved in sodium phosphate buffer (pH 7.0; 10 mM). Resveratrol was dissolved in distilled water in order to yield a 52 μM stock solutions. Fluorescence spectra were recorded in a Perkin Elmer LS 55 luminescence spectrometer (Perkin Elmer, Cambridge, UK) using a 10 mm quartz Suprasil fluorescence cuvette (Hellma, Germany). In order to quantify the potential interaction between resveratrol and HSA, the latter were titrated in cuvette by successive additions of resveratrol (0–45 mM; final concentrations). Fluorescence emission spectra were recorded from 300 to 400 nm with excitation at 280 nm. The excitation and emission slits were both set to 10 nm and scanning speed was 500 nm/min. All experiments were carried out at 22 ± 1 °C. Fluorescence intensity was read at protein emission maximum of 337 nm. The bimolecular quenching rate constant (Kq) was calculated using the Stern–Volmer equation:

$$F0/F = +1\ Kq\tau 0[Q]$$

where F0 and F are the fluorescence intensities of the protein solutions in the absence and presence of the quencher, respectively; [Q] is the quencher concentration, and τ0 (≈5 × 10^{9} s) is the lifetime of the fluorophore in the absence of the quencher.

2.4. *Reaction between HSA and α-dicarbonyls*

Six different types of reaction units containing HSA (5 mg/mL) in sodium phosphate buffer (pH 7.4; 100 mM) were prepared as follows: CONTROL included only HSA as negative control samples; GO included HSA and glyoxal (0.4 mM; MGO included HSA and methylglyoxal (0.4 mM), RES included only the resveratrol (2.5 μM), GO+RES included HSA, glyoxal (0.4 mM) and resveratrol (2.5 μM) and finally, MGO + RES included HSA, methylglyoxal (0.4 mM) and resveratrol (0.4 mM). All concentrations are final concentrations in the protein suspensions and the levels of reactants (GO, MGO and RES) simulate the concentration of the α-dicarbonyls in plasma from diabetic patients [22] and the concentration of RES in human plasma following consumption of red wine [23]. All reaction units were prepared in triplicate (6 × 3 = 30 in total) and incubated at 37 °C for 48 h with constant stirring. The headspace of the reaction vials was filled with nitrogen to minimize the impact of residual oxygen. Samples were taken at fixed times (3, 6, 12 24 and 48 h) and subsequently analyzed for the concentration of AAS and GGS, free thiols, AGEs and tryptophan fluorescence quenching.

2.5. *Analysis of ROS concentration in experimental units*

To check the potential formation of ROS in the experimental units, superoxide radical was assessed in experimental units following the method reported by Susanto, Firdhaosh and Dond-An [24] as follows. For each experimental unit, three reaction mixtures were prepared i) one with SOD (100 U/mL), ii) other with DETCA (3 mM), and finally iii) with NADH (100 mM) and DETCA (3 mM). The test tubes were incubated for 1 h at 37 °C with regular shaking. The supernatant was mixed with cytochrome c (20 μM) and absorbance was immediately read at 550 nm

using a Hitachi U-2000 spectrophotometer. The absorbance differences between sample with or without SOD were used to calculate the release of superoxide radical by using the molecular extinction coefficient for cytochrome c of 21 mM^{-1} cm^{-1}.

2.6. *Analysis of free thiols by spectrophotometry*

Free thiols were quantified following the procedure described by Rysman et al. [25]. Two hundred µL of protein suspension was mixed with 1.25 mL of 6 M GuHCl in 1 M citric acid buffer (pH 4.5) and 250 µL of 4-DPS solution (4 mM 4-DPS in 12 mM HCl). The absorbance was measured at 324 nm against 6 M GuHCl in 1 M citric acid buffer (pH 4.5) before the addition of 4-DPS (A_{pre}) and after 30 min of reaction with 4-DPS in the dark at room temperature (A_{post}). A mixture of 1.25 mL of 6 M GuHCl in 1 M citric acid buffer (pH 4.5) and 250 µL of 4DPS solution was prepared as a blank sample (A_{blank}). The absorbance corresponding to the thiol concentration was calculated by subtracting A_{pre} and A_{blank} from A_{post}. The thiol concentration was calculated on the basis of a five-point standard curve ranging from 2.5 to 500 µM L-cysteine in 6 M GuHCl in 1 M citric acid buffer (pH 4.5). The thiol content was expressed as µM.

2.7. *Analysis of AAS and GGS by HPLC-FLD*

The procedure described by Utrera et al. [26] was followed for the quantification of AAS and GGS using HPLC-FLD. Five hundred µL of the protein suspensions were dispensed in 2 mL screw-capped eppendorf tubes and treated with 1 mL of cold 10% TCA solution. Each eppendorf was vortexed and then proteins were precipitated with centrifugation at 5000 g for 5 min at 4 °C. The supernatant was removed, and the resulting pellet was treated again with 1.5 mL of cold 5% TCA solution. A new centrifugation was performed at 5000 g for 5 min at 4 °C for protein precipitation. The supernatant was removed, and then the pellets were treated with the following freshly prepared solutions: 0.5 mL of 250 mM MES buffer pH 6.0 containing 1% SDS and 1 mM DTPA, 0.5 mL of 50 mM ABA in 250 mM MES buffer pH 6.0, and 0.25 mL of 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. The derivatization was completed by allowing the mixture to react at 37 °C for 90 min. The samples were stirred every 30 min. The derivatization was stopped by adding 0.5 mL of cold 50% TCA followed by a centrifugation at 5000 g for 5 min. The pellet was then washed twice with 10% TCA and diethyl ether−ethanol (1:1). Centrifugations at 5000 g for 10 min were performed after each washing step. The pellet was treated with 6 N HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. The hydrolysates were dried under constant nitrogen flow. Finally, the generated residue was reconstituted with 200 µL of Milli-Q water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 µm pore size, Pall Corporation) for HPLC analysis.

Samples were eluted in a Cosmosil 5C18-AR-II RP-HPLC column (5 µm, 150 mm × 4.6 mm) equipped with a guard column (10 mm × 4.6 mm) packed with the same material (Nacalai Inc., USA). A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corp., Kyoto, Japan), equipped with a quaternary solvent delivery system (LC-20 CE), DGU-20AS online degasser, SIL-20A autosampler, RF-10A XL fluorescence detector, and CBM-20A system controller, was used. Sodium acetate buffer (50 mM, pH 5.4) (eluent A) and acetonitrile (ACN) (eluent B) were used as eluents. A low-pressure gradient program was used, varying B concentration from 0% (min 0) to 8% (min 20). The volume injection was 0.5 µL, the flow rate was kept at 1 mL/min, and the temperature of the column was maintained constant at 30 °C. Excitation and emission wavelengths were set at 283 and 350 nm, respectively. Standards (0.2 µL) were run and analyzed under the same conditions. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times with those from the standard compounds. The peaks corresponding to AAS-ABA

and GGS-ABA were manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mM. Results are expressed as nmol of carbonyl per mg of protein.

2.8. *Analysis of AGEs by fluorescence spectroscopy*

AGEs was analyzed using a LS-55 Perkin-Elmer fluorescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.). Prior to the analysis, human proteins solutions were diluted with 100 mM sodium phosphate buffer, pH 7.4. AGEs was excited at 350 nm, and the emitted fluorescence was recorded from 400 to 500 nm. The excitation and emission slits were both set to 10 nm and scanning speed was 500 nm/min.Results are expressed as fluorescence intensity (Area units).

2.9. *Detection of resveratrol and adducts with HESI-Orbitrap-MS*

Samples (48 h of incubation under the aforementioned conditions) were prepared by diluting (1:40) the suspensions with MS-grade acetonitrile and methanol (1:1). A direct-infusion of the samples was carried on a Q-Exactive quadrupole Orbitrap instrument (Thermo Fisher Scientific, Bremen, Germany) using a syringe pump (11 Plus, Harvard Apparatus, Holliston, MA, USA) and a 500 μL syringe (Hamilton, Reno, NV, USA) at a flow of 5 μL min^{-1}. Analyses were carried out by using the Ion Max source from Thermo Fisher Scientific operating in the negative ion HESI detection mode and applying the following parameters: sheath gas flow 5 arbitrary units and capillary temperature of 320 °C. The AGC target was set to $1e^6$ and the maximum injection time to 50 ms; the spray voltage was operated at 3.5 kV, and the in-source fragmentation at 40.0 eV. The S-lens RF level was set to 50.0 and the scan range to m/z 50–1000 in the negative ion polarity mode. MS data analysis was carried out using the Trace Finder software from Thermo Fisher Scientific.

2.10. *Statistical analysis*

All experimental units were prepared in triplicate and each individual sample at each sampling time was analyzed three times for each measurement. The effect of the addition of the reactants on the concentration of free thiols, protein carbonyls and AGEs was analyzed by an Analysis of Variance (ANOVA). The effect the incubation time was assessed by a repeated messures ANOVA. The Tukey's test was used for multiple comparisons of the means. The significance level was set at $p < .05$.

Table 1

Concentration (nmol/mg protein) of γ-glutamic semialdehyde (means ± standard deviation) in HSA (5 mg/mL) during incubation at 37 °C during 48 h in the presence of GO (25 μg/mL),

	3 h	6 h	12 h	24 h	48 h	p_A
Control	$0.40^{b} \pm 0.05$	$0.44_{xy,b} \pm 0.04$	$0.55^{yz,a} \pm 0.06$	$0.53^{y,a} \pm 0.10$	$0.60^{z,a} \pm 0.06$	*
GO	$0.47^{b} \pm 0.08$	$0.48_{xy,b} \pm 0.04$	$0.52^{z,b} \pm 0.06$	$0.78^{x,a} \pm 0.09$	$0.87^{xy,a} \pm 0.08$	***
MGO	$0.44^{c} \pm 0.07$	$0.46_{xy,c} \pm 0.05$	$0.51^{z,bc} \pm 0.08$	$0.61^{y,ab} \pm 0.08$	$0.76^{y,a} \pm 0.07$	***
RES	0.41 ± 0.05	$0.36^{y} \pm 0.05$	$0.35^{w} \pm 0.04$	$0.34^{z} \pm 0.07$	$0.37^{w} \pm 0.03$	ns
GO+RES	$0.48^{c} \pm 0.06$	$0.53^{xy,bc} \pm 0.06$	$0.62^{y,b} \pm 0.04$	$0.59^{y,b} \pm 0.07$	$0.77^{y,a} \pm 0.07$	***
MGO + RES	$0.41^{d} \pm 0.06$	$0.60^{x,c} \pm 0.03$	$0.79^{x,b} \pm 0.05$	$0.81^{x,b} \pm 0.05$	$0.94^{x,a} \pm 0.07$	***
p_B	ns	**	***	***	***	

MGO (25 μg/mL) and resveratrol (4 μg/mL).

Different superscripts ($^{a-d}$) within the same experimental unit (rows) denote significant differences between sampling times in repeated measured ANOVA^{A} (statistical significance; *: $p < 0.05$; ***: $p < 0.001$; ns: non significant).

Different superscripts ($^{x-w}$) within the same sampling time (columns) denote significant differences between experimental units in ANOVA^{B} (statistical significance, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant).

3. Results and discussion

3.1. *Chemical modifications induced in HSA by GO and MGO*

The incubation of HSA at 37 °C for 48 h led to basal, yet, significant chemical modifications in the CONTROL experimental units. In these samples, the concentration of GGS and AAS increased (Tables 1 and 2, respectively) while free thiols decreased over time (Table 3). No significant changes were observed for the concentration of AGEs (Table 4). Since no pro-oxidants were deliberated added to these experimental units, the oxidative changes observed may have been caused by reactive species formed from molecular oxygen (~20.5%) in the headspace of the vials. According to our calculations, the concentration of superoxide radical was around 5 nM in all samples with this concentration being around thousand times lower than in Fenton-reaction systems [27]. Biomolecules and biological systems with high susceptibility to oxidation such as HSA and other proteins and emulsions, may suffer oxidative modifications in an oxygenated environment as previously documented by Akagawa, Shigemitsu & Suyama [28], Akagawa et al. [29] and Gürbüz, Kauntola, Ramos Diaz, Jouppila, & Heinonen [30]. In those studies, the induction of oxidation by reactive species led to a more severe oxidative conditions as compared to the CONTROL counterparts, which is consistent with the present results. The addition of MGO and GO at levels found in diabetic patients led to significant increases of the semialdehydes in HSA with the concentration of both being significantly higher than the in CONTROL counterparts at the end of the assay (48 h). As expected, the α-dicarbonyls induced the oxidative deamination of lysine, arginine and/or proline residues to yield the corresponding semialdehydes. To our knowledge, this is the first time to report the formation of specific carbonyls in HSA upon incubation with GO and MGO at pathological concentrations. Akagawa et al. [5] and Akagawa, Sasaki, Kurota & Suyama [31] found consistent results after incubating bovine serum albumin with assorted reducing sugars and reactive α-dicarbonyls. The quantitative differences between studies (3–4 times lower in Akagawa studies) may have been caused by differences in the incubation conditions. Since similar ROS concentrations were

calculated in all samples, the proposed mechanism may involve the Maillard-mediated pathway already reported by Akagawa et al. [5]. The formation of the semialdehydes from the susceptible amino acid residues is depicted in Fig. 1 showing the oxidative deamination of lysine residues by GO as an example. Firstly, MO condenses with protein-bound lysine residues to form a Schiff base adduct (an iminoketone). Subsequently, the ε-proton of the lysine moiety is abstracted by basic media, and the enolization leads to the formation of an iminoenaminol. In the electron transfer process, it is assumed that a transition metal or any other electron acceptor, stabilizes the iminoenaminol through the formation of a coordination complex. While this was not studied in the present work, Akagawa et al. [5] and Villaverde & Estévez [32] among others, observed the catalytic effects of Cu and Fe ions on the carbonylation of BSA and food proteins by reducing sugars and α-dicarbonyls. Finally, the iminoenaminol undergoes a spontaneous hydrolysis leading to the release of an enaminol and the formation of AAS. The proposed mechanism is also applicable to explain the formation of GGS from arginine and proline residues. GO appeared to be more effective than MGO in inducing carbonylation of HSA which is consistent with the studies reporting a more reactive nature of the former compared to the latter [33]. While the pathological role of these semialdehydes in diabetes requires further elucidation, both AAS and its oxidation end-product, the AAA, have been highlighted as biomarkers of diabetes and related pathological complications. Fan et al. [13] found significantly higher AAS levels in diabetic patients and found strong evidences of the implication of α-dicarbonyls-mediated

Table 2

Concentration (nmol/mg protein) of α-aminoadipic semialdehyde (means ± standard deviation) in HSA (5 mg/mL) during incubation at 37 °C during 48 h in the presence of GO (25 μg/mL), MGO (25 μg/mL) and resveratrol (4 μg/mL).

	3 h	6 h	12 h	24 h	48 h	p
Control	$0.63^{c,y}$ ± 0.07	$0.83^{b,y}$ ± 0.05	$0.92^{ab,y}$ ± 0.12	$0.98^{ab,y}$ ± 0.14	$1.05^{a,v}$ ± 0.08	**
GO	$0.99^{c,x}$ ± 0.16	$0.90^{c,xy}$ ± 0.07	$1.20^{b,x}$ ± 0.09	$1.14^{b,y}$ ± 0.16	$1.45^{a,z}$ ± 0.15	***
MGO	$0.66^{c,y}$ ± 0.07	$0.83^{b,y}$ ± 0.08	$0.84^{b,y}$ ± 0.11	$1.11^{a,y}$ ± 0.07	$1.23^{a,w}$ ± 0.08	***
RES	0.50^{z} ± 0.04	0.42^{z} ± 0.06	0.42^{z} ± 0.11	0.50^{z} ± 0.08	0.49^{u} ± 0.05	ns
GO+RES	$0.75^{d,y}$ ± 0.0.09	$1.06^{c,x}$ ± 0.08	$1.21^{c,x}$ ± 0.14	$1.68^{b,x}$ ± 0.21	$2.20^{a,x}$ ± 0.20	***
MGO + RES	$0.64^{c,y}$ ± 0.07	$1.00^{b,x}$ ± 0.05	$1.10^{b,xy}$ ± 0.07	$1.19^{b,y}$ ± 0.18	$1.73^{a,y}$ ± 0.17	***
p	**	***	***	***	***	

Different superscripts ($^{a-d}$) within the same experimental unit (rows) denote significant differences between sampling times in repeated measured ANOVA^{A} (statistical significance; *: $p < 0.05$; ***: $p < 0.001$; ns: non significant).

Different superscripts ($^{x-u}$) within the same sampling time (columns) denote significant differences between experimental units in ANOVA^{B} (statistical significance, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant).

Table 3

Concentration (μM) of free thiols (means ± standard deviation) in in HSA (5 mg/mL) during incubation at 37 °C during 48 h in the presence of GO (25 μg/mL), MGO (25 μg/mL) and resveratrol (4μg/mL).

	3 h	6 h	12 h	24 h	48 h	p
Control	$63.45^{a,x}$ ± 1.57	$57.26^{b,x}$ ± 2.55	$55.74^{b,xy}$ ± 3.57	$57.54^{b,x}$ ± 3.89	$54.12^{b,x}$ ± 2.22	*
GO	$63.47^{a,x}$ ± 3.81	$54.45^{bc,xy}$ ± 2.48	$58.83^{b,x}$ ± 2.71	$51.75^{c,y}$ ± 2.74	$48.53^{c,y}$ ± 3.86	***
MGO	$64.89^{a,x}$ ± 4.77	$60.12^{ab,x}$ ± 4.70	$59.73^{b,x}$ ± 1.16	$47.50^{c,y}$ ± 2.71	$48.33^{c,y}$ ± 1.35	***
RES	53.16^{y} ± 1.34	50.97^{y} ± 2.96	53.94^{y} ± 1.34	53.63^{y} ± 3.34	51.25^{x} ± 2.79	ns
GO+RES	20.32^{z} ± 1.93	19.67^{z} ± 1.61	21.99^{z} ± 3.12	20.33^{z} ± 4.25	22.64^{z} ± 1.93	ns
MGO + RES	25.73^{z} ± 4.25	23.03^{z} ± 3.86	24.57^{z} ± 4.09	29.21^{z} ± 2.04	24.57^{z} ± 0.77	ns
p	***	***	***	***	***	

Different superscripts ($^{a-c}$) within the same experimental unit (rows) denote significant differences between sampling times in repeated measured ANOVA^{A} (statistical significance; *: $p < 0.05$; ***: $p < 0.001$; ns: non significant).

Different superscripts ($^{x-u}$) within the same sampling time (columns) denote significant differences between experimental units in ANOVA^{B} (statistical significance, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant).

oxidation of lysyl residues in the nuclear sclerosis of human crystalline. To similar conclusions came Sell et al. [12] who found accumulation of AAA in the skin of diabetic patients and linked the in vivo formation of AAS in the presence of α-dicarbonyls

with diabetes complication such as renal failure. More recently, Saremi et al. [14] reported strong associations between lysine oxidation and AAA, on the extent of coronary artery calcification in patients with type 2 diabetes. Thus, the oxidative deamination of the lysine residue may be implicated in the development of diabetic complications at the molecular level, as speculated for AGEs.

In addition to the formation of specific semialdehydes, GO and MGO induced the depletion of free thiols (Table 3). HSA features just one free thiol, Cys34, with this moiety comprising ~80% of the total free thiols in plasma. Considering the concentration of HSA in our samples, the calculated free thiols in CONTROL, GO AND MGO units at the beginning of the assay is consistent with the theoretical concentration of free thiols (~70 μM). Cys34 is known to be highly reactive and readily interacts with ROS and reactive nitrogen species (RNS), ability to which the redox properties of HSA is ascribed [34]. The incubation with GO and MGO for 48 h caused a depletion of 23% and 25% of free thiols in HSA while in CONTROL samples the loss was limited to 14%. While the interaction between α-dicarbonyls with nitrogen substituents in lysine, arginine and proline, is well documented, the glycation of protein thiols is much less understood. Zeng & Davies [35] hypothesized that Cys residues in proteins, as powerful nucleophiles, should also be targets of glycation. These authors observed that the loss of free thiols in creatine kinase incubated with GO occurred concomitantly with the formation of S-carboxymethylcysteine, providing strength to their hypothesis. They also detected cross-links formation between N-acetylcysteine and the Lys-rich protein histone H1, demonstrating the formation of thiol−glyoxal−amine cross-links. As facile targets of glycation, protein thiols with irrelevant biological significance may play a role as antiglycation agents similarly to the sacrificial protection already attributed to these moieties against ROS [35, 36]. In agreement with the present results, Aćimović, Stanimirović & Mandić [37] also observed a significant depletion of Cys34 in HSA incubated in the presence of MGO. The authors concluded that thiol glycation played an important role in protein modification by MG and confirmed the formation of crosslinking via condensation of the product of reaction between SH-group and MG with lysine side-chain amino groups. Both free and proteinbound carbonyls have been found to inhibit lysosomal cysteine proteases through glycation of the cysteine in their active site, which provides explanation to the accumulation of damaged proteins in a large number of human pathologies, including aging, diabetes and Parkinson [7, 38, 39]. Hence, the depletion of thiols in HSA as observed in the present study may be a molecular indicator of the harmful consequences of elevated GO and MGO in diabetic patients. Finally, GO and MGO induced the formation of AGEs in HSA (Table 4) with this being one of the most remarkable and well-known chemical manifestations of the α-dicarbonyls stress on proteins [7]. While the formation of AGEs in proteins by GO and MGO responds to assorted biochemical pathways (Reviewed by Vistoli et al. [40]), the formation of hydroimidazolone from the reaction between arginine and α-dicarbonyls is the most quantitatively and functionally important AGEs in physiological systems [7]. As well as the semialdehydes previously reported, AGEs are increased in tissues affected by α-dicarbonyls stress and directly linked to the onset of diabetic-related disorders such as cataract formation, nephropathy and vascular complications [41, 42]. In fact, once generated, protein carbonyls may be precursors of protein cross-linking and AGEs, hence contributing to the pathological effects of the latter. Two AAS residues condense with each other via aldol condensation or with lysine residue via Schiff base formation to form various inter- and intramolecular cross-links spontaneously [42]. The protein cross-linking leads to increasing resistance to removal by proteolytic means as well as impeding protein functionality [43].

Table 4

AGEs (means ± standard deviation) in in HSA (5 mg/mL) during incubation at 37 °C during 48 h in the presence of GO (25 μg/mL), MGO (25 μg/mL) and resveratrol (4 μg/mL).

	3 h	6 h	12 h	24 h	48 h	p
Control	$129^{y} \pm 18$	$132^{y} \pm 25$	$134^{yz} \pm 26$	$142^{y} \pm 31$	$135^{zw} \pm 29$	ns
GO	$165^{d,x} \pm 20$	$175^{cd,x} \pm 33$	$189^{bc,x} \pm 29$	$215^{b,x} \pm 38$	$302^{a,x} \pm 35$	***
MGO	$154^{c,x} \pm 21$	$160^{c,x} \pm 31$	$180^{bc,x} \pm 31$	$198^{ab,x} \pm 35$	$223^{a,y} \pm 42$	*
RES	$110^{z} \pm 16$	$125^{y} \pm 26$	$121^{z} \pm 24$	$115^{z} \pm 29$	$128^{w} \pm 25$	ns
GO+RES	$131^{y} \pm 23$	$133^{y} \pm 23$	$141^{y} \pm 28$	$145^{y} \pm 36$	$152^{z} \pm 31$	ns
MGO + RES	$126^{y} \pm 21$	$132^{y} \pm 26$	$145^{y} \pm 30$	$144^{y} \pm 34$	$149^{z} \pm 39$	ns
p	**	**	***	***	***	

Different superscripts ($^{a-d}$) within the same experimental unit (rows) denote significant differences between sampling times in repeated measured ANOVA^{A} (statistical significance; *: $p < 0.05$; ***: $p < 0.001$; ns: non significant).

Different superscripts ($^{x-w}$) within the same sampling time (columns) denote significant differences between experimental units in ANOVA^{B} (statistical significance, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant).

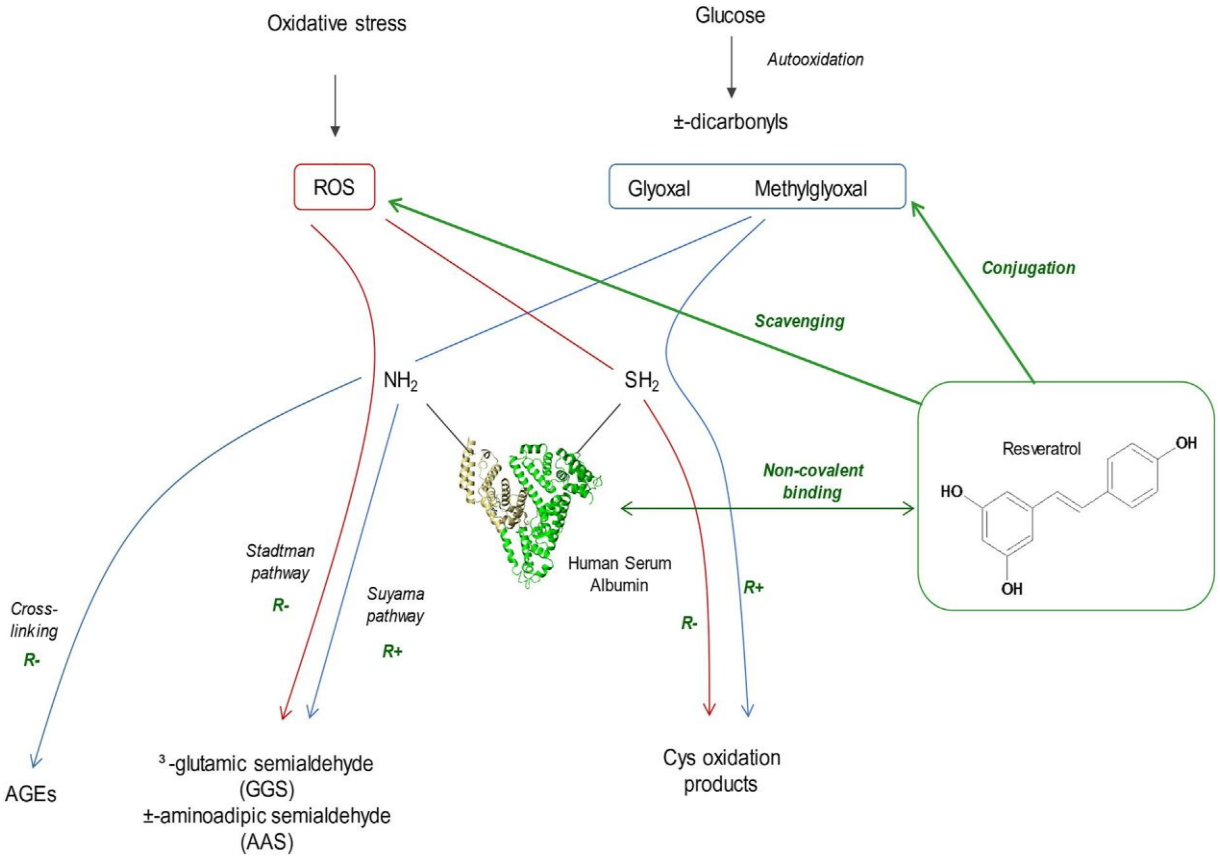

Fig. 1. Mechanisms of resveratrol against the pro-oxidative action of ROS and α-dicarbonyls on NH_2 and SH_2 groups in HSA. Red arrows denote ROS-mediated pathways. Blue arrows denote α-dicarbonyls-mediated pathways. Green arrows denote resveratrol-mediated actions. Resveratrol inhibiting a particular pathway is denoted by R-. Resveratrol promoting a particular pathway is denoted by R+.

3.2. *Effect of resveratrol on HSA*

According to the aforementioned, inhibiting the formation of AAS, GGS and AGEs in human proteins under hyperglycemic conditions requires understanding of the chemistry behind the formation of such harmful species and these are issues of the utmost scientific interest. Given the profuse literature describing the ability of resveratrol to alleviate diabetes-induced dysfunctions [44, 45] the present study covers the effect of physiological plasma levels of resveratrol on the chemical modifications suffered by HSA in the presence of pathological concentrations of GO and MGO. Both, antioxidant and pro-oxidant effects were observed depending on the presence/absence of α-dicarbonyls and the chemical modification under assessment. Though several mechanisms may occur concomitantly, for a better comprehension, proposed antioxidant and pro-oxidant mechanisms are described in different sections as follows. Fig. 1

3.3. *Antioxidant protection of RES on HSA*

The effect of resveratrol on the formation of GGS, AAS, depletion of free thiols and accumulation of AGEs is shown in Tables 1–4, respectively. The formation of semialdehydes during incubation of HSA at 37 °C for 48 h was completely inhibited in the presence of resveratrol. The addition of the phenolic compound kept the levels of both protein carbonyls at basal levels with the concentration of AAS being significantly lower in RES experimental units than in the CONTROL counterparts at all sampling times. Since the most plausible mechanism implicated in the formation of protein carbonyls in CONTROL samples is the Stadtman pathway (radical-mediated), the antioxidant effect of resveratrol against the formation of AAS and GGS in HSA may be attributed to its well-known radical-scavenging ability. In fact, numerous studies have shown the capacity of resveratrol to scavenge both hydroxyl and superoxide anion radicals [46]. Other authors such as Olas, Nowak, Kolodziejczyk, Ponczek & Wachowicz [47] and Pandey & Rizvi [48] previously reported the in vitro protective effect of resveratrol against carbonylation of plasma proteins. However, the previous studies used unspecific spectrophotometric/luminescence methods and this is, to our knowledge, the first time to report quantitative data of particular protein carbonyls, AAS and GGS, in HSA. While the anti-radical ability of resveratrol may be attributed to the occurrence of hydrogen-donating hydroxyl groups and the electron-delocalization abilities of the phenolic rings, the protection of proteins against oxidation may also be affected by the molecular interactions between the polyphenol and the biomolecule. The quenching of the natural fluorescence emitted by tryptophan residues in HSA by eight increasing resveratrol concentrations ($R > 0.95$), revealed a bimolecular quenching rate constant between resveratrol and HSA of Kq: $1.69 \times 10^5\ M^{-1}$. The binding affinity calculated in the present study is consistent with that previously reported by N′soukpoé-Kossi et al. [21] ($2.56 \times 10^5\ M^{-}1$) and both lower to that calculated by Nair [17] (Kq: $4.47 \times 10^6\ M^{-1}$). Using additional tools to study molecular docking (FTIR and Circular Dichroism), the former authors concluded that most resveratrol-protein binding is made through H-bonding between polypeptide C]O, CeN, and NH groups. This may contribute to explaining the ability of resveratrol to hindering the oxidative deamination of ε-NH groups by reactive species.

In the presence of α-dicarbonyls (GO+RES and MGO + RES experimental units) RES had contradictory effects on HSA: formation of AGEs was inhibited (Table 4) while protein carbonylation and thiols depletion were significantly promoted as compared to the GO and MGO systems (Tables 1, 2 and 3). The latter pro-oxidant effects will be explained in the following section. The ability of RES to inhibit AGEs under hyperglycemic conditions (or α-dicarbonyls stress) has been observed in both in vitro and in vivo studies [45, 49–51]. These studies discussed the biological pathways and mechanisms implicated in the protective effect of RES at the cell or animal level while the actual and

precise molecular mechanism by which RES may counteract reactive αdicarbonyls was not identified [52] described for the first time the ability of flavonoids such as quercetin, luteolin, and apigenin to scavenge MGO by forming adducts with the reactive α-dicarbonyl under simulated physiological conditions. While some of the structural requirements for flavonoids to scavenge methylglyoxal may be applicable to RES, stilbenes were not considered in that study. First, Gu, Wang & Liu [53] and more recently, Shen, Xu & Sheng [54] originally reported the conjugation of MGO by RES and the structures of the adducts were tentatively identified by HPLC-ESI-MS^n. Both studies agreed on the identification of RES as m/z 227 $[M-H]^-$ and the corresponding adduct with MGO as m/z 299 $[M-H]^-$ which matches with the deprotonated molecular ion of one resveratrol (m/z 228) with one MGO (m/z 72) detected in MS in the negative mode. The direct infusion of the present samples on a Q-Exactive Orbitrap equipment confirmed the detection of resveratrol as m/z 227 $[M-H]^-$ in RES experimental units and the formation of m/z 299 $[M-H]^-$ adducts in the experimental units containing resveratrol and methylglyoxal. Furthermore, we found in GORES experimental units a molecular ion with m/z 285 $[M-H]^-$ that was tentatively identified as the conjugation of one resveratrol (m/z 228) molecule with one glyoxal (m/z 58). The fragmentation of the aforementioned parental ions led to the identification of fragments that reasonably match the molecular structures proposed by Gu, Wang & Liu [52] (Fig. 2). Therefore, it seems reasonable to consider that the nucleophilic-based addition of RES to the reactive α-dicarbonyls hindered the attack of the latter on arginine NH_2 moieties, hence, inhibiting the formation of AGEs in HSA. While these findings are revealing, the lack of quantitative data on the adducts and the absence of knowledge on the stability and/or further reactivity of such conjugates prevent from concluding potential beneficial effects of this mechanism in living organisms.

3.4. *Pro-oxidant actions of RES on HSA in the presence of GO and MGO*

The protective effect of RES against the formation of AGEs, already described in literature, contrast with the pro-oxidant effect of the stilbene on the formation of GGS and AAS in HSA when GO and/or MGO were added to the experimental units. At 48 h, the concentration of GGS in HSA containing MGO-RES was significantly higher than in proteins with MGO (Table 1). Likewise, the addition of RES to experimental units containing GO and MGO led to significant increases in the concentration of AAS at the last sampling time (Table 2). After 48 h of incubation in the presence of GO and RES, the concentration of GGS and AAS in HSA triplicated the physiological concentration of total carbonyls in plasma (1 nmol/mg protein) as reported in literature [55, 56]. The present results indicate that RES promoted the oxidative deamination of lysine, arginine and/or proline to yield their corresponding and specific semialdehydes, and this pro-oxidant effect only occurred in the presence of α-dicarbonyls. Additionally, the concentration of free thiols dramatically decreased from first sampling in GO-RES and MGO-RES experimental units with these low levels (20–25 μM) remaining stable during the remaining sampling times. These results suggest the pro-oxidant effect of the combination of RES with GO/MGO on HSA derives from the molecular interaction between the stilbene and the α-dicarbonyls. As mentioned above, only two reaction pathways lead to the formation of AAS and GGS from the susceptible amino acid residues: the direct attack of free radicals on the εamino group of protein residues (Stadtman pathway) and the oxidative deamination of the same protein moieties by reactive α-dicarbonyls through a Maillard-type mechanism (Suyama pathway). Given that the addition of RES and GO/MGO did not lead to the formation of additional radicals in the present samples, the first pathway may be unlikely yet, should not be discarded: α-dicarbonyls have been found to yield ROS in biological systems [57] and the method

employed for the detection of radical species in the present study has documented limitations [24]. On the other hand, phenolic compounds have been found to induce the oxidative deamination of lysine residues and hence, promote the formation of AAS in BSA through the Suyama pathway [28]. This pro-oxidant action on proteins requires a previous oxidation of the polyphenols into their corresponding quinones as the oxidized polyphenols eventually induce the oxidative deamination of lysine through a Maillard-type mechanism [28]. The hypothetical carbonylation of HSA induced by an oxidized form of RES may also be unlikely since resveratrol is not oxidized into quinones [58] and actually none of the resveratrol oxidation end-products reported in literature were detected by MS in the present samples. Since the promotion of HSA carbonylation requires the presence of both RES and α-dicarbonyls, the implication of the conjugates identified in our experimental units (Fig. 1) may be a plausible hypothesis. In fact, in the absence of quinone structures, the RES-dicarbonyl adduct feature a carbonyl moiety that may enable a nucleophilic attack on SH and ε-NH groups in HSA, triggering in the latter the oxidative deamination process that leads to the formation of the corresponding semialdehyde. The phenolic structure of the adduct may facilitate its interaction with HSA (as previously explained for mono-resveratrol) prior to the nucleophilic addition and the subsequent oxidation of the SH and ε-NH groups. This hypothesis would explain why the combination of RES and α-dicarbonyls was more effective at promoting carbonylation and depleting cysteine than the αdicarbonyls alone. If our hypotheses are correct, the conjugation of RES with reactive α-dicarbonyls may protect against the formation of AGEs, while favors the formation of specific protein carbonyls from lysine, arginine and/or proline. It is worth noticing that AGEs formation requires of a free α-dicarbonyl: one carbonyl moiety performs the initial nucleophilic attack while the second establishes further interaction with other amino groups leading to subsequent cross-linking. Quenching the α-dicarbonyl by resveratrol neutralize one carbonyl moiety and hence, would hinder the formation of AGEs. Yet, the remaining carbonyl moiety may be accessible for the nucleophilic attack on SH and ε-NH groups. This action may be facilitated to a reactive carbonyl moiety attached to the phenolic structure of RES, that is known to bind to HSA. In accordance to our results, the resulting GGS and AAS may not be involved in the formation of AGEs (as previously hypothesized). Consistently, Akagawa et al. [5] recognized the possibility of AAS being involved in the formation of AGEs but stated that such structures have never been identified in human plasma. The prooxidant effects of resveratrol have been previously described and the alleged mechanism usually involves the generation of ROS and/or modulation of gene expression and related biochemical processes [60]. Hence, the present study originally proposes RES-dicarbonyl adducts as promoters of carbonylation in human plasma proteins with this prooxidant mechanism being well-matched with the well-recognized ability of resveratrol to inhibit AGEs formation. In the absence of further knowledge, it is not possible to state the physiological consequences of these chemical mechanisms but it is worth to highlight that certain pro-oxidant actions of resveratrol and other polyphenols have been identified as beneficial. According to Plauth et al. [58], prooxidative resveratrol induces cellular responses leading to increased endogenous Nrf2-mediated antioxidant defenses which, in turn, protects against oxidative stress and aging. Whether carbonylation of HSA by RES-dicarbonyl adducts may be playing a role as cell signaling to activate physiological responses against the hyperglycemia, is currently unknown. However, this hypothesis is possible since protein carbonylation has been recently identified as a ‘novel mechanism of redox signaling’ [59]. Like this, the two-sides effect of resveratrol in the presence of α-dicarbonyls, protecting against AGEs and promoting carbonylation of specific amino acid residues may be regarded as chemical modifications of overall positive consequences. Nevertheless, it is worth recalling that AAS and its end product AAA (not identified here) have been directly implied in

numerous diabetes-induced pathogenesis [12–14]. The chemical mechanisms discussed here lead to hypothesis of worthy investigation given the relevant consequences of α-dicarbonyl stress and protein carbonylation on human health.

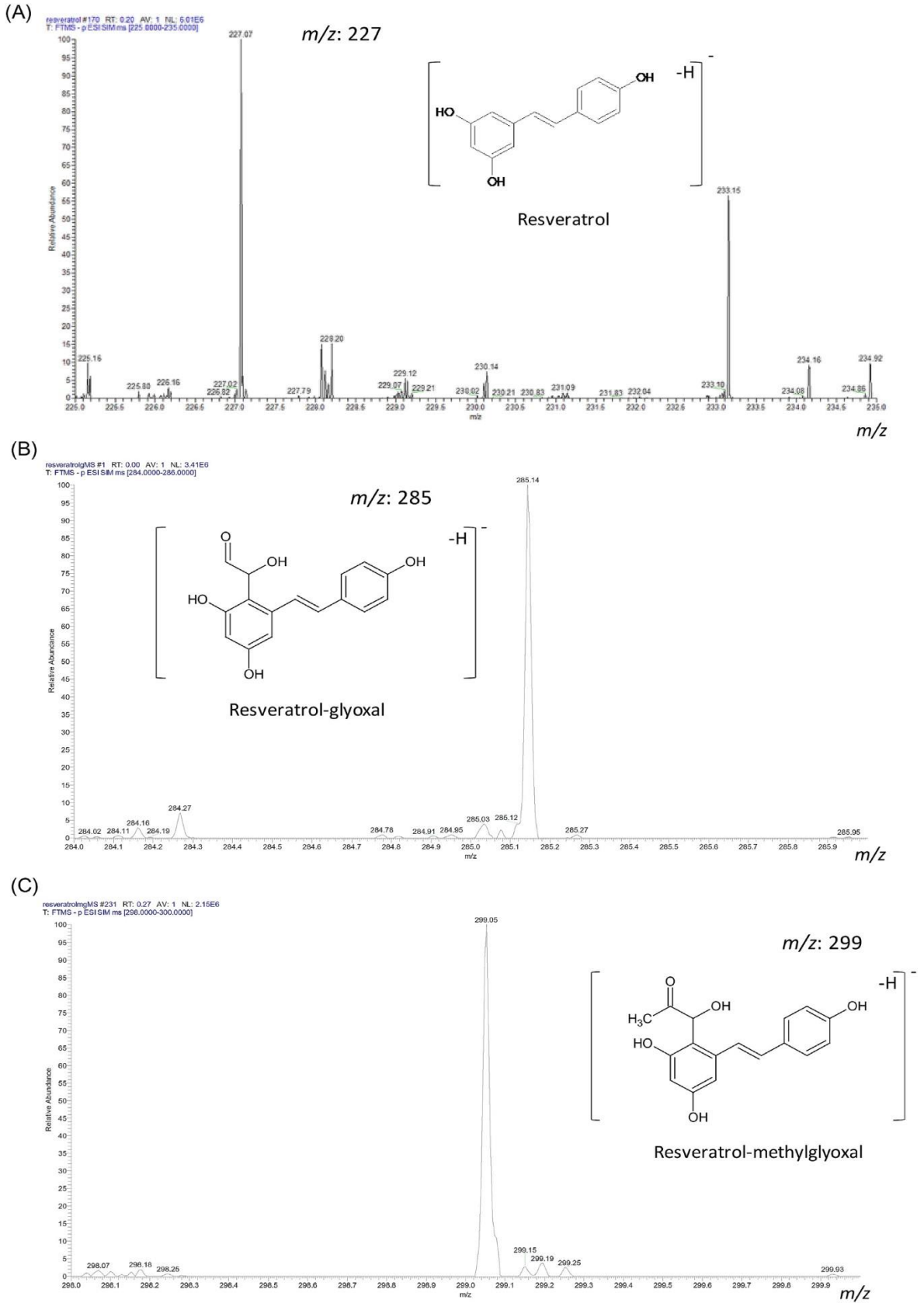

Fig. 2. Molecular ions detected by Q-Exactive Orbitrap after direct infusion of samples.
A) Resveratrol, B) resveratrol-glyoxal conjugate; C) Resveratrol-methylglyoxal conjugate.

4. Conclusions

The analysis of the molecular interactions between resveratrol and α-dicarbonyls reveals redox mechanisms leading to both antioxidant and pro-oxidant actions on HSA. Besides the well-known formation of AGEs in glycated proteins, more precise chemical modifications induced by α-dicarbonyls on HSA, such as the depletion of the unique Cys34 residue and the formation of particular protein carbonyls (GGS/ AAS) may be used as more precise molecular biomarkers of α-dicarbonyl stress and related physiological disorders. Furthermore, the plausible pro-oxidative role of the adduct between resveratrol and αdicarbonyls should be further investigated to clarify whether this action leads to positive or harmful clinical consequences. The biological relevance of human protein carbonylation as a redox signaling mechanism and/or as a reflection of oxidative damage and disease should also be studied in future works.

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Effects of resveratrol on hydrogen peroxide (H_2O_2**) - induced oxidative stress in *Lactobacillus reuteri* PL503**

N.O. Arcanjo[1], M.J. Andrade[2], P. Padilla[2,3], A. Rodríguez[2], M. S. Madruga[1], M. Estévez[3]♣

[1] *Paraiba Federal University, João Pessoa, Paraíba, Brazil*

[2] *Meat and Meat Products Research Institute, Food Hygiene and Safety, University of Extremadura, 10003, Cáceres. Spain.*

[3] *Meat and Meat Products Research Institute, Food Technology, University of Extremadura, 10003, Cáceres. Spain.*

♣ Corresponding author:
Mario Estévez (mariovet@unex.es)
Tel. +34927257100 (Ext. 51390)
Fax. +34927257110
Meat and Meat Products Research Institute, Food Technology,
University of Extremadura, 10003, Spain.

Abstract

Understanding of the mechanisms implicated in the response of probiotic bacteria to oxidative stress is of the utmost scientific interest. This study provides original insight into the genetic and molecular basis of the responses of *Lactobacillus reuteri* PL503 against hydrogen peroxide (H_2O_2) -induced oxidative stress. Six experimental groups were considered depending on the addition and concentration of H_2O_2 and resveratrol: 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 µM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 µM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 µM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 µM resveratrol). These experimental groups were incubated at 37 °C for 24 h in microaerophilic conditions at sampled at 12, 16, 20 and 24 hours and analysed in triplicate. The NADH-dependent-oxidoreductase encoded by the *dhaT* gene is a likely candidate to be strongly implicated in the antioxidant response of *L. reuteri*. Resveratrol (100 µM) is found to protect *L. reuteri* against protein carbonylation plausibly through various mechanisms including direct scavenging of reactive oxygen species (ROS), upregulation of the *dhaT* gene and promoting the synthesis of sulfur containing compounds. The hypothesis formulated on the ability of *L. reuteri* to detoxify H_2O_2 and its underlying mechanism needs to be clarified. Furthermore, protein carbonylation as a reflection of oxidative damage to bacteria and its consequences, as well as the role of protein carbonyls as signaling molecules implicated in the responses of bacteria to oxidative stress needs to be further investigated.

Keywords: Probiotic bacteria, stress response, protein oxidation, RNA analysis, phenolic compound

1. INTRODUCTION

The colon is known to be particularly susceptible to oxidative stress owing to an intense generation of reactive oxygen species (ROS) that frequently exceed antioxidant capabilities [1]. Some dietary components and, very particularly, red meat has been identified as a source of pro-oxidant species [2] and has been further found linked to the onset of colorectal cancer (CRC) by the IARC [3]. In this scenario, ROS and other reactive species naturally present in red meat (hydrogen peroxide, transition metals, heme molecules…) or formed during its digestion (carbonyls, nitroso-compounds,…) cause oxidative and nitrosative damage to proteins and lipids from the intestinal mucosa [4]. An enduring chemical injury at this location leads to pathological conditions in which oxidative stress is known to play a relevant role, including inflammatory bowel disease (IBD), ulcerative colitis and the aforementioned CRC [5,6]. In relation to the onset of oxidation-driven diseases in human individuals, the oxidation of proteins is highlighted as a remarkable molecular feature of most pathological conditions as protein oxidation leads to dysfunction, impairment of physiological patterns and inflammation [7]. Recently, Estévez & Luna [4] emphasized the transfer of dietary protein oxidation products from the lumen (luminal oxidative stress) to the intestinal mucosa and from there to the bloodstream (organic oxidative stress), leading to alteration in target internal organs. In both, foods and living systems, the hydroxyl radical typically formed from the Fenton reaction in the presence of hydrogen peroxide (H_2O_2) and transition metals (Fe, Cu, …) is identified as the most efficient radical species in biological systems and causes a variety of oxidative modifications in proteins, including carbonylation, cross-linking and aggregation, loss of functional groups, among others [7].

Probiotic bacteria are a natural protective barrier between potential harmful effects of dietary components and the intestinal mucosa. The protective role of microbiota on this and other processes is gaining increasing interest owing to their abilities to counteract the luminal oxidative stress in humans [8]. Probiotics are designated as “live microbes which, when administered in adequate amounts, confer a health benefit to the host” [9]. *Lactobacillus reuteri* is a natural colonizer of the gastrointestinal tract in humans and animals and has been widely used as a dietary supplement to enhance human gut health [10]. Oral administration of *L. reuteri* reduces gastrointestinal disorders

and infections and contributes to a balanced colonic microbiota [10]. According to the mechanisms related to its probiotic effects, *L. reuteri* has been reported to protect against oxidative stress and inhibits the accretion of oxidation products in the lumen, but probiotic effects are specific to a particular strain [11]. While the benefits of *L. reuteri* against oxidative stress and gut disorders are documented [12], the molecular mechanisms implicated in the responses of this probiotic bacteria under specific pro-oxidant conditions (such as those induced by a hydroxyl-radical generating system) are not defined yet.

On the other hand, certain dietary components with antioxidant properties may also be used to neutralize the pro-oxidant action of ROS and avoid and/or alleviate the symptoms of bowel disorders [8]. Phytochemicals with assorted bioactivities have been profusely studied for their potential health benefits in the gut. The review article rendered by Biasi et al. [13] includes scientific evidences of the ability of wine components to regulate inflammation and redox-signaling in intestinal cells. Phenolic compounds such as resveratrol, are able to act directly, as free-radical scavengers and indirectly, modulating gene expression and promoting the endogenous antioxidant defenses and protection against IBD and CRC through the activation of the NF-kB transcription factor. Interestingly, wine phenolic compounds have also been praised as prebiotics as they may be able to exert benefits to the colonic bacterial population. Hence, both wine phenolics and probiotics may protect against luminal oxidative stress and reduce intestinal injury.

The study of the molecular interactions between dietary oxidation products, phytochemicals and human probiotic bacteria requires innovative methods in molecular biology. Proteins act as modulators and executors of most biological functions and hence, their oxidative damage induced by ROS, leads to dysfunction and hence, to impaired physiological processes, and depending on extent of damage, to a pathological condition [14]. From a medical perspective, the study of protein oxidation is essential to understand the molecular basis of diseases in which oxidative stress plays a major role. To this regard, little information is available on the biological responses of probiotic bacteria to specific oxidative species and in particular, in the presence of bioactive compounds such as resveratrol. On the same line, the analysis of gene expression is a valuable and highly specific tool to understand how external factors precisely influence on particular biological functions and

metabolic routes. For instance, advanced genomic tools have been crucial to reveal the molecular basis of the beneficial effects of resveratrol on tumorigenic colon cells [15] and also to understand the antiproliferative effects of *L. reuteri* on human myeloid leukemia-derived cells [16]. This study is a preliminary approximation to the understanding of the protective effect that the combination of the probiotic *L. reuteri* bacterium and the resveratrol may have against H_2O_2-induced oxidative stress. To provide mechanistic insight, molecular and genetic responses of *L. reuteri* to the oxidative threat were analyzed.

2. MATERIAL AND METHODS

2.1. Chemicals and raw material

All chemicals and reagents used in this study were of ACS analytical grade and purchased from Sigma Chemicals (Sigma-Aldrich, Germany), Scharlab S.L. (Spain), Pronadisa (Conda Laboratory, Spain), Applied Biosystems (USA), Epicentre (USA) and Acros Organics (Spain). *L. reuteri* PL503 was isolated from pig faeces and identified by 16S rRNA by Ruiz-Moyano et al. 17]

2.2. Experimental setting

The strain used in this study, *L. reuteri* PL503, was maintained as glycerol freezer stocks at -80 °C. For preparing working cultures, this strain was consecutively grown twice in MRS broth (Conda Laboratory) plus 0.5% acetic acid 10% (v/v) at 37 °C for 24 h each. Six experimental groups were considered depending on the addition and concentration of H_2O_2 and resveratrol: 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 µM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 µM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 µM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 µM resveratrol). Three replicates were carried out for each treatment. Experimental tubes were inoculated with 100 µL of the last overnight culture of *L. reuteri* PL503 in MRS broth and incubated at 37 °C for up to 24 h in microaerophilic conditions. Samples of the cultures were collected in four times (12, 16, 20 and 24 h) from the inoculation. For further protein

analyses, culture medium was removed by washing with a phosphate buffered saline (PBS, pH 7.4) solution twice.

2.3. Gene expression

After incubation time, 1 mL of each treatment was immediately frozen and stored at −80 °C until RNA extraction procedure, which was performed using the MasterPure™ RNA purification kit (Epicentre, USA) following manufacturer's instructions. To remove genomic DNA contamination, samples were diluter to a concentration of 0.1 µg/µL and treated with DNase I (Thermo Fisher Scientific, USA). Pure RNA was eluted in 35 µL buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and kept at −80 °C until required. The RNA concentration (ng/µL) and purity (A_{260}/A_{280} ratio) were spectrophotometrically determined using a 1.5 µL aliquot on the Nanodrop 2000 (Thermo Scientific, USA).

Next, two-step reverse transcription real-time PCR (RT-qPCR) were performed to conduct gene expression studies. For this, the synthesis of cDNA was firstly performed using about 500 ng of total RNA following manufacturer instructions of the PrimeScript™ RT Reagent kit (Takara Bio Inc., Japan). The obtained cDNA was stored at −20 °C until further use in qPCR reactions.

The qPCR reactions were carried out on an Applied Biosystems ViiA™ 7 Real-Time System (Applied Biosystems) in MicroAmp optical 96-well reaction plates sealed with optical adhesive covers (Applied Biosystems). The SYBR Green technology was used. The reaction mixtures of a final volume of 12.5 µL consisted of 2.5 µL of cDNA template, 6.25 µL of SYBR® *Premix Ex Taq*™ (Takara Bio Inc.), 0.625 µL of ROX™ Reference Dye (Takara Bio Inc.), 2.37 µL of sterile ultrapure water and 300 mM of each primer pair (**Table 1**). The qPCR methods for amplification of *UspA*, *dhaT* and 16S rRNA genes were conducted with the following thermal cycling conditions: a single step of 8 min at 95 °C, 40 cycles at 95 °C for 10 s, 53 °C for 10 s and 72 °C for 25 s. After the final qPCR cycle, melting curves were performed by heating the products to 60-99 °C and the values of the melting temperatures were checked to ensure the truthfulness of the results and specificity of the primer pairs. Threshold cycle (Ct) values represent the PCR cycle in which an increase in fluorescence,

over a defined threshold, first occurred for each amplification plot. All samples were analyzed in triplicate, including control sample consisting of adding sterile ultrapure water instead of template DNA, and qPCR reactions were repeated at least twice. The expression ratio was calculated using the $2^{-\Delta\Delta CT}$ method reported by Livak & Schmittgen [18]. The 16S rRNA gene was used as endogenous control for the relative quantification of the expression of the *UspA* and *dhaT* genes. The experimental group CONTROL was used as calibrator at each sampling time.

2.4. Synthesis of α-aminoadipic semialdehyde (AAS) standard compound

N-Acetyl-L-AAS was synthesized from Nα-acetyl-L-lysine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al. [19]. Briefly, 10 mM Nα-acetyl-L-lysine was incubated with constant stirring with 5 g egg shell membrane in 50 mL of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane then was removed by centrifugation and the pH of the solution adjusted to 6.0 using 1M HCl. The resulting aldehydes were reductively aminated with 3 mmol ABA (4- aminobenzoic acid) in the presence of 4.5 mmol sodium cyanoborohydride ($NaBH_3CN$) at 37 °C for 2 h with stirring. Then, ABA derivatives were hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C to dryness. The resulting AAS-ABA was purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as elution solvent. The purity of the resulting solution and authenticity of the standard compounds obtained following the aforementioned procedures have been checked by using MS and ^{1}H NMR [19,20].

2.5. Quantification of AAS

Five hundred microliters of culture were dispensed in 2 mL microtubes and treated with cold 10% TCA (Trichloroacetic acid) solution. Each microtube was vortexed and then subjected to centrifugation at 5000 rpm for 5 minutes at 4 ºC. The supernatants were removed and the pellets were incubated with the following freshly prepared solutions: 0.5 mL 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL 50 mM ABA in 250 mM MES buffer pH 6.0 and 0.25 mL 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. The tubes were vortexed and then incubated in water bath at 37 °C for 90

174 min. The samples were stirred every 15 min. After derivatization, samples were treated with a cold
175 50% TCA solution and centrifuged at 10000 rpm for 10 min. The pellets were then washed twice with
176 10% TCA and diethyl ether-ethanol (1:1). Finally, the pellets were treated with 6 N HCl and kept in
177 an oven at 110 ºC for 18 hours until completion of hydrolysis. The hydrolysates were dried *in vacuo*
178 in a centrifugal evaporator. The generated residue was reconstituted with 200 µL of milliQ water and
179 then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 µm pore size,
180 Pall Corporation, USA) for HPLC analysis.

181 A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Kyoto, Japan), equipped with a
182 quaternary solvent delivery system (LC-20AD), a DGU-20AS on-line degasser, a SIL-20A auto-
183 sampler, a RF-10A XL fluorescence (FLD) detector, and a CBM-20A system controller, was used.
184 An aliquot (1 µL) from the reconstituted protein hydrolysates was injected and analyzed in the above
185 mentioned HPLC equipment. AAS-ABA was eluted in a Cosmosil $5C_{18}$-AR-II RP-HPLC column (5
186 µm, 150 x 4.6 mm) equipped with a guard column (10 x 4.6 mm) packed with the same material. The
187 flow rate was kept at 1 mL/min and the temperature of the column was maintained constant at 30
188 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm,
189 respectively. Standards (0.1 µL) were run and analyzed under the same conditions. Identification of
190 both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their
191 retention times with those from the standard compounds. The peak corresponding to AAS-ABA was
192 manually integrated from FLD chromatograms and the resulting areas plotted against an ABA
193 standard curve with known concentrations that ranged from 0.1 to 0.5 mM. Results were expressed
194 as nmol of carbonyl compound per mg of protein.

195 **2.6. Analysis of protein thiols**

196 To avoid possible contamination with thiols from the medium, 250 µL culture was washed twice with
197 PBS and with ethanol:ethyl acetate (1:1), the pellet was resuspended in 250 µL of guanidine
198 hydrochloride and added to the cuvette in a final volume of 1.250 µL of guanidine hydrochloride.
199 Absorbance was measured at 324 nm, pre and post addition of 250 µL of 4 DPS (4,4′-Dipyridyl
200 disulfide) in 12 mM HCl. Results were expressed as µmol of free thiol groups per mg of protein.

2.7. Statistical analysis

Data from the analysis (n=3) were collected and subjected to statistical analyses. In order to assess the effect of different concentrations of resveratrol in the presence of hydrogen peroxide on the genetic and chemical expression of the bacteria an analysis of variance (ANOVA) was applied (SPSS v. 15.5). A Tukey test was applied when ANOVA found significant differences between treatments. The statistical significance was set at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Genetic responses of *L. reuteri* to H_2O_2-induced oxidative stress

In the present study, these actions were firstly assessed by the analysis of the expression of stress-related genes. **Figure 1A** shows the relative expression ($2^{-\Delta\Delta C_T}$) of the *UspA* gene during the incubation period (37 ºC/24 h) of *L. reuteri* PL503 challenged with H_2O_2 and resveratrol. The universal stress protein A (*UspA*) superfamily comprehends an ancient and conserved group of proteins found in assorted microorganisms, insects and plants. Although the regulation of the *UspA* gene has been tried to be ascertained in bacteria (namely *Escherichia coli*), the precise roles of Usp proteins remain unclear; yet, they seem to be linked to struggle against DNA-damaging agents [21]. Oxidative stress may be considered as a major cause of cell damage through various mechanisms including metabolic pathway disruptions, spontaneous mutations and bacteriostatic/bactericidal effects. Anaerobic bacteria are particularly sensitive to ROS such as H_2O_2 due to the lack of catalases and other enzymes associated with the defense against oxidative stress. Unexpectedly, the incubation of *L. reuteri* PL503 in the presence of H_2O_2 led to a down regulation of the *UspA* gene during the entire assay. This observation is, however, consistent with findings obtained by Oberg et al. [22] who observed a significant inhibition of the *UspA* gene expression in *Bifidobacterium longum* exposed to a H_2O_2-induced stress. These authors only found upregulation of an ATP-dependent matallo-protease which was hypothesized to act in response to oxidative damage of membrane proteins. To similar conclusions came Jin et al. [23] who studied the tolerance of the same bifidobacterium to organic acids using a genomic approach. Unfortunately, results from these previous studies did not provide insight into the underlying antioxidant mechanisms of bacteria. Based on our initial

hypothesis of resveratrol being a antioxidant compound and potential enhancer of the probiotic effects of bacteria, this phytochemical was tested at two biologically relevant concentrations (20 and 100 µM) alone or in combination with H_2O_2. The incubation of *L. reuteri* PL503 with resveratrol also led to a downregulation of the *UspA* gene though the effect was not so remarkable as for H_2O_2. No dose effect was observed in this regard though the high concentration of resveratrol (100 µM) led to a suppression of the *UspA* gene similar to that displayed by H_2O_2. Only the combination of H_2O_2 with 100 and 20 µM of resveratrol at 20 and 24 h of incubation, respectively, led to a significant increase of the *UspA* gene expression, reflecting a yet indefinite potential synergistic effect of both chemical species on the response of *L. reuteri* PL503 to a pro-oxidative stress.

Unlike the heterogeneous group of proteins encoded by the *UspA* gene, the expression of the other gene under study (*dhaT*) leads to the synthesis of a single protein with definite function, the 1,3 propanediol oxidoreductase (1,3-PDO). This enzyme has lately attracted considerable attention given its ability to produce 1,3 propanediol (1,3-PD) from glycerol under anaerobic conditions (**Figure 2A**). In a first step, a coenzyme B12-dependent glycerol dehydratase, catalyzes the formation of 3-hydroxypropionaldehyde (3-HPA) from glycerol and in a second step, 3-HPA is reduced to 1,3-PD. It is worth highlighting that, in this latter reaction, NADH is regenerated into NAD^+, which is required to ferment glucose through its main carbohydrate fermentation pathway (6-phosphogluconate/phosphoketolase; 6-PG/PK). Hence, supplementation with glycerol and the activation of this route have been found to stimulate the ability of *L reuteri* to grown on the presence of carbohydrates [24]. Furthermore, this route plays a major role in the probiotic properties of *L. reuteri* as the intermediate product (3-HPA) is also referred as to reuterin, which is known to be excreted in copious amounts to the surrounding environment, imparting strong antimicrobial properties [25].

Highly remarkable changes in the expression of this gene were observed during the incubation of *L. reuteri* PL503 as affected by the addition of H_2O_2 and two concentrations of resveratrol (**Figure 1B**). During the first 16 h, the effects shifted between no significant influence on the *dhaT* gene expression (i.e. LRES at 12 and 16 h) to a moderate downregulation of such gene (i.e. H_2O_2-HRES at 12 h). During the second half of the incubation assay, the effect of the incubation conditions changed

dramatically as an upregulation of the gene under study was observed in all the experimental groups (except LRES at 24 h). The sole addition of the pro-oxidant threat (0.5 mM H_2O_2) led to an overexpression of the *dhaT* gene which is a reflection that 1,3-PDO may be implicated in some kind of response to counteract the harmful effect of H_2O_2. Interestingly, *L. reuteri* was grown in the absence of glycerol in the present study, so the activation of the route previously described (**Figure 2A**) to generate NAD^+ from 3-HPA seems unlikely. While the compound identified as the main substrate of the 1,3-DPO was plausibly absent, the gene encoding for such enzyme was being upregulated under oxidative stress conditions. We propose that the NAD+-dependent activity of the 1,3-PDO may be able to detoxify H_2O_2in the presence of $NADH_2$ in accordance to the pathway proposed in **Figure 2B**. Whereas this extent may need *in vitro* confirmation, this would be, to our knowledge the first time that the *dhaT* gene and 1,3-DPO are reported to be implicated in the antioxidant defense of bacteria against H_2O_2. Kang et al. [26] highlighted a NADH oxidase-NADH peroxidase system as a common oxidative stress resistance mechanism for *Lactobacillus panis* grown in aerobic culture. In such conditions and using O_2 as electron acceptor, an NADH oxidase enzyme produced up to 100 µM of H_2O_2, with this pro-oxidant species being subsequently detoxified by a NADH peroxidase activity that authors ascribed to be decoded by an *npx* gene. While the *in vitro* NADH peroxidase activity was intense in aerobic culture (ca. 200 µM H_2O_2 decomposed per minute) and negligible in microaerobic conditions (non-detected) the expression of the *npx* gene did not seem to be affected by the culture conditions (relative expression *npx* gene: 1.00±0.10 vs 1.16±0.28 in microaerobic and aerobic conditions, respectively), suggestive of the lack of connection between such peroxidase activity and the gene expression. Interestingly, the authors found limited 1,3 PD production by *L. panis* in the aerobic conditions even though glycerol was supplied. It is reasonable to hypothesize that a H_2O_2-dependent peroxidase activity of 1,3-DPO with would have contributed to detoxify H_2O_2while the usage of 3-HPA (from glycerol metabolism) as preferential substrate played a secondary role. The lack of specific substrate in NADH-dependent enzymes has been described in literature when involved in redox-reactions [27] and other lactic acid bacteria such as *B. longum* has been found to lack NADH peroxidase and employs a NADH oxidoreductase to protect itself against H_2O_2-induced oxidative stress [28]. These observations provide strength to our hypothesis.

The incubation of *L. reuteri* PL503 with resveratrol induced an overexpression of the *dhaT* gene to a larger extent than H_2O_2 (20 h) and no dose-dependent effect was observed (**Figure 1B**). The ability of resveratrol to protect against oxidative stress in biological systems has been profusely documented and the underlying mechanisms involve not only its radical-scavenging activity but also modulation of gene expression to improve the endogenous antioxidant and immune defenses of the biological system [13]. The impact of resveratrol on the redox homeostasis of the gut is of particular relevance and authors have described the ability of this phytochemical to promote the growth and health of *Bifidobacterium* and *Lactobacillus* spp. at the expense of pathogens such as *Clostridium* [29]. The upregulation of the *dhaT* gene by resveratrol was extraordinarily enlarged when this phytochemical was provided together with H_2O_2. The relative expression of the *dhaT* gene was 11 and 7 times higher in H_2O_2-HRES at 20 h and H_2O_2-LRES at 24 h than the CONTROL group, illustrating the clear protective effect of resveratrol against oxidative stress through gene expression regulation and that such protective effect is dependent on the concentration of both, $H_2O_2$and resveratrol. The striking resveratrol-driven response of *L. reuteri* PL503 against H_2O_2 was more intense and appeared earlier (20 h) when the concentration of the phytochemical was higher (100 µM). The gene expression responses observed in the present study may account for some of the prebiotic effects attributed to resveratrol. Authors such as Jang & Surth [30], Konyalioglu et al. [31] and Bosutti & Degens [32] have proven the protective effect of resveratrol on H_2O_2-induced oxidative stress in assorted human and rat cells, and alike the present study, observed a dose-dependent interaction of both molecules and reported the ability of the phytochemical to strengthen the endogenous antioxidant defenses by gene expression regulation.

3.2. Chemical responses of *L. reuteri* to H_2O_2-induced oxidative stress

Oxidative stress causes damage to biomolecules and proteins are preferential targets of ROS. The damage caused to proteins by ROS under pro-oxidative conditions has been highlighted as one of the most remarkable causes of aging and disease in humans [4, 33]. In a recent review, Ezraty et al. [33] reported that oxidative damage has a devastating effect on the structure and functionality of proteins and may even lead to cell death. This is particularly applicable to anaerobic bacteria as they lack catalases and other regular antioxidant enzymes to neutralize the pernicious effects of ROS

[35]. Interestingly, the oxidative damage to proteins is also involved in the bacterial response to oxidative stress [34] as proteins are activated by oxidative means to trigger specific antioxidant mechanisms. Despite the undeniable significance of protein oxidation in bacterial biology, scientific literature lacks the description of the oxidative damage to bacterial proteins challenged under pro-oxidant conditions. In the present study we aimed to quantify AAS as a specific marker of the oxidative damage to proteins and free thiols as relevant redox-active moieties in proteins.

3.2.1. Protein carbonylation

The evolution of the concentration of AAS in *L. reuteri* PL503 during the incubation assay (37ºC/24 h) is shown in **Table 2**. AAS is formed in biological systems as a result of the oxidative deamination of lysine residues in proteins, is emphasized as the most abundant protein carbonyl and commonly used in biology and medicine as a marker of oxidative stress, aging and disease [36, 37]. Ballesteros et al. [38] also proposed protein carbonylation as a reflection of bacterial senescence and that, as occurs in eukaryotes, oxidized proteins accumulate in non-proliferating bacteria. This may explain the evolution of AAS in CONTROL group of *L. reuteri* PL503. The concentration of AAS increased up to 1.48 nmol/mg protein at 20 h following incubation and decreased afterwards, by the end of the assay. While the increase of AAS precisely reflects the oxidation of lysine residues by reactive species [20, 37], the further decline may respond to the involvement of this carbonyl in subsequent reactions, more specifically in the formation of Schiff base structures by reaction with other protein amino groups [14]. As expected, the incubation with H_2O_2 promoted the oxidative deamination of lysine residues to yield the corresponding carbonyl compound. The concentration of AAS in these colonies was significantly higher than in the CONTROL counterparts at all sampling times (except initial point). H_2O_2 has been efficiently used to promote *in vitro* the carbonylation of assorted proteins such as bovine serum albumin [37], human serum albumin [39], α-lactoglobulin [40], myoglobin [41], and myofibrillar proteins [42], among others. In the presence of transition metals, H_2O_2 yields hydroxyl radical which is able to abstract an electron from the carbon neighboring the amino group in lysine residues, triggering the subsequent deamination [37]. To our knowledge, this is the first attempt to quantify AAS in bacteria challenged with H_2O_2 as previous studies have used the routine and more unspecific dinitrophenylhydrazine (DNPH) method to estimate the total amount of protein

carbonyls [38]. While AAS has been reported in bacteria before [43], never was it related to oxidative stress. The addition of resveratrol had contradictory effects on the formation of AAS as low concentrations had a pro-oxidant action and the high concentration led to a significant reduction of protein carbonylation as compared to the CONTROL (20 and 24 h). Both pro- and antioxidant actions of resveratrol have been reported in literature and the dose in known to play a relevant role. While both concentrations were able to activate the *dhaT* gene, only the highest dose displayed an antioxidant action. While pro-oxidant effects are ascribed to the involvement of resveratrol in ROS generation, the protective effect observed at high concentrations may be principally attributed to direct redox effects of the resveratrol such as its ability to neutralize ROS [44]. In clear connection with the gene expression regulation observed for *dhaT*, the addition of resveratrol to *L. reuteri* PL503 challenged with H_2O_2 led to a significant protection of proteins against carbonylation. The fact that the concentration of AAS in LRES is higher than that in H_2O_2-LRES proves that the underlying mechanisms of resveratrol as a protective molecule in *L. reuteri* PL503 are highly dependent on the presence of H_2O_2. While the protection of *L. reuteri* PL503 against portein carbonylation by resveratrol in H_2O_2-containing cultures was noticiable already at 12h, the remarkable effect of resveratrol on *dhaT* took place in subsequent stages of the incubation assay (**Figure 2A**). In clear connection with the interesting considerations made by Ezraty et al. [34] the accumulation of carbonyls, as a irresversible modification in oxidized proteins, triggered the H_2O_2-induced *dhaT* upregulation by resveratrol (**Figure 2A**). Carbonyls may be not only the irresversible products of the oxidative stress on bacterial proteins, they may also act as signalling molecules to induce the activation of specific pathways aimed to control senescence and impaired functionality. Likewise, resveratrol (both concentrations) may not only be effective against protein carbonylation by neutralizing ROS in challenged cultures, the stilbene, resveratrol, may also promote the expression of genes related to endogenous antioxidant defenses at particular carbonyl concentrations (> 1 nmol/mg protein) (**Figure 3**).

3.2.2. Free thiols

The amino acids cysteine (Cys) and methionine (Met) are particularly sensitive to oxidation owing to the electron-rich sulfur atom in their side chain. While thiol depletion is a typical feature in oxidized

proteins and this may lead to dysfunction, sulfur-containing amino acids are known to act as antioxidant moieties in proteins due to its sacrificial oxidation in the presence of ROS [7]. According to this theory, thiols with irrelevant biological significance are readily oxidized under oxidative stress and as a result, avoid the oxidative degradation of other valuable residues, namely amino groups from lysine [7]. Furthermore, unlike lysine oxidation and the formation of AAS, certain oxidation mechanisms involving free thiols are reversible and hence, under reducing environments, proteins may be repaired to preserve its integrity and functionality [34]. In bacteria, this mechanism of proteins repair may be related to virulence: counteracting the pernicious effect of ROS in proteins allows their survival [34]. In *L. reuteri* PL503 and other potential probiotic bacteria, this mechanism may also be linked to their ability to neutralize reactive species and protect themselves and the host against their toxic effects.

Table 3 shows the concentration of free thiols in *L. reuteri* during the incubation assay (37 ºC/24 h). No significance changes in thiol concentration was observed in CONTROL samples reflecting that the lack of external sources of oxidative stress enabled a paired balance between thiol oxidation and repair/de novo synthesis of proteins that allowed a stable quantity of sulfur-containing compounds during the entire assay. Conversely, the incubation in the presence of H_2O_2 caused a gradual and significant loss of free thiols. H_2O_2 can directly react with Cys and Met to yield sulfenic acid and methionine sulfoxide, respectively, or be decomposed through the Fenton reaction into hydroxyl radical which readily and efficiently oxidizes sulfur amino acids [7,34]. The depletion of thiols, however, was not so intense to compromise the survival of *L. reuteri* PL503, in accordance to the counts shown in **Table 1**. Even if free thiols could have acted as antioxidants and be lost as a sacrificial protection, when protein redox capabilities are wasted, other amino acid residues are oxidized, which is compatible with the increase of lysine-derived protein carbonyls shown in **Table 2**. In general, growing *L. reuteri* PL503 with resveratrol diminished the depletion of thiols which emphasizes the protective effect against oxidative stress. Only a depletion of thiols in the first stages of the incubation in LRES denotes a pro-oxidant effect which is again compatible with the results observed for this incubation system in **Table 2**. At the end of the incubation assay (24 h), the concentration of thiols in LRES and HRES is similar to that in CONTROL. In H_2O_2-LRES and H_2O_2-

HRES, the evolution of free thiols followed a different trend: an initial depletion at 12 h was followed by a significant increase so that the concentration of free thiols in H_2O_2-HRES and H_2O_2-LRES was significantly higher than in CONTROL at 20 and 24 h, respectively. The timely coincidence between the accretion of thiol groups in these experimental groups with the remarkable expression of the *dhaT* gene in the same groups suggest a plausible connection between the genetic response of *L. reuteri* PL503 against the H_2O_2-induce oxidative stress and *the novo* synthesis of sulphur containing proteins/peptides (**Figure 3**). While the implicated genes and underlying molecular mechanisms remain indefinite, the hypothesis of considering thiol accretion as means to protect against oxidative stress is highly plausible given the well-known role of such moieties as redox-active compounds and elements of antioxidant protection in biological systems, including lactic acid bacteria [28].

4. CONCLUSIONS

This study provides preliminary but sound and original findings on the genetic and molecular basis of the responses of *L. reuteri* PL503 against H_2O_2-induced oxidative stress. The NADH-dependent-oxidoreductase encoded by the *dhaT* gene is a plausible candidate to be strongly implicated in the antioxidant response of *L. reuteri* PL503. Resveratrol (100 µM) is found to protect *L. reuteri* PL503 against protein carbonylation plausibly through various mechanisms including direct scavenging of ROS, upregulation of the *dhaT* gene and promoting the synthesis of sulfur containing compounds with protective effect. Along with this novel information, the present study provides grounds for future studies as the hypothesis formulated on the ability of *L. reuteri* PL503 to detoxify $H_2O_2$and its underlying mechanism needs to be clarified. Furthermore, protein carbonylation as a reflection of oxidative damage to bacteria and its consequences, as well as the role of protein carbonyls as signaling molecules implicated in the responses of bacteria to oxidative stress needs to be further investigated.

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FIGURE CAPTIONS

FIGURE 1. Relative expression of the *UspA* (A) and *dhaT* (B) genes in *Lactobacillus reuteri* PL503 during an incubation period for up to 24 h.

Blue line at $2^{-\Delta\Delta C_T}$= 1 denotes standarized expression rate for CONTROL group at each sampling time (Calibrator). $2^{-\Delta\Delta C_T} < 1$ denotes supression of gene expression; $2^{-\Delta\Delta C_T} > 1$ denotes activation of gene expression.

Experimental groups:

■ CONTROL (*L. reuteri* in MRS broth);

□ H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2);

⊡ LRES (*L. reuteri* in MRS broth + 20 µM resveratrol);

⊡ HRES (*L. reuteri* in MRS broth + 100 µM resveratrol);

⊟ H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 µM resveratrol);

▤ H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 µM resveratrol).

FIGURE 2. Production of 3-HPA (reuterin) and 1,3 DP from glycerol in *Lactobacillus reuteri* PL503 in accordance to the description made by Talarico et al. [24] (A) and proposed mechanism by which *dhaT*–encoded 1,3-PD-NAD+ oxidoreductase may detoxify hydrogen peroxide (B).

FIGURE 3. General scheme of the proposed mechanisms by which resveratrol may protect *Lactobacillus reuteri* PL503 proteins against H_2O_2-induced oxidative stress.

TABLE 1. Sequences of primers used for reverse transcription real-time PCR assays to conduct gene expression analyses.

Primers	Gene	Nucleotide sequence (5´- 3´)	Annealing temperature	References
uspALr-F1	*UspA*	CTTGGGTAGCGTTCACCATT	60 °C	This study
uspALr-R1		TGAAAAAGCGGTTGACACTG	60 °C	This study
LS67	*dhaT*	TGACTGGATCCTAATTTGGTCCTGGTGTTATTGC	60 °C	Schaefer et al., 2010
LS68		TGACTGAATTCTTCCGGATCTTAGGGTTAGG	60 °C	Schaefer et al., 2010
Lr16S_F	16S rRNA	CCGCTTAAACTCTGTTGTTG	55 °C	This study
Lr16S_R		CGTGACTTTCTGGTTGGATA	55 °C	This study

TABLE 2. Concentration of α-aminoadipic semialdehyde (AAS; nmol/mg protein) in *Lactobacillus reuteri* PL503 during incubation assay (37ºC/24 h).

	CONTROL	H_2O_2	LRES	HRES	H_2O_2-LRES	H_2O_2-HRES	p^A
0 h	0.30^{v}±0.03	0.31^{v}±0.04	0.32^{v}±0.03	0.27^{v}±0.04	0.29^{v}±0.03	0.27^{v}±0.04	ns
12 h	$0.32^{d,v}$±0.04	$1.89^{a,x}$±0.23	$0.50^{c,w}$±0.12	$0.41^{cd,w}$±0.10	$0.99^{b,w}$±0.15	0.95^{x}±0.29	***
16 h	$0.45^{d,vw}$±0.13	$2.84^{a,y}$±0.31	$1.06^{b,x}$±0.21	$0.58^{cd,wx}$±0.18	$0.96^{b,w}$±0.18	$0.62^{c,w}$±0.22	***
20 h	$1.48^{b,x}$±0.29	$3.44^{a,z}$±0.55	$1.77^{b,y}$±0.39	$0.79^{c,x}$±0.22	$1.54^{b,x}$±0.39	$1.84^{b,y}$±0.41	***
24 h	$0.71^{b,w}$±0.23	$1.27^{a,w}$±0.28	$0.99^{b,x}$±0.31	$0.38^{c,w}$±0.08	$0.81^{b,w}$±0.21	$0.85^{b,wx}$±0.24	**
p^B	***	***	***	**	***	***	

Data expressed as means ± standard deviations of three replicates.

Experimental groups : 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 µM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 µM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 µM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 µM resveratrol).

[A] Significance from ANOVA denotes differences between means from the same row. ** : p≤0.01 ; *** : p≤0.001 ; ns : no significant.

[B] Significance from ANOVA denotes differences between means from the same column. ** : p≤0.01 ; *** : p≤0.001.

Superscripts (a-d) within the same row denote significant differences between experimental groups.

Superscripts (v-z) within the same column denote significant differences between sampling times.

TABLE 3. Concentration of free thiols (µmol/mg protein) in *Lactobacillus reuteri* PL503 during incubation assay (37ºC/24 h).

	CONTROL	H_2O_2	LRES	HRES	H_2O_2-LRES	H_2O_2-HRES	p^A
0 h	10.97±0.41	11.58^{x}±0.56	11.50^{x}±0.65	11.04^{x}±0.84	11.14^{x}±0.95	11.10^{x}±0.75	ns
12 h	9.76^{bc}±0.51	$9.63^{bc,y}$±0.48	$8.80^{c,z}$±0.43	$11.01^{a,x}$±0.78	$9.93^{b,y}$±0.65	$9.58^{bc,y}$±0.64	**
16 h	10.05^{bc}±0.45	$9.18^{c,yz}$±0.59	$9.51^{bc,yz}$±0.57	$10.78^{ab,x}$±0.45	$10.17^{bc,xy}$±0.68	$11.07^{a,x}$±0.58	*
20 h	10.84^{b}±0.65	$8.71^{c,yz}$±0.61	$10.28^{b,y}$±0.61	$10.61^{b,xy}$±0.65	$10.58^{b,x}$±0.64	$12.11^{a,x}$±0.78	**
24 h	10.20^{bc}±0.55	$8.48^{d,z}$±0.51	$10.11^{bc,y}$±0.59	$9.94^{c,y}$±0.44	$11.13^{a,x}$±0.71	$10.87^{ab,xy}$±0.47	***
p^B	ns	***	**	**	**	***	

Data expressed as means ± standard deviations of three replicates.

Experimental groups : 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 µM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 µM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 µM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 µM resveratrol).

[A] Significance from ANOVA denotes differences between means from the same row. * : p≤0.05 ; ** : p≤0.01 ; *** : p≤0.001 ; ns : no significant.

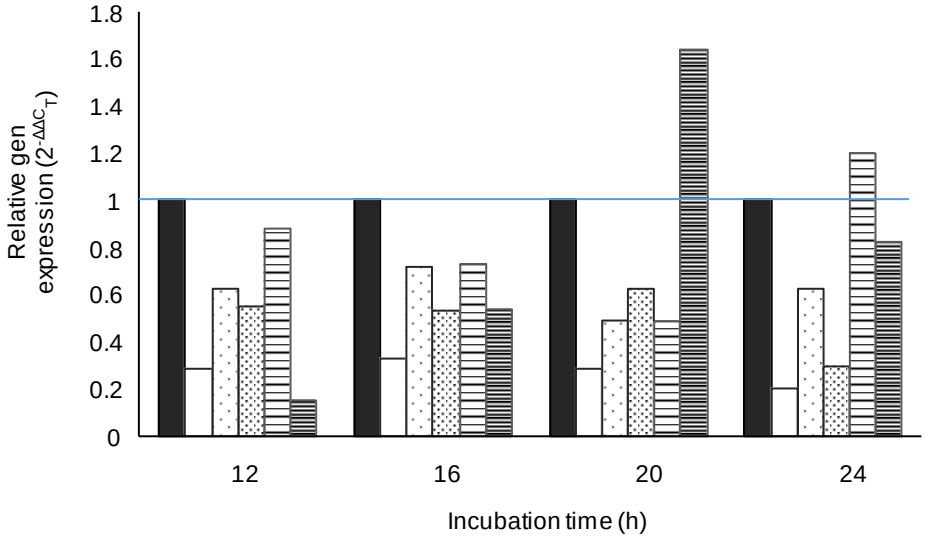
[B] Significance from ANOVA denotes differences between means from the same column. * : p≤0.05 ; ** : p≤0.01 ; *** : p≤0.001.

Superscripts (a-d) within the same row denote significant differences between experimental groups.

Superscripts (x-z) within the same column denote significant differences between sampling times.

FIGURE 1

(A)


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative gen expression ($2^{-\Delta\Delta C_T}$)
12
16
20
24
Incubation time (h)

(B)

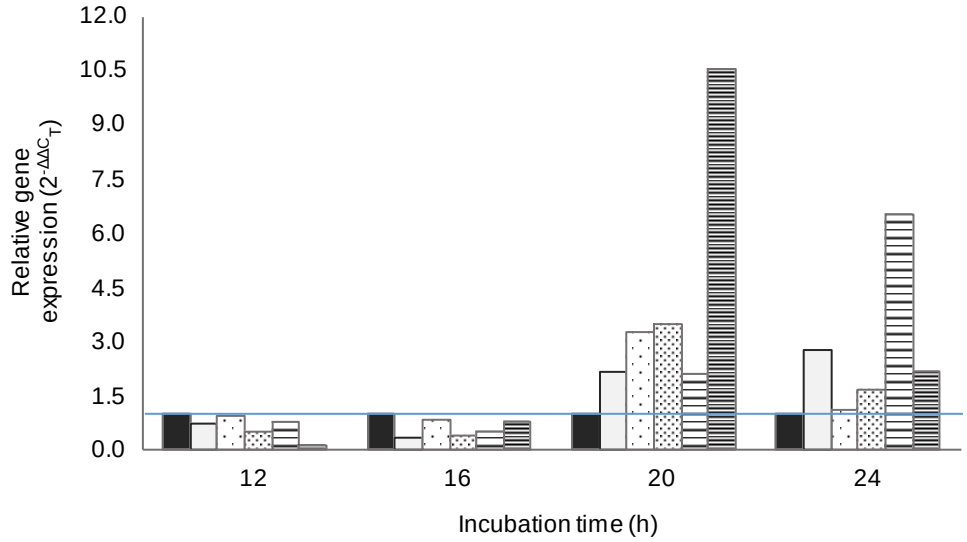

12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
0.0
Relative gene expression ($2^{-\Delta\Delta C_T}$)
12
16
20
24
Incubation time (h)

FIGURE 2

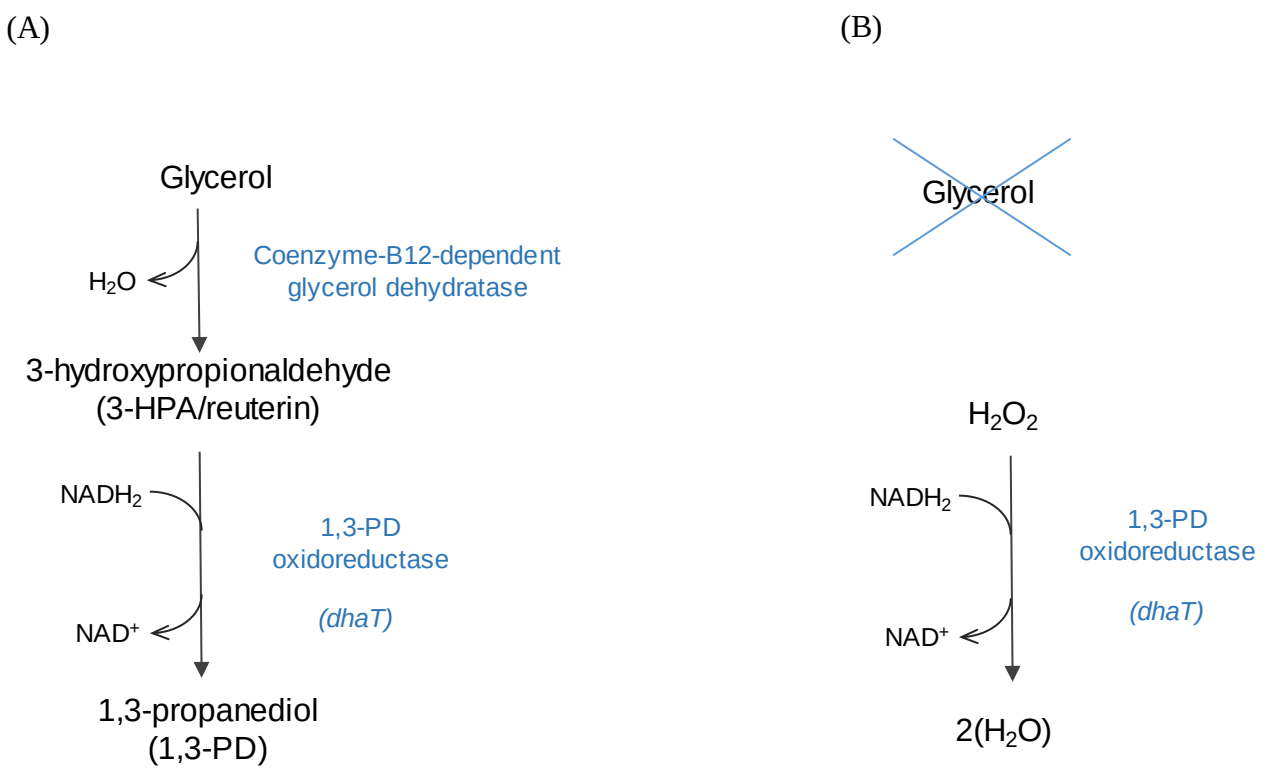
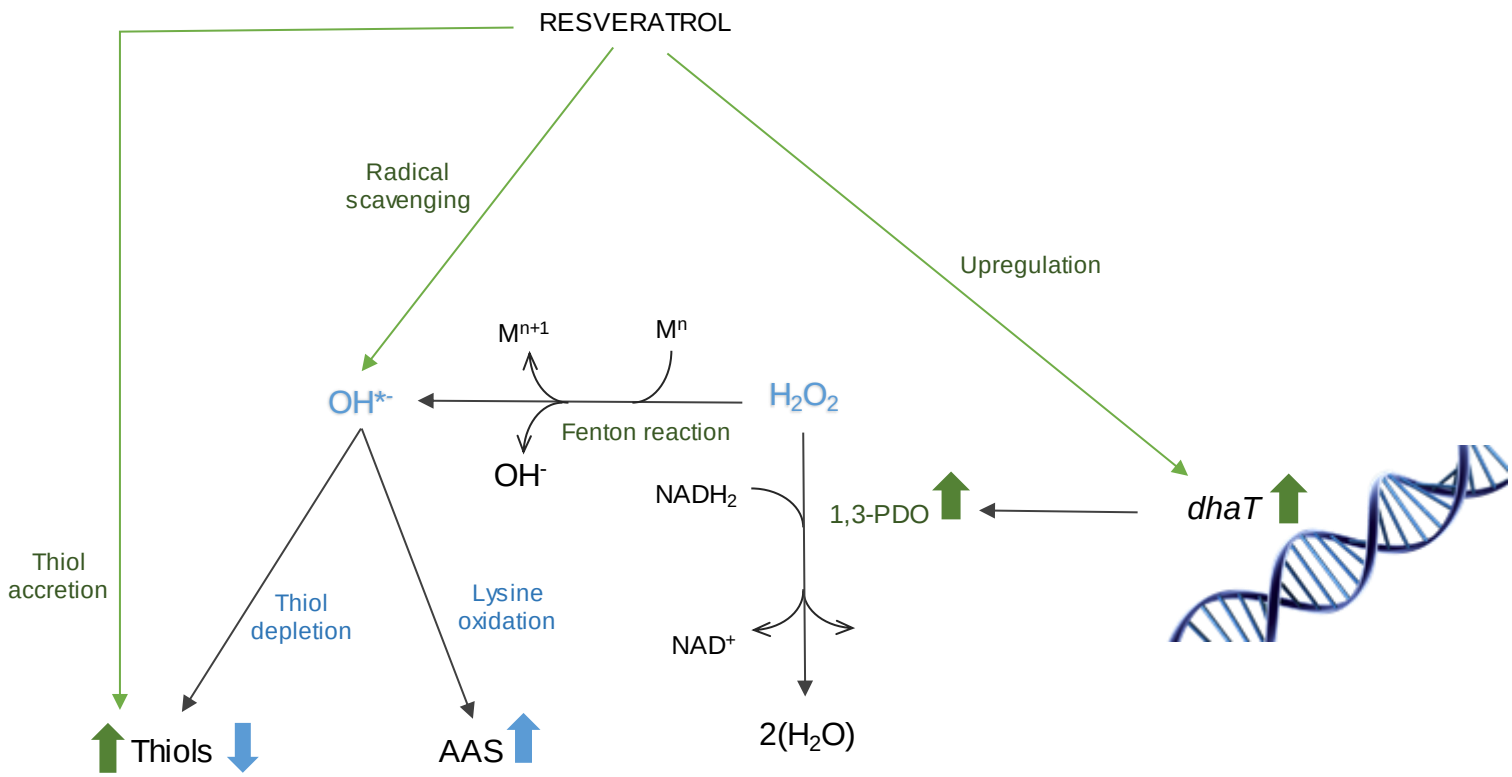

(A)
Glycerol
H2O
Coenzyme-B12-dependent
glycerol dehydratase
3-hydroxypropionaldehyde
(3-HPA/reuterin)
NADH2
NAD+
1,3-PD
oxidoreductase
(dhaT)
1,3-propanediol
(1,3-PD)
(B)
Glycerol
H2O2
NADH2
NAD+
1,3-PD
oxidoreductase
(dhaT)
2(H2O)

FIGURE 3


RESVERATROL
Radical scavenging
Upregulation
M^{n+1}
M^{n}
OH^{*-}
H_2O_2
Fenton reaction
OH^{-}
$NADH_2$
1,3-PDO
dhaT
Thiol accretion
Thiol depletion
Lysine oxidation
NAD^{+}
Thiols
AAS
$2(H_2O)$

5 CONCLUSÕES GERAIS

Conforme os resultados obtidos dos experimentos pode-se concluir que:

- O processo de marinação com vinho tinto demonstra diversas ações positivas para os bifes bovinos, principalmente contra reações de oxidação de lipídeos e proteinas, além disso melhora alguns aspectos sensorias como a textura e cor, e atua contra o crescimento microbiano. De modo particular, específicos componentes do vinho apresentam influência protetora contra detrioração do bifes, com destaque para o ácido cafárico e o ácido hidroxicinâmico abundante no vinho Isabel.

- Nos bifes assados, o efeito da marinação com o vinho ISA demonstra a potencial capacidade em inibir a oxidação de proteínas, limitando os potenciais efeitos adversos das proteínas carbonilas, ditirosinas e produtos de oxidação do triptofano na saúde do consumidor, quando comparado aos demais marinados.

- Portanto, os dados apresentados sustentam o benefício tecnológico de usar vinho para marinar bifes bovinos. E, vinho produzidos com uvas Isabel vinho de baixa qualidade quando comparado aos vinhos produzidos com uvas *Vitis Vinífera* podeser utilizado com sucesso como ingrediente funcional de produtos cárneos.

- Investigações dever ser desenvolvidas com a finalidade de verificar o papel pró-oxidativo do aducto entre resveratrol e α-dicarbonil e assim trazer esclarecerimentos se esta ação leva a conseqüências clínicas positivas ou prejudiciais. Mecanismos de potenciais reações redox e danos oxidativos da carbonilação proteína no plasma humano, bem como possível desenvolvimento de doenças e/ou o aumento da gravidade de doenças também devem ser considerados em estudos futuros.

- O estudo desenvolvido fornece resultados preliminares, sólidos e originais, sobre a base genética e molecular das respostas de *L. reuteri* contra o estresse oxidativo induzido por H2O2. O resveratrol (100 µM) protege a L. reuteri contra a carbonilação proteica de forma plausível

através de vários mecanismos, incluindo eliminação direta de ROS, regulação positiva do gene dhaT e promoção da síntese de compostos contendo enxofre com efeito protetor.

- O presente estudo fornece bases para estudos futuros como a hipótese formulada sobre a capacidade de *L. reuteri* para desintoxicar o H_2O_2 e seu mecanismo subjacente precisa ser esclarecido. Além disso, a carbonilação de proteínas como reflexo do dano oxidativo às bactérias e suas conseqüências, bem como o papel das proteínas carbonilas como moléculas sinalizadoras implicadas nas respostas das bactérias ao estresse oxidativo, precisam ser mais investigadas.