UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

DIETARY CONDENSED TANNINS TO IMPROVE THE RUMINANT ANTIOXIDANT STATUS AND QUALITY OF THEIR EDIBLE PRODUCTS – ELUCIDATION OF ACTION MECHANISMS

DAVID ANTÓNIO BRAGA SOLDADO

Orientadores: Doutora Eliana Alexandra Sousa Jerónimo Alves
Professor Doutor Rui José Branquinho de Bessa

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias
na Especialidade de Produção Animal

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Acknowledgments

My most special thanks:

To Fundação para a Ciência e Tecnologia (FCT) for my individual PhD scolarship.

To Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL and Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA) for hosting me while developing my work, providing all the resources, expertise, means and solutions needed to solve my problems and develop my research.

To Pólo de Inovação da Fonte Boa – Estação Zootécnica Nacional, Instituto Nacional de Investigação Agrária e Veterinária I. P., for the opportunity to host my animal experiments, the *Cistus ladanifer* grinding and all the *know-how* and help for animal management and processing.

To my supervisors, Doutora Eliana Jerónimo and Professor Doutor Rui Bessa for the opportunity and challenge to develop this PhD project and thesis. I sincerely thank you for your invaluable guidance, support and encouragement throughout this process. Your patience and mentorship helped me to grow as a researcher and to shape the way I approach challenges and critically think. I'll always be grateful for the time and effort you have invested in my development.

To everyone that directly and indirectly helped me in the animal management, with a special thanks to Pedro Garcia, from São Paulo State University (UNESP), for being an invaluable colleague while the trial was taking place. To Doutor José Santos-Silva, for all the guidance while I was at Pólo de Inovação da Fonte Boa. Also from Fonte-Boa, to Eng.º António Sequeira, José Batista, Diogo Henriques, Doutor João Almeida and Paula Santos.

To all my colleagues at CEBAL, a big thank you for all the support and moments we shared together. A special thanks to Olinda Guerreiro for the invaluable assistance in all the situations I needed it.

To all my close friends and family, whom, caused by recent events, proved to be essential to my life, supporting my development, caring for my well-being and always being tender to me. Thank you from the heart, mom and dad, it is because of you I'm here. This is also you PhD.

To my brother Daniel, my grandmothers Maria and Luzia, I thank you for all the kind and uplifting words, I’m still here because of you.

Finally, to you, Liliana. You’ve been my guiding light in this sea of unknown, we’re sailing together. Thank you for your unconditional love.

This work was financed by Fundação para a Ciência e Tecnologia (FCT) through the individual fellowship SFRH/BD/145814/2019

This work was also co-financed by Alentejo2020 program with the project “CistusRumen - Sustainable use of Rockrose (*Cistus ladanifer* L.) in small ruminants - Increase of the competitiveness and reduction of the environmental impact” (ALT20-03-0145-FEDER-000023) through Regional Development European Fund.

Resumo

Taninos condensados nas dietas para melhorar o estado antioxidante dos ruminantes e a qualidade dos seus produtos edíveis – elucidação dos mecanismos de ação

Os taninos condensados (TC) são produtos do metabolismo secundário das plantas, com propriedades antioxidantes benéficas para a saúde animal e para a qualidade dos seus produtos. Têm sido utilizados em dietas de ruminantes para melhorar o estado antioxidante dos animais e a estabilidade oxidativa dos seus produtos. No entanto, esta estratégia nutricional tem apresentado resultados inconsistentes. Os mecanismos de ação pelos quais os TC exercem a sua atividade antioxidante nos ruminantes são ainda pouco compreendidos, no entanto a evidência sugere que eles podem atuar através de mecanismos diretos e/ou indiretos, de um modo integrado e sinérgico. O objetivo desta tese foi explorar os possíveis mecanismos de ação antioxidantes dos TC em pequenos ruminantes, quando incorporados em dietas de borregos. A suplementação da dieta com TC permitiu proteger a carne da oxidação lipídica e aumentar a atividade antioxidante no rúmen e abomaso, sem afetar a performance produtiva e as características físico-químicas da carne dos borregos. Os TC foram também capazes de limitar as mudanças da cor da carne ao longo do tempo de conservação. Não foi possível identificar monómeros de TC no músculo, o que não permitiu validar um mecanismo direto de ação. O conteúdo de α-tocoferol no músculo foi mais alto, apesar de um menor consumo pela dieta, o que sugere que os TC podem influenciar o metabolismo do α-tocoferol. No entanto, não foi verificada interação entre os TC e o α-tocoferol, quando suplementados em conjunto, não permitindo validar uma relação sinérgica entre ambos os antioxidantes. Foi também verificado que a suplementação de TC nas dietas dos ruminantes pode eficazmente substituir doses elevadas de α-tocoferol, enquanto mantém a estabilidade lipídica da carne, sobretudo em dietas com níveis elevados de pro-oxidantes.

Palavras-chave: taninos condensados; atividade antioxidante; mecanismos de ação; ruminantes.

Abstract

Dietary condensed tannins to improve the ruminant antioxidant status and quality of their edible products – elucidation of action mechanisms

Condensed tannins (CTs) are secondary plant metabolites, with antioxidant properties which are beneficial for animal health and the quality of their products. They have been used in ruminant diets to improve the animal antioxidant status and the oxidative stability of their products. However, this nutritional strategy has shown inconsistent results. The action mechanisms by which dietary CTs exerts the antioxidant effect on ruminants are still poorly understood, although evidence suggests they act through direct and/or indirect mechanisms, in an integrated and synergistic way. The aim of this thesis was to explore the possible CTs antioxidant action mechanisms in small ruminants, by CTs incorporation in lambs' diets. Supplementation of diets with CTs was able to protect meat from lipid oxidation and increase the antioxidant activity in the rumen and abomasum, while not affecting lamb's productive performance and meat physicochemical traits. Also, CTs were able to limit meat colour changes through storage time. CTs monomers were not identified in muscle, making it impossible to demonstrate a direct action mechanism. α-tocopherol content in muscle was higher, despite a lower dietary consumption, suggesting that dietary CTs may influence α-tocopherol metabolism. However, the interaction between CTs and α-tocopherol was not verified when supplemented together, not allowing the validation of a synergistic relation between both antioxidants. It was also verified that CT supplementation in ruminant diets can effectively replace high doses of α-tocopherol, maintaining a controlled meat lipid oxidative stability, especially in diets with high pro-oxidant levels.

Keywords: condensed tannins; antioxidant activity; action mechanisms; ruminants.

Resumo alargado

Taninos condensados nas dietas para melhorar o estado antioxidante dos ruminantes e a qualidade dos seus produtos edíveis – elucidação dos mecanismos de ação

A produção animal, nomeadamente a de pequenos ruminantes, está sujeita a vários desafios que provocam situações de stress oxidativo nos animais. O stress oxidativo leva à destruição de componentes celulares vitais, com consequente deterioração de funções fisiológicas e impacto no crescimento, reprodução e imunidade, que condiciona a saúde e bem-estar dos animais e causa prejuízos económicos aos produtores. Condições ambientais, tais como o aumento médio das temperaturas globais; condições intrínsecas à produção, como as condições de alojamento, de maneio, de transporte e a contaminação de alimento são situações que podem levar ao aumento de stress oxidativo em pequenos ruminantes. Além disso, uma estratégia largamente utilizada para melhorar o valor nutricional da carne dos ruminantes – a suplementação de dietas com ácidos gordos polinsaturados (PUFA) – é mais um fator que pode aumentar o desequilíbrio entre os agentes antioxidantes e pró-oxidantes nos animais, levando também a situações de stress oxidativo. Para minimizar o desequilíbrio pró-oxidante causado pela suplementação das dietas com fontes lipídicas ricas em PUFA, é frequente a incorporação de antioxidantes nas dietas. No entanto, a maioria dos antioxidantes utilizados com este propósito são sintéticos, sobre os quais tem aumentado a preocupação dos consumidores em relação à sua segurança. Deste modo, o interesse na utilização de antioxidantes naturais para inclusão nas dietas dos animais tem sido crescente.

Um dos grupos de antioxidantes mais abundantes na natureza são os taninos condensados (TC), provenientes do metabolismo secundário das plantas. A sua utilização em dietas de ruminantes tem sido feita quer pela aplicação direta de plantas e coprodutos, quer através de extratos obtidos a partir das mesmas fontes, ricos em TC. Uma fonte de TC amplamente disponível em Portugal e cuja valorização industrial é muito baixa é a Esteva – *Cistus ladanifer* L. No que diz respeito à eficácia dos TC como antioxidantes, quer para melhorar o estado oxidativo dos animais, quer para garantir a estabilidade oxidativa dos seus produtos, os poucos estudos que abordaram este tema obtiveram resultados contraditórios. Por exemplo, num estudo, a aplicação de grainha de uva em dietas de ruminantes permitiu melhorar o estado antioxidante no músculo dos animais, reduzir a oxidação lipídica e aumentar a capacidade antioxidante total, bem como a atividade da enzima antioxidante GPx4. No entanto, em outro estudo, a inclusão de grainha de uva na dieta não teve efeito na estabilidade oxidativa de carne. Do mesmo modo, a inclusão de sorgo rico em TC conseguiu reduzir a oxidação lipídica de carne embalada a vácuo, mas não conseguiu evitar o seu aumento

quando exposta ao ar. As diferenças na estrutura química dos TC, a concentração na dieta, bem como a diversidade das dietas base usadas e a presença de outros compostos antioxidantes na dieta, podem ajudar a explicar os resultados inconsistentes que se obtém ao utilizar plantas e coprodutos diretamente nas dietas dos ruminantes. De modo a isolar o efeito antioxidante dos TC, a solução passa por obter extratos ricos em TC. O uso de quebracho teve resultados variáveis: enquanto alguns estudos demonstraram um efeito protetor contra a oxidação lipídica na carne, outros não viram efeito na concentração de malondialdeído. Do mesmo modo, o extrato de grainha de uva conseguiu melhorar o estado antioxidante de borregos, mas não teve efeito na limitação da oxidação lipídica da carne. De um modo geral, apesar dos extratos de TC poderem melhorar o estado antioxidante do animal e a estabilidade oxidativa dos produtos, os resultados são variáveis e contraditórios, o que revela a necessidade de compreender os mecanismos pelos quais os TC da dieta podem exercer atividade antioxidante nos animais e nos seus produtos. Têm sido apontados como possíveis mecanismos diretos – através da absorção e deposição dos TC nos tecidos – e/ou mecanismos indiretos – através do efeito antioxidante dos TC ao longo do trato gastrointestinal e/ou da interação dos TC com outros componentes do sistema antioxidante endógeno. Assim, o objetivo desta tese foi explorar os possíveis mecanismos de ação antioxidante dos TC em pequenos ruminantes, quer pela sua ação direta, através da possível absorção e deposição nos tecidos, quer pela sua ação indireta, ao longo do trato gastrointestinal ou pela interação com outras componentes do sistema antioxidante endógeno.

A parte aérea de *Cistus ladanifer* L. foi utilizada anteriormente em ensaios com borregos, nos quais a sua incorporação nas dietas melhorou a estabilidade oxidativa da carne. No entanto, esta maior proteção antioxidante conferida pela Esteva, pode dever-se ao facto de que a planta possui diversos componentes antioxidantes além dos TC, tais como flavonoides, terpenos e vitaminas, como a vitamina E. Assim, para clarificar qual a papel dos TC no efeito antioxidante da Esteva, foi testada a hipótese, num primeiro trabalho, de que a incorporação de um extrato de TC de Esteva na dieta de ruminantes poderia induzir a mesma proteção antioxidante que a incorporação da parte aérea da planta. Deste modo, delineou-se um ensaio com borregos, no qual foram utilizadas duas formas de fornecimento de TC (em extrato vs. diretamente pela planta) e três níveis de incorporação (sem suplementação de TC, nível médio e alto de suplementação de TC), o que resultou em 6 dietas experimentais. A incorporação de TC, em ambas formas, resultou num aumento da capacidade antioxidante das dietas, existindo uma maior capacidade nas dietas que foram suplementadas com a parte aérea da planta, devido à presença de outros antioxidantes além dos TC. No entanto as dietas com maiores níveis de TC, apresentaram menores concentrações de α-tocoferol. Um dos possíveis mecanismos de ação dos TC é a sua ação local direta, ao longo do trato gastrointestinal dos ruminantes. Neste trabalho foi avaliada a atividade antioxidante nos conteúdos do rúmen e abomaso e nas fezes – a primeira vez que tal foi feito, tanto quanto sabemos. A atividade antioxidante no rúmen e abomaso aumentou linearmente com o aumento da concentração de TC nas dietas, em ambas as formas de suplementação. Já nas fezes, a suplementação das dietas com TC não teve efeito na atividade antioxidante, o que pode indicar a transformação dos TC ao longo do trato gastrointestinal. Os TC não tiveram efeito na estabilidade oxidativa da carne dos borregos, mas foram responsáveis por algumas diferenças encontradas nos parâmetros de cor. Apesar de o consumo de α-tocoferol ter diminuído com o aumento de TC nas dietas, no músculo foram encontrados níveis iguais de α-tocoferol, o que poderá ser indicativo de que os TC interagem com o seu metabolismo, promovendo uma sua maior disponibilidade no músculo. Este último resultado, a interação do TC com o metabolismo da vitamina E, está de acordo com um dos possíveis mecanismos de ação já descritos na bibliografia, mecanismos que decidimos compreender melhor num trabalho subsequente.

Neste segundo trabalho, foi estudada a interação dos TC com a vitamina E. A hipótese estudada neste trabalho é a de que a combinação de ambos os antioxidantes, TC e vitamina E, confere uma maior proteção antioxidante do que os antioxidantes suplementados individualmente. Para tal, foi delineado um ensaio *in vivo* com borregos onde se suplementaram dietas com TC (extrato obtido a partir de Esteva, como no ensaio anterior), com vitamina E e com ambos, o que resultou na formulação de quatro dietas experimentais. Neste ensaio pretendeu-se promover condições de pressão oxidativa através da inclusão na dieta base de uma fonte de AGPI altamente suscetíveis à oxidação lipídica, como os AGPI n-3 de cadeia longa, para tal todas as dietas continham *Nannochloropsis oceannica*. A suplementação das dietas com os TC e vitamina E não afetou a performance produtiva dos animais, as características da carcaça nem a qualidade da carne. As características sensoriais foram afetadas pelos tratamentos, sobretudo na dieta com apenas TC. A suplementação das dietas com TC de Esteva e vitamina E, tanto individualmente como em conjunto, mostrou-se eficaz para limitar a oxidação lipídica na carne ao longo do tempo de conservação. Neste trabalho não se verificou o aumento de α-tocoferol no músculo nas dietas com TC, como observado anteriormente, não sendo possível validar a interação entre TC o α-tocoferol.

Uma vez que o consumidor procura cada vez mais produtos de origem natural, que consigam de igual modo ser eficazes e seguros em termos nutricionais, para além de todas as pressões que o sistema de produção animal está sujeito, é necessário encontrar boas fontes de antioxidantes para suplementar nas dietas e assim garantir quer o bem-estar dos animais, quer a segurança alimentar dos seus produtos. A utilização de TC de Esteva, na forma de extrato, parece ser uma fonte promissora de antioxidantes naturais para a alimentação de ruminantes, uma vez que consegue melhorar o estado antioxidante dos animais e garantir a estabilidade oxidativa dos seus produtos. No entanto, para garantir que a sua aplicação nas dietas promova o desejado efeito antioxidante no animal e nos seus produtos, é imperativo desenvolver métodos precisos de identificação e caracterização estrutural dos TC, de modo a encontrar respostas mais conclusivas sobre os mecanismos antioxidantes de ação, e assim, tornar a sua aplicação mais direcionada e eficiente.

List of publications

This thesis was based on the following publications:

Soldado, D., Bessa, R.J.B., Jerónimo, E. Condensed tannins as antioxidants in ruminants – Effectiveness and action mechanisms to improve the animal antioxidant status and oxidative stability of products. *Animals* **2021**, 11, 3243. https://doi.org/10.3390/ani11113243

Soldado, D., Guerreiro, O. Fialho, L., Cachucho, L., Francisco, A., Santos-Silva, J., Bessa, R.J.B., Jerónimo, E. Inclusion of the *Cistus ladanifer* L. plant and its condensed tannin extract in lamb diets – Effects on animal antioxidant status and oxidative stability of meat. *Animal Feed Science and Technology* **2024**, 316, 116070. https://doi.org/10.1016/j.anifeedsci.2024.116070

Soldado, D., Garcia, P., Vitor, A. C, Francisco, A., Almeida, J. M., Guerreiro, O., Alves, S. P., Santos-Silva, J., Bessa, R.J.B., Jerónimo, E. Vitamin E and condensed tannins from *Cistus ladanifer* L. improve meat oxidative stability in lambs given n-3 long chain polyunsaturated fatty acids. (In advanced preparation to be submitted)

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%	Percentage
ºC	Degree Celsius
µL	microlitre
µmol	micromol
*a**	Redness (CIELAB colour dimension)
ADF	Acid Detergent Fiber
ADG	Average Daily Gain
ADL	Acid Detergent Lignin
*b**	Yellowness (CIELAB colour dimension)
C*	Chroma – colour saturation
C	Carbon
CAT	Catalase
CL0	Basal diet without *C. ladanifer* inclusion
CLH	Basal diet with medium level of *C. ladanifer* inclusion
CLM	Basal diet with high level of *C. ladanifer inclusion*
CT	Condensed Tannins
DAD	Diode Array Detector
DM	Dry Matter
DMI	Dry Matter Intake
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Ex0	Basal diet without *C. ladanifer* CT extract inclusion
ExM	Basal diet with medium level of *C. ladanifer* CT extract inclusion
ExH	Basal diet with high level of *C. ladanifer* CT extract inclusion
FCR	Feed conversion ratio
FRAP	Ferric Reducing Antioxidant Power
g	gram
g	Relative centrifuge force
GIT	Gastrointestinal tract

GSH	Glutathione
GPx	Glutathione peroxidase
GPx4	Glutathione peroxidase 4
H*	Hue angle
h	Hour
HCl	Hydrochloric acid
HT	Hydrolysable Tannins
kg	kilogram
*L**	Lightness
LT	*Longissimus thoracis*
MDA	Malondialdehyde
mDP	mean degree of polymerization
mg	milligram
mL	milliliter
mRNA	messenger ribonucleic acid
MS/MS	Tandem Mass Spectrometry
n-3 PUFA	n-3 Polyunsaturated Fatty Acids
NDF	Neutral Detergent Fiber
nm	Nanometer
NO	Basal diet NO
NOVE	Basal diet NOVE
NrF2	Nuclear factor erythroid 2-related factor 2
OH	Hydroxide
PC	Procyanidin
PD	Prodelphinidin
pH	Potential of hydrogen
PUFA	Polyunsaturated fatty acids
P-SH	Protein thiol groups
PT	Phlorotannins
RNS	Reactive nitrogen species
ROS	Reactive oxygen Species

rpm	Rotations per minute
s	Seconds
SAS	Statistical analysis system (software package)
SEM	Standard Error of the Mean
SOD	Superoxide dismutase
TAE	Tannic acid equivalents
TBARS	Thiobarbituric Acid Reactive Substances
TC	Tanino condensado
TEAC	Trolox Equivalent Antioxidant Capacity
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
T-SH	Total thiol groups
UPLC-MS/MS	Ultra Performance Liquid Chromatography–Tandem Mass Spectometry
uHPLC	ultra-High Performance Liquid Chromatography

Introduction

Oxidative stress takes place when normal cellular metabolism is disrupted, created by an unbalance between pro-oxidants and antioxidants (Lushchak 2014; Sies 2018). In this scenario, the production of reactive oxygen species is increased, leading to oxidative damage of cells and tissues, affecting the physiological function of livestock species (Miller et al. 1993; Surai et al. 2019). Several factors can induce suitable conditions to the development of oxidative stress in farm animals, such as diet lipid supplementation (Bessa 2015; Hess et al. 2008; Shingfield et al. 2013), nutritional imbalances and feed contaminations (Surai et al. 2019), high environmental temperatures (Guo et al. 2021), animal management, transportation, mechanical injuries and diseases (Deters 2020; Puppel et al. 2015). Animal antioxidant status is reflected in the quality of their products (Soldado et al. 2021). To counter the harmful effects of oxidative stress, an adaptative endogenous antioxidant response is triggered (Lushchak 2014), however in some situations that response may not be enough to limit the oxidative damage (Surai et al. 2019). Synthetic antioxidants are commonly used in animal nutrition (Salami et al. 2016), but their use is becoming unsightly to consumers, which subsequently impels the application of natural antioxidants. Condensed tannins (CTs), plant secondary metabolites, provide various positive biological functions, with the antioxidant action being one of the most relevant (Huang et al. 2018; Rauf et al. 2019; Unusan 2020; Yang et al. 2018). Nonetheless, CTs supplementation in ruminant diets yielded inconsistent results in terms of the animal's antioxidant status and the oxidative stability of its products. To have a better understanding of CT's antioxidant effects in ruminants, the chemical structure and action mechanisms should be investigated further.

Condensed tannins are oligomers and polymers composed of interlinked flavan-3-ol units, creating molecules with high degrees of polymerization, from two to more than fifty flavan-3-ol units (Girard et al. 2020; Khanbabaee and van Ree 2001; Tao et al. 2019). Their use as antioxidants in ruminant diets has been greatly tested, including direct application of plants (shrubs, legumes and trees) and by-products with high CT contents (Dey and De 2014; García et al. 2019; Gravador et al. 2015; Jerónimo et al. 2012; Lee et al. 2017; Pathak et al. 2017; Tayengwa et al. 2020; Zhao et al. 2018). However, by directly applying plants and by-products rich in CTs to ruminant diets, there are other phenolic compounds that might be present and influence the overall antioxidant activity. To better understand the specific antioxidant effect of CTs in ruminant's diets, many researchers opted to obtain CT-rich extracts from these sources and apply it in ruminant dietary solutions (Biondi et al. 2019; Buccioni et al. 2017; Gladine et al. 2007b; Guerra-Rivas et al. 2016; Lobón et al. 2017; Lopéz-Andrés et al. 2013; Luciano et al. 2011; Salas et al. 2024; Santos et al. 2022; Staerfl et al. 2011).

Regardless, even using CTs extracts, the results are uneven. The elucidation of CTs antioxidant action mechanisms becomes a key problem that may deemed critical to understanding the inconsistent findings affecting CT supplementation in ruminant diets.

As other antioxidants, CTs antioxidant function involves various mechanisms such as free radical scavenging, transition metal chelation and pro-oxidative enzymes inhibition (Koleckar et al. 2008) but also by synergizing with endogenous antioxidants and endogenous phenolics and by interacting with the metabolism of other antioxidants (Iglesias et al. 2012). Some action mechanisms appear to be feasible, involving direct and indirect mechanisms, such as CTs or their metabolites absorption, allowing for direct action in the animal body and tissues; an antioxidant action in the gastrointestinal tract (GIT), and the interaction with other antioxidant compounds or with the endogenous antioxidant system.

This thesis aims to explore the possible antioxidant action mechanisms of dietary CTs in small ruminants, either by their direct action – through a possible absorption and deposition in the tissues – or through their indirect action, along the GIT or by the interaction with other antioxidants. The findings of this thesis may serve to answer various problems, which, when combined, may contribute to improving (or discarding) the hypotheses proposed on the mechanisms of CTs antioxidant effect.

This thesis in structured in 4 chapters. Chapter 1, is based on a review paper, already published, fulfilling the scientific background needed for the preparation of this thesis. On this review paper, we introduce the problematic of oxidative stress underlying livestock production, pointing out some causes and solutions, namely the use of natural antioxidants in ruminant diets. Then, we introduce CTs as a promising natural antioxidant source, briefly overviewing their chemical characteristics, followed by presenting and discussing a fair amount of scientific works using either plant-rich CT sources or CT extracts as nutritional strategies in ruminants. After realizing that a common problematic among these studies is the inconsistent outcomes, we then proceed to carefully analyze a fundamental aspect, the CTs mechanisms of action. This overview guides us to the conclusion that CTs can improve animal antioxidant status and give rise to edible products with increased oxidative stability, however, the effectiveness of CTs use in ruminant nutrition still need further research.

Chapter 2 is based on an already published scientific paper. Previous research from our group showed that *Cistus ladanifer* is a promising source to use in ruminant nutrition with several health benefits (Francisco et al. 2018; Francisco et al. 2015; Guerreiro et al. 2020; Guerreiro et al. 2016a; Jerónimo et al. 2012; Jerónimo et al. 2020a; Jerónimo et al. 2020b). This work (Chapter 2) was outlined to explore if the incorporation of *C. ladanifer* CT extract could induce the same antioxidant protection as the *C. ladanifer* plant, since, as described before, the antioxidant effect of CT-plant sources may not only be attributed to CTs itself. Furthermore, how CTs induces improvements in the antioxidant status of animals and the oxidative stability of meat was also explored in this work. Contrary to our expectations, *C. ladanifer* plant and CT extract did not improve the lipid stability and antioxidant status of meat, but both CT sources enhanced the meat colour stability over storage time. We also did not find CT monomers in muscle, which did not allow us to validate a possible direct antioxidant action mechanism involving absorption and deposition in the tissues. Both CTs sources increased the antioxidant activity in the contents of rumen and abomasum, but had a minor impact on fecal antioxidant activity, suggesting that CTs may be transformed in the GIT. Interestingly, similar levels of α-tocopherol were found in muscle, despite reduced α-tocopherol intake with increasing dietary CT level, suggesting that dietary CTs may facilitate better availability of α-tocopherol.

These findings, which were consistent with previous research on CT's interactions with other antioxidant compounds, prompted us to conduct another study to further investigate a possible indirect antioxidant action mechanism (Chapter 3). In this study we assessed the efficacy of dietary vitamin E and *C. ladanifer* CT extract supplementation in improving the oxidative stability of lamb meat, as well as the hypothesis that a dietary combination of vitamin E and *C. ladanifer* CTs extract outperforms the individual antioxidants, using *Nannochloropsis oceanica* to create a strong pro-oxidant diet. It wasn't possible to verify the interaction between α-tocopherol and *C. ladanifer* CTs extract. However, we were able to verify that the *C. ladanifer* CT extract alone was able to limit meat lipid oxidation, during storage time.

The last chapter (chapter 4) summarizes and discusses the findings from each chapter of the thesis, integrating them to meet the research aims. Main conclusions and futures perspectives are also reported in this chapter.

1. CHAPTER 1 | Condensed tannins as antioxidants in ruminants – Effectiveness and action mechanisms to improve animal antioxidant status and oxidative stability of products

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Adapted from *Animals,* 11, November 2021, 3243
(Available online at https://doi.org/10.3390/ani11113243)

Abstract

Condensed tannins (CTs) are widely distributed in plants, and due to their recognized antioxidant activity are considered as possible natural antioxidants for application in ruminant diets. A wide range of CT-rich sources has been tested in ruminant diets, and their effects on animal antioxidant status and oxidative stability of their products are reviewed in the present work. Possible mechanisms underlying the CT antioxidant effects in ruminants are also discussed, and the CT chemical structure is briefly presented. The utilization of CT-rich sources in ruminant feeding can improve the animals' antioxidant status and oxidative stability of their products. However, the results are still inconsistent. Although poorly understood, the evidence suggests that CTs can induce an antioxidant effect in living animals and in their products through direct and indirect mechanisms, which can occur by an integrated and synergic way involving: i) absorption of CTs with low molecular weight or metabolites, despite CTs' poor bioavailability; ii) antioxidant action on the gastrointestinal tract; and iii) interaction with other antioxidant agents. Condensed tannins are alternative dietary antioxidants for ruminants, but further studies should be carried out to elucidate the mechanism underlying the antioxidant activity of each CT source to design effective antioxidant strategies based on the use of CTs in ruminant diets.

1.1. Introduction

Livestock species are frequently exposed to oxidative stress, generated by overproduction of free radicals that cannot be handled by the body's antioxidant defense capacity, resulting in oxidative damage of several vital cell components and deterioration of many physiological functions, such as growth, reproduction, and immunity (Miller et al. 1993; Surai et al. 2019). Oxidative damage in cells and tissues results from the action of several reactive molecular species, generally designated as reactive oxygen (ROS) and nitrogen species (RNS), although some non-radical reactive derivatives of oxygen and nitrogen are also included (Surai et al. 2019) that can induce serious injuries in molecules, such as deoxyribonucleic acid (DNA), lipids, proteins and carbohydrates (Lushchak 2014). A normal cellular metabolism generates and eliminates ROS, both processes being closely related, resulting in a very low level of ROS in living organisms (Lushchak 2014). However, this balance can be disturbed under several circumstances (Lushchak 2014) and an imbalance between oxidants and antioxidants in favor of the oxidants results in a status known as oxidative stress (Morrissey et al. 1998; Sies 2018).

Several factors can create favorable conditions for the development of oxidative stress in farm animals. Lipid supplementation of ruminants' diets has been used for a long time to increase their energy density, and more recently to improve the fatty acid profile of ruminant products by inclusion of lipid sources rich in polyunsaturated fatty acids (PUFA) in diets (Bessa 2015; Hess et al. 2008; Shingfield et al. 2013). However, due to PUFAs' high susceptibility to oxidation, these nutritional strategies expose the animals to oxidative stress conditions, while enhancing the susceptibility of products to oxidation which limits its quality and acceptability (Morrissey et al. 1998). Nutritional imbalance, feed contamination, and the presence of mycotoxins are other oxidative stress factors (Surai et al. 2019). High environmental temperatures are also associated with increased oxidative stress (Guo et al. 2021), which constitutes a major concern as to the possible impact of expected climate change with alarming temperature increases and more frequent and intense heat waves. In addition to diet and environmental aspects, other factors such as housing condition, animal management, transportation, mechanical injuries, disease and the physiological state of animals can contribute to oxidative imbalance (Deters 2020; Puppel et al. 2015; Surai et al. 2019). These oxidative stress conditions lead to increased production of free radicals above the capacity of the antioxidant system to eliminate them. The excessive free radicals are available to cause oxidative damage to various cell macromolecules, a process by which more free radicals are generated, triggering a chain of oxidative destruction (Rahal et al. 2014).

The animals' antioxidant status is also reflected in the quality of their products, determining a higher susceptibility or resistance to oxidative deterioration. Animal-derived foods are subjected to oxidative reactions, resulting in deterioration of color and development of undesirable odor and flavor, loss of nutrients such as essential fatty acids, vitamins, and other bioactive compounds, and production of compounds harmful to human health, a major cause of the quality loss in food (Dominguez et al. 2019; Papuc et al. 2017). These oxidative reactions are influenced by intrinsic and extrinsic factors that can promote or inhibit them. Processing and storage conditions are the external factors that mostly affect the oxidative stability of foods (Dominguez et al. 2019; Samples 2013). The lipid content and composition are one of the intrinsic parameters which great influence food's oxidative stability, with foods containing high amounts of unsaturated fatty acids, such as foods naturally rich or enriched in n-3 PUFA, highly susceptible to lipid oxidation (Jacobsen 2015; Secci and Parisi 2016). Foods with high levels of prooxidants (e.g., hemeproteins, metals such as Fe and Cu) are also particularly prone to oxidation (Dominguez et al. 2019). In contrast, the presence of antioxidants can limit oxidative reactions and improve the shelf-life of foods (Samples 2013).

When oxidative stress occurs, an adaptive stress response is triggered to minimize the damaging effects of free radicals, increasing the capability of antioxidant systems to eliminate the free radicals (Lushchak 2014). The endogenous antioxidant system – composed by antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), and by non-enzymatic antioxidants such as glutathione (GSH), alpha-lipoic acid, Coenzyme Q, ferritin, uric acid and bilirubin – plays crucial roles in maintaining the redox balance in the body (Aguilar 2016). Antioxidant compounds such as vitamins E, C and β-carotene, which are naturally present in greater or lesser amounts in animal feed, are also strongly involved in the mechanisms of protection against free radicals in the living organism and products (Morrissey et al. 1998). However, under certain circumstances, the endogenous antioxidant system and antioxidant compounds naturally present in the feedstock may not be enough to prevent oxidative damage, being fundamental to increase the levels of antioxidant compounds in the diet to ensure animal welfare and productivity, as well as the quality of products (Surai et al. 2019). Synthetically manufactured antioxidants are largely used in animal nutrition (Salami et al. 2016), but to address consumer concerns on safety of these products there has been an increasing interest in the use of natural antioxidants in the diets of various farm animals.

As plant secondary metabolites, condensed tannins (CTs) are part of the plant chemical defense system against biotic and abiotic stressors (Huang et al. 2018; Rauf et al. 2019). Moreover, CTs present several biological activities with benefits for human and animal health and food quality. Antioxidant, anticancer, antidiabetic, anti-inflammatory, anthelmintic, antimicrobial, immunostimulant and cardio- and neuro- and eye-protective are some of the beneficial health properties of CTs (Koleckar et al. 2008; Rauf et al. 2019; Tao et al. 2019; Unusan 2020; Yang et al. 2018). Although they are also known for their antinutritional properties, several CT sources has been applied in livestock species diets with neutral and beneficial effects, such as prevention of bloating, control of internal parasites, reduction of methane production, improvement of the digestive utilization of feed proteins, the fatty acid composition of products, or the productive performance in ruminant animals (Jerónimo et al. 2016; Piluzza 2014; Waghorn 2008). Improved gastrointestinal health and growth performance are some of the beneficial effects of dietary CTs reported in monogastric animals (Caprarulo et al. 2021; Huang et al. 2018).

Probably due to the association of various diseases with oxidative stress, the antioxidant activity has been one of the most studied biological effects of CTs, in both *in vitro* and *in vivo* models. The strong antioxidant capacity of CTs is demonstrated in several *in vitro* assays (Beninger and Hosfield 2003; Hagerman et al. 1998; Zhang et al. 2016), and CT extracts have been successfully applied to retard lipid oxidation in foods (Lorenzo et al. 2013; Serra et al. 2021). Moreover, the antioxidant efficacy of CTs has also been verified in human and animal studies, including both ruminants and monogastric farm animals (Jerónimo et al. 2016; Rauf et al. 2019; Serra et al. 2021; Tao et al. 2019; Yang et al. 2018). Due to their recognized antioxidant activity and wide distribution among the plant kingdom, CTs are appointed as good natural antioxidant candidates for application in ruminant diets. However, if the antioxidant effect of CTs is fully proven in *in vitro* studies and when they are directly added to food products, their *in vivo* and post-mortem antioxidant effects when applied in ruminant diets have been contradictory, with doubts remaining as to the effectiveness of this approach, as well as the action mechanisms. So, the main objective of the current review is to critically analyze the available knowledge on the effect of CT sources' incorporation in ruminant diets on animal antioxidant status and oxidative stability of their products and discuss the CT antioxidant mechanisms in ruminants. Tannins' chemical structure will also be briefly reviewed.

1.2. Condensed tannin's chemical structure

Tannins are a heterogeneous group of phenolic compounds that can be classified into 3 major groups based on their chemical structures: hydrolysable tannins (HTs), condensed tannins (CTs) and phlorotannins (PTs) (Huang et al. 2018; Singh and Kumar 2019). The HTs and CTs are found in terrestrial plants, while PTs only appears in brown seaweeds (Huang et al. 2018; Singh and Kumar 2019). Hydrolysable tannins contain a central unit of glucose or other polyols esterified with gallic acid, named gallotannins, or with heaxahydroxydiphenic acid, named ellagitannins (Koleckar et al. 2008). Phlorotannins comprise a distinct set of polymers, structurally like terrestrial tannins, being formed by polymeric chains of phloroglucinol, connected via C-C and/or C-O-C bonds (Shrestha et al. 2021).

For the purposes of this review, our focus will be on the CTs. Known also as proanthocyanidins, CTs are oligomers and polymers composed of flavan-3-ols linked through C4-C8 and/or C4-C6 bonds, both called B-type CTs, and doubly linked with an additional ether bond at C2 → O → C7 or C2→ O → C5, designed as an A-type CT (Girard et al. 2020; Tao et al. 2019). The size of the CT molecule is characterized by its degree of polymerization, which varies between two and greater than fifty flavan-3-ols subunits (Khanbabaee and van Ree 2001). The majority of CTs present in common forage species are mainly composed by four monomeric flavan-3-ol subunits, catechin and epicatechin, gallocatechin and epigallocatechin (Zeller 2019) (Figure 1A), which differ in the number and stereochemistry of hydroxyl groups and the relative stereochemistry of the substituents on the C-ring (i.e. *cis* or *trans*-configuration) (Desrues et al. 2017; Zeller 2019). The catechin and epicatechin differ from each other only in their stereochemistry of the hydroxyl (OH) group at the C3 carbon on the C-ring. The gallocatechin and epigallocatechin also differ from each other by the spatial orientation of the OH group at C3 on the C-ring (Zeller 2019). The stereochemistry of the C2 and C3 substituents in the C-ring also differ between flavan-3-ol subunits, in which epicatechin and epigallocatechin show *cis* orientation, whereas catechin and gallocatechin show *trans* orientation (Zeller 2019). Moreover, catechin and epicatechin have two OH groups on the B-ring, and gallocatechin and epigallocatechin three OH groups on the B-ring (Desrues et al.

2017). In addition, few plants have CTs containing flavan-3-ol subunits that are modified with galloyl groups at the C3 hydroxyl position (Figure 1B), giving rise to catechin gallate, epicatechin gallate, gallocatechin gallate and epigallocatechin gallate (Zeller 2019). According to flavan-3-ol subunit composition, CTs can be classified into different subgroups. The two major CT subgroups are procyanidin tannins (PC), composed by catechin and epicatechin and prodelphinidin tannins (PD), which have gallocatechin and epigallocatechin (Figure 1A) (Girard et al. 2020; Zeller 2019). The CTs in common forage plants typically contain a complex mixture of PC and PD, while few plants only synthesize PC or PD (Klongsiriwet et al. 2015).

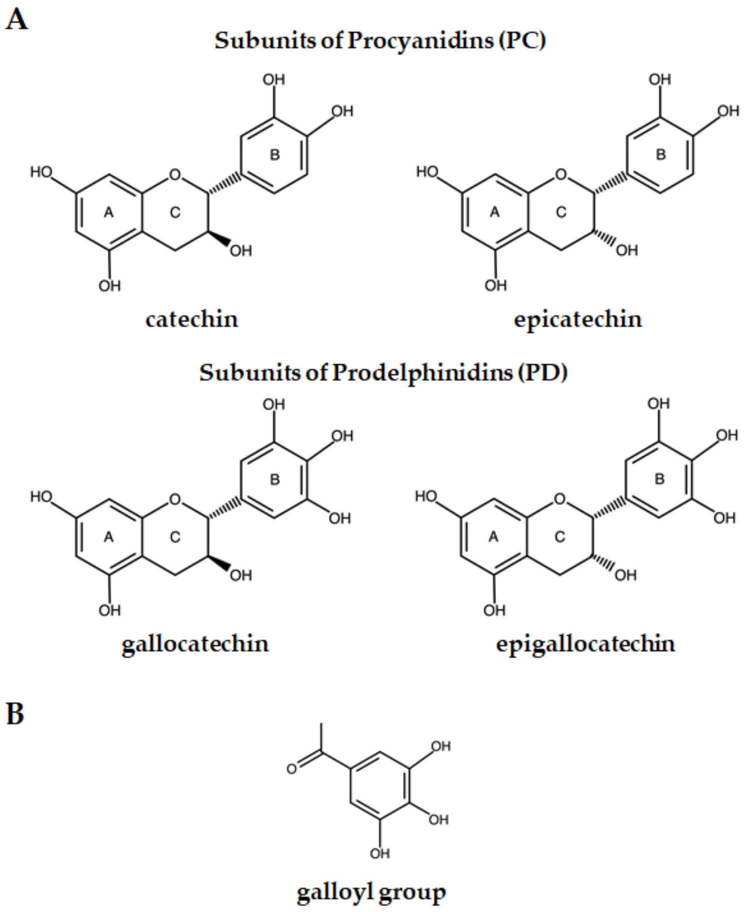

Figure 1.1. A) Monomeric proanthocyanidins, subunits of procyanidins – catechin and epicatechinand subunits of prodelphinidin – gallocatechin and epigallocatechin; (B) galloylgroup.

Taking into account the different pattern of hydroxylation, types of interflavan-3-ol linkages, stereochemistry of the C2 and C3 and degree of polymerization, a great structural diversity of CTs is found in plants (Girard et al. 2020). Their structure has relevant influence on the chemistry and biological features of CTs, affecting their absorption and metabolism as well as the protein binding affinity, and the anthelmintic and antioxidant activities (Desrues et al.

2017; Unusan 2020). For example, the protein precipitation increases with the CT size (Zeller 2019), and it is reported that the antioxidant activity of CTs is positively related to increase of the polymerization degree (Es-Safi et al. 2006; Hagerman et al. 1998; Zhou et al. 2011; Zhou et al. 2014).

1.3. Condensed tannins as antioxidants in ruminant diets

A wide range of CT sources has been extensively tested in ruminant diets, as modulator of the ruminal biohydrogenation in order to improve the fatty acid profile of meat and milk fat (Frutos et al. 2020; Jerónimo et al. 2016), and as natural agents to reduce methane emissions, protect the protein against ruminal degradation, prevent bloating, control internal parasites, and improve animals' antioxidant status and oxidative stability of their products (Huang et al. 2018; Makkar et al. 2007; Mueller-Harvey et al. 2019; Piluzza 2014; Vasta and Luciano 2011; Waghorn 2008; Zeller 2019). The CT sources applied in these nutritional strategies for ruminants include plants (legumes, trees and shrubs) and by-products containing high CT contents or extracts with variable CT purity obtained from these plants and by-products. Despite the large number of CT sources applied in ruminant diets, only a few of them have been tested in terms of their efficacy to improve the animal antioxidant status and limit the oxidative deterioration of products. Standing out are by-products from of wine industry, which are used in their original form (grape pomace) or in the form of extracts (e.g., grape seed extract), by-products from other fruits (carob pulp and peanut skin) and from the forest (pine bark), foliage and extracts from endemic shrubs and trees (e.g., *C. ladanifer*, quebracho and mimosa), and widespread legumes forages (sainfoin and sorghum). Plants or by-products rich in CTs may represent a greater proportion of the diet (50-750 g/kg dry matter (DM), Table 1.1) and provide other nutrients and bioactive compounds in addition to CTs, while CT extracts, which contain high levels of CTs (more than 40% and reaching 95%), are usually incorporated into diets at lower levels (Table 2). So, the antioxidant impact of CT sources applied in ruminant diets will be presented and discussed according to the applied strategy, separating the strategies based in use of CT-rich plants or by-products from CT extracts.

1.3.1. Condensed tannin-rich plants and agro-industrial by-products

Grape pomace, consisting of grape seeds, skin and pulp, is one of the most studied CT sources in terms of its efficacy to improve ruminants' antioxidant status and the oxidative stability of their products. Inclusion of 50 and 100 g/kg DM of red wine grape pomace in lamb diets resulted in the improvement of several muscle antioxidant status parameters (Zhao et al. 2018). A reduction of lipid oxidation, indicated by lower levels of malondialdehyde (MDA), one of the most abundant aldehydes derived from the oxidation of PUFA, and increased total antioxidant capacity and activity of antioxidant enzyme GPx4 in muscle were observed when 100 g/kg DM of grape pomace was incorporated in lamb diets (Zhao et al. 2018). A lower grape pomace level – 50 g/kg DM – was enough to reduce the reactive oxygen species (ROS) content and to increase the SOD activity in muscle (Zhao et al. 2018). In the same experiment, authors also evaluated the effect of dietary grape pomace inclusion on antioxidant capacity in the testes of ram lambs maintained under housing situations that can lead to increased oxidative stress (Zhao et al. 2017). The results showed that grape pomace inclusion in diets reduced the ROS and MDA contents in tests of confined lambs to values similar to those found in lambs raised under free-range conditions (Zhao et al. 2017). Moreover, higher activity of CAT, SOD and GPx4 was also observed in testes of confined lambs that received grape pomace (Zhao et al. 2017). Inclusion of 50 and 100 g/kg DM of grape pomace in lactating ewes' diet was also effective in preventing the lipid oxidation and metmyoglobin formation in suckling lamb meat packaged under high oxygen atmospheres (80% of O_2 and 20% of CO_2) after 10 days of storage in retail conditions (Vieira et al. 2022). Conversely, in lamb meat samples also packaged under high O_2:CO_2 atmosphere and stored at 2 °C during 14 days, there was no improvement of the lipid and myoglobin stability when 50 g/kg DM of pomace grape was included in the concentrate supplied to the lambs (Guerra-Rivas et al. 2016).

Higher levels of grape pomace in lamb diets than those tested by other authors (50, 100, 150 and 200 g/kg DM) were used in experiments by Chikwanha et al. (2019). The muscle antioxidant activity, evaluated by ferric reducing ability (FRAP) assay decreased over 9 days of storage independently of the grape pomace level in the diet; however, during the first 3 days of storage higher values of antioxidant activity were observed in muscle from lambs fed diets containing 150 and 200 g/kg DM of grape pomace than those fed diets with lower levels of grape pomace. From day 5 to day 9, the difference between the diets was less expressive, but during all storage periods the diet containing of 200 g/kg DM of grape pomace resulted in higher muscle antioxidant activity than a control diet without grape pomace (Chikwanha et al. 2019). Consistently, feeding 200 g/kg DM of grape pomace also resulted in the lowest MDA and carbonyl contents in meat from the 5th day of storage (Chikwanha et al. 2019). Reduction of the MDA and carbonyl contents and increase of the antioxidant activity (FRAP assay) were also observed in beef when 150 g/kg DM of grape pomace was included in steers diets (Tayengwa et al. 2020).

Table 1.1 – Antioxidant effect of utilization of condensed tannin-rich plants and agro-industrial by-products in ruminant diets

Animal	Source		CT level in diets (g/kg DM)	Basal diet	Sample	Effect	Reference
	Plant	Level in diet (g/kg DM)					
Lamb	Grape pomace	50 and 100[1]	-	corn, soybean meal, wheat bran, oil cake of flax seed, naked oats straw and potato rattan	muscle and testes	↓ ROS and MDA levels; ↑ Total antioxidant capacity; ↑ activity of GPx4 and SOD in muscle and testes; ↑ activity of CAT in testes	Zhao et al. 2017; Zhao et al. 2018
Lamb	Grape pomace	51.7 and 103	2.21 and 4.41	forage:concentrate (40:60) supplemented with 2.7 % of linseed oil supplied to dams	muscle	↓ MDA levels and MMb % in suckling lambs	Vieira et al. 2021
Lamb	Grape pomace	50	2.23[4]	barley straw and concentrate *ad libitum*; grape pomace included in concentrate	muscle	= MDA levels and MMb %	Guerra-Rivas et al. 2016
Lamb	Grape pomace	50, 100, 150 and 200[2]	2.7; 3.5; 4.9 and 6.0[5] 0.8; 1.2; 1.8 and 2.6[6]	Concentrate: lucerne meal (80:20)	muscle	↑ FRAP values from day 1 to day 3 in the diets with 150 and 200 g/kg grape pomace; ↓ MDA levels from day 5 onward in the diets with 200 g/kg grape pomace; ↓ carbonyl content at days 5 and 7 of storage	Chikwanha et al. 2019
Steers	Grape pomace	150[2]	50.7[5] 24.1[7]	wheat straw:concentrate:lucerne (9:80:10)	muscle	↑ FRAP values; ↓ MDA and Carbonyls levels	Tayengwa et al. 2020
Cow	Grape pomace silage	50, 75 and 100	-	silage:concentrate (60:40); grape pomace silage replace partially the corn silage in forage	milk	↑ reducing power; = production of conjugated diene hydroperoxides	Santos et al. 2014
Lamb	Carob pulp	240 and 350[2]	3.4 and 4.5[8]	concentrate:dehydrated lucerne (80:20); carob pulp replaces partially barley of the concentrate	muscle	= MDA levels, free thiol and carbonyl levels and MMb %	Gravador et al. 2015
Lamb	Carob pulp	0 and 20%	1.45 and 18.9[13]	Concentrate + barley straw *ad libitum;* carob pulp replaces partially barley and wheat of the concentrate	muscle	= MDA levels	Bottegal et al. 2024
Goat	Pine bark	-	130[4]	rye grass pasture supplemented with mixture of pine bark with molasses (6% w/w) and alfalfa (5% w/w) containing 130 g/kg DM of CT	muscle	= MDA levels	Lee et al. 2017
Goat	Peanut skin	250; 500 and 750[2]	39; 78 and 117[2]	concentrate (cracked corn, soybean meal, soy hull and molasses) and peanut skin	muscle	= MDA levels in diets with 500 and 750 g/kg peanut skin	Kafle et al. 2021

Lamb	*Cistus ladanifer* L.	250	20.9[9]	dehydrated lucerne supplemented with 0 or 6% of a blend of sunflower and linseed oils (1:2, v/v)	muscle	↓ MDA levels after lipid oxidation induction	Jerónimo et al. 2012
Lamb	*Cistus ladanifer* L.	50, 100 and 200	2.7; 6.9 and 15,6[9]	concentrate:dehydrated lucerne (50:50); supplemented with 0, 4 and 8% of a blend of soybean and linseed oils (1:2, v/v); *Cistus ladanifer* replaces partially the forage	muscle	↓ MDA levels; ↓ MDA levels after lipid oxidation induction; ↑ α-tocopherol content; = total phenolic content, FRAP and TEAC values	Francisco et al. 2015; Jerónimo et al. 2020
Lamb	*Cistus ladanifer* L.	150	3.5 – 5.6[9]	concentrate: dehydrated lucerne (50:50); supplemented with 5-6% of soybean oil; *Cistus ladanifer* replaces partially the forage	muscle	= MDA levels	Francisco et al. 2018
Goat	*Larrean divaricata*	125[2]	1.74	alfalfa hay, corn and soybean meal	muscle	= DPPH values; = total phenolic content; ↓ MDA levels in meat stored at 4°C over 6 days, at 26°C for 6 h and at - 18°C for 30 days	García et al. 2019
	Acacia aroma	125[2]	5.63				
Goat	*Acacia farnesiana*	100, 200 and 300	-	Lucerne hay and concentrate	milk	↑ total phenolic content; = catechin concentration; ↑ ORAC and FRAP values	Delgadillo-Puga et al. 2019
Lamb	*Acacia mearnsii*	50, 100, 150 and 200	0.8, 1.66, 3.51, 7.33	Total mixed ration (wheat bran, *Zea mays*, lucerne hay, molasses); *A. mearnsii* replacing wheat bran	muscle	↑ FRAP activity; ↓ MDA and carbonyls levels	Ushona et al. 2023
Cow	*Ficus infectoria*	119[2]	15[4]	rice straw, maize green and concentrate (maize, mustard cake and rice bran); *Ficus infectoria* leaves included in concentrate replacing rice bran	erythrocytes	↑ SOD and CAT activity; ↑ GSH levels; ↓ MDA levels; ↑ total total thiol levels	Dey et al. 2014
Lamb	*Ficus infectoria*	106, 159 and 212[2]	10; 15 and 20[4]	Wheat straw, green fodder, and concentrate; *Ficus infectoria* leaves replacing partially the wheat bran of the concentrate	erythrocytes	↑ SOD and CAT activity; ↑GSH levels; ↓ MDA levels; ↑ total tiol and protein thiol levels	Dey et al. 2015
Lamb	*Ficus infectoria* and *Psidium guajava* (70:30)	96; 144 and 192[3]	10; 15 and 20[4]	Wheat straw, oat hay and concentrate (maize, wheat bran, deoiled soybean meal); Leaf meal mixture replace the concentrate	erythrocytes	↑ SOD, GPx and CAT activity; ↑ GSH and GST levels; ↑ total thiol and protein thiol levels; = MDA levels§§	Pathak et al. 2017
Goat	Oak (*Quercus leucotrichophora*)	-	33.5[4]	concentrate: oak leaves as roughage	erythrocytes	↑ SOD and CAT activity; ↑ GSH levels	Chaurasiya et al. 2018
Lamb	Sainfoin (*Onobrychis viccifolia*)	-	5.59 - 6.71[4, 8]	silage mixture of timothy and sainfoin (50:50) *ad libitum*, straw (60-80g/d) and barley (229 g/d)	muscle	= MDA levels in raw meat; ↓ MDA levels under pro-oxidant conditions (cooking and incubation with pro-oxidant catalysts)	Luciano et al

Lamb	Sainfoin (*Onobrychis viccifolia*)	-	21.9[10]	sainfoin pasture supplied to dams	muscle	↓ MDA levels suckling lambs	Lobón et al. 2017
Steers	high-tannin sorghum	383 - 765	17.3 - 34.6[11]	silage:concentrate (10:90); high-tannin sorghum replace partially the corn of the concentrate	muscle	↓ MDA levels in vacuum-packaged beef; ↑ MDA levels in displayed beef over 6, 10 and 15 days; = SOD, CAT and GPx activity	Larraín et al. 2008
Lamb	Sorghum grain	100; 200 and 400	8.2; 16.4 and 24.5[11]	Forage (*Aneurolepidium Chinense* hay and alfalfa hay): concentrate (corn grain and soybean meal) (42:58). Stepwise replacement of corn grain by sorghum grain	muscle	= MDA levels; ↑ tannin levels	Zhong et al. 2016
Steers	Sorghum	0, 100, 200, 300 and 400	0.5, 0.7, 0.7, 0.8, 1.0[5]	Total mixed diet (wheat bran, lucerne hay, wheat straw, soybean meal, molasses)	muscle	↑ FRAP values; tendency to ↑ MDA	Njisane et al. 2024

[1] Expressed on air-dry matter basis; [2] expressed on an as-fed basis; [3] calculated according to CT levels of leaf meal mixture; [4] CT levels in CT source or in supplement mixture; [5] Total tannins expressed as g gallic acid equivalent/kg DM; [6] CT expressed as g cyanidin chloride equivalates/kg DM; [7] CT expressed as % leucocyanidin equivalents; [8] Total tannins expressed as g tannic acid equivalents/kg DM; [9] CT quantified using *C. ladanifer* purified CT as standards; [10] CT expressed as g cyanidin equivalent/kg DM; [11] CT expressed as g catechin equivalents/kg DM; [12] CT expressed as g leucocyanidin equivalent; [13] CT expressed as in g of the equivalent CT purified from carob pulp/kg of DM of feed; CAT – catalase; DPPH - 2,2-Diphenyl-1-picrylhydrazyl assay; FRAP - reducing ability assay; GPx - glutathione peroxidase; GSH - glutathione; MDA - malondialdehyde; MMb – metmyoglobin; ROS - reactive oxygen species; SOD - superoxide dismutase; TEAC - radical scavenging ability.

In previous studies, the grape pomace was previously dried after incorporation into the diets. Ensiling the grape pomace is an alternative way to conserve this by-product. Inclusion of grape pomace silage (50, 75 and 100 g/kg DM) in the soybean oil supplemented diet (40 g/kg DM of soybean oil) of dairy cows increased the reducing power of milk to reduce the ferric ions (Santos et al. 2014). However, the improvement of antioxidant capacity in milk did not result in reduction of the production of conjugated diene hydroperoxides, a primary product of lipid oxidation (Santos et al. 2014).

Carob pulp is an agro-industrial by-product widely available in the Mediterranean area and commonly used in animal feed (Vasta et al. 2008), which is characterized by its high levels of CTs (Rodríguez-Solana et al. 2021). However, the effectiveness of carob pulp to improve the oxidative stability of lipids and proteins of lamb meat was not proven when two doses of carob pulp (240 and 350 g/kg) were included in diets (Gravador et al. 2015). Bottegal et al. (2024) found no differences in meat MDA levels from lambs fed carob pulp as a partial replacement for concentrate's barley and wheat.

Other agro-industrial by-products rich in CTs were also tested in ruminant diets, although the results on their antioxidant effect are still limited. Supplementation of goat diets with pine bark, a by-product of the timber industry containing up to 130 g/kg DM of CTs, also did not affect the meat MDA levels (Lee et al. 2017). Meat lipid oxidation was also not affected by inclusion in goat diets of high levels of peanut skin (500 and 750 g/kg), a by-product of the peanut industry (Kafle et al. 2021).

The utilization of endemic species due to their richness in CTs has been another approach to supply CTs in ruminant diets. The aerial part of *Cistus ladanifer*, a perennial shrub very abundant in the Mediterranean area rich in CTs (32-161 g/kg DM of CTs (Jerónimo et al. 2020a), has been applied in lamb diets with an approach to limit meat lipid oxidation. Incorporation of 250 g/kg DM of *C. ladanifer* (leaves and soft stems) in a high-forage diet, supplemented or not with 6% of a blend of soybean and linseed oils (1:2, v/v), reduced the MDA levels in meat after lipid oxidation induction (Jerónimo et al. 2012). As expected, lipid oxidation increased throughout the 7 days of storage at 2 °C, but lower MDA levels were observed in meats from lambs fed with *C. ladanifer*, showing the effectiveness of the dietary *C. ladanifer* to enhance the meat's resistance against lipid oxidation, even in PUFA-enriched meat (Jerónimo et al. 2012). Increased resistance against lipid oxidation of lamb meat was confirmed in another work, where increasing levels of *C. ladanifer* (50, 100 and 200 g/kg DM) were incorporated in a forage and concentrate diet (1:1) supplemented with 0, 40 and 80 g/kg DM of soybean and linseed oils (1:2, v/v) (Jerónimo et al. 2020b). These results were obtained using a methodology where the lipid oxidation products (MDA) were determined after the lipid oxidation induction (Mercier et al. 2004), not allowing evaluation of the real lipid damage in meat. Nevertheless, the effectiveness of the dietary *C. ladanifer* to reduce the lipid oxidation in lamb meat without oxidative induction has also been proven (Francisco et al. 2015; Jerónimo et al. 2020b). These papers report that, independently of the dietary oil supplementation level, increasing levels of *C. ladanifer* (50, 100 and 200 g/kg DM) in diets gradually reduced the MDA levels in meat samples stored for 7 days at 2 °C (Jerónimo et al. 2020b). Despite that, the muscle antioxidant status evaluated by ferric reducing ability (FRAP assay) and radical scavenging ability (TEAC assay) and the total phenolic content of muscle was unaffected by increasing levels of *C. ladanifer* in the diet (Jerónimo et al. 2020b). In this experiment, all diets were supplemented with the same levels of vitamin E (22.5 mg/kg), and even with the expected prevention of the lipid oxidation in meat by vitamin E, the *C. ladanifer* resulted in a significant protection of the meat against lipid oxidation. In a more recent work, the partial replacement of dehydrated lucerne with 150 g/kg DM of *C. ladanifer* in oil supplemented diets (50-60 g/kg DM of soybean oil) composed by 1:1 forage and concentrate did not reduce the MDA levels in lamb meat (Francisco et al. 2018). In this experiment, all diets were also supplemented with vitamin E (22.5 mg/kg), which may have masked a possible beneficial of *C. ladanifer* on lipid stability. Differences in basal diets, levels and type of lipid supplement and inclusion of other antioxidant compounds in diets might have created a different antioxidant–pro-oxidant balance among experiments, leading to the inconsistent results on the antioxidant effect of the dietary *C. ladanifer* (Jerónimo et al. 2020a).

The inclusion of two different CT-rich woody species – *Larrea divaricate* (125 g/kg DM of leaves) and *Acacia aroma* (125 g/kg DM of leaves) – in goat diets composed by forage and concentrate did not change the antioxidant activity (DPPH assay) and total phenols content in meat, but reduced the MDA levels in meat stored at 4 °C for 6 days, at -18 °C for 30 days and at 26 °C for 6 h (García et al. 2019). Delgadillo-Puga et al. (2019) also did not observe differences in milk antioxidant activity evaluated by DPPH assay in dairy goats fed a diet composed of forage:grain concentrate (60:40) containing increasing levels of *Acacia farnesiana* pods (0, 100, 200 and 300 g/kg DM). On the other hand, when the FRAP and oxygen radical absorbance capacity (ORAC) assays were used, an increase of the antioxidant activity of milk was reported, reaching similar or higher values than those presented in the milk of goats fed exclusively on pasture. Increasing levels of total phenols in goats' milk with increasing amounts of *A. farnesiana* pods in diets was also observed; however, the catechin levels remained unchanged. Recently, Uushona et al. (2023) found an increased FRAP activity and lower MDA concentration in the muscle of lambs fed *Acacia mearnsii*, in replacement of wheat bran.

The impact of some native species on animal antioxidant status was evaluated by analyses of several antioxidant parameters in the erythrocytes, including enzymatic and non-enzymatic antioxidants and lipid peroxidation parameters. In cows, the dietary replacement of rice bran by dried and ground leaves of *Ficus bengalensis* (119 g/kg in concentrate), a tanniferous native tree from the Indian Subcontinent (Dey and De 2014), seems to enhance the animal antioxidant status, increasing the intracellular GSH and the activity of antioxidant enzymes SOD and CAT, while reducing the MDA levels and increasing the level of total thiol (T-SH) groups that act as intracellular antioxidants by scavenging free radicals through enzymatic reactions (Dey and De 2014). Similar results were also verified in lambs, when increasing levels of ground leaves of *Ficus infectoria* (evergreen tree abundant in northern parts of India) were included in their diets (106, 159 and 212 g/kg) (Dey et al. 2015). Inclusion of *F. infectoria* and *Psidium guajava* leaf mixture (70:30) in concentrate feed (100, 150 and 200 g/kg) supplied to lambs enhanced the erythrocytic antioxidant status, increasing the intracellular activity of SOD and CAT and the levels of GST, GSH and total and protein thiol (P-SH) (Pathak et al. 2017). Increased levels of GSH and SOD activity in the blood were also observed in goats that received oak leaves (*Quercus electrophori*) containing 3.35% of CT and 3.10% of HT, suggesting an improvement in the antioxidant status of those animals (Chaurasiya et al. 2018).

Forage legumes with high levels of CTs, such as sainfoin and high-tannin sorghum, were also evaluated in terms of their effectiveness to limit meat lipid oxidation when incorporated in ruminant diets. Feeding lambs with silages containing sainfoin improved the lipid oxidative stability of meat subjected to pro-oxidant conditions (cooking and incubation with pro-oxidant catalysts) compared to grass silages (Luciano et al. 2019). Consistently, Lobón et al. (2017) reported lower levels of lipid oxidation from day 7 to day 14 of storage at 4 °C in meat of suckling lambs when dams grazed on a sainfoin pasture compared to meat of suckling lambs from dams fed a total mixed ration. The protective effect of high-tannin sorghum against lipid oxidation in meat was contradictory, reducing the MDA levels in vacuum-packaged beef, while increased the lipid oxidation during aerobic display of the beef (14 days at 4 °C) (Larraín et al. 2008). In this work, the activity of antioxidant enzymes in muscle was not affected by high tannin sorghum. In a recent work, Njisane et al. (2024) found increased meat FRAP levels in beef from steers fed sorghum as a replacement for maize.

Differences in CT chemical structure and concentration in the diet, as well as the diversity of basal diets used and the presence of other bioactive compounds with antioxidant activity in the diet, can help to explain the inconsistent results on the antioxidant effect of CT-rich plants and by-products, either between different CT sources, or with the same CT source. It is also important to note that within each CT source type, the CT levels can be variable among experiments, since the biosynthesis of phenolic compounds, such as CTs, depends on several factors, including variety, plant/fruit development stage, environmental temperature and water availability (Guerreiro et al. 2016b; Mangan 1988; Rauf et al. 2019; Skogsmyr and Fagerström 1992). Thus, the utilization of the same level of a certain plant or by-product may not correspond to an equal CT amount in the diet. Another important aspect when CTs is provided to animals through the inclusion of plants or by-products in diets is the presence of other antioxidant compounds in addition to CTs in the vegetable material, such as other phenolic compounds and vitamins, which makes it impossible to isolate the contribution of CTs to the antioxidant effect.

1.3.2. Condensed tannin extracts

Utilization of CT extracts allows a better understanding of the specific effect of dietary CTs on ruminant antioxidant status and oxidative stability of their products. Quebracho, mimosa and grape seed have been the main CT extracts applied in ruminant diets, as possible sources of antioxidants (Table 1.2).

The protective effect of dietary quebracho CTs against lipid oxidation was verified by Lobón et al. (2017b) in meat of suckling lambs when dams fed pasture or hay were supplemented with 300 g/head of concentrate containing 10% of quebracho. Conversely, the supplementation of fattening concentrate of the weaned lambs with 50 g/kg of quebracho did not affect the MDA levels in meat over 14 days of storage (Lobón et al. 2017). No effect of dietary supplementation with quebracho on lamb meat lipid oxidation was also reported by Brogna et al. (2014), when including 80 g/kg of quebracho in lamb diets, and by Luciano et al. (2009a), who supplemented the barley-based concentrate with 89 g/kg DM of quebracho. However, in this last study, Luciano et al. (2009a) also observed that supplementation of lamb diets with quebracho reduced the metmyoglobin formation and produced an improvement in the muscle antioxidant capacity evaluated using FRAP and TEAC assays and increased the concentration of total phenolic compounds in muscle (Luciano et al. 2011). Improvement of the liver overall antioxidant status by dietary supplementation with quebracho CT was also observed by Lopéz-Andrés et al. (2013), who reported higher values of FRAP and total phenolic content in the liver of lambs fed a concentrate containing 64 g/kg of quebracho in contrast to the control ones. In turn, Buccioni et al. (2017) found that supplementation of concentrate with 52.8 g/kg DM of quebracho was not able improve the oxidative status in lactating grazing ewes, when they determined the MDA levels in plasma of ewes fed CT-supplemented concentrate.

In lambs, the incorporation of 25 g/kg DM of grape seed extract in a high-forage diet supplemented or not with a blend of vegetable oils (sunflower and linseed oils, 1:2 (v/v)) reduced the MDA levels in meat stored for 3 and 7 days at 4 °C and subjected to lipid oxidation induction (Jerónimo et al. 2012). Consistently, Mu et al. (2020) reported that the MDA content decreased linearly in lamb meat with increasing levels of grape seed extract (0, 20, 30 and 40 mg/kg body weight/day) in the high-concentrate diet. Furthermore, the increasing levels of grape seed extract in the diet also resulted in a linear increase of the total antioxidant capacity and activity of the CAT, SOD and GPx4 in muscle (Mu et al. 2020). Improvement of the total antioxidant capacity, evaluated in plasma by TEAC assay, was also reported by (Gladine et al. 2007b) when grape seed extract was infused directly into rumen. Conversely, the plasma antioxidant activity (FRAP assay) of dairy goats and sheep was not affected by concentrate supplementation with grape seed extract (74 g/kg DM) (Leparmarai et al. 2019). Feeding lambs with concentrate diet supplemented with 50 mg/kg DM of grape seed extract also did not affect the MDA and metmyoglobin contents in meat packaged under high O_2 atmosphere (O_2:CO_2 , 80:20) and stored at 4 °C for 14 days (Guerra-Rivas et al. 2016). Contents of MDA and protein carbonyl in PUFA n-3 enriched meat stored in a modified atmosphere (O_2:CO_2 , 70:30) over 12 days at 4 °C was also not affected by supplementation of lamb diets with 900 mg of red wine extract per kg of concentrate (Muíño et al. 2014).

The efficacy of dietary supplementation with mimosa (*Acacia mearnsii*) CT extract to protect lamb meat against lipid oxidation was tested in two studies, where 40 g/kg of mimosa extract was included in concentrate (Biondi et al. 2019; Valenti et al. 2019). In both experiments the supplementation of diet with mimosa extract did not affect the MDA levels in raw meat stored at 4 °C over 7 days (Biondi et al. 2019; Valenti et al. 2019), not even in meats subjected to pro-oxidant conditions (cooking and meat homogenate with pro-oxidant catalysts) (Valenti et al. 2019). However, the effect of mimosa CT extract on oxidation of myoglobin in meat was contradictory: one of these works reported the reduction of metmyoglobin formation (Biondi et al. 2019), while the other showed that supplementation of diets with mimosa CT extract did not affect the myoglobin oxidation (Valenti et al. 2019). Lipid oxidation in salted and sun-dried meat from bulls fed an oil-supplemented (42.5 g/kg DM of soybean oil) diet composed of 40:60 forage and concentrate was also not affected by increasing levels of mimosa extract in the diets (10, 30 and 50 g/kg DM) (Gesteira et al. 2019). Similar results were also found by Staerfl et al. (2011), who reported that supplementation of maize silage with concentrate containing 141 g/kg of mimosa extract did not improved the oxidative stability of perirenal fat of bulls. Avila et al. (2020) supplemented the diets of lactating dairy cows with increasing levels of *A. mearnsii* CT extract (6.12, 12.25; 18.42 and 24.58 g/kg DM) and found no differences in the MDA milk levels. However, they found increasing diene conjugates concentration, which indicates a greater lipid peroxidation, with increasing CT levels. More recently, Brunetto et al. 2024 using *Acacia mearnsii* extract in lamb diets, in a dose of 2.5 g/kg DM, observed lower lipid peroxidation in both serum and meat from lambs receiving the diet supplemented with the CT extract. The same result was found by Santos et al. 2024, who supplemented bulls' diets with increasing levels of *Acacia mearnsii* extract (10, 30 and 50 g/kg DM) and found lower lipid oxidation in meat from the animals that received the diets containing the CT extract. By contrary, both Jacondino et al. 2022 and Salas et al. 2024, supplementing lambs' diets with *Acacia mearnsii* extract did not observed effects on meat lipid oxidation.

Although the results point to the effectiveness of CT extracts in improving the overall antioxidant status of ruminants and the oxidative stability of their products, the results are controversial, and for some CT extracts, antioxidant effect in ruminants have not yet been verified, as reported for the mimosa extracts. The inconsistent antioxidant effect in ruminants of the various CT extracts may be related to the chemical structure of CTs, once the CT structure differs markedly according to its origin (Tao et al. 2019; Unusan 2020). Moreover, several other factors can contribute to inconsistent antioxidant effect of CT extracts, such as CT concentration in the diets, composition of basal diet, presence of pro- and antioxidant compounds, or other uncontrolled factors as environmental temperature and stress-inducing situations, that might create a different balance between antioxidant and pro-oxidant agents. In addition, the interpretation of results is hampered by the diversity of analytical techniques and standards used to quantify the CTs, and in some studies, such information is not displayed. The CT content in sources and diets has been quantified through butanol-HCl (Chikwanha et al. 2019; Dey and De 2014; Dey et al. 2015; Francisco et al. 2015; Gravador et al. 2015; Jerónimo et al. 2012; Jerónimo et al. 2020b; Tayengwa et al. 2020) and vanillin (Larraín et al. 2008; Zhong et al. 2016) assays and using different standards, such as cyanidin chloride, leucocyanidin, catechin, tannic acid or CT purified from the vegetal material under study (see footnotes of Tables 1 and 2). Some studies only reported the total tannins content, or the CT levels supplied by the manufacturer without identification of the procedures used for CT quantification, and in others, the CT levels in diets are not reported. The lack of information on the CT contents and the non-standardization of methodologies and standards used for quantification make comparisons between studies difficult.

If the occurrence of antioxidant compounds other than CTs can contribute to the antioxidant effect when CT-rich plants and by-products are used, the improvement in antioxidant status and oxidative stability observed by the inclusion of the CT extracts in principle are directly related to CT action. However, the mechanisms by which CTs are able to exert an antioxidant effect in ruminants are unclear. So, elucidation of the CT antioxidant mechanisms is a relevant topic that might be useful to understand the controversial results on the antioxidant effect of dietary CTs in ruminants, and to design effective antioxidant strategies for these species based on incorporation of the CT sources in their diets.

Table 1.2 – Antioxidant effect of utilization of condensed tannin extracts in ruminant diets

Animal	**Source**			**CT level** (g/kg DM)	**Basal diet**	**Sample**	**Effect**	**Reference**
	Plant	**CT levels of extract** (g/kg DM)	**CT extract levels** (g/kg DM)					
Lamb	Quebracho	-	50^{1}	3.7^{6}	Concentrate (corn, soybean meal, wheat and barley) + straw *ad libitum*. Quebracho extract included in concentrate	muscle	= MDA and MMb levels	Lobón et al. 2017
Lamb	Quebracho	750^{1}	100^{1}	$75^{1, 5}$	Dietary treatments supplied to dams. Pasture vs forage diets supplemented with concentrate. Quebracho extract included in concentrate.	muscle	↓ MDA levels; ↑ α-tocopherol levels in muscle of suckling lambs; = MMb levels	Lobón et al- 2017b
Lamb	(Quebracho) *Schinopsis lorentzii*	-	89	40.4^{2}	High-concentrate diet (barley and soyabean meal) and lucerne hay. Quebracho extract included in concentrate and forage mixture	muscle	= MDA levels; ↑ total phenols levels; ↑ FRAP and TEAC values; ↓ MMb %	Luciano et al. 2009; Luciano et al. 2011
Lamb	(Quebracho) *Schinopsis lorentzii*	-	95.7	64	High-concentrate diet (barley and soyabean meal) and lucerne hay. Quebracho extract included in concentrate and forage mixture	liver	= total phenolic content; ↑ FRAP values in raw samples; = total phenolic content and FRAP values in SPE samples	López-Andrés et al. 2013
						plasma	↑ total phenolic content and FRAP values in raw samples; = total phenolic content and FRAP values in SPE samples	
Sheep	(Quebracho) *Schinopsis lorentzii*	456^{2}	52.8^{5}	16 g/kg DM intake	250 g Chopped grass hay + 800 g concentrate/day	plasma	= MDA levels	Buccioni et al. 2017
Sheep	*Aspidosperma quebracho* (Quebracho)	-	80^{1}	-	Dried beet pulp supplemented with 2% of vegetable oil	muscle	= MDA levels	Brogna et al. 2014
Lamb	Quebracho (*Schinopsis lorenzii*)	700^{1}	-	10, 30, 60 g/kg DM intake	Silage:concentrate	muscle	= MDA levels	Santos et al. 2022
Cow	Chestnut:quebracho 60:40	621^{2}	150g/head	135.3	Concentrate + pasture (Ver)	plasma cheese	↓ ROM in plasma ↑ DPPH activity in cheese	Santillo et al. 2022
Lamb	Grape seed (*Vinis vitifera*)	950	25	14.1^{7}	Dehydrated lucerne supplemented with 0 or 6% of a mixture of sunflower and linseed oils (1:2, v/v)	muscle	↓ MDA levels after lipid oxidation induction	Jerónimo et al. 2012

Sheep	Grape peel and seed (*Vinis vitifera*)	> 800	10% DM intake	-	concentrate (barley, beet pulp, soybean meal, molasses): meadow hay (30:70)	plasma	↑ TEAC values; ↑ the length of the lag phase of conjugated dienes generation; presence of five different phenolic compounds, including epicatechin	Gladine et al. 2007
Lamb	Grape seed	-	10, 20 and 40 mg/kg BW/day	-	Total mixed feed with concentrate (corn, soybean meal, cottonseed meal, wheat bran): forage (corn and millet straw) (70:30)	muscle	↑ total antioxidant capacity, ↑ activity of CAT, SOD and GPx4; ↓ MDA levels	Mu et al. 2020
Lamb	Grape seed	413	50 mg extract/kg DM[5]	-	barley straw and concentrate (barley, soya and molasses) *ad libitum,* grape seed extract included in concentrate	muscle	= MDA levels and MMb %	Guerras-Rivas et al. 2016
Sheep Goat	Grape seed	-	74[5]	7.3 – 7.5[4]	forage:concentrate:dried sugar beet pulp (51:46:3), grape seed included in concentrate	milk plasma	= FRAP values in plasma; ↑ total phenol concentration in plasma and milk	Leparmarai et al. 2019
Lamb	Red wine extract	-	900 mg extract/kg feed[5]	-	Barley + concentrate (corn meal, barley, wheat, soybean meal, sunflower meal) supplemented with extruded linseed and deodorised fish oil. Red wine extract included in concentrate	muscle	= MDA and protein carbonyl levels; = total phenols content	Muíño et al. 2019
Lamb	Mimosa (*Acacia mearnsii*)	-	40[1]	22.3[2]	concentrate (barley, wheat bran, soybean meal, molasses): dehydrated lucerne (85:15)	muscle	= MDA levels; ↓ MMb %	Biondi et al. 2019
Lamb	Mimosa (*Acacia mearnsii*)	881[3]	40[1]	22.3[2]	concentrate (barley, wheat bran, soybean meal, molasses): dehydrated lucerne (85:15)	muscle	= MDA levels in raw and cooked meat and in meat homogenates with Fe3+/Asc; did not after colour stability	Valenti et al. 2019
Bull	Mimosa (*Acacia mearnsii*)	720[3]	10, 30 and 50	-	concentrate (corn, soybean meal, soybean oil (4.3%)): Tifton-85 hay (60:40)	muscle	= MDA levels in salted and sun-dried meat	Gesteira et al. 2019
Bull	Mimosa (*Acacia mearnsii*)	700	141[1,5]	-	maize silage:concentrate; mimosa extract included in concentrate	perirenal fat	= oxidative stability evaluated by rancimat test	Staerfl et al. 2011
Cow	Mimosa (*Acacia mearnsii*)	805[1]	6.1; 12.2; 18.4 and 24.6	5; 10; 15 and 20	Concentrate: forage (80:20)	milk	= MDA levels and reducing power; ↑ diene conjugates concentration	Avila et al. 2020

Lamb	Black wattle (*Acacia mearnsii)*	735 ou 986	2.5	2.82g intake per animal/day	Concentrate:silage (55:45)	muscle serum	↓ MDA levels	Brunneto et al. 2024
Lamb	Black wattle (*Acacia mearnsii)*	-	40 g/kg	16.7	Concentrate (soybean hulls, soybean meal, corn)	muscle	= MDA levels	Jacondino et al. 2022
Lamb	Black wattle (*Acacia mearnsii)*	700	1.75, 3.5, 5.25	0.25, 0.5, 0.75 %in diet	?? (Corn, soybean meal, oat hay)	muscle	↑ phenolic content = MDA levels	Salas et al. 2024
Bull	*Acacia mearnsii*	833	-	10, 30, 50 g/kg	Total mixed ration (roughage:concentrate 40:60)	muscle	↓ MDA levels	Santos et al. 2024

[1] as fed basis; [2] total tannins expressed as g of tannic acid equivalents/kg DM; [3] total tannins; [4] tannins expressed as g of tannic acid equivalents/kg DM; [5] condensed tannin levels in CT source or supplement mixture; [6] condensed tannins expressed as g of cyanidin equivalents/kg DM; [7] CT quantified using grape seed purified CT as standards; CAT – catalase; FRAP - reducing ability assay; GPx - glutathione peroxidase; MDA - malondialdehyde; MMb – metmyoglobin; SPE – solid-phase extraction; SOD - superoxide dismutase; TEAC - radical scavenging ability

1.4. Condensed tannins' antioxidant mechanisms

As with other polyphenols, the antioxidant function of CTs involves diverse mechanisms, such as scavenging of free radicals, transition metals chelation and the inhibition of pro-oxidative enzymes (Koleckar et al. 2008). Moreover, antioxidant synergies between exogenous phenolics and endogenous antioxidants have been reported, and it is known that CTs can interfere with the metabolism of other antioxidant compounds (Iglesias et al. 2012). The antioxidant activity of CTs is well established in *in vitro* models, as well as when directly applied to food products. However, the mechanisms by which CTs can induce antioxidant activity *in vivo* are still unclear. Unlike other phenolic compounds with low molecular weight, the polymeric nature and high structural complexity of CTs limit their absorption into circulation and the direct antioxidant effect in living animal and post-mortem, making it more difficult to explain the mechanisms by which CTs has antioxidant effect *in vivo*. Several action mechanisms, involving direct and indirect mechanisms, seem to be possible, such as: i) absorption of CTs or metabolites allowing direct action in animal body and tissues; ii) antioxidant action in the gastrointestinal tract (GIT); and iii) interaction with other antioxidant compounds and endogenous antioxidant system.

The presence of the CTs in circulation and deposition in tissues would allow a direct antioxidant action. However, the bioavailability (usually defined as the concentration of a given compound or its metabolites that reaches the systemic circulation (Holst and Williamson 2008)) of CTs depends highly on the degree of polymerization and molecular weight (Tao et al. 2019; Unusan 2020). Condensed tannins are characterized by their polymeric nature and high molecular weight, which limit their bioavailability once only monomers and oligomers up to tetramers can be absorbed and transported via circulation to various organs (Ou and Gu 2014; Sallam et al. 2021; Tao et al. 2019). Moreover, between monomers and oligomers that can be absorbed, there are differences in absorption rates, depending on the molecular weight, chemical structure and stereochemical configuration (Sallam et al. 2021; Tao et al. 2019; Yang et al. 2018). The flavan-3-ol monomers show different absorption rates according to chemical structure and stereochemical configuration, decreasing the absorption rate from (−) − epicatechin > (+) − epicatechin = (+) − catechin > (−) − catechin (Ottaviani et al. 2011). In addition, the absorption rates of CT dimers, trimers and tetramers decrease with their increasing molecular size and number of hydrophilic hydroxyl groups (Sallam et al. 2021). Although the CT chemistry varies between plant sources, generally it is mainly composed of oligomers and polymers, and low molecular weight CTs are present in low concentrations (Tao et al. 2019; Unusan 2020). So, the contribution of native CT monomers and small oligomers to generate antioxidant effects through their absorption may be limited.

The degradation of CTs into compounds with a lower degree of polymerization or even to monomers throughout the GIT would allow greater availability of absorbable compounds, but the CT metabolization in the GIT is still poorly understood. Despite the conflicting results, several reports have shown that CTs are not inert during transit through the GIT, instead they are subject to extensive biotransformation processes (Abia and Fry 2001; Déprez et al. 2000; Ou and Gu 2014; Tao et al. 2019). Along the GIT, there are conditions like gastric acidity and microorganisms that could promote CT changes. However, CTs are characterized by their high resistance to degradation induced by acid conditions and by most microorganisms (Mena et al. 2015), which can limit the CT transformation in the GIT. Results on CT degradation under acidic conditions are inconsistent. Degradation of procyanidin oligomers from cocoa and apple with the production of oligomers with a lower degree of polymerization and monomers was observed after *in vitro* incubations under acid conditions (Gültekin-Özgüven et al. 2016; Kahle et al. 2011; Spencer et al. 2000). On the other hand, simulating the gastric conditions, Li et al. (2015) observed only a slight decrease in the degree of polymerization of CTs from *Choerospondias axillaris* peels after incubation. Gültekin-Özgüven et al. (2016) reported that monomers and dimers from cocoa remained stable under acidic conditions. In humans, it was also demonstrated that cocoa CT remained quite stable during *in vitro* gastric digestion (Rios et al. 2002). So, due to their high resistance to acidic conditions and low absorption in the small intestine, it is widely accepted that most of the CTs reaches the colon intact, where they are metabolized through the action of the intestinal microbiota with the production of various low molecular weight compounds that can be absorbed into the circulation (Cires et al. 2017; Mena et al. 2015; Sallam et al. 2021). The transformation of CTs by gut microbiota has been reviewed, with a wide variety of compounds reported to be generated, mainly aromatic acids and valerolactones, which can also contribute to the health effects of CTs (Cires et al. 2017; Mena et al. 2015; Ou and Gu 2014; Sallam et al. 2021; Tao et al. 2019). Although CTs might undergo extensive metabolization during transit through the GIT, the presence of CT monomers, oligomers, and polymers in feces of animals that received CT sources (Choy et al. 2013; Choy et al. 2014; Jimenez-Ramsey et al. 1994) suggests that their biotransformation is incomplete (Choy et al. 2013).

The studies on the fate of CTs in the GIT have been performed mainly using *in vitro* models and in humans and animals such as rats and pigs (Cires et al. 2017; Tao et al. 2019), while in ruminants the literature available on CT metabolism throughout GIT is scarce. Recently, Quijada et al. (2018) reported a large disappearance of CTs from sainfoin and hazelnut skin between feed and feces (61–85%), suggesting that CTs may be structurally modified, degraded, or absorbed along the GIT. Consistently, the recovery of CTs in feces from sainfoin-fed cows was less than would be expected if the CTs remained inert throughout the

GIT (Desrues et al. 2017). Moreover, a reduction of the mean degree of polymerization (mDP) of CTs in the digestive tract was observed in these cows fed sainfoin (Desrues et al. 2017). Recently, Girard et al. (2020) also reported that only 46% and 78% of CTs were recovered in the large intestine of the lambs fed sainfoin or birdsfoot trefoil silages, respectively. In both lambs fed sainfoin or birdsfoot trefoil silages, soluble CTs were detected in the digesta when the Acetone-HCl-butanol assay was used, but a large part of soluble PC and PD were not detected by UPLC-MS/MS analysis, suggesting the occurrence of chemical transformations of CTs in the digestive tract in compounds that could not be detected by the specific MS/MS method for analysis of PC and PD. Using 14C-labeled CTs, a substantial disappearance of CTs from sheep and goat gastrointestinal tracts was also identified (Perez-Maldonado and Norton 1996; Terrill et al. 1994). Gladine et al. (2007b) reported the presence of epicatechins in the plasma of sheep that received grape peel and skin extract directly into the rumen. Considering the minor proportion of monomeric compounds in grape peel and skin extract, Gladine et al. (2007b) associated the presence of epicatechins in the plasma of sheep with the possible biodegradation of polymeric CTs by the ruminal microorganisms. Conversely, Makkar et al. (1995a); Makkar et al. (1995b) could not demonstrate the degradation of CTs by rumen microorganisms. However, it could be hypothesized that CTs can also be metabolized in the rumen and in the intestine of ruminants, similarly to what happens in the colon of humans (Girard et al. 2020), and further studies using advanced techniques of CT analysis can help to elucidate the fate of CTs in the GIT of ruminants.

Condensed tannins are known for their ability to form complexes with various types of molecules, primarily with proteins and to a lesser extension with polysaccharides, nucleic acids and metal ions (Makkar 2003a). These complexes are generally unstable depending on numerous factors, including pH, and the pH variation along the ruminants GIT defines the behavior of CTs. Condensed tannins form stable complexes with proteins under the rumen pH conditions (pH 5.5 to 7.0), releasing under the acid conditions of the abomasum (pH 2.5 to 3,5), and in alkaline conditions of the distal small intestine (pH ≈ 7.5) (Jones and Mangan 1977; Mueller-Harvey 2006b). So, the GIT conditions can conditionate the accessibility to CTs and, therefore, their reactivity and bioavailability.

Despite the conflicting results on CT metabolization and absorption, increasing evidence supports that CTs undergo transformation along the GIT. The discrepancy in these results can be attributed to several factors, such as type of CT (degree of polymerization, chemical structure), possible interactions with food/feed matrix (Tao et al. 2019), and animal species. Furthermore, methodologies that have been used over time to assess the concentration and composition CTs and their metabolites have different sensitivities, which may also help to explain the conflicting results.

Several studies reported an increase of the total phenolic content, determined using Folin-Ciocalteau reagent, in plasma, muscle and milk of ruminants fed diets contain CTs (shown in Tables 1 and 2), which could suggest a possible transfer of dietary CTs to plasma, muscle and milk. However, the Folin-Ciocalteau reagent is not specific to phenolic compounds and can react with other reducing agents (Sánchez-Rangel et al. 2013), so these results should be interpreted with caution. Presence of CT monomers or small oligomers in plasma, muscle and milk of ruminants fed CT sources only was reported by Gladine et al. (2007b), who identified epicatechins in the plasma of sheep fed supplements with grape peel and skin extract. Conversely, no phenolic compounds were detected in liver and plasma of lambs supplemented with quebracho CT extract (Lopéz-Andrés et al. 2013). The level of catechins in milk also did not change when increasing levels of *Acacia farnesiana* were added to goats' diets (Delgadillo-Puga et al. 2019).

Although the CTs in intact form or their metabolites can be absorbed and transported to organs by blood inducing beneficial effects, the great proportion of CTs and their metabolites that are excreted in feces is an indicator of their poor bioavailability (Tao et al. 2019). So, the contribution of dietary CTs to animal antioxidant status and oxidative stability of products through absorption is probably very limited, although it cannot be excluded. Other action mechanisms can be involved in the antioxidant activity of CTs *in vivo*.

The GIT has been proposed as the main site of CTs' action, where they and/or their metabolites can directly exert several biological activities, as antioxidant activity. In the intestine, CTs can scavenge free radicals, chelate metals, and reduce lipid peroxidation and the production of lipid oxidation derivatives and toxic compounds, which could result in improvements of the animal's overall antioxidant status (Halliwell et al. 2005; Kerem et al. 2006). Moreover, several studies have shown that CTs are also able to protect other antioxidant molecules from oxidation, such as vitamins. This would increase the amounts of antioxidant compounds along the GIT, contributing to enhance the animal's antioxidant status (Iglesias et al. 2012). Yamamoto et al. (2006) reported the increase of vitamin E levels in the large intestine mucosa of rats fed green tea catechins. The effect of CTs on vitamin E levels does not appear to be limited to GIT alone and increases in vitamin E concentrations have been reported in tissues from animals fed CT sources. The inclusion of grape extract in rat diets increased the vitamin E content by 24% in the liver (Gladine et al. 2007a). Furthermore, it has been reported that several phenolic compounds, including catechins and epicatechins, increase the vitamin E levels in the plasma and liver of rats (Frank 2005). Inclusion of 200 g/kg DM of *C. ladanifer* in lamb diets also resulted in increased levels of α-tocopherol in muscle (Jerónimo et al. 2020b). Although *C. ladanifer* can be a source of vitamin E, other mechanisms that may be responsible for these higher levels of α-tocopherol in muscle cannot be excluded. Various mechanisms by which phenolic compounds might induce such an increase have been hypothesized, such as by protecting vitamin E against oxidation, restoring the vitamin from oxidation, and inhibition of metabolism and enhancement of absorption (Frank et al. 2004).

Moreover, CTs are also able to protect other antioxidant compounds in addition to vitamin E, such as ascorbic acid (Iglesias et al. 2012). Iglesias et al. (2012) demonstrated the strong ability of grape CT to repair oxidized α-tocopherol and to delay the ascorbic acid depletion in muscle tissues of fish. Regeneration of α-tocopherol from tocoperoxyl radical was also observed by Facino et al. (1998) by the addition of grape procyanidins to phosphatidylcholine liposomes and red blood cells. So, the interaction with other antioxidant compounds in GIT, increasing their availability for absorption or to exert antioxidant activity in GIT, or even the interaction of CTs that can be absorbed with other antioxidant compounds in circulation or deposited in tissues, are possible mechanisms by which CTs can exert antioxidant activity indirectly.

Moreover, it has also been shown that CTs' beneficial effects may be related to an ability to modulate the cell signaling pathways, thus affecting the expression of specific genes (Unusan 2020). Supplementation of sheep diets with grape skin increases the gene expression of SOD in plasma (Sgorlon et al. 2006). More recently, Mu et al. (2020) reported increased GPx4 and SOD mRNA abundance in *longissimus* muscle from lambs fed on grape seed CT. Higher antioxidant enzymes gene expression was also observed in human hepatoblastoma cells incubated with grape seed procyanidins (Puiggros et al. 2005). Consistently, increased activity of antioxidant enzymes was observed in animals fed sources of CT, as presented in previous Sections 1.3.1 and 1.3.2. Increased transcription factor Nrf2 (nuclear factor erythroid 2–related factor 2) was observed in the muscle of lambs fed diets containing wine grape pomace (Zhao et al. 2018) or grape seed CT extract (Mu et al. 2020). Nrf2 is a major regulator of antioxidant protective genes, regulating the expression of proteins involved in the antioxidant defense system (da Costa et al. 2019). The Nrf2 pathway is activated by oxidative stress (excessive levels of ROS), but also by the presence of electrophilic compounds (da Costa et al. 2019; Suraweera et al. 2020). It is reported that some polyphenolic compounds can induce endogenous antioxidant defense mechanisms by modulating Nrf2 (da Costa et al. 2019; Martinez-Huelamo et al. 2017; Suraweera et al. 2020; Tebay et al. 2015; Upadhyay and Dixit 2015). Among many other phenolic compounds, epicatechin and catechin have already been described as capable of activating the Nrf2 pathway (Martinez-Huelamo et al. 2017). Despite the poor bioavailability of CTs, the increased expression and activity of antioxidant enzymes and transcription factor Nrf2 observed in ruminants fed CT sources suggest that activation of cell signaling pathways may be another mechanism by which CTs can improve the antioxidant response in living animals.

1.5. Conclusions

The results showed that inclusion of CT-plants and plant extracts in ruminant diets can improve the animal antioxidant status and produce edible products with better oxidative stability. However, the antioxidant effect of dietary CT sources in ruminants has not been proven in all works, which can be due to several factors, such as CT chemical structure and concentration in the diets, composition of basal diet, presence of pro- and antioxidant compounds, or other uncontrolled factors that might create different balance between antioxidant and pro-oxidant agents. Evidence supports that the antioxidant effects of CTs, which are observed both in living animals and post-mortem by preventing oxidative damage in products, may be due to multiple mechanisms of action, which could occur in an integrated and synergic way. The direct antioxidant effect, which implies the absorption of CTs or their metabolites and presence in circulation and tissues, probably is not the most relevant CT antioxidant mechanism considering the poor bioavailability of CTs. However, this mechanism cannot be excluded and can contribute to overall antioxidant status when CT sources contain or originate higher levels of absorbable CTs. The antioxidant action of CTs in the GIT is the most consensual method, which can also occur in different ways, reducing oxidation reactions and protecting other antioxidant compounds. Interaction with other enzymatic and non-enzymatic components of the antioxidant system seems to be another possibility by which CTs can exert antioxidant activity. In addition to other factors, the chemical structure of CTs seems to be an important factor in determining the antioxidant action mechanisms of CTs *in vivo*, so the way CTs exert antioxidant activity, and its effectiveness probably depend on the CT's origin. However, the chemical structure of several CT sources used in animal nutrition is poorly characterized, and their characterization should be improved. In addition to better knowledge of the chemistry of each CT source, it is important to carry out further studies in ruminants to understand the action mechanism by which each CT source exerts antioxidant activity in living animals and in their products, which will allow design of effective antioxidant strategies based on the application of CT sources in diets.

Funding

This research was funded by the Fundação para a Ciência e Tecnologia (FCT) through research grants to D.S. (SFRH/BD/145814/2019) and the projects UIDB/00276/2020 from Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon and UIDB/05183/2020 from MED – Mediterranean Institute for Agriculture, Environment and Development; and by the Alentejo2020 program for financial support to the project "CistusRumen – Sustainable use of Rockrose (*Cistus ladanifer* L.) in small ruminants—

Increase of the competitiveness and reduction of the environmental impact" (ALT20-03-0145-FEDER-000023) co-funded by European Regional Development Fund.

Author contributions

Conceptualization, D.S. and E.J.; writing—original draft preparation, D.S. and E.J.; writing – review and editing, D.S., E.J. and R.J.B.B.; supervision, E.J. and R.J.B.B. All authors have read and agreed to the published version of the manuscript.

2. CHAPTER 2 | Inclusion of the *Cistus ladanifer L.* plant and its condensed tannin extract in lamb diets – Effects on animal antioxidant status and oxidative stability of meat

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Adapted from *Animal Feed Science and Technology*, 316, October 2024, 116070
(Available online at https://doi.org/10.1016/j.anifeedsci.2024.116070)

Abstract

This study evaluated whether the incorporation of condensed tannins (CT) extract from *Cistus ladanifer* in lamb diets induces the same antioxidant protection as the *C. ladanifer* plant. How dietary CT can enhance the antioxidant status of animals, and the oxidative stability of their products was also explored. Thirty-six lambs were used to evaluate the effect of three levels of *C. ladanifer* CT (without CT enrichment, medium, and high level of CT) and two ways of CT supply (*C. ladanifer* plant or *C. ladanifer* CT extract) on meat lipid and colour stability, and antioxidant status of muscle, rumen, abomasum, and faeces. The presence of CT monomers in muscle and CT content in digestive contents and faeces was evaluated. *Cistus ladanifer* plant and CT extract did not improve the lipid stability and antioxidant status of meat, but both CT sources enhanced the meat colour stability over storage time. Condensed tannin monomers were not identified in muscle. Intake of α-tocopherol decreased with inclusion of CT sources in diets. However, muscle α-tocopherol content was not affected by diets, suggesting that CT increase the α-tocopherol availability in muscle. Both CT sources increased antioxidant activity in the digestive contents, although, in the abomasum, *C. ladanifer* plant resulted in higher antioxidant activity than CT extract for the same CT level in the diet. No CT were detected in the faeces and both CT sources had a minor effect on the antioxidant activity, which could indicate the CT transformation in the gastrointestinal tract.

2.1. Introduction

Due to the antioxidant properties of condensed tannins (CT), CT-rich sources have been tested as alternatives to synthetic antioxidants in animal feed. Although the results are still inconsistent, the application of several CT-rich sources in ruminant diets has been shown to be effective in improving the animals' antioxidant status and limiting oxidative deterioration of their products (Soldado et al. 2021).

Cistus ladanifer L. (rockrose) is a Mediterranean endemic perennial shrub containing high CT levels. Values between 32–161 g/kg dry matter (DM) of CT were reported in the *C. ladanifer* collected in Portugal (Dentinho et al. 2005; Guerreiro et al. 2016a). Due to its high CT levels and abundance in the Mediterranean area, leaves and soft stems of *C. ladanifer* have been applied as a source of antioxidant compounds in lamb diets with positive effects on the lipid oxidative stability of meat (Francisco et al. 2015; Jerónimo et al. 2012; Jerónimo et al. 2020b).

The ability of the *C. ladanifer* plant (leaves and soft stems) to improve the meat oxidative stability was associated with the increment of antioxidant compounds in diets due to its high contents of phenolic compounds, particularly in CT (Jerónimo et al. 2012). However, the *C. ladanifer* plant has a combination of antioxidant compounds, including flavonoids, phenolic acids and CT, but also terpenes, and vitamins such as tocopherols and ascorbic acid (Barrajón-Catalán 2010; Barros 2013; Gomes et al. 2005; Guerreiro et al. 2022a; Guimarães et al. 2010), which can contribute to the antioxidant effect. The inclusion of the *C. ladanifer* plant in lamb diets increased the α-tocopherol content in muscle, suggesting that the *C. ladanifer* protective effect against meat lipid oxidation can be related to the higher levels of α-tocopherol in muscle (Jerónimo et al. 2020b). Nevertheless, it is unclear whether the higher α-tocopherol content in the muscle of lambs fed *C. ladanifer* resulted from the direct transfer of α-tocopherol from *C. ladanifer* to the muscle or from a possible interaction between polyphenolic compounds of *C. ladanifer* with α-tocopherol promoting its higher availability (Gladine et al. 2007a; Gobert et al. 2010).

The contribution of several antioxidant compounds of *C. ladanifer*, including CT, to limit meat lipid oxidation is unclear. To clarify the role of CT from *C. ladanifer* we designed a study to test the hypothesis that incorporation of CT extract from *C. ladanifer* in lamb diets might induce the same antioxidant protection as the *C. ladanifer* plant. Furthermore, the aim of the present study was also to explore how CT induces improvements in the antioxidant status of animals and the oxidative stability of meat. The present experiment evaluated the effect of three levels of *C. ladanifer* CT (without CT enrichment, medium level, and high level of CT) and two ways of CT supply (*C. ladanifer* plant or *C. ladanifer* CT extract) in lamb diets on meat lipid and colour stability and antioxidant status of muscle, rumen, and abomasum digestive contents and faeces. The presence of CT monomers in muscle was also researched.

2.2. Material and methods

2.2.1. Animals, diets and management

Details on the animal experiment, management, diet and experimental design have been already reported by Guerreiro et al. (2020). The experiment was approved by the "Organisation Responsible for the Welfare of Experimental Animals" (ORBEA) of Instituto Nacional de Investigação Agrária e Veterinária (INIAV I. P.), Portugal. In this experiment, thirty-six Merino Branco x Romane lambs, with an average body weight of 19.9 ±1.86 kg (mean, s. d.), were individually housed and randomly assigned to the six dietary treatments (6 lambs/treatment). Six diets were formulated, considering a completely randomized experimental design with a 3 × 2 factorial arrangement of treatments with three levels of *C. ladanifer* CT (without CT enrichment (0), medium level of CT (M), and high level of CT (H)) and two ways of supplying *C. ladanifer* CT (*C. ladanifer* plant (leaves and soft stems) (CL) vs *C. ladanifer* CT extract (Ex)). The diets were: CL0 – basal diet without the *C. ladanifer* plant inclusion; CLM – basal diet with 125 g of the *C. ladanifer* plant/kg; CLH – basal diet with 250 g of the *C. ladanifer* plant/kg; Ex0 – basal diet without *C. ladanifer* CT extract inclusion; ExM –

basal diet with 20.5 g of *C. ladanifer* CT extract/kg; ExH – basal diet with 41 g of *C. ladanifer* CT extract/kg. The diets containing *C. ladanifer* plant and *C. ladanifer* CT extract were formulated to obtain similar CT contents. Basal diet consisted of dehydrated lucerne supplemented with 60 g/kg DM of soybean oil. The trial lasted for 35 days, after an adaptation period of 7 days to experimental conditions. The animals were weighed, just before feeding, on days 1, 8, 18, 28 and 35 of the trial. The experimental diets were offered *ad libitum* in the morning period, and the intake was daily registered.

The leaves and soft stems of *C. ladanifer* harvested in southern of Portugal (37º43′33.5”N 8º21′42.5”W), were dried at room temperature and milled to a final particle size of 1 mm. *Cistus ladanifer* CT extract was prepared using the procedure described by Guerreiro et al. (2020), which consist in double extraction of ground *C. ladanifer* with acetone:water solution (70:30, v/v) at 1:5 of solid:liquid ratio. For preparation of the diets, the leaves and soft stems of *C. ladanifer* or *C. ladanifer* CT extract were mixed with milled dehydrated lucerne and soybean oil. During the feeding trial, 3 samples of each experimental diet were collected and analyzed for DM, crude protein, fibre fraction, ash and ether extract as described by Guerreiro et al. (2020). The analyses of α-tocopherol content in diets followed the procedures described in Santos-Silva et al. (2022). Total phenols and CT contents were determined following the methodology described in 2.3. section. Chemical composition of the diets was presented in Table 2.1.

Table 2.1 – Chemical composition (g/kg DM) of the experimental diets.

	Diets[1]					
	Cistus ladanifer plant			*Cistus ladanifer* CT extract		
	CL0	CLM	CLH	Ex0	ExM	ExH
Dry matter (DM)[2]	916	915	918	916	917	914
Crude protein	162	147	133	162	154	155
NDF	447	441	411	447	440	434
ADF	326	322	301	326	326	315
ADL	62	64	62	62	63	64
Ash	113	110	104	113	115	112
Ether extract	77.0	82.7	98.3	77.0	80.0	70.0
Total phenols[3]	5.75	12.7	23.0	5.75	10.9	15.8
Condensed tannins[4]	8.13	22.6	27.9	8.13	22.3	29.3
α-tocopherol[5]	14.1	13.0	12.5	14.1	12.8	12.6

[1] CL0 – basal diet; CLM – CL0 + 125 g of *C. ladanifer*/kg; CLH – CL0 + 250 g of *C. ladanifer*/kg; Ex0 – basal diet: ExM – Ex0 + 20.5 g of *C. ladanifer* condensed tannin extract/kg; ExH – Ex0 + 41 g of *C. ladanifer* condensed tannin extract/kg.

[2] g/kg feed.

[3] Tannic acid equivalents.

[4] Condensed tannins quantified using purified *C. ladanifer* condensed tannins as standard.

[5] α-tocopherol, μg/g dry matter

2.2.2. Slaughter and sample collection

Lambs were stunned and slaughtered by exsanguination in the INIAV I. P. experimental abattoir. Immediately after the slaughter, the whole rumen and abomasum digestive contents were collected from each lamb. Faeces were collected immediately after the slaughter, directly from the rectum. After collection, digestive contents and faeces were frozen at -80 ºC, freeze-dried, milled, and re-stored at -80 ºC until analysis.

Immediately after slaughter, a sub-sample of *Longissimus thoracis* (LT) muscle was also collected at the 12th vertebra, frozen with liquid nitrogen, and thereafter stored at -80 ºC until analysis of overall antioxidant status and examined for CT monomers.

Carcasses were stored at 10 ºC for 24 h and then refrigerated at 2 ºC until the 72 h after slaughter. After carcass preparation, three 1.5 cm thick sub-samples of the remaining LT muscle were collected for analysis of colour and lipid oxidative stability during 0, 4 and 7 days of storage. On the first day of storage (day 0), the colour parameters were determined after 1 h of blooming and samples were vacuum packed and stored at -80 ºC until analysis. The remaining samples were placed individually in polystyrene trays. The trays were then wrapped with oxygen permeable polyvinyl chloride film and stored at 2 ºC in an illuminated cooler. After 4 and 7 days of storage, the meat colour was evaluated after 1 h of blooming, and samples were vacuum packed and stored at -80 ºC until lipid oxidation analyses.

2.2.3. Diet, digestive contents and faeces

2.2.3.1. Total phenols and antioxidant activity

Total phenols content and antioxidant activity were evaluated in previously extracts of diets, digestive contents, and faeces. Extracts were prepared according to Julkunen-Tiitto (1985) and Makkar (2003b), with slight changes. After lyophilization, samples were ground to a 1 mm particle size. Then, in duplicate, to 200 mg of sample, 10 mL of aqueous acetone (70 %) was added. The resulting solution was maintained in an ultrasound bath for 10 min with ice. Then, samples were centrifuged at 1600 *g* for 30 min at 4 ºC. The supernatant was recovered and stored in micro tubes, kept at -20 ºC until further analysis.

Total phenols were determined using the Folin–Ciocalteau assay according to Julkunen-Tiitto (1985) as described by Makkar (2003b). Antioxidant activity was measured using the ferric reducing antioxidant power (FRAP) assay according to Fawole and Opara (2013) and Trolox equivalent antioxidant capacity (TEAC) assay according to the method based on Re et al. (1999) and described by Luciano et al. (2011).

2.2.3.2. Condensed tannins

Condensed tannins in diets, digestive contents and faeces were quantified according to the method of Grabber et al. (2013) with slight modifications. Briefly, 10 mg of previously lyophilized and ground samples were weighed into screw cap tubes to which 10 mL of acetone-butanol-HCl reagent was added. The reagent was prepared daily, by dissolving 40 mg of ammonium ferric sulphate dodecahydrate in 3.3 mL of distilled water and 5 mL of 12 M HCl and adding 42 mL of butanol and 50 mL of acetone. The tubes were left at room temperature for 1 h, then they were heated at 70 ºC for 2.5 hours and cooled for 45 min at room temperature. The absorbance peak for all the samples was recorded at 550 nm in a spectrophotometer (Double-beam UV–Vis scanning spectrophotometer, Helios alpha spectrophotometer, Thermo Scientific, Bremen, Germany). The concentration of CT was quantified using purified *C. ladanifer* CT extract as a standard, which was prepared using Sephadex LH-20 column chromatography (GE Healthcare Bio-Science, Uppsala, Sweden), according to Strumeyer and Malin (1975).

2.2.4. Muscle analysis

2.2.4.1. Overall antioxidant status

To evaluate the global antioxidant status of the muscle, an extract was previously prepared according to Luciano et al. (2011), with slight changes. Briefly, while frozen, muscle was chopped into small pieces. Then, in duplicate analyses, 500 mg of muscle were placed into a 50 mL tube and 10 mL of distilled water were added. Samples were homogenized for 60 s at 9500 rpm with an Ultra-Turrax (T25 digital homogenizer, IKA Werbe GmbH & Co. KG, Staufen, Germany), while kept in a cold bath, followed by 6 min (3 min twice, with a 2 min break) in an ultrasound bath (Bransonic Ultrassonic 3510E-DTH, Branson Ultrasonics Corporation, Danbury, USA). Then, samples were centrifuged for 15 min, at 1600 g and 4 ºC. The resulting aqueous phase was filtered through Whatman 541 paper filters and stored in microtubes at -80 ºC for analyses.

In the extracts obtained were then applied the Folin-Ciocalteau, Ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays following the methodologies described by Luciano et al. (2011).

2.2.4.2. α-tocopherol content

Sample preparation for quantification of the α-tocopherol content in LT muscle was performed in accordance with a procedure described by Prates et al. (2006). The analysis carried out in a Dionex Ultimate 3000 uHPLC (Thermo Fisher Scientific) equipped with a normal-phase silica column (Zorbax RX-Sil, with the matching 12.5 mm analytical guard column, 4.6 mm ID, 250 mm, 5 µm particle size, Agilent Technologies Inc., Palo Alto, CA, USA) as described by Jerónimo et al. (2020b).

2.2.4.3. Condensed tannin monomers

Research on CT monomers (catechin, epicatechin, gallocatechin and epigallocatechin) in muscle were performed according to a procedure described by Ganguly et al. (2016), with slight modifications. Briefly 500 mg of minced muscle were homogenized with 2.5 mL ascorbate-EDTA solution with an Ultra-Turrax (T25 digital homogenizer, IKA Werbe GmbH & Co. KG, Staufen, Germany). Then, 200 µL of ethanol and 500 µL of dichloromethane were added. The resulting mixture was vortexed and centrifuged at 19000 *g* for 5 min, at room temperature. The upper aqueous phase was collected, 2 mL of water were added to the lower organic phase and another extraction was performed. To the pooled aqueous solutions, 2 mL of ethyl acetate were added, the solution was vortexed and centrifuged at 2200 *g* for 20 min. The resulting upper ethyl acetate layer was collected and after a second extraction, the pooled ethyl acetate extracts were evaporated to dryness with N_2. The dried sample was re-suspended in acetonitrile 15 % containing 0.2 % acetic acid, and the tubes were centrifugated at 19000 *g* for 10 min. Approximately 1 mL of the resulting solution was filtered with a 0.45 µm hydrophobic membrane into a vial and stored at -20 ºC until analysis. The analysis was performed using a Dionex Ultimate 3000 uHPLC (Thermo Fisher Scientific) and pentafluorophenyl phase column (Luna® 3 µm PFP (2) 100 Å, 150 ×4.6 mm, with the correspondent Security Guard ™ Cartridge PFP 4×3 mm, Phenomenex), kept at 40 ºC. Elution was performed according to Ganguly et al. (2016), with a mobile phase composed by (A) an aqueous solution of 0.2 % acetic acid and (B) acetonitrile with 0.2 % acetic acid, at a 0.8 mL/min flow rate. Samples (30 µl) were injected into a gradient, which began with 98 % A, ramping down to 70 % A in 4 min and kept at 70 % A until 7 min. At 7 min, it was then ramped to 2 % A until 10 min, and kept for 8 min at 2 % A. After that the system was re-equilibrated for 7 min, reaching 98 % A, before a new sample injection. The recorded wavelength (226 nm) was previously determined using a Diode Array Detector (DAD), which corresponded to the maximum absorbance peak of the used standards.

2.2.5. Meat lipid oxidation

For the evaluation of the meat lipid stability over refrigerated storage for 0, 4 and 7 days was quantified the thiobarbituric acid reactive substances (TBARS), following the general methodology of Grau et al. (2000) and detail described by Francisco et al. (2015).

2.2.6. Meat colour

Meat colour coordinates over storage time were measured employing CIE L^*, a^*, b^* system using a Minolta CR-300 chromometer (Konica Minolta, Tokyo, Japan), where L^* is lightness, a^* redness and b^* yellowness. Measurements were taken by applying the C illuminant and 2º standard observers. Hue angle (H*) was calculated as $\tan^{-1}(b^*/a^*) \times (180/\pi)$ and colour saturation (chroma, C*) as $(a^{*2} + b^{*2})^{1/2}$ (King et al. 2023). Overall colour variation between each day of storage and the day 0 measurements was calculated as the square root of the sum of squared differences between the L^*, a^*, and b^* coordinates on days 4 and 7 compared to day 0 according to equation (King et al., 2023):

$$\Delta E = [(L^*_{(4\ \mathrm{or}\ 7)} - L^*_0)^2 + (a^*_{(4\ \mathrm{or}\ 7)} - a^*_0)^2 + (b^*_{(4\ \mathrm{or}\ 7)} - b^*_0)^2]^{1/2}$$

Equation 2.1 – Overall colour variation between day 0 and each storage day (4 or 7)

2.2.7. Statistical analysis

This trial was conducted using a 3 × 2 factorial treatment arrangement in a completely randomized design, where the 2 factors were level of CT supplied (without CT enrichment (0), medium level (M), and high level of CT (H)) and CT source (*C. ladanifer* leaves and soft stems (CL) or *C. ladanifer* CT extract (Ex)). Lamb was the experimental unit. Data were analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The variance homogeneity was tested at a level of $P = 0.01$, and when significant, the variance heterogeneity was accommodated in the model, using the group option of repeated statement of Proc Mixed. The models used considered the CT level and CT source and their interaction (CT level × CT source) as fixed effects. To estimate the individual daily intake of dry matter and nutrients, a repeated measure model was applied to the average individual daily intake per week, considering a first order autoregressive (AR (1)) covariance structure. The colour and lipid oxidation were studied with a similar model including the day of sampling as the repeated measurement. The level of statistical significance was set at $P < 0.05$ and trends towards significance at $0.05 < P < 0.10$.

2.3. Results

2.3.1. Antioxidant activity of diets and intake

The results of diets antioxidant activity and intake of dry matter and nutrients of lambs are presented in Table 2.2. The inclusion of CT sources in diets increased the antioxidant activity of diets. In both antioxidant activity assays, we found significant CT Level × CT Source interactions ($P < 0.05$), because the *C. ladanifer* plant resulted in higher FRAP and TEAC values in diets than *C. ladanifer* CT extract for the same level of CT inclusion in the diets.

Both the CT source as the level did not affect dry matter intake (DMI). However, the DMI for the diet CLH diet was numerically lower than in other dietary treatments (-28,7 %). Increasing CT levels in the diet increased the intake of total phenols. For intake of CT an interaction between CT source and CT level was observed ($P = 0.012$), with progressive increase of the CT intake with increasing levels of *C. ladanifer* CT extract, while with diets with the *C. ladanifer* plant CT intake was similar for M and H levels, with an average 11.7 g/day in CL0 diet and an average 28.9 g/day in CLM and CLH diets. Intake of α-tocopherol decreased ($P = 0.006$) with inclusion of both CT sources in diets, with an average 20.7 mg/day in diet without CT enrichment and an average 15.9 mg/day in diets enriched with CT (diets M and H).

Table 2.2 – Effect of increasing levels of *C. ladanifer* plant and *C. ladanifer* condensed tannin (CT) extract in lamb diets on antioxidant activity of diets and intake.

	Diets[1]						SEM	P value		
	C. ladanifer plant			*C. ladanifer* CT extract				Level	Source	Level*Source
	CL0	CLM	CLH	Ex0	ExM	ExH				
Antioxidant activity										
FRAP[2]	36.2^{a}	191^{c}	411^{e}	36.2^{a}	134^{b}	237^{d}	7.38	<0.001	<0.001	<0.001
TEAC[3]	6.00^{a}	17.8^{c}	33.2^{d}	6.00^{a}	13.2^{b}	22.0^{c}	1.49	<0.001	0.001	0.009
Intake										
Dry matter (g/day)	1323	1223	905	1358	1150	1290	113.0	0.210	0.099	0.109
Total phenols[4] (g/day)	8.33	17.1	22.9	8.53	13.8	22.4	1.55	<0.001	0.334	0.457
CTs[5] (g/day)	11.7^{a}	30.2^{b}	27.6^{b}	12.0^{a}	27.6^{b}	41.3^{c}	2.65	<0.001	0.071	0.012
α-tocopherol (mg/day)	20.4	17.4	12.3	20.9	16.1	17.8	1.613	0.006	0.243	0.113

[1] CL0 – basal diet; CLM – CL0 + 125 g of *C. ladanifer*/kg; CLH – CL0 + 250 g of *C. ladanifer*/kg; Ex0 – basal diet: ExM – Ex0 + 20.5 g of *C. ladanifer* condensed tannin extract/kg; ExH – Ex0 + 41 g of *C. ladanifer* condensed tannin extract/kg.

[2] Ferric reducing antioxidant power, μmol of Fe^{2+} equivalents/g dry matter.

[3] Trolox equivalent antioxidant capacity, μmol of trolox equivalents/g dry matter.

[4] Tannic acid equivalents.

[5] Condensed tannins quantified using purified *Cistus ladanifer* condensed tannins as standard.

Means within a row with different letters are significantly different ($P < 0.05$).

SEM – standard error of mean.

2.3.2. Condensed tannins and antioxidant status in digestive contents and faeces

The effect of CT source and level on total phenol and CT contents and antioxidant status of rumen and abomasum digestive contents and faeces is presented in Table 2.3. Regardless of the CT source, the total phenols content increased in the rumen with increasing levels of CT in diets ($P < 0.001$), averaging 3.5, 3.9 and 4.3 TAE/g DM in diets 0 and M and H, respectively. The antioxidant activity in rumen, determined from FRAP and TEAC assays, was also affected by the diet CT levels. The FRAP values were higher ($P < 0.001$) in diets H (avg. 26.8 µmol Fe^{2+} equivalents/g DM) than in diets 0 and M (avg. 22.2 µmol Fe^{2+} equivalents/g DM). The TEAC was higher ($P = 0.042$) in diets H (avg. 47.9 µmol Trolox equivalents/g DM) compared to diets 0 (avg. 43.0 µmol Trolox equivalents/g DM), while diets M showed intermediate values. The concentration of CT in rumen showed an interaction between CT level and CT source ($P = 0.001$). Increasing levels of *C. ladanifer* plant in the diets resulted in progressive increases of the CT contents in the rumen digesta, whereas for the *C. ladanifer* CT extract both diets with medium and high CT level resulted in similar increases of the CT content in the rumen digesta, with an average 8.6 mg/g DM in diet Ex0 and an average 13.9 mg/g DM in diets ExM and ExH.

In the abomasum digesta, a significant interaction was observed between CT level and CT source for total phenols and CT contents and antioxidant activity ($P < 0.05$). Total phenols content, TEAC and FRAP values increased progressively with increasing levels of the *C. ladanifer* plant in diets, whereas the inclusion of both levels of *C. ladanifer* CT extract resulted in similar increase of the total phenols content (avg. 7.27 vs. 11.4 mg/g DM in diets Ex0 and ExM/ExH, respectively), FRAP (avg. 42.1 vs. 66.5 µmol Fe^{2+} equivalents/g DM in diets Ex0 and ExM/ExH, respectively), and TEAC values in abomasum (avg. 37.0 vs. 58.4 µmol Trolox equivalents/g DM in diets Ex0 and ExM/ExH, respectively). The inclusion of both sources of CT in diets increased the CT content in the abomasum, however, for the same level of CT inclusion the *C. ladanifer* plant resulted in higher CT contents in the abomasum than the *C. ladanifer* CT extract.

Total phenols content was higher ($P = 0.049$) in faeces from lambs fed diets with CT sources (avg. 4.93 mg TAE/g DM) than in those fed diets without CT supplementation (avg. 4.46 mg TAE/g DM). Moreover, the faeces tended ($P = 0.083$) to have higher total phenols content in diets with *C. ladanifer* CT extract (avg. 4.92 mg TAE/g DM) than in diets with *C. ladanifer* plant (avg. 4.36 mg TAE/g DM). The antioxidant activity of faeces determined by FRAP and TEAC assays was affected by CT source ($P < 0.05$), with higher FRAP and TEAC values in lambs fed diets with *C. ladanifer* CT extract (avg. 26.6 µmol Fe^{2+} equivalents/g DM and 46.1 µmol Trolox equivalents/g DM) than in those fed diets with *C. ladanifer* plant (avg. 24.1 µmol Fe^{2+} equivalents/g DM and 41.9 µmol Trolox equivalents/g DM). No CT was detected in the faeces.

Table 2.3 – Effect of increasing levels of *C. ladanifer* plant and *C. ladanifer* condensed tannin (CT) extract in lamb diets on overall antioxidant status, and condensed tannin and α-tocopherol content of digestive contents and faeces

	Diets[1]						SEM	P value		
	C. ladanifer plant			*C. ladanifer* CT extract				CT Level	CT Source	CT Level * CT Source
	CL0	CLM	CLH	Ex0	ExM	ExH				
					Rumen					
Total phenols[2]	3.41	3.86	4.33	3.64	3.94	4.18	0.146	<0.001	0.669	0.429
FRAP[3]	20.4	23.3	27.3	21.2	23.8	26.3	1.361	<0.001	0.933	0.769
TEAC[4]	42.8	45.6	47.7	43.2	47.7	48.2	1.926	0.042	0.544	0.893
CTs[5]	8.65^{a}	18.3^{c}	22.1^{d}	8.56^{a}	12.8^{b}	14.9^{b}	0.904	<0.001	<0.001	0.001
					Abomasum					
Total phenols[2]	7.17^{a}	11.8^{b}	17.4^{c}	7.27^{a}	10.2^{b}	12.6^{b}	0.891	<0.001	0.007	0.030
FRAP[3]	40.6^{a}	70.7^{b}	99.9^{c}	42.1^{a}	59.2^{b}	73.8^{b}	5.173	<0.001	0.008	0.040
TEAC[4]	34.0^{a}	61.8^{b}	89.4^{c}	37.0^{a}	52.8^{b}	63.9^{b}	5.337	<0.001	0.023	0.041
CTs[5]	8.46^{a}	23.0^{c}	30.9^{d}	8.20^{a}	15.5^{b}	20.3^{c}	1.609	<0.001	<0.001	0.010
					Faeces					
Total phenols[2]	4.36	4.71	4.78	4.56	5.15	5.04	0.206	0.049	0.083	0.819
FRAP[3]	22.5	24.8	25.1	24.8	27.1	28.0	1.480	0.126	0.046	0.971
TEAC[4]	41.2	43.2	41.2	43.4	48.7	46.2	2.435	0.331	0.044	0.773

[1] CL0 – basal diet; CLM – CL0 + 125 g of *C. ladanifer*/kg; CLH – CL0 + 250 g of *C. ladanifer*/kg; Ex0 – basal diet: ExM – Ex0 + 20.5 g of *C. ladanifer* condensed tannin extract/kg; ExH – Ex0 + 41 g of *C.ladanifer* condensed tannin extract/kg.

[2] mg tannic acid equivalents (TAE)/g of dry matter.

[3] Ferric reducing antioxidant power, µmol of Fe2+equivalents/g of dry matter.

[4] Trolox equivalent antioxidant capacity, µmol of trolox equivalents/g of dry matter.

[5] mg/ g dry matter, using purified *C.ladanifer* condensed tannins as standard.

Means within a row with different letters are significantly different (*P* < 0.05).

SEM – standard error of mean.

2.3.3. Overall antioxidant status of muscle and presence of catechin monomers in muscle

The effect of CT source and level on the overall antioxidant status of LT muscle is presented in Table 2.4. Inclusion of both levels and sources of CT in diets did not affect the reducing ability (FRAP) and α-tocopherol content in muscle (P > 0.05). Total phenol content tended (P = 0.096) to be lower in diets H (avg. 0.35 mg TAE/g of muscle) than in diets 0 and M (avg. 0.41 mg TAE/g of muscle). The radical scavenging ability (TEAC) in muscle was lower (P = 0.005) in diets H than in diets 0 and M (avg. 8.28 vs. 10.4 µmol of Trolox equivalents/g of muscle).

Table 2.4 – Effect of increasing levels of *C. ladanifer* plant and *C. ladanifer* condensed tannin (CT) extract in lamb diets on overall antioxidant status and α-tocopherol content of muscle

	Diets[1]						SEM	P value		
	C. ladanifer plant			*C. ladanifer* CT extract				CT Level	CT Source	CT Level * CT Source
	CL0	CLM	CLH	Ex0	ExM	ExH				
Total phenols[2]	0.40	0.41	0.37	0.41	0.40	0.33	0.020	0.096	0.972	0.932
FRAP[3]	1.91	1.74	1.71	1.79	1.78	1.65	0.143	0.482	0.708	0.855
TEAC[4]	9.80	10.1	8.04	11.7	10.1	8.52	0.706	0.005	0.188	0.424
α-tocopherol[5]	1.09	1.45	1.17	1.01	1.07	1.02	0.158	0.392	0.133	0.610

[1] CL0 – basal diet; CLM – CL0 + 125 g of *C. ladanifer*/kg; CLH – CL0 + 250 g of *C. ladanifer*/kg; Ex0 – basal diet: ExM – Ex0 + 20.5 g of *C. ladanifer* condensed tannin extract/kg; ExH – Ex0 + 41 g of *C. ladanifer* condensed tannin extract/kg.
[2] mg tannic acid equivalents (TAE)/g of muscle.
[3] Ferric reducing antioxidant power, µmol of Fe^{2+} equivalents/g of muscle.
[4] Trolox equivalent antioxidant capacity, µmol of trolox equivalents/g of muscle.
[5] µg/g of muscle.
Means within a row with different letters are significantly different (*P* < 0.05).
SEM – standard error of mean.

Condensed tannin monomers (catechin, epicatechin, gallocatechin, and epigallocatechin) were investigated in muscle samples, however, none of the monomers was identified in muscle samples (Fig. 2.1).

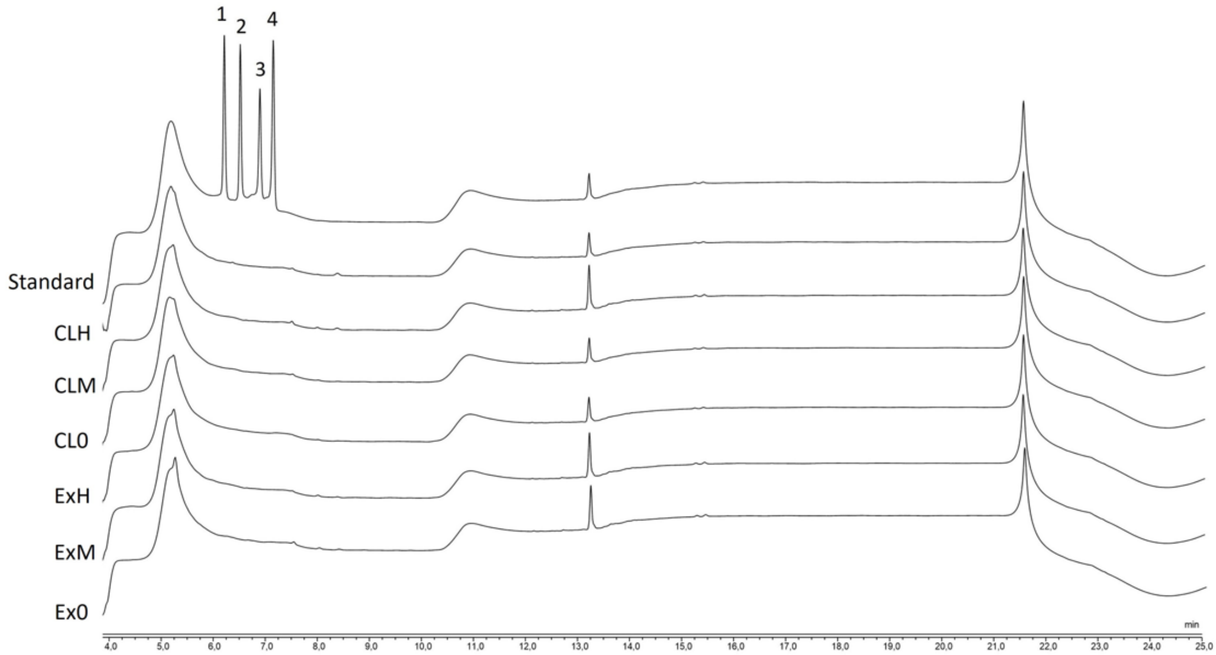

Figure 2.1. Chromatograms of gallocatechin (1); epigallocatechin (2); catechin (3); and epicatechin (4) in standard and in muscle from lambs fed: CL0 – basal diet; CLM – CL0 + 125 g of *C. ladanifer*/kg; CLH – CL0 + 250 g of *C. ladanifer*/kg; Ex0 – basal diet: ExM – Ex0 + 20.5 g of *C. ladanifer* condensed tannin extract/kg; ExH – Ex0 + 41 g of *C. ladanifer* condensed tannin extract/kg.

2.3.4. Lipid and colour stability during storage

The effects of storage time, and the inclusion of the *C. ladanifer* plant or *C. ladanifer* CT extract in diets on lipid and colour stability in LT muscle are presented in Table 2.5. Meat lipid oxidation was not affected by the CT level (P = 0.802) or source (P = 0.765). Independently of the dietary treatment, the TBARS values in meat increased in the first 4 days of storage (P < 0.001), from 0.09 mg malondialdehyde (MDA)/kg meat at day 0 to 0.37 mg MDA/kg meat at day 4, a value that remained unchanged through the 7th day of storage.

Conversely, some meat colour parameters were affected by CT level or CT source. Lightness (*L**) was higher (P = 0.018) in meats from diets with a high level of CT (avg. 46.5) than in those from diets without CT enrichment or with a medium CT level (avg. 44.1). The incorporation of the *C. ladanifer* plant in lamb diets resulted in meats with higher yellowness (*b**) values (P = 0.028; avg. 9.13) compared to CT extract (avg. 8.59). The ΔE at day 4 was not affected by dietary treatment, but after 7 days of storage, the ΔE values were lower in diets containing *C. ladanifer* CT extract (avg. 5.37) than in those containing the *C. ladanifer* plant (avg. 6.46).

As expected, the meat colour parameters were affected by storage time. Lightness (*L**) values increased during the first 4 days of storage, but between days 4 and 7 of storage remained unchanged (P < 0.001). The yellowness (*b**) values almost doubled through the 4th day of storage and then decreased slightly (P < 0.001). The redness (*a**) and the intensity of the red meat colour (C*) showed interaction between CT level and storage day (P = 0.033 and P = 0.033, respectively). The *a** and C* values reduced between days 0 and 7 of storage in diets without or with medium CT enrichment, while in the diets with high CT level, the *a** and C* values were more stable between days 0 and 7 of storage (Fig. 2.2 A and B, respectively).

Table 2.5 – Effect of increasing levels of *C. ladanifer* plant and *C. ladanifer* condensed tannin (CT) extract in lamb diets on meat colour parameters and lipid oxidation

	*L**	*a**	*b**	Chroma	Hue angle	∆E4	∆E7	Lipid oxidation[1]
Effect of storage time								
0 days	43.8^{a}	17.9^{c}	5.92^{a}	18.9^{b}	18.3^{a}	-	-	0.09^{a}
4 days	45.4^{b}	16.0^{b}	10.8^{c}	19.3^{b}	33.9^{b}	-	-	0.37^{b}
7 days	45.5^{b}	14.6^{a}	9.90^{b}	17.7^{a}	34.1^{b}	-	-	0.40^{b}
SEM	0.422	0.225	0.155	0.236	0.430	-	-	0.039
P value	<0.001	<0.001	<0.001	<0.001	<0.001	-	-	<0.001
Effect of condensed tannin level								
Without condensed tannin enrichment	44.3^{a}	16.5	8.79	18.9	28.1	5.79	5.66	0.26
Medium condensed tannin level	43.9^{a}	16.1	8.69	18.5	28.5	6.30	6.39	0.30
High condensed tannin level	46.5^{b}	15.9	9.10	18.5	29.7	5.84	5.70	0.29
SEM	0.678	0.284	0.208	0.298	0.583	0.291	0.298	0.050
P value	0.018	0.318	0.363	0.597	0.155	0.412	0.167	0.802
Effect of condensed tannin source								
Cistus ladanifer plant	45.2	16.4	9.13	18.9	29.3	6.22	6.46	0.29
Cistus ladanifer extract	44.6	16.0	8.59	18.3	28.2	5.73	5.37	0.28
SEM	0.554	0.232	0.170	0.243	0.476	0.237	0.243	0.041
P value	0.429	0.290	0.028	0.092	0.116	0.153	0.004	0.765
Interactions								
CT level * CT Source	0.865	0.434	0.759	0.396	0.992	0.153	0.849	0.722
CT level * Day	0.439	0.033	0.332	0.033	0.219	-	-	0.957
CT Source * Day	0.136	0.113	0.852	0.326	0.233	-	-	0.186

[1] mg malondialdehyde (MDA)/kg of muscle.

Means within a column with different superscripts differ ($P < 0.05$).

SEM – standard error of mean

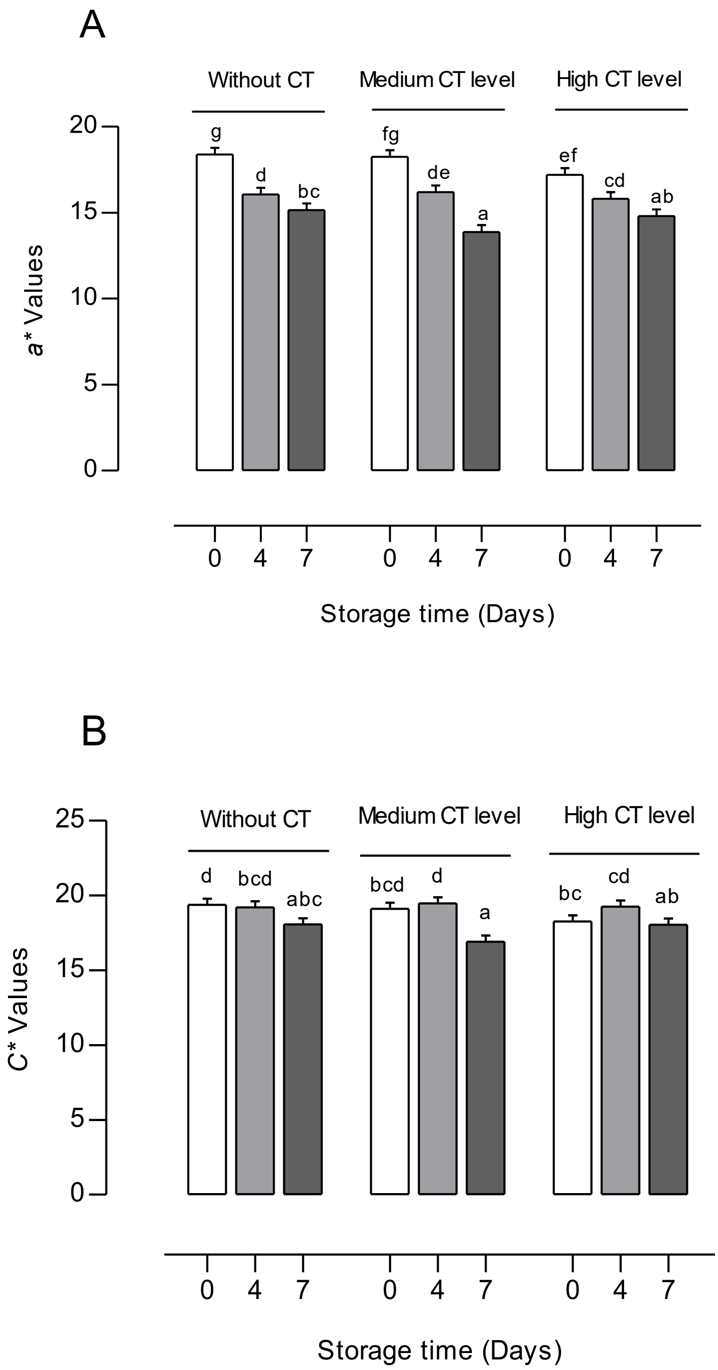

Figure 2.2. Effect of the condensed tannin (CT) level in the diets and time of storage on (A) redness (a*) and (B) Chroma (C*) values in lamb meat. Values are means, with SD represented by vertical bars. Values with different superscripts are significantly different ($P < 0.05$).

2.4. Discussion

2.4.1. Antioxidant activity of diets and nutrient intake

As shown in the companion paper (Guerreiro et al. 2020), the *C. ladanifer* plant induced a more pronounced depression in average daily gain and feed conversion ratio than *C. ladanifer* CT extract for the same dietary CT levels. *Cistus ladanifer* plant is characterized by low contents of crude protein, moderate levels of neutral detergent fibre, and low organic matter digestibility (Dentinho et al. 2005; Guerreiro et al. 2016a). So, due to its poor nutritional value, the inclusion of high levels of *C. ladanifer* plant in diets may reduce the nutritional value of the diet, feed intake, and growth performance. In the present work, the *C. ladanifer* plant affected the diet chemical composition, particularly the crude protein content, which progressively reduced with increasing levels of the *C. ladanifer* plant in diets, resulting in a lower crude protein intake for the CLM diet than in other diets (Guerreiro et al. 2020). So, *C. ladanifer* CT extract instead of the plant can be a good solution to provide the same level of CT in diets without relevant effect on feed intake and growth performance. However, replacing the *C. ladanifer* plant with the C*. ladanifer* CT extract will only be advantageous if the CT extract has an antioxidant effect like the *C. ladanifer* plant.

The *C. ladanifer* plant and CT extract progressively increased the antioxidant activity of the diets with increasing levels of CT in the diets. Nevertheless, the *C. ladanifer* plant resulted in diets with higher antioxidant activity than the *C. ladanifer* CT extract for the same CT level. The presence of antioxidant compounds other than CT in the plant, such as other phenolic compounds, terpenes and vitamins (Barrajón-Catalán 2010; Barros 2013; Gomes et al. 2005; Guerreiro et al. 2022b; Guimarães et al. 2009) may explain the higher antioxidant activity of diets with the C*. ladanifer* plant than in diets with CT extract.

In the present work, the CT quantification in the diets was carried out using a modified assay developed by Grabber et al. (2013), run with acetone-butanol-HCl-iron reagent (designed as acetone-butanol-HCl assay). This modified quantitative colorimetric assay was optimized for quantification of free and bound CT, allowing a higher CT recovery than the traditional butanol-HCL assay (Porter et al. 1986). So, using acetone-butanol-HCl assay, we found higher CT levels in diets and higher CT intake than reported in previous papers (Guerreiro et al. 2020; Guerreiro et al. 2022a). The CT values measured in the present work were particularly higher in diets with *C. ladanifer* CT extract compared to the previously reported values using the traditional butanol-HCl assay. It is recognized that the butanol-HCl assay has limitations in quantifying the bound CT, underestimating its levels (Makkar et al. 1999). Therefore, the present results reinforce the occurrence of greater complexation of CT with other dietary components when CT was provided by *C. ladanifer* extract than by inclusion of the *C. ladanifer* plant in the diets as suggested by Guerreiro et al. (2020). Although the modified assay developed by Grabber et al. (2013) resulted in higher CT levels in all experimental diets, a progressive increase in CT content would be expected with increasing levels of *C. ladanifer* plant or *C. ladanifer* extract in the diets. However, between medium and high levels of CT in diets, we expected a more expressive increase of the CT content. Condensed tannins type and the reactivity of CT with other dietary compounds may have interfered with the CT quantification (Grabber et al. 2013). Despite this, increasing levels of CT extract in diets progressively increase the CT intake. Conversely, due to lower numerical DMI in the CLH diet than in other dietary treatments, the CT intake did not increase gradually with increasing levels of the *C. ladanifer* plant in diets, being the CT intake similar in both diets with the *C. ladanifer* plant (CLM and CLH diets).

The inclusion of both CT sources in the diets slightly decreased the α-tocopherol contents of the diets. *Cistus ladanifer* plant is a source of tocopherols (vitamin E), with α-tocopherol representing the most abundant member of this class of compounds (Guerreiro et al. 2022b; Guimarães et al. 2010). However, the replacement of dehydrated lucerne by the *C. ladanifer* plant was not enough to maintain or even increase the α-tocopherol content in the diets. Condensed tannin-enriched diets resulted in a lower α-tocopherol contents, which, associated with numerically lower DMI in the CLH diet, led to a particular lower α-tocopherol intake in lambs fed the CLH diet (-39.7 % than CL0 diet).

2.4.2. Condensed tannins contents and antioxidant activity in gastrointestinal digestive contents and faeces

Condensed tannins and their possible metabolites can directly exert antioxidant activity in the gastrointestinal tract, promoting the improvement of the animal's overall antioxidant status (Halliwell et al. 2005; Kerem et al. 2006). To the best of our knowledge, the current study presents for the first time the measurement of the antioxidant capacity in the ruminal and abomasal digestive contents from lambs fed CT-enriched diets. Antioxidant capacity of rumen and abomasum digesta, assessed by the Folin-Ciocalteau, FRAP and TEAC assays, increased progressively with increasing levels of the *C. ladanifer* plant and *C. ladanifer* CT extract in diets. The CT extract was shown to be able to confer antioxidant activity in the gastrointestinal tract such as the *C. ladanifer* plant, although in the abomasum, the *C. ladanifer* plant resulted in higher antioxidant activity than CT extract for the same CT level in diet, which will be related to the presence of other antioxidant compounds in plant in addition to CT.

In faeces, only the total phenols content increased with the inclusion of either CT sources in diets, although this increase was less pronounced (9 % in the *C. ladanifer* plant and 12 % in CT extract) than that observed in rumen (21 %) and abomasum (143 % in the *C. ladanifer* plant and 57 % in CT extract) digesta. On the other hand, the antioxidant activity of faeces evaluated by the FRAP and TEAC assays only increased in CT extract diets. The higher FRAP and TEAC values in faeces in CT extract than in *C. ladanifer* plant diets can be related to contents of total phenols that tend to be higher in faeces from CT extract diets, and total phenols content is positively correlated with FRAP (r = 0.920, P < 0.001) and TEAC values (r = 0.812, P < 0.001). Unfortunately, the antioxidant activity was not evaluated in small and large intestine digestive contents, which would verify the antioxidant activity changed throughout the intestinal tract for both CT sources. However, the lower total phenol contents and FRAP and TEAC values in faeces than in abomasum for both dietary CT sources suggest that a large part of antioxidant compounds from both CT sources, at least in their native form, are lost in the digestive process. Consistently with our results, Molino et al. (2018), evaluating the antioxidant capacity of quebracho and chestnut extracts through *in vitro* gastrointestinal digestion and microbial fermentation, showed the reduction of antioxidant activity of tannins after microbial fermentation, with very low antioxidant activity values in the solid fraction obtained after microbial fermentation, which correspond to undigestible and nonabsorbable fraction. The metabolism of CT along gastrointestinal tract is still controversial, and few studies are available on the fate of CT in the gastrointestinal tract of ruminants. However, the results from studies performed *in vitro* or *in vivo* in humans, rats, pigs, and ruminants increasingly support that CT undergo transformations throughout the gastrointestinal tract (Abia and Fry 2001; Cires et al. 2017; Desrues et al. 2017; Girard et al. 2020; Molino et al. 2018; Perez-Maldonado and Norton 1996; Quijada et al. 2018; Tao et al. 2019; Terrill et al. 1994). After *in vitro* digestion of quebracho CT, several compounds were identified in the supernatant fraction, including CT oligomers from dimer to tetramer, catechins, gallocatechin, epicatechin, and other small metabolites such as vanillic acid and hydroxyphenyl acetic acid (Molino et al. 2018). Moreover, other studies also reported that a wide variety of CT derivative compounds are produced by gut microorganisms (Cires et al. 2017; Mena et al. 2015; Molino et al. 2018). So, the reduction of antioxidant activity observed in faeces can be related to the transformation and absorption of CT, which appears to have occurred mainly in the intestine.

There are a few studies that have evaluate changes in CT concentrations over the gastrointestinal tract of ruminants. Consistent with other reports (Desrues et al. 2017; Quijada et al. 2018), we also found higher CT concentrations in the abomasum than in the rumen, which can be due to the ability of CT to form complexes with other molecules, mainly with proteins (Makkar 2003a). The stability of these CT complexes depends on numerous factors, including pH. Under the rumen pH conditions (pH 5.5–7.0) the CT form stable complexes with proteins, while under the acid conditions of the abomasum (pH 2.5–3.5) and in alkaline conditions of the distal small intestine (pH ≈ 7.5) these complexes are dissociated (Mueller-Harvey 2006a). As suggested by Quijada et al. (2018), the CT released could be more accessible and reactive for quantification by acetone-butanol-HCl assay, which can explain the higher concentrations of CT in abomasum than in rumen. Moreover, the favourable condition for CT to form stable complexes with proteins in rumen than in abomasum could also be the explanation for the higher antioxidant activity in the abomasum versus the rumen digesta.

Our results are opposite to those reported by Desrues et al. (2017) and Quijada et al. (2018), who quantified the CT in faeces from lambs fed sainfoin pellets and hazelnut skins and calves fed sainfoin pellets, using the acetone-butanol-HCl assay. However, in both works the faeces CT concentrations were lower than expected. The transformation of CT along the gastrointestinal tract could be an explanation for the lower levels or absence of CT in faeces. In addition, as suggested by Desrues et al. (2017) the CT may be involved in reactions with digesta components, forming products that are not detected by acetone-butanol-HCl assay. Indeed, using the thiolysis assay, which mainly detects “free” CT, the CT concentrations in the small and large intestine and faeces of cattle fed sainfoin were very low, and in some animals the CT were not detected in the small and large intestine (Desrues et al. 2017).

An interesting result of the present work was the distinct effect of the *C. ladanifer* plant and *C. ladanifer* CT extract in faeces antioxidant activity, with increase of the antioxidant capacity in lambs fed CT extract diets, while the *C. ladanifer* plant did not affect the FRAP and TEAC values in faeces. The present result suggests that the way in which CT are included in the diet may affect the fate of CT along the gastrointestinal tract, which could have consequences on the CT bioactivity.

2.4.3. Muscle overall antioxidant status and presence of condensed tannin monomers

In accordance with previous work where the *C. ladanifer* plant was included in lamb´s diets (Jerónimo et al. 2020b), in the present work both the *C. ladanifer* plant and CT extract did not increase the muscle total phenols content and FRAP values. Conversely, diets with a high CT level resulted in muscle samples with lower TEAC values, which may be related to the contents of total phenols, which tend to be lower in these diets. In fact, a positive correlation is found between TEAC and total phenols content in muscle ($r = 0.534$, $P < 0.001$). Inconsistent results may occur between assays of antioxidant capacity, since the methods are based on detection the different target substrates, as reviewed by Echegaray et al. (2021).

In previous work we verified that the inclusion of 200 g/kg DM of the *C. ladanifer* plant in lamb diets increased the muscle content of α-tocopherol (Jerónimo et al. 2020b). Conversely, in the present work, the muscle contents of α tocopherol were not affected either by the *C. ladanifer* plant or by the CT extract. Despite the reduction of the α-tocopherol intake with increasing levels of CT in diets, the muscle contents of α-tocopherol were equal between the diets. This result suggests that CT-enriched diets exert favourable effect on α-tocopherol concentrations, resulting in equal availability of α-tocopherol in the muscle despite the lower intake. Indeed, several studies reported the increase of α-tocopherol in tissues and milk when polyphenol-rich plant extracts, including CT extracts, are included in animal diets (Gladine et al. 2007a; Gobert et al. 2010; Lobón et al. 2017b). Inhibition of metabolism and increased absorption, protection against oxidation, and restoring the vitamin E from oxidation are proposed as possible mechanisms by which phenolic compounds might interact with vitamin E (Frank et al. 2004). Interaction with other antioxidant agents increasing its availability is suggested as a possible indirect mechanism by which CT can induce an antioxidant effect in living animals and in their products (Soldado et al. 2021).

In addition to interaction with antioxidant components, other mechanisms by which CT exert antioxidant effect in animals and their products has been postulated and are reviewed by Soldado et al. (2021). The transfer of CT with low molecular weight or metabolites from feed to animal body and products is a possibility. Condensed tannins are oligomers and polymers composed of flavan-3-ols subunits, with the catechin, epicatechin, gallocatechin and epigallocatechin the most widespread subunits (Patra and Saxena 2011; Zeller 2019). The presence of epicatechins in the plasma of sheep that received grape peel and skin extract directly into the rumen was reported by Gladine et al. (2007b). Conversely, in the present work, the presence of catechin, epicatechin, gallocatechin and epigallocatechin was not detected in muscle samples (Fig. 2). Similar to our findings, in the liver and plasma of lambs fed a quebracho-enriched diet, phenolic compounds were not detected, suggesting that the tannins from quebracho might not be degraded or absorbed in the gastrointestinal tract (Lopéz-Andrés et al. 2013). The possible CT absorption has generated a great controversy, since only monomers and oligomers up to tetramers are known to be absorbed (Ou and Gu 2014; Sallam et al. 2021; Tao et al. 2019) and the CT degradation in the gastrointestinal tract into absorbable compounds is not clearly established (Soldado et al. 2021).

2.4.4 Meat lipid and colour stability during storage

Due to the undesirable effects of lipid oxidation on meat sensory and nutritional attributes (Bekhit et al. 2013; Dominguez et al. 2019), several nutritional strategies have been explored with the aim of limiting oxidative deterioration, including the application of natural antioxidants in animal diets. In this context the first objective for the present work was to examine if the dietary *C. ladanifer* CT extract is able to induce the same protection against lipid oxidation in lamb meat as reported previously for the *C. ladanifer* plant (Francisco et al. 2015; Jerónimo et al. 2012; Jerónimo et al. 2020b). Contrary to our expectations, neither the *C. ladanifer* plant nor the *C. ladanifer* CT extract affected meat lipid oxidation. The levels of the

C. ladanifer plant used in the present work (125 and 250 g/kg DM) were chosen considering previous results, which showed that levels higher than 100 g/kg DM of the *C. ladanifer* plant in the diet improve the resistance of lamb meat against lipid oxidation. However, only levels equal to or greater than 200 g/kg DM of the *C. ladanifer* plant produced consistent results (Francisco et al. 2018; Francisco et al. 2015; Jerónimo et al. 2012; Jerónimo et al. 2020b). So, considering previous results, our expectation was that, at least for the highest level of the *C. ladanifer* plant (250 g/kg DM) in the diet, it would be possible to find a protective effect against lipid oxidation in lamb meat.

Regarding the efficacy of dietary CT extracts in improving lipid oxidative stability of ruminant products, as recently reviewed by Soldado et al. (2021), the results remain inconsistent. For example, Lobón et al. (2017b) verified the reduction of MDA levels in meat of suckling lambs when their dams' diet was supplemented with quebracho, a widely used commercially available CT containing feed supplement (Soldado et al. 2021). Conversely, other reports showed that supplementation of lamb diet with quebracho did not affect the lipid oxidative stability of meat (Brogna et al. 2014; Lobón et al. 2017; Luciano et al. 2009a). The efficacy of grape seed extract in limiting meat lipid oxidation is inconsistent, with reports showing the reduction of lipid oxidation in meat (Jerónimo et al. 2012; Mu et al. 2020) and another that did not observe such effect when grape seed extract was included in lamb diets (Guerra-Rivas et al. 2016). Furthermore, supplementation of diets with *Acacia mearnsii* CT extract has not been shown to be able to reduce lipid oxidation levels in meat, fat, and milk of ruminants (Avila et al. 2020; Biondi et al. 2019; Gesteira et al. 2019; Staerfl et al. 2011; Valenti et al. 2019). The inconsistent antioxidant effect of CT sources may be related to several factors, such as CT structure and concentration in diets and the composition of basal diet, which contains variable levels of other antioxidant compounds but also pro-oxidant agents (Soldado et al. 2021).

Regardless of the dietary treatment, the TBARS values found in meat samples stored over 7 days (0.40 g MDA/kg of fresh meat) were much lower than those reported in other works for lamb meat stored over 7 days under the same conditions (Dentinho et al. 2023; Francisco et al. 2020; Santos-Silva et al. 2019), including studies with inclusion of the *C. ladanifer* plant in diets (values close to or greater than 1 g MDA/kg of fresh meat) (Francisco et al. 2018; Jerónimo et al. 2020b). The meat also showed high lipid stability over storage, without significant changes in TBARS values between 4th and 7th day of storage. The natural antioxidants present in dehydrated lucerne, such as tocopherols and polyphenols (Rafínska et al. 2017), may have conferred a baseline level of antioxidant protection enough to ensure low levels of lipid oxidation independently of the *C. ladanifer* plant or CT extract inclusion in diets. Moreover, in the present experiment, the oxidative pressure may not have exceeded the antioxidant capacity of meat, hindering observation of a possible antioxidant effect of the *C. ladanifer* plant and CT extract in meat.

The oxidative stability of meat depends on a complex balance between antioxidant defenses and pro-oxidant factors (Bekhit et al. 2013). The composition of polyunsaturated fatty acids (PUFA) in meat is a factor that can greatly compromise the oxidative stability of meat, since the susceptibility of PUFA to oxidation increases with increasing unsaturation degree (Scislowski et al. 2005). With the aim of increasing the intramuscular fat content in health-beneficial FA, including PUFA, in the present work, all diets were supplemented with 60 g/kg DM of soybean oil, a vegetable oil rich in PUFA, particularly in linoleic (18:2n-6) and linolenic (18:3n-3) acids (White 2000). Independently of the CT source, increasing levels of CT in diets tended to increase the contents of total PUFA (P = 0.081), total n-6 PUFA (P = 0.075) and 18:2n-6 (P = 0.068) in intramuscular fat (Table 8). However, using the fatty acid composition of lamb intramuscular fat, previously reported by Guerreiro et al. (2020), we verified that the content of highly oxidizable PUFA and the peroxidation index (PI) were not affected by dietary treatment (Table 2.6), suggesting that the meats of the various dietary treatments do not present a different propensity to oxidation due to its composition in fatty acids.

In red meat, colour is one of the most important sensory attributes in the purchase decision, with the bright red colour associated with meat freshness and superior meat quality (Bellés et al. 2019). Regardless of dietary treatments, the meat colour parameters changed along the 7 days of refrigerated storage, with increase of the lightness (*L**), yellowness (*b**), and hue angle (H*) and decrease of the redness (*a**) and chroma (C*), changes that reflect the typical meat discolouration pattern. A more realistic perspective of the meat browning over time can be given by H* evaluation than by analysis of single colour coordinates (Luciano et al. 2009b). The increase of H* values up to day 4, then remaining stable until day 7, indicates that the meat colour deterioration occurred mainly during the first 4 days of storage. However, *C. ladanifer* CT extract limits the meat colour changes after 7 days of storage compared to *C. ladanifer* plant, as indicated by the lower value of ΔE7, which measures the overall variation of meat colour between day 0 and 7 of storage. The ΔE7 in meat from lambs fed *C. ladanifer* CT extract was also below 6, the threshold value from which meat colour changes are detectable by the consumer with the naked eye (Abril et al. 2001; Echegaray et al. 2021; Julkunen-Tiitto 1985; Khliji et al. 2010; White 2000). In addition, the *a** and C* values were more stable in the diets with CT supplementation, decreasing over storage time in diets without CT enrichment, suggesting that both CT sources help to maintain the colour in meat over time. Luciano et al. (2009a) also reported the improvement of meat colour stability in lambs fed quebracho supplemented diets.

Only meat *L** values were affected by dietary CT level, with meat from lambs fed the diets with a high CT level having higher L* values than meat from lambs fed diets without CT enrichment and with a medium CT level. Our results are in agreement with other works that reported the lighter-coloured meat in lambs fed CT-rich plants, such as sulla, carob pulp, *Acacia cyanophylla* and *Acacia mearnsii* (Priolo et al. 2005; Priolo et al. 2002; Priolo et al. 1998; Uushona et al. 2023). Conversely, in previous works, inclusion of *C. ladanifer* plant in lamb diets did not affect the meat colour (Francisco et al. 2018; Francisco et al. 2015; Jerónimo et al. 2012). Other factor that may help explain this result is the lower carcass weight obtained with diets with a high CT level, as reported in the companion paper (Guerreiro et al. 2020). Sanudo et al. (1996) and Santos-Silva et al. (2002) reported that carcass weight affects meat colour, with higher *L** values in lighter carcasses. On the other hand, the *b** values of meat were affected by CT source, with higher values in lambs fed *C. ladanifer* plant than in those fed *C. ladanifer* CT extract, which may be due to *C. ladanifer* plant pigments (Maoka 2020).

Table 2.6 – Effect of increasing levels of *C. ladanifer* plant and *C. ladanifer* CT extract in lamb diets on intramuscular pro-oxidant fatty acids

	Diets[1]						SEM	P value		
	C. ladanifer plant			*C. ladanifer* CT extract				CT Level	CT Source	CT Level * CT Source
	CL0	CLM	CLH	Ex0	ExM	ExH				
Total Lipids[2]	6.82	7.30	5.72	7.18	6.31	5.94	0.611	0.139	0.777	0.485
Total PUFA[3,4]	70.6	94.2	132	95.7	110	119	18.03	0.081	0.532	0.553
Total *n*-6 PUFA[3,4]	60.1	80.1	111	80.2	92.0	100	14.87	0.075	0.567	0.562
18:2 *n*-6[3]	53.4	69.8	90.7	66.3	76.1	83.0	11.11	0.068	0.675	0.645
n-6 LC-PUFA[3,5]	6.69	10.3	20.3	13.9	16.0	17.0	4.28	0.157	0.369	0.421
Total *n*-3 PUFA[3,4]	10.5	14.2	20.8	15.5	18.0	18.8	3.45	0.159	0.426	0.564
18:3 *n*-3[3]	7.21	9.15	10.8	8.57	10.1	10.7	1.33	0.112	0.503	0.856
n-3 LC-PUFA[3,5]	4.45	6.47	12.4	8.91	10.2	9.92	2.536	0.217	0.370	0.336
HP-PUFA[3,6]	17.9	25.5	43.1	30.9	35.7	37.1	7.91	0.154	0.382	0.443
PI[7]	10.9	14.8	22.1	16.2	18.5	19.5	3.39	0.117	0.448	0.473

[1] CL0 – basal diet; CLM – CL0 + 125 g of *C. ladanifer*/kg; CLH – CL0 + 250 g of *C. ladanifer*/kg; Ex0 – basal diet: ExM – Ex0 + 20.5 g of *C. ladanifer* condensed tannins extract/kg; ExH – Ex0 + 41 g of *C. ladanifer* condensed tannins extract/kg.

[2] g/100 g muscle dry matter.

[3] mg/g total fatty acids.

[4] PUFA – Sum of polyunsaturated fatty acids.

[5] LC-PUFA – Sum of long chain polyunsaturated fatty acids (≥ C20).

[6] HP-PUFA – Sum of polyunsaturated fatty acids with three or more unsaturated bonds.

[7] PI – Peroxidability index = (% dienoic acid × 1) + (% trienoic acid × 2) + (% tetraenoic acid × 3) + (% pentaenoic acid × 4) + (% hexaenoic acid × 5).

Means within a row with different letters are significantly different ($P < 0.05$).

SEM – standard error of mean.

2.5. Conclusion

Although the *C. ladanifer* plant and CT extract in lamb diets did not improve the meat lipid stability and the muscle antioxidant status, both CT sources, and particularly the CT extract, contributed to the meat colour stability over storage time. Due to the absence of effect on protection against meat lipid oxidation, it is not possible to clarify if the incorporation of CT extract from *C. ladanifer* in lamb diets might induce the same protection against lipid oxidation as the *C. ladanifer* plant as we hypothesized. The similar levels of α-tocopherol in the muscle, when CT-enriched diet decreased its intake, suggest that dietary CT affect the α-tocopherol metabolism promoting its greater availability in muscle. Both the *C. ladanifer* plant and CT extract increased the antioxidant activity in rumen and abomasum. The minor effect of both CT sources on antioxidant activity of faeces and the absence of CT are consistent with CT transformation in the gastrointestinal tract.

Funding

This research was funded by Alentejo2020 program through European Regional Development Fund (ERDF) under the project "CistusRumen - Sustainable use of Rockrose (*Cistus ladanifer* L.) in small ruminants - Increase of the competitiveness and reduction of the environmental impact" (ALT20–03–0145-FEDER-000023), and by National Funds through FCT – Foundation for Science and Technology through PhD scholarships of D. Soldado (SFRH/BD/145814/2019) L. Cachucho (2020.05712.BD) and L. Fialho (2020.04456.BD) and projects UIDB/05183/2020 and UIDP/05183/2020 to MED, UIDB/00276/2020 to CIISA, LA/P/0121/2020 to CHANGE and LA/P/0059/2020 to AL4AnimalS.

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3. CHAPTER 3 | Vitamin E and condensed tannin from *Cistus ladanifer* L. improve meat oxidative stability in lambs given n-3 long-chain polyunsaturated fatty acids

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Abstract

The effectiveness of dietary vitamin E and *Cistus ladanifer* condensed tannins (CT) extract alone or combined was tested in twenty lambs, distributed in four dietary treatments: C – basal diet NO; VE – basal diet NOVE; CT – basal diet NO supplemented with CT extract from *C. ladanifer*; and VECT – basal diet NOVE supplemented with CT extract from *C. ladanifer*. All the diets included 125 g/kg DM of *Nannochloropsis oceanica*. Growth performance, feed intake, carcass traits, meat quality, sensorial traits and meat colour, lipid oxidation and α-tocopherol content during refrigerated storage were determined. Dietary treatment did not affect growth performance and feed intake. Hot and cold carcass weight, dressing percentage, and kidney knob channel fat percentage were also not affected by dietary treatments. No differences were found among dietary treatments concerning meat pH, cooking loss, and shear force. Meat from CT diet fed lambs scored lower in sensorial attributes than meat from diets without CT-extract supplementation. Colour coordinates b^*, C^* and H^* values increased ($P < 0.001$) between days 0 and 4 of storage, remaining stable until day 7. Interactions between vitamin E level and CT supplementation were observed for L^* values ($P = 0.009$). Meat lipid oxidation increased over the storage time, but only in the C diet. α-tocopherol meat content increased with vitamin E supplementation. Although no interaction between CT and vitamin E, this work found that dietary supplementation with *C. ladanifer* CT extract limits meat lipid oxidation, paving the way for its usage as an alternative to synthetic vitamin E.

3.1. Introduction

Lamb diet supplementation with polyunsaturated fatty acids (PUFA) rich sources is a well-known feeding strategy to improve lamb meat's nutritional value (De Marzo et al. 2023; Musati et al. 2024; Shingfield et al. 2013). However, increasing levels of PUFA in ruminant diets contribute to increased oxidative pressure (Scislowski et al. 2005; Siphambili et al. 2022) which may negatively affect animal health and welfare (Ayemele et al. 2021; Dursun and Guler 2023; Khan et al. 2023), as well as produce undesirable side effects in their products (Guo et al. 2021; Ponnampalam et al. 2022; Tatar 2024). Dietary supplementation with antioxidants is an effective strategy to limit the prooxidative effect of PUFA-enriched diets (Cimmino et al. 2018; Kyriakaki et al. 2022; Ponnampalam et al. 2022). Vitamin E is a lipophilic antioxidant (Niki 2014) that has been successfully used in the prevention of oxidation processes in ruminant meat (Bellés et al. 2019; Gonzalez-Calvo et al. 2015; Lauzurica et al. 2005) while being fundamental to animal growth and development (Shastak and Pelletier 2024). Growing consumer preference for natural products has driven the use of natural antioxidants as replacements for synthetic ones in ruminant feeds (Ponnampalam et al. 2022; Tsiplakou et al.

2021).Condensed tannins (CT) are a group of plant phenolic compounds known for their antioxidant properties (Amarowicz and Pegg 2024; Unusan 2020), however, their antioxidant effect and action mechanisms in animals remain unclear (Soldado et al. 2021). In addition to a possible direct antioxidant effect of CT on the animal body and their products, it has been proposed that dietary CT may exert its antioxidant effect indirectly, through interaction with other antioxidant agents such as antioxidant enzymes and antioxidant compounds. Increased vitamin E levels have been reported in meat from lambs-fed CT sources (Jerónimo et al. 2020b; Lobón et al. 2017b). Furthermore, a mixture of antioxidant sources seems to be more efficient than individual antioxidants (Bayram and Decker 2023).

So, the present experiment was designed to evaluate, in lambs fed PUFA-rich diets, the effectiveness of dietary vitamin E and *C. ladanifer* CT extract alone to improve the oxidative stability of lamb meat and test the hypothesis that a dietary combination of vitamin E and *C. ladanifer* CT extract has a better antioxidant performance than individual antioxidants. Furthermore, it is also the objective of this work to investigate the possible interaction between CT and vitamin E. Therefore, the present experiment explores the effects of diet supplementation with vitamin E and *C. ladanifer* CT extract alone and its combination on lamb growth performance, carcass and meat quality traits, including meat α-tocopherol content and oxidative stability.

3.2. Material and methods

3.2.1 Animals, diets and management

The trial followed strict international guidelines on the use of production animals in experimentation (EU 2010) and was carried out at the experimental facilities of Polo de Inovação da Fonte Boa – Instituto Nacional de Investigação Agrária e Veterinária (INIAV I. P.), Portugal. The trial was approved by INIAV I. P. Animal Welfare Responsible Organism (ORBEA – INIAV).

Twenty Romane ram lambs, approximately 60 days old with an average body weight of 19.9 ± 0.86 kg (mean, standard deviation (SD)), were randomly placed into individual pens with wood shavings bedding. Five lambs were assigned per each experimental treatment, according to a completely randomized experimental design with a 2 × 2 factorial arrangement of treatments. The first factor was vitamin E supplementation – low vitamin E level vs. high vitamin E level, and the second factor was CT supplementation – without CT supply vs. with CT supply. Two basal diets were produced, one composed of 450 g/kg DM of barley, 376 g/kg DM of dehydrated lucerne, 125 g/kg DM of *N. oceanica*, vitamins and minerals (basal diet NO); and another composed of 450 g/kg DM of barley, 7 g/kg DM of soybean meal, 367.33 g/kg DM of dehydrated lucerne, 125 g/kg DM of *N. oceanica*, 1.67 g/kg DM tocopheryl acetate (3a700

Vitamin E, 500 mg/g), vitamins and minerals (basal diet NOVE) (Table 3.1). The vitamin and mineral premix used in both basal diets provided 22.5 g/kg of α-tocopherol. Basal diets were prepared in the Feed Compounds Unit of Polo de Inovação da Fonte Boa (INIAV I. P.).

The 2 × 2 factorial arrangements resulted in 4 dietary treatments: C – basal diet NO; VE – basal diet NOVE; CT – basal diet NO supplemented with CT extract from *C. ladanifer*; and VECT – basal diet NOVE supplemented with CT extract from *C. ladanifer*. Each day, 60 g of CT extract were added to the diets of animals receiving CT and VECT treatments, corresponding to 28.1 g CT/head/day. Diets were provided *ad libitum* once daily in the morning, calculated by a 10% increase from the previous day's intake. Grass hay was also supplied, at a rate of 15% based on the previous day's intake, and no leftovers were found. Animals had access to clean water daily. Weekly samples of both basal diets and hay were collected. At the end of the trial, three pooled samples of each basal diet and hay were analysed for chemical characterization, which is presented in Table 3.1.

The microalgae *N. oceanica* used in the present trial was prepared and supplied by the company ALLMICROALGAE – Natural Products SA (Pataias, Portugal) in the form of spray-dried biomass, containing 387 and 129 g/kg of protein and total lipids. Eicosapentaenoic acid (20:5 n-3) represented 22.7 g/100 g of total lipids. CT extract was prepared at CEBAL Lab from leaves and soft stems of *C. ladanifer* (known as rockrose) collected in Ourique, southern Portugal (37°43′33.5” N8°21′42.5”W), during April and May 2022. After drying at room temperature, leaves and soft stems of *C. ladanifer* were milled to a final particle size of 1 mm, and the CT extract was prepared following the methodology described by Guerreiro et al. (2020). The CT content of the CT extract was 469 g/kg.

The trial started after a 7-day adaptation period to experimental conditions and lasted for 35 days. During the trial, daily feed intake was monitored, and lambs were weighed weekly before diet distribution.

Table 3.1 – Ingredients and chemical composition of basal diets

	NO	NOVE	Hay
Ingredients (g/kg dry matter, DM)			
Barley	450	450	
Soybean meal	-	7	
Dehydrated lucerne	376	367	
Nannochloropsis oceanica	125	125	
Calcium carbonate	13	13	
Sodium bicarbonate	20	20	
Di-calcium phosphate	9	9	
Salt	4	4	
Premix[1]	3	3	
Vitamin E	-	1.67	
Chemical composition (g/kg dry matter, DM)			
Dry matter (g/kg product)	903	910	927
Ash	122	121	92
Crude Protein	167	169	49
Ether extract	12	15	6
NDF	343	333	773
ADF	176	178	489
ADL	37	41	58
Starch	315	273	-
Sugar	53	48	-

[1] Premix composition /kg – vitamins A: 4000000 UI, D3: 1100000 UI, E: 7500 mg, B1 and B2: 250mg; trace elements: zinc – 35 g; Iron – 12.5 g; copper – 250 mg; manganese – 17.5 g; iodine – 200 mg; cobalt – 250 mg; selenium – 100 mg.

3.2.2. Slaughter, carcass evaluation and sample collection

At the end of the trial, 24 h before slaughter, lambs were transported to the commercial abattoir MLA – Matadouro do Litoral Alentejano, S. A., located 192 km away from the trial facilities. Lambs were slaughtered by exsanguination, after electrical stunning, respecting the European regulations on the protection of animals at the time of slaughter (EU 2010). After skinning and evisceration, the carcasses were kept at 5 ºC for 70 min in a fast-cooling tunnel and then at 2 ºC for 24 h after slaughter. They were then transported to the experimental slaughterhouse of Polo de Inovação da Fonte Boa (INIAV I. P.) and stored at 2 ºC until 48 h after slaughter. Carcasses were weighed 48 h after slaughter to determine the cold carcass weight and then prepared as described by Jerónimo et al. (2009). The left shoulder from each carcass was collected and dissected to determine the proportions of muscle, bone, subcutaneous fat, and intermuscular fat.

Samples of *Longissimus thoracis* (LT) muscle from the left part of the carcasses were minced with a food processor, vacuum-packed and frozen at – 20 °C for evaluation of the pH and fatty acid (FA) composition. Still, on the left side of the carcass, 3 samples of LT muscle with 1.5 cm thickness were collected for monitoring the lipid and colour stability and the α-tocopherol content of raw meat over refrigerated storage at 4 ºC over 0, 4 and 7 days. The muscle sample from day 0 was vacuum-packed, while the other 2 muscle samples were placed on polystyrene trays and wrapped with oxygen-permeable film. Following colour measurement, at each conservation period, each sample was vacuum-packed and kept at − 80 ºC until lipid oxidation analysis. Loin joints, containing the *Longissimus lumborum* (LL), from both sides of the carcass were individually vacuum-packed and refrigerated at – 2 °C for 7 days and then frozen at − 20 °C. To evaluate the cooking loss and sensory attributes, the LL from the left side of the carcass was used, while the LL from the right side was used for shear force analysis. 3.2.3. Feed chemical analysis.

3.2.3. Feed chemical analyses

Basal diets and hay were characterized for contents of dry matter (DM) (ISO 6496 1999) ash (ISO 5984 2002), ether extract (ISO 6492 1999), crude protein (ISO 5983 1997), sugar and starch (Clegg 1956), neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) (Goering and Van Soest 1970). The NDF was analysed without sodium sulphite and α-amylase and results were expressed as residual ash. The CT content *in C. ladanifer* extract was quantified according to the general procedure of Grabber et al. (2013), as described in detail by Soldado et al. (2024). Purified *C. ladanifer* CT extract was used as a standard, which was prepared using Sephadex LH-20 column chromatography (GE Healthcare Bio-Science, Uppsala, Sweden), according to Strumeyer and Malin (1975).

3.2.4. Meat physicochemical and sensory analysis

Meat pH analysis was carried out according to Dentinho et al. (2023) with a pH meter (Metrohn 744, Herisau, Switzerland) equipped with a combined glass electrode. Instrument calibration was performed at room temperature and using two buffer solutions (pH 4 and 7). For the FA analysis of intramuscular fat, the methodologies and chromatographic conditions described by Vitor et al. (2023) were applied.

For the analysis of meat shear force, cooking loss and sensory traits, frozen loin samples were thawed at 2 ± 1 °C, 24 h before analysis. The samples were then prepared by removing the bone and fat residue, followed by cooking in an electric oven at 170 ± 5 °C, remaining in the oven until the internal temperature reached 71 °C, measured at the thermal centre with a T-type thermocouple (Thermometer, Omega RDXL4SD, Manchester, USA).

Samples used for shear force and cooking loss analysis were washed with cold water, to remove precipitated exudates and initiate rapid cooling, and stored at 4 ± 0.5 °C for 20 h. Samples were weighed, and cooking loss was calculated as a percentage of the initial weight by comparing the weights before and after cooking. A Texture Analyzer (TA-XT2 Texture Analyzer; Stable Micro Systems, Surrey, England), calibrated with a 30 kg compression load cell, was utilized to assess the meat shear force as described by Guerreiro et al. (2020). In each sample, 14–20 measurements were performed.

Meat sensory quality was analyzed by a nine-member trained sensory panel, in a total of six sessions. All the panel members agreed to participate in the meat sensory analysis and provided informed consent. In each session, samples from each dietary treatment were randomly selected, and every panel member tested a sample from each treatment. After cooking, the LL samples were stabilized for 10 min at 40 °C, sectioned into 1 × 1 × 1 cm subsamples, and kept in heated dishes maintained at 40 °C until tasting. The meat was evaluated in terms of tenderness, juiciness, odour intensity, flavour intensity, flavour acceptance and global acceptance. A numeric scale from 1 to 8 was used for evaluation, where 8 corresponded to “extremely odorous, juicy, tender, flavoured and pleasant” and 1 corresponded to “extremely inodorous, dry, tough, unflavoured and unpleasant”.

3.2.4.1. Meat colour

Meat colour was evaluated in the same raw meat samples used for lipid oxidation analysis, on days 0, 4 and 7 of storage at 4 ºC. At day 0 of storage, the colour coordinates were evaluated after 1 hour of blooming. In samples stored for 4 and 7 days, the colour measurement occurred 1 hour after the film removal. Measurements were made with a CR-400 Chroma Meter (Konica Minolta, Tokyo, Japan), in compliance with the CIE L^*, a^*, b^* colour space, where L^* stands for lightness, a^* redness and b^* yellowness. C illuminant and 2º standard observers were applied to all measurements. Hue angle (H*) was calculated as $\tan^{-1}(b^*/a^*) \times (180/\pi)$ and colour saturation (chroma, C*) as $(a^{*2} + b^{*2})^{1/2}$. Overall colour change between day 0 and each storage day was calculated as the square root of the sum of the squared differences between L^*, a^* and b^* coordinates on days 4 and 7 compared to day 0, according to the equation:

$$\Delta E = [(L^*_{(4\text{ or }7)} - L^*_0)2 + (a^*_{(4\text{ or }7)} - a^*_0)2 + (b^*_{(4\text{ or }7)} - b^*_0)^2]^{1/2}$$

Equation 3.1 – Overall colour variation between day 0 and each storage day (4 or 7)

3.2.4.1. Meat lipid oxidation

Lipid oxidation was determined in raw meat stored over 0, 4, and 7 days at 4 ºC, through the quantification of thiobarbituric acid reactive substances (TBARS), following the general procedure of Grau et al. (2000), described in detail by Francisco et al. (2015).

3.2.4.2. Meat α-tocopherol content

α-tocopherol content in LT muscle was evaluated through storage time, on days 0, 4 and 7, according to the extraction and quantification procedure described by Prates et al. (2006). HPLC analysis was carried out in a Dionex Ultimate 3000 uHPLC (Thermo Fisher Scientific) equipped with a normal-phase silica column (Zorbax RX-Sil, with the matching 12.5mm analytical guard column, 4.6 mm ID, 250 mm, 5 µm particle size, Agilent Technologies Inc., Palo Alto, CA, USA) as described by Jerónimo et al. (2020b).

3.2.5. Statistical analysis

Data was analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) as a completely randomized design, using a 2 × 2 factorial treatment design, where the 2 factors were vitamin E level (Vit E, low and high) and CT supplementation (CT, without CT extract and with CT extract supplementation). The interaction between vitamin E level and CT supplementation was included (Vit E × CT). Lambs were considered the experimental unit. Variance heterogeneity among treatments was assessed at a significance level of P = 0.01. When significant, the variance heterogeneity of variances was accommodated into the model using the “group” option within the repeated statement of the MIXED procedure of SAS.

The lambs' live weights measured 6 times during the 5 weeks of the experiment were used to estimate the average daily gain (ADG), through a random intercept regression model. To estimate the individual daily intake of DM and nutrients, the individual daily intake was averaged per week and analyzed using repeated measures over time, using a first-order autoregressive (AR(1)) covariance structure. Live slaughter weight and hot and cold carcass weight were adjusted to the initial live weight. For the analysis of shoulder tissue composition and the percentage of kidney knob channel fat in the carcass, data was adjusted to hot carcass weight. The panelist and tasting session were considered a random effect, when analyzing the sensory quality of the meat.

Data on muscle colour, lipid oxidation, and α-tocopherol levels were analyzed using a repeated measure over time in each sample, with a first-order autoregressive (AR(1)) covariance structure. The model considered the effects of vitamin E level (Vit E), CT supplementation (CT), storage time (T) and Vit E × CT, Vit E × T, CT × T and Vit E × CT × T interactions. The least squares means (LSMeans) and standard error of the mean (SEM) are presented in Tables and Figures.

3.3. Results

3.3.1. Growth performance, feed intake and carcass traits

The results on the effect of dietary treatments on growth performance, feed intake and carcass traits are presented in Table 3.2. Dietary treatment did not affect the lamb's live weight at slaughter, average daily gain (ADG) and feed conversion ratio (FCR), which averaged 28.2 ± 3.03 kg, 213.8 ± 28.3 g/day and 6.42 ± 2.477 (mean ± SD), respectively. Dry matter intake of concentrate feed was not affected by treatments, averaging 1082 ± 268.8 g/day. The intake of CT from *C. ladanifer* extract was 24.1 and 26.6 g/day in CT and VECT diets, respectively.

Hot and cold carcass weight, dressing percentage, and kidney knob channel fat percentage were also not affected by dietary treatments, averaging 12.4 ± 1.60 kg, 12.1 ± 1.57 kg, 44.1 ± 3.28 %, and 1.49 ± 0.466 %, respectively. Shoulder tissue composition was also unaffected by dietary treatments.

Table 3.2 – Effect of dietary vitamin E level and *C. ladanifer* condensed tannin extract supplementation on growth performance, dry matter intake and carcass traits of lambs

	Diets[1]				SEM	P value		
	C	VE	CT	VECT		Vit E	CT	Vit E ×CT
Growth Performance								
Initial live weight (kg)	21.2	22.0	21.3	21.1	-	-	-	-
Slaughter live weight (kg)	28.5	28.2	26.6	29.7	1.37	0.342	0.911	0.260
Average daily gain (g/d)	207	181	224	243	28.3	0.861	0.774	0.680
Dry matter intake	1124	1095	1027	1138	105.5	0.698	0.797	0.507
Feed conversion ratio	6.20	7.63	6.40	5.44	1.142	0.840	0.398	0.311
Carcass traits								
Hot carcass weight (kg)	12.9	12.7	12.1	12.4	0.80	0.925	0.499	0.815
Cold carcass weight (kg)	12.6	12.5	11.9	12.1	0.79	0.925	0.498	0.814
Dressing (%)	44.8	46.0	45.5	41.3	2.09	0.479	0.347	0.217
Kidney knob channel fat (%)	1.43	1.51	1.40	1.48	0.230	0.705	0.889	0.984
Shoulder composition (%)								
Muscle	56.3	57.3	58.6	58.3	1.52	0.793	0.290	0.662
Bone	25.4	24.7	24.7	22.2	0.827	0.058	0.058	0.244
Subcutaneous fat	5.23	5.64	572	4.95	0.781	0.807	0.897	0.423
Intermuscular fat	11.9	10.4	9.40	12.6	1.485	0.540	0.904	0.114

[1] C – Basal diet containing spray-dried *N. oceanica* biomass (NO); VE – Basal diet containing spray-dried *N. oceanica* biomass and with high levels of vitamin E (NOVE); CT – Basal diet NO supplemented with *C. ladanifer* CT extract; and VECT – Basal diet NOVE supplemented with *C. ladanifer* CT extract.

SEM – standard error of the mean

3.3.2. Meat physicochemical and sensorial traits

The results of meat quality traits are presented in Table 3.3, while the results of the FA composition of the intramuscular fat are presented in Table 3.4. No differences were found among dietary treatments concerning meat pH, cooking loss, and shear force, which averaged 5.84 ± 0.163, 28.3 ± 3.58 g/100 g, and 29.2 ± 7.05 N, respectively. Interactions between vitamin E level and CT supplementation were observed for most of the sensory attributes analyzed in meat ($P < 0.05$, Table 3). Meat from the CT diet had lower scores for tenderness, juiciness, flavour intensity, flavour and overall acceptability, and a higher odour intensity score than meat from diets without CT-extract supplementation. The tenderness, juiciness, flavour and overall acceptability scores of meats from VE diet were intermediate between CT and C/VECT diets and did not differ between the last two. Meat flavour intensity was equal in meat from C, VE, and VECT diets. Meat odour intensity was only affected by the vitamin E level ($P = 0.007$), with lower scores in meat from animals receiving a higher level of vitamin E (2.15 vs 2.50). Intramuscular fat FA composition was not affected by dietary vitamin E level or CT supplementation. Additionally, the meat content of highly oxidizable PUFA and peroxidable index (PI) remained unaffected by the dietary treatments.

Table 3.3 – Effect of dietary vitamin E level and *C. ladanifer* condensed tannin extract supplementation on meat quality traits

	Diets[1]				SEM	P value		
	C	VE	CT	VECT		Vit E	CT	Vit E × CT
pH	5.78	5.81	5.98	5.80	0.079	0.354	0.227	0.167
Cooking loss (g/100 g)	27.4	30.1	27.3	28.3	1.85	0.295	0.589	0.633
Shear force (N)	27.1	32.0	32.9	25.3	3.42	0.679	0.892	0.066
Sensory characteristics								
Tenderness	5.37^{c}	5.09^{b}	4.61^{a}	5.46^{c}	0.172	0.004	0.064	<0.001
Juiciness	4.69^{c}	4.40^{b}	4.33^{a}	4.92^{c}	0.154	0.154	0.489	<0.001
Odour intensity	2.30	2.17	2.70	2.14	0.259	0.007	0.173	0.106
Flavour	2.55^{a}	2.54^{a}	3.02^{b}	2.26^{a}	0.239	0.006	0.06	0.009
Flavour acceptability	4.92^{bc}	4.89^{b}	4.60^{a}	5.15^{c}	0.123	0.002	0.739	<0.001
Overall acceptability	4.92^{bc}	4.82^{b}	4.31^{a}	5.13^{c}	0.167	0.001	0.266	< 0.001

[1] C – Basal diet containing spray-dried *N. oceanica* biomass (NO); VE – Basal diet containing spray-dried *N. oceanica* biomass and with high levels of vitamin E (NOVE); CT – Basal diet NO supplemented with *C. ladanifer* CT extract; and VECT – Basal diet NOVE supplemented with *C. ladanifer* CT extract.
Means within a column with different superscripts differ ($P < 0.05$).
SEM – standard error of the mean.

Table 3.4 – Effect of dietary vitamin E level and *C. ladanifer* CT extract supplementation on intramuscular FA composition (mg/g total fatty acids)

	Diets[1]				SEM	P value		
	C	VE	CT	VECT		Vit E	CT	Vit E × CT
L-SFA[2]	417	430	429	421	13.9	0.829	0.932	0.443
BCFA[3]	5.07	5.50	4.38	5.48	0.626	0.204	0.545	0.569
MUFA[4]	356	364	337	359	16.9	0.357	0.435	0.659
PUFA[5]	166	153	175	165	18.2	0.511	0.538	0.957
n-6 PUFA[5]	125	113	132	126	15.0	0.519	0.493	0.850
18:2n-6	87.1	78.8	85.5	86.2	8.85	0.645	0.730	0.587
n-6 LC-PUFA[6]	37.3	33.8	45.9	38.8	7.31	0.444	0.329	0.793
n-3 PUFA[5]	26.0	26.8	29.5	25.9	3.47	0.545	0.569	0.616
18:3n-3	6.97	7.03	6.57	6.79	0.575	0.791	0.548	0.556
n-3 LC-PUFA[6]	19.1	18.6	23.0	19.1	3.30	0.495	0.482	0.580
HP-PUFA[7]	65.8	62.0	78.7	67.3	10.94	0.463	0.379	0.711
PI[8]	30.4	28.1	34.3	30.7	4.16	0.478	0.433	0.831

[1] C – Basal diet containing spray-dried *N. oceanica* biomass (NO); VE – Basal diet containing spray-dried *N. oceanica* biomass and with high levels of vitamin E (NOVE); CT – Basal diet NO supplemented with *C. ladanifer* CT extract; and VECT – Basal diet NOVE supplemented with *C. ladanifer* CT extract.

[2] Sum of linear saturated fatty acids.

[3] Sum of branched-chain fatty acids.

[4] Sum of monounsaturated fatty acids.

[5] Sum of polyunsaturated fatty acids.

[6] LC-PUFA – Sum of long chain polyunsaturated fatty acids (≥ C20).

[7] HP-PUFA – Highly oxidizable polyunsaturated fatty acids, sum of polyunsaturated fatty acids with three or more unsaturated bonds.

[8] PI – Peroxidability index = (∑ dienoic acid × 1) + (∑ trienoic acid × 2) + (∑ tetraenoic acid × 3) + (∑ pentaenoic acid × 4) + (∑ hexaenoic acid × 5).

3.3.3. Meat colour, lipid oxidation and α-tocopherol content during storage time

The effect of storage time, dietary vitamin E level, and CT supplementation on colour and lipid stability in LT muscle are presented in Table 3.5. Meat colour parameters *L**, *b**, C* and H* varied over storage time. Regardless of the dietary treatments, *b**, C* and H* values increased (P < 0.001) between days 0 and 4 of storage, remaining stable until day 7. Interactions between vitamin E level and CT supplementation were observed for *L** values (P = 0.009, Figure 3.1). In VE and CT diets, the *L** values increased up to day 4 and then remained unchanged, while in C and VECT diets no changes were observed in the *L** values during storage time. The overall colour variation indices determined after 4 (Δ*E4*) and 7 (Δ*E7*) days of storage, remained below 5 and were not affected by the dietary treatments. For meat lipid oxidation, we found an interaction between vitamin E level, CT supplementation, and storage time (P < 0.001). On the first storage day (day 0) the TBARS values did not differ among diets (0.01 mg MDA/kg muscle). Over the storage time, TBARS values only increased in the C diet, reaching 0.40 and 1.11 mg MDA/kg muscle on days 4 and 7 of storage, respectively. By contrast, the TBARS values in the other diets remained unchanged throughout the 7 days of storage. The α-tocopherol content of meat varied over the storage period according to dietary vitamin E level (interaction between vitamin E level and storage time, *P* = 0.047). In all storage days, meat from diets with high vitamin E levels had higher α-tocopherol contents than meat from diets with low vitamin E levels. In the meat from diets with high vitamin E levels, the α-tocopherol content decreased from 4.96 to 3.24 mg/kg muscle between days 0 and 4 of storage, remaining stable between days 4 and 7. On the other hand, in diets with low vitamin E levels, the meat α-tocopherol content did not vary over the 7 days of storage (1.53 α-tocopherol mg/kg muscle).

Table 3.5 – Effect of dietary vitamin E level and *C. ladanifer* CT extract supplementation on colour, lipid oxidation and α-tocopherol content of lamb meat over storage time

	*L**	*a**	*b**	C*	H*	∆E4	∆E7	TBARS[1] (mg/kg muscle)	α-tocopherol (mg/kg muscle)
Effect of storage time (T)									
0 days	40.0^{a}	12.6	3.90^{a}	13.2^{a}	17.2^{a}	-	-	0.01^{a}	3.46^{a}
4 days	42.8^{c}	12.9	7.66^{b}	15.1^{b}	30.8^{b}	-	-	0.13^{b}	2.26^{b}
7 days	42.1^{b}	12.6	7.72^{b}	14.8^{b}	31.7^{b}	-	-	0.36^{c}	2.25^{b}
SEM	0.45	0.35	0.230	0.34	0.940	-	-	0.041	0.176
P value	<0.001	0.159	<0.001	<0.001	<0.001	-	-	<0.001	<0.001
Effect of vitamin E level (Vit E)									
0 g/kg DM	41.7	12.8	6.24	14.3	25.9	4.59	4.18	0.30	1.53
1.67 g/kg DM	42.3	12.6	6.61	14.4	30.8	4.66	4.69	0.04	3.79
SEM	0.61	0.45	0.306	0.45	1.226	0.233	0.280	0.036	0.140
P value	0.487	0.816	0.394	0.949	0.440	0.838	0.209	<0.001	<0.001
Effect of CT supplementation (CT)									
0 g/kg DM	41.6	12.7	6.66	14.5	27.3	4.58	4.45	0.28	2.56
60 g/kg DM of CT extract/day	42.4	12.7	6.19	14.2	25.8	4.69	4.42	0.05	2.76
SEM	0.61	0.45	0.306	0.45	1.126	0.233	0.280	0.036	0.140
P value	0.352	0.941	0.264	0.703	0.397	0.772	0.942	<0.001	0.311
P values interactions									
Vit E × CT	0.184	0.941	0.981	0.920	0.784	0.128	0.573	<0.001	0.124
Vit E × T	0.730	0.642	0.771	0.798	0.684	-	-	<0.001	0.047
CT × T	0.844	0.473	0.410	0.511	0.669	-	-	<0.001	0.184
Vit E × CT × T	0.009	0.167	0.824	0.157	0.682	-	-	<0.001	0.309

[1] mg malondialdehyde (MDA)/kg of muscle.

Means within a column with different superscripts differ ($P < 0.05$).

SEM – standard error of mean.

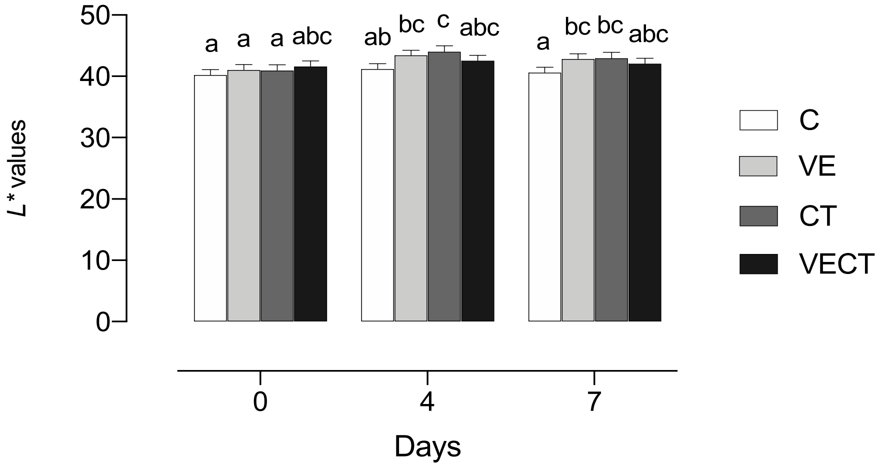

Figure 3.1. Effect of vitamin E level and *C. ladanifer* condensed tannins extract supplementation and storage time on L* values. C – Basal diet containing spray-dried *N. oceanica* biomass (NO); VE – Basal diet containing spray-dried *N. oceanica* biomass and with high levels of vitamin E (NOVE); CT – Basal diet NO supplemented with *C. ladanifer* CT extract; and VECT – Basal diet NOVE supplemented with *C. ladanifer* CT extract. Bars represent the least square means, with their respective standard error of the mean. Different superscript values are significantly different (P < 0.05).

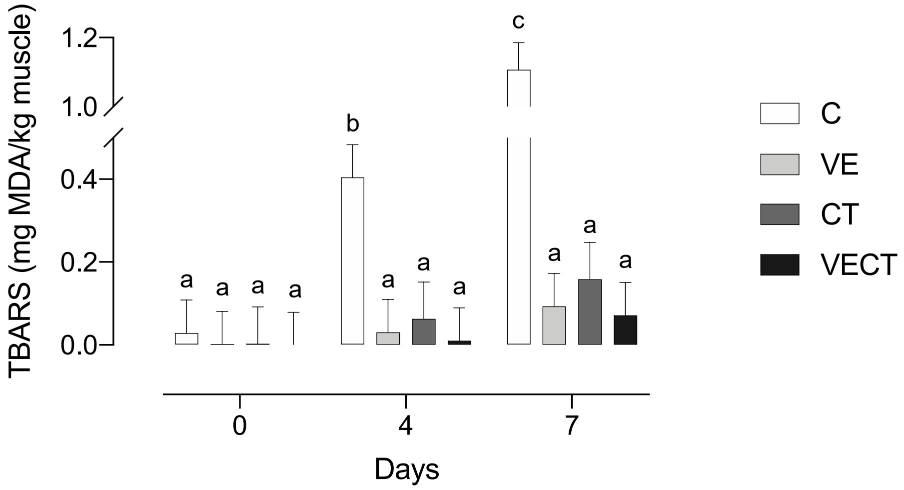

Figure 3.2. Effect of vitamin E level and *C. ladanifer* condensed tannins extract supplementation and storage time on muscle lipid oxidation. C – Basal diet containing spray-dried *N. oceanica* biomass (NO); VE – Basal diet containing spray-dried *N. oceanica* biomass and with high levels of vitamin E (NOVE); CT – Basal diet NO supplemented with *C. ladanifer* CT extract; and VECT – Basal diet NOVE supplemented with *C. ladanifer* CT extract. Bars represent the least square means, with their respective standard error of the mean. Different superscript values are significantly different (P < 0.05).

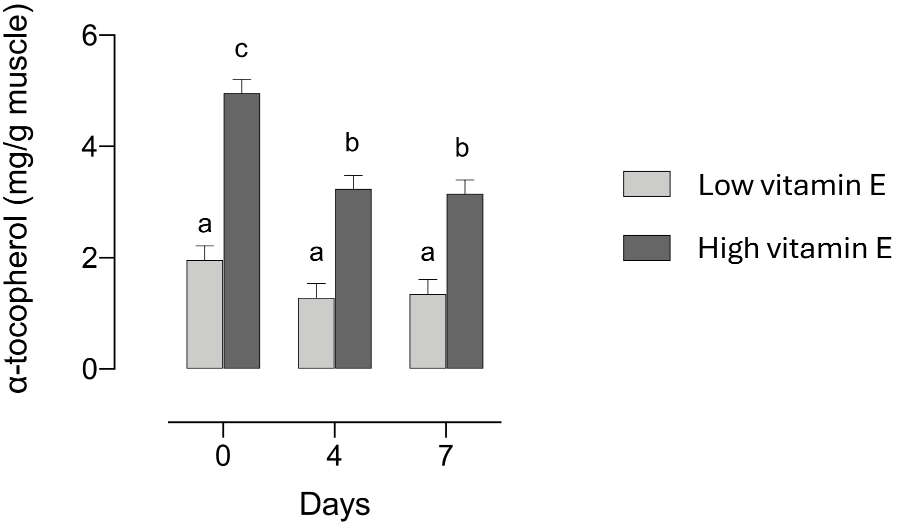

Figure 3.3. Effect of vitamin E level in the diet and storage time on muscle α-tocopherol content. Bars represent the least square means, with their respective standard error of the mean. Different superscript values are significantly different ($P < 0.05$).

3.4. Discussion

The present experiment was designed to improve knowledge about the individual effect of CT and the interaction between CT and vitamin E on the oxidative stability of meat from lambs fed PUFA-rich diets. *Nannochloropsis oceanica*, a marine microalgae known for its high content of 20:5 n-3, was included in all dietary treatments to increase oxidative pressure in meat, promoting an imbalance between pro-oxidants and antioxidant agents. Adding microalgae to animal diets raises concerns about potential adverse effects on feed intake due to its low palatability (Lamminen et al. 2017; Pappadopoulos et al. 2002; Urrutia et al. 2016). A previous study conducted by our research team (Vitor et al. 2023) demonstrated that including 125 g/kg DM of *N. oceanica* in lamb diets did not negatively affected feed intake or lamb performance. The feed intake observed in the current experiment was similar to what was previously reported for lambs fed *N. oceanica* in the form of spray-dried biomass, which was 1017 g DM/day (Vitor et al. 2023). Several studies demonstrated that supranutritional doses of vitamin E in lamb diets do not affect animal growth performance (Bellés et al. 2019), as observed in our study. Conversely, CT are known for their potential negative impact on feed intake, nutrient digestibility, and animal performance (Makkar 2003). Condensed tannins can bind to various molecules, such as proteins, polysaccharides, and minerals, forming indigestible complexes, which reduces nutrient digestibility and feed intake (Makkar 2003). Moreover, the astringency caused by CT binding to salivary proteins can also reduce feed intake (Lesschaeve and Noble 2005). In the present experiment, the *C. ladanifer* CT extract did not affect the feed intake or the growth performance of lambs. This finding is consistent with other works on lambs, where their diets were supplemented with various CT extracts (Biondi et al. 2019; Brunetto et al. 2024; Mu et al. 2020). In contrast, Guerreiro et al. (2020) found that including 41 g/kg DM of *C. ladanifer* CT extract in lamb diets, which corresponded to an intake of 28.8 g of CT per day, affected the lambs' growth performance impairing the ADG, FCR and carcass weight. Jacondino et al. (2022) also reported lower DMI and reduced cold carcass weight when 40 g/kg DM of *Acacia mearnsii* CT extract alone or in combination with α-tocopherol was included in lamb diets. Indeed, the effects of CT on feed intake and growth performance are controversial. The concentration of CT in the diet and its chemical composition, composition of basal diet, animal species and metabolic status are factors that may contribute to discrepancies in the results (Besharati et al. 2022; Mueller-Harvey et al. 2019).

Regarding the quality parameters of lamb meat, we only observed dietary effects on sensory characteristics. Meat samples from the lambs that received *C. ladanifer* CT extract were perceived by the sensory panel as less tender and juicy and with greater flavour intensity, obtaining lower scores in flavour and overall acceptability than meat from the other diets. According to our results, Francisco et al. (2018) also reported that the inclusion of *C. ladanifer* plant in lamb diets reduces the meat tenderness, juiciness and overall acceptability. Moreover, higher scores of flavour intensity and off-flavour intensity were found in meat from lambs fed *C. ladanifer* plant (Francisco et al. 2018). In contrast, Guerreiro et al. (2020) reported an increase in meat acceptance among panellists in response to increased levels of *C. ladanifer* plant or *C. ladanifer* CT extract in lamb diets, while in other work the consumer panel did not detect the effect of *C. ladanifer* inclusion in diets on lamb meat sensory proprieties (Jerónimo et al. 2012). It was suggested that phenolic compounds derived from *C. ladanifer* might interfere in early postmortem proteolysis of meat reducing the meat tenderness (Francisco et al. 2018). However, when associated with high levels of vitamin E in the diet, the *C. ladanifer* CT extract did not impair the meat sensorial attributes, with the meat from the VECT diet being given the highest overall acceptability score. The impact of the *C. ladanifer* plant or its CT extract on the sensory characteristics of lamb meat has been inconsistent, like findings from other CT sources (Priolo et al. 1998; Whitney et al. 2011). This inconsistency may be related to several factors, including the composition of the diet, CT source and level, and animals used in the experiments.

We also verified that high levels of vitamin E in the diet reduced the meat odour intensity. The lower intensity of meat odour found in high vitamin E diets may be related to lower production of odorous compounds derived from lipid oxidation since in these diets was observed reduced levels of lipid oxidation. All dietary treatments resulted in meat that can be considered tender, with shear force values significantly lower than the threshold of 49 N set as an indication of acceptable tenderness in sheep meat (Hopkins et al. 2006). Furthermore, the redness (*a**) and lightness (*L**) values of the meat, measured on the first day and throughout the storage period, were higher than 9.5 and 34, respectively, values from which consumers considered the lamb meat colour acceptable (Khliji et al. 2010). Despite good indicators regarding the consumer acceptability of meat colour throughout the storage period, in the first 4 days of storage it was verified an increase in the meat colour parameters – yellowness (*b**), chroma (C*) and hue angle (H*), consistent with meat discoloration. However, the variation in meat luminosity (*L**) over storage time was dependent on the dietary treatment, increasing only in the VE and CT diets. It is widely accepted that supplementing diets with vitamin E is an effective strategy to prevent colour deterioration in red meats (Herrera et al. 2024; Lynch et al. 1999; Stubbs et al. 2002). It has also reported that CT intake helps to preserve the colour of meat (Luciano et al. 2009a; Soldado et al. 2024). In the present study, we did not observe a protective effect of vitamin E and CT supplementation on meat colour when applied alone, and the combination of both antioxidant agents maintains meat *L** values unchanged over storage time. Nonetheless, the colour changes observed in the meat over the storage period do not seem to be detectable by consumers with the naked eye, as the overall colour variation indices (Δ*E4* and Δ*E7*) remained below 6 (Abril et al. 2001).

The main goal of this study was to evaluate whether the combination of *C. ladanifer* CT extract and vitamin E in the diet offers better antioxidant efficiency under pro-oxidative conditions, compared to using each antioxidant separately. *Cistus ladanifer* CT extract and vitamin E, separated or combined were able to protect the meat from lipid oxidation and kept it under the 2 mg MDA/kg muscle threshold (Campo et al. 2006), used as an indicator of off-flavour development, during 7 days of refrigerated storage. This result showed for the first time that dietary *C. ladanifer* CT extract has a protective effect against lipid oxidation in lamb meat. The ability of *C. ladanifer* plant to limit lipid oxidation in lamb meat had been previously reported (Francisco et al. 2015; Jerónimo et al. 2012; Jerónimo et al. 2020a; Jerónimo et al. 2020b). The antioxidant effect of the *C. ladanifer* plant was attributed to its high levels of CT (Jerónimo et al. 2012). However, in the work carried out by Soldado et al. (2024), it was not possible to confirm the antioxidant effect of *C. ladanifer* CT extract, since in this study neither the *C. ladanifer* plant nor the *C. ladanifer* CT extract affected meat lipid oxidation. The discrepancy between the works may be related to different imbalances between pro-oxidant and antioxidant components promoted by basal diets. In the present work, the daily intake of CT was slightly lower than the 28.8 g CT/day reported by Soldado et al. (2024). The basal diet used by Soldado et al. (2024) consisted solely of dehydrated lucerne, which supplies natural antioxidants like tocopherols and polyphenols (Rafínska et al. 2017). These antioxidant components may have provided a sufficient baseline level of antioxidant protection, helping to maintain low levels of lipid oxidation, regardless of the inclusion of CT extract in the diets. On the other hand, in the present work, the basal diet is susceptible to induce increased oxidative pressure in meat due to the presence of *N. oceanica*. The FA composition of lamb meat remained unchanged regardless of the dietary treatments, with high proportions of n-3 long-chain PUFA (n-3 LC-PUFA) in intramuscular fat (18.6 – 23.0 mg/g total FA). This high proportion of n-3 LC-PUFA makes the meat prone to lipid oxidation, as the susceptibility of PUFA to oxidation increases with a greater degree of unsaturation (Scislowski et al. 2005). Additionally, the levels of highly oxidizable PUFA and the peroxidation index (PI) were unaffected by the dietary treatments, suggesting that the oxidative pressure derived from the FA composition was consistent across all diets. The meat's oxidative stability depends on the balance between antioxidant agents and pro-oxidant factors (Bekhit et al. 2013), and under pro-oxidant conditions created in the present work, the antioxidant effect of *C. ladanifer* CT extract could have been more evident.

However, the effectiveness of CT extracts in improving meat oxidative stability has been inconsistent. Only a few studies using CT extract have found positive results as a dietary strategy to limit meat lipid oxidation (Brunetto et al. 2024; Jacondino et al. 2022; Jerónimo et al. 2012; Lobón et al. 2017b; Muíño et al. 2014), while others show neutral results (Biondi et al. 2019; Brogna et al. 2014; Buccioni et al. 2017; Lobón et al. 2017; Luciano et al. 2009a; Salas et al. 2024; Valenti et al. 2019).

As expected, α-tocopherol levels in meat increased with supranutritional doses of vitamin E in the diet (Bellés et al. 2019), reaching 4.96 mg/kg of meat on day 0, a value 2.5-fold higher than that found in meat from lambs fed diets with low levels of vitamin E. Due to numerous factors that affect the meat's susceptibility to oxidation, it is difficult to establish the minimum vitamin E levels in meat that inhibits lipid oxidation (Bellés et al. 2019). Under prooxidant conditions verified in the present work, the supranutritional doses of vitamin E in the diet (adding 835 mg/kg DM of diet) were essential to prevent meat lipid oxidation. In contrast, the C diet, supplemented only with 22.5 mg/kg DM of α-tocopherol, resulted in a meat α-tocopherol content of 1.96 mg/kg. Although this level of vitamin E supplementation is close to the recommended dosage for growing-finishing lambs of 20–30 kg of body weight (Council 1985) it was not enough to prevent meat lipid oxidation. Such result agrees with other reports that warn of the need for a greater supply of antioxidants when diets containing high levels of highly oxidizable PUFA are used (Gobert et al. 2009; Soldado et al. 2018; Vitor et al. 2023).

Regarding the mechanisms of action by which CT exerts an antioxidant effect on the animal body and its products, there is still great controversy, as extensively discussed by Soldado et al. (2021). An indirect antioxidant action through interaction with other antioxidant agents such as antioxidant enzymes and antioxidant compounds has been appointed as a possible mechanism. Increased vitamin E levels in the liver were found in rats fed grape extract (Gladine et al. 2007a). Dietary phenolic compounds, such as the CT monomers – catechins and epicatechins, also increased vitamin E levels in the plasma and liver of rats (Frank 2005). In lamb meat, increased levels of α-tocopherol were also reported when *C. ladanifer* plant and quebracho were included in the diets of lambs or their mothers, respectively (Jerónimo et al. 2020b; Lobón et al. 2017b). These results suggested that dietary CT interacts in some way with vitamin E increasing its muscle availability. Vitamin E protection against oxidation, its recycling, or enhanced absorption has been appointed as possible mechanisms by which phenolic compounds may interact with vitamin E (Frank et al. 2004). However, in the present work, higher α-tocopherol levels were not found in meat from lambs that received the combination of *C. ladanifer* CT extract and vitamin E. Moreover, it was not possible to validate the hypothesis that the combination of *C. ladanifer* CT extract and vitamin E has a better antioxidant performance than individual antioxidants. In agreement with our results, it was reported that the combination of carob pulp or *Acacia mearnsii* tannin extract with higher vitamin E levels in the diets also did not result in increased α-tocopherol deposition or improved lipid stability in lamb meat when compared to the use of CT-sources and vitamin E separately (Bottegal et al. 2024; Jacondino et al. 2022). Supranutritional vitamin E levels in the diets could have masked a possible interaction between CT and vitamin E. Indeed, in the works that reported increased meat levels of α-tocopherol in lambs fed CT sources, there was either no supplementation, or the diets were supplemented with significantly lower levels of vitamin E.

3.5. Conclusion

Although it has not been verified an interaction and a synergic effect between CT and vitamin E, this work found that the dietary supplementation with *C. ladanifer* CT extract limits meat lipid oxidation over 7 days of storage, as occurred with a high dose of vitamin E. Present results may create the conditions to the use of large-scale adoption of *C. ladanifer* CT extract, as an alternative to synthetic vitamin E additives, without impairing animal development and efficiency.

Funding

This research is funded by National Funds through FCT - Foundation for Science and Technology through PhD scholarships of D. Soldado (SFRH/BD/145814/2019) and projects UIDB/05183/2020 (MED, https://doi.org/10.54499/UIDB/05183/2020), UIDB/00276/2020 (CIISA; https://doi.org/10.54499/UIDB/00276/2020), LA/P/0121/2020(CHANGE;

https://doi.org/10.54499/LA/P/0121/2020), and LA/P/0059/2020 (AL4AnimalS; https://doi.org/10.54499/LA/P/0059/2020).

Acknowledgements

The authors would like to thank to the CEBAL staff to support in the *Cistus ladanifer* collection and sampling, namely Liliana Cachucho, Letícia Fialho, Patrícia Lage and Andreia Silva. The authors would also like to thank to the INIAV staff of Fonte Boa, namely Paula Santos, for the support in carcass evaluation and sample collection and to José Batista for the chemical analysis.

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4. CHAPTER 4 | General discussion, future perspectives and conclusion

There has been an upsurge in scientific research into natural antioxidants, notably CT, for ruminant feeding strategies, with the goal of enhancing animal production and product quality, while using local resources, promoting production system resilience and the circular economy. Condensed tannins have been widely used for ruminant nutrition as feed-added antioxidants; however, their overall effect has inconsistent results.

Covid-19 pandemic matched the beginning of my PhD scholarship, making it impossible to begin the programmed experimental work. So, to make good use of the available time, we started to gather all the info we could about the use of CTs in ruminant nutrition, with the antioxidant effect in the animal and its products as our main target. That resulted in the first scientific review on the use of CTs in ruminant nutrition, regarding their antioxidant effect (Chapter 1). This review would become an unvaluable resource, as it allowed us to unite not only the different nutritional strategies using CTs and the underlying issues, but also to critically address the possible action mechanisms by which CTs exert antioxidant effects in animal body and its products.

Our research group had previously used *C. ladanifer* in ruminant diets, with positive and promising results on meat oxidative stability (Francisco et al. 2015; Jerónimo et al. 2012; Jerónimo et al. 2020b). In this thesis, we opted to continue utilizing *C. ladanifer* as a CT-rich plant for our trials, because it is a local resource, and our group is familiar with its utilization. As most of the CT-rich plants used in ruminant nutrition, their antioxidant effect may not only be attributed to the CT fraction. To explore the whether the *C. ladanifer* CT extract has the same antioxidant effect than the *C. ladanifer* plant (found in previous works) we prepared a trial using both CT sources (Chapter 2), while also exploring CT mechanisms of action. We found that, in this trial, neither *C. ladanifer* plant nor *C. ladanifer* CT extract were able to protect meat from lipid oxidation and increase muscle antioxidant status. This result didn't allow us to clarify if the *C. ladanifer* CT extract can induce the same antioxidant effect than the *C. ladanifer* plant. Direct antioxidant action, comprehending absorption and deposition in the tissues, is one of the action mechanisms by which CTs can exert their antioxidant action. Since CTs are large molecules, impossible to be absorbed by the tissues, they must undergo transformation. We looked for their flavan-3-ol subunits in the muscle, but they were not detected. This study was the first to measure the antioxidant activity in the ruminal and abomasal digestive contents. As expected, increasing levels of CTs in the diets progressively increased the antioxidant activity and the CTs levels in both GIT compartments. In faeces, the antioxidant activity increase was less pronounced, and CT weren't possible to be quantified, suggesting that CT may undergo transformation in the GIT. Dietary α-tocopherol content decreased with the inclusion of both *C. ladanifer* CT sources in the diets, reducing the α-tocopherol intake.

However, the α-tocopherol content in muscle was similar among all the diets, suggesting that dietary CT affects α-tocopherol metabolism, enhancing its availability in the muscle. This result was consistent with that described by Jerónimo et al. (2020b) and Lobón et al. (2017b), who reported increased levels of α-tocopherol in lamb meat when the *C. ladanifer* plant or quebracho were included in the diets of lambs or their mothers, respectively, which led us to search for this interaction even further.

A new trial was conducted (Chapter 3), were the efficacy of dietary *C. ladanifer* CT extract and vitamin E supplementation in improving the oxidative stability of meat was assessed. This trial also explored if the dietary combination of both antioxidants was able to outperform the individual antioxidants. In this trial, *N. oceanica* microalga was included in the basal diet to increase the oxidative pressure, in an attempt to highlight the antioxidant effect of vitamin E and *C. ladanifer* CT extract in a more adverse context. Both vitamin E and *C. ladanifer* CT extract alone or when combined were able to protect meat lipid oxidation during storage time. The discovery that *C. ladanifer* CT extract offers the same level of protection as vitamin E supplementation is significant. This finding could lead to the widespread adoption of *C. ladanifer* CT extract as a substitute for additional vitamin E, without negatively impacting animal production. In this trial there wasn't observed an interaction between *C. ladanifer* CT extract and α-tocopherol, regarding α-tocopherol content in muscle and in meat lipid oxidation. We also couldn't validate the hypothesis that both antioxidants combined can perform better than by themselves.

Overall, the results showed that *C. ladanifer* CTs, especially in extract form, can be an efficient dietary antioxidant supplement, improving the antioxidant status of the animal and the oxidative stability of their products, without compromising animal growth performance and their product characteristics.

The antioxidant action mechanisms of CTs require additional research because they are difficult to validate. The composition of CTs greatly varies depending on the plant source. Furthermore, the interaction with the basal diet can determine how freely they can interact with the GIT when consumed. This will affect their antioxidant activity action mechanisms. An important and much-needed next step is to characterize the CT chemical structure of each extract or plant prior to their use in dietary strategies, as well as when coupled with the basal diet, as their bioavailability can be altered. This may help us understand why their action mechanisms are so inconsistent.

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