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COLLEGE OF SCIENCE, ENGINEERING AND FOOD SCIENCE

SCHOOL OF FOOD AND NUTRITIONAL SCIENCES

Head of School: Professor Paul McSweeney

Supervisors: Professor Joseph P. Kerry (UCC) & Dr. Michael N. O'Grady (UCC)
Professor Alessandro Priolo (University of Catania, Italy)
Dr. Giuseppe Luciano (University of Perugia, Italy)

REDUCING THE IMPACT OF FEEDSTUFF TRANSPORT BY A BETTER UTILIZATION OF PLANT WASTES FOR LIVESTOCK: A WAY TO IMPROVE MEAT QUALITY?

THESIS

Presented by

SAHEED AYODEJI SALAMI, B.Tech, M.Sc

For the degree of

DOCTOR OF PHILOSOPHY

In Food Science and Technology

January 2019

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Certified by:



Saheed A. Salami

January 2019

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Abstract

The effect of feeding plant by-products (cardoon meal (CM), dried corn gluten feed (CGF), corn (CDGS) and wheat (WDGS) distillers' grains with solubles, and citrus pulp (DCP)) on the quality of meat from ruminant animals (lambs and beef cattle) was investigated. The first experiment examined the response of rumen function to diets rich in phenolic compounds (i.e. tannins), which are secondary metabolites prevalent in several plant by-products. Concentrate diet supplemented with 4% of two hydrolysable (chestnut and tara) and condensed (mimosa and gambier) tannin extracts did not impair ruminal fermentation traits and microbiome in lambs. The second and third experiments indicated that replacement of 15% dehydrated alfalfa with CM did not influence lamb performance and carcass traits. Feeding CM promoted ruminal biohydrogenation of fatty acids (FA) through a modulation of the rumen bacterial community and consequently, reduced the proportion of *t*-11 18:1 and *c*-9,*t*-11 conjugated linoleic acid (CLA) in lamb meat. Moreover, dietary CM did not influence the oxidative stability of lamb meat whereas a phenolic-rich CM extract reduced lipid oxidation in an ovine muscle model system. The fourth, fifth and sixth experiments assessed the quality indices of beef from grass silage-fed steers offered concentrate supplements in which barley/soybean meal was replaced with varying levels of CGF (25%, 50% and 75%), CDGS and WDGS (80%), and DCP (40% and 80%), respectively. The inclusion of up to 75% CGF improved the FA profile of beef by decreasing the proportion of C14:0 and increasing CLA, C18:3 *n*-3, C20:5 *n*-3 and C22:5 *n*-3 without negatively influencing the shelf-life and eating quality of beef. Dietary CDGS or WDGS increased the proportion of CLA and total polyunsaturated fatty acids (PUFA) in beef but decreased beef shelf-life by increasing lipid oxidation and discolouration in fresh beef patties stored in modified atmosphere packs (80% O_2:20% CO_2). The inclusion of up to 80% DCP improved the FA profile of beef by increasing the percentage of CLA and

PUFA in beef without compromising the oxidative stability and consumer acceptability of beef.

Keywords: Animal diet, Ruminal biohydrogenation, fatty acids, Beef, Lamb meat, Oxidative stability.

List of Publications

PEER-REVIEWED ARTICLES:

Salami, S.A., G. Luciano, M.N. O'Grady, L. Biondi, C.J. Newbold, J.P. Kerry and A. Priolo (2018). Sustainability of feeding plant by-products: a review of the implications for ruminant meat production. Submitted to *Animal Feed Science and Technology*.

Salami, S.A., B. Valenti, M. Bella, G. Luciano, M.N. O'Grady, J.P. Kerry, E. Jones, A. Priolo and C.J. Newbold (2018). Characterization of the ruminal fermentation and microbiome in lambs fed hydrolysable and condensed tannins. *FEMS Microbiology Ecology*, 94: DOI: 10.1093/femsec/fiy061.

Salami, S.A., B. Valenti, G. Luciano, M. Lanza, J.P. Kerry, M.N. O'Grady, C.J. Newbold and A. Priolo (2018). Effects of dietary dehydrated lucerne or cardoon meal on the modulation of ruminal biohydrogenation and microbial community in lambs. Submitted to *Frontiers in Microbiology*.

Salami, S.A., B. Valenti, M.N. O'Grady, J.P. Kerry, M. Simona, G. Licitra, G. Luciano and A. Priolo (2018). Influence of dietary cardoon meal and cardoon extract on s selected quality parameters of lamb meat. Submitted to *Meat Science*.

Salami, S.A., M.N. O'Grady, G. Luciano, A. Priolo, M. McGee, A.P. Moloney and J.P. Kerry (2018). Concentrate supplementation with dried corn gluten feed improves the fatty acid profile of beef from steers offered grass silage. Submitted to *Food Chemistry*.

Salami, S.A., M.N. O'Grady, G. Luciano, A. Priolo, M. McGee, A.P. Moloney and J.P Kerry (2018). Fatty acid composition, shelf-life and eating quality of beef from steers fed corn or wheat dried distillers' grains with solubles in a supplement to grass silage. Submitted to *Meat Science*.

Salami, S.A., M.N. O'Grady, G. Luciano, A. Priolo, M. McGee, A.P. Moloney and J.P. Kerry (2018). Quality indices and sensory attributes of beef from steers offered grass silage and a concentrate supplemented with dried citrus pulp. Submitted to *Meat Science.*

CONFERENCE ABSTRACTS AND PRESENTATIONS:

Salami, S.A., M.N. O'Grady, G. Luciano, A. Priolo, S. Onakuse, M. McGee, A.P. Moloney and J.P. Kerry (2018). Fatty acid composition and antioxidant potential of beef from steers fed corn or wheat dried distillers' grains in a supplement to grass silage. 2018 ASAS-CSAS Annual Meeting and Trade Show, 8 to 12 July 2018, Vancouver, Canada.

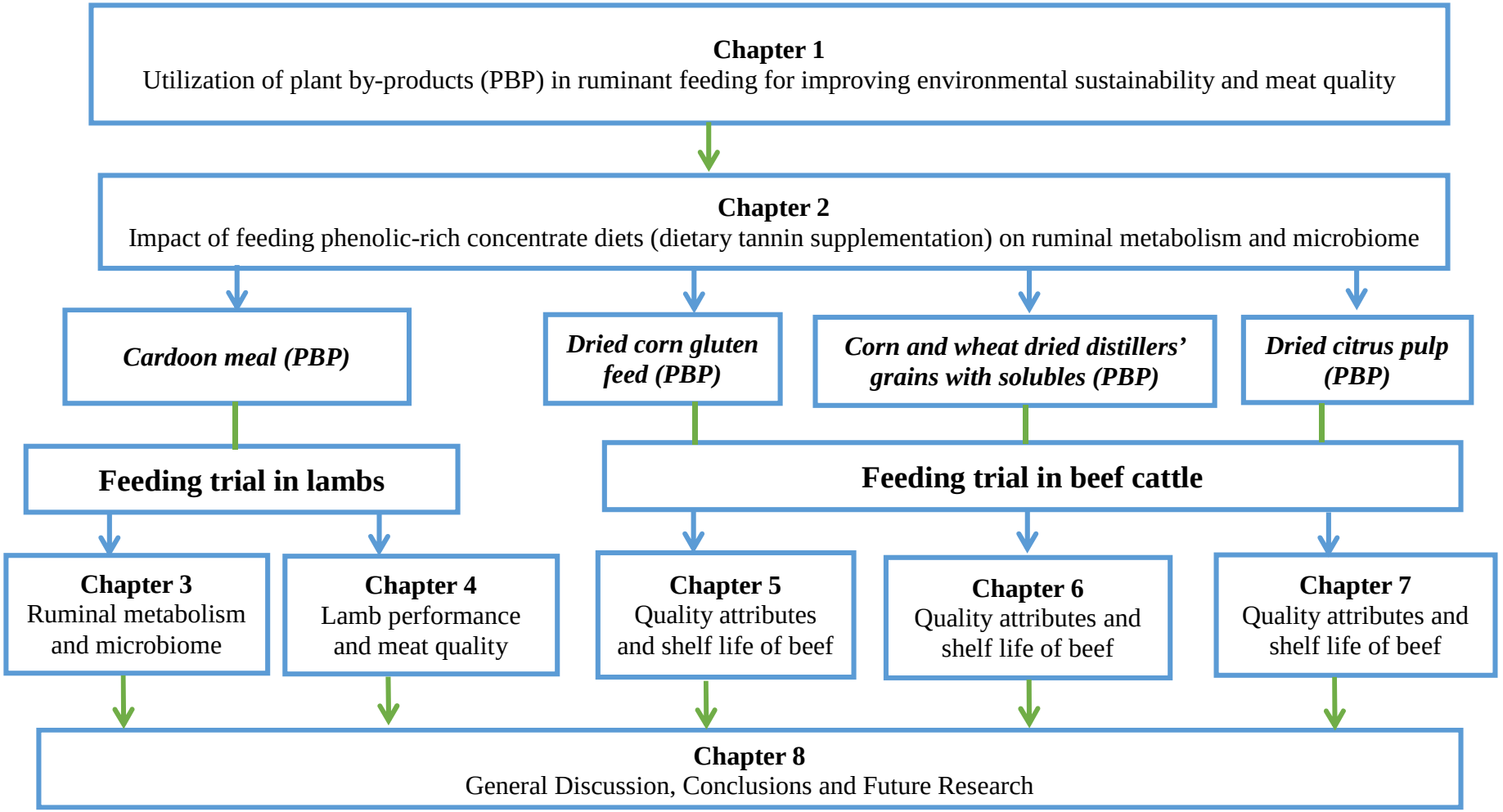
Salami, S.A., M.N. O'Grady, G. Luciano, A. Priolo, S. Onakuse, M. McGee, A.P. Moloney and J.P. Kerry (2018). Inclusion of dried corn gluten feed in a concentrate supplement for grass silage-fed steers improves the fatty acid profile of beef. 2018 ASAS-CSAS Annual Meeting and Trade Show, 8 to 12 July 2018, Vancouver, Canada.

Salami, S.A., B. Valenti, L. Campidonico, G. Luciano, M.N. O'Grady, J.P. Kerry, E. Jones, A. Priolo and C.J. Newbold (2017). Molecular characterization of the rumen microbiome in lambs fed hydrolysable and condensed tannins. 68th Annual Meeting of the European Federation of Animal Science, 28th August to September 2017, Tallinn, Estonia.

Salami, S.A., B. Valenti, G. Luciano, M.N. O'Grady, J.P. Kerry, C.J. Newbold and A. Priolo (2017). Cardoon meal as a novel alternative feed: effect on performance and ruminal microbiome in lambs. 68th Annual Meeting of the European Federation of Animal Science, 28 August to 1 September 2017, Tallinn, Estonia.

Salami, S.A., B. Valenti, G. Luciano, M.N. O'Grady, J.P. Kerry and A. Priolo (2017). Comparative effects of dehydrated alfalfa versus cardoon meal on tissue fatty acid profiles in lambs. 63rd International Congress of Meat Science and Technology, 13 to 18 August 2017, Cork, Ireland.

Schematic Overview of the Thesis



List of Abbreviations

a* - Redness

AD - Atypical dieonic fatty acids

ADF - Acid detergent fibre

ADG - Average daily weight gain

b* - Yellowness

BCFA - Branched chain fatty acids

BW - Body weight

C* - Chroma (colour saturation)

CBP - Carob pulp

CCA - Canonical correspondence analysis

CDGS - Corn distillers' grains with solubles

CF - Crude fibre

CGF - Corn gluten feed

CIE - Commission Internationale de l´Eclairage

CLA - Conjugated linoleic acid

CO_2 - Carbon dioxide

CP - Crude protein

CSM - Cottonseed meal

CT - Condensed tannins

CT-g - Gambier condensed tannin

CT-m – Mimosa condensed tannin

CVD - Cardiovascular disease

DC – Decanter cake

DCP - Dehydrated citrus pulp

DGS - Distillers' grains with solubles

DM - Dry matter

DMI - Dry matter intake

DNA - Deoxyribonucleic acid

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EC - European Commission

EU - European Union

EUROP - Carcass classification system

FA - Fatty acids

FB - feed block

FAME - Fatty acid methyl esters

FAO - Food and Agriculture Organization of the United Nations

FDA - Food and Drug Administration

FID - Flame ionization detector

FICA - Ferrous ion chelating activity

FRAP - Ferric reducing antioxidant power

GBS - Grassland-based system

GC - Gas chromatography

GHG - Greenhouse gas

H_2 - Hydrogen

HPLC - High-performance liquid chromatography

HT - Hydrolysable tannins

HT-c - Chestnut hydrolysable tannin

HT-t - Tara hydrolysable tannin

IMF - Intramuscular fat

L* - Lightness

LCA - Life cycle assessment

LC-PUFA - Long chain polyunsaturated fatty acids

LM - *Longissimus* muscle

LPS - Landless production system

LT – *Longissimus thoracis muscle*

LTL - *Longissimus thoracis et lumborum muscle*

MAP - Modified atmosphere packs

MDA - Malondialdehyde

ME - Metabolizable energy

MFS - Mixed farming systems

MUFA - Monounsaturated fatty acids

***n*-3 -** Omega-3 fatty acids

***n*-6 -** Omega-6 fatty acids

NDF - Neutral detergent fibre

O_2 - Oxygen

OBCFA - Odd- and branched-chain fatty acids

PBP - Plant by-products

PCoA - Principal coordinate analysis

pH - Potential of hydrogen

PKC - Palm kernel cake

PKM - Palm kernel meal

PUFA - Polyunsaturated fatty acids

RBH - Ruminal biohydrogenation

RMPP - Relative methane production potentials

RNA - Ribonucleic acid

rDNA - Ribosomal deoxyribonucleic acid

rRNA - Ribosomal ribonucleic acid

SBP - Sugar beet pulp

SDB - Sage distillation by-product

SEM - Standard error of mean

SFA - Saturated fatty acids

Spp. – Species

TBARS - Thiobarbituric acid reactive substances

TCA - Trichloroacetic acid

TFA - *Trans* fatty acids

TPA - Texture profile analysis

TPC - Total phenol content

UFA - Unsaturated fatty acids

UK - United Kingdom

US - United States

VFA - Volatile fatty acids

WBSF - Warner-Bratzler shear force

WDGS – Wheat distillers' grains with solubles

CHAPTER 1 – Literature Review

This chapter is a substantial part of a manuscript submitted for publication in *Animal Feed Science and Technology* (2018):

Saheed A. Salami, Giuseppe Luciano, Michael N. O'Grady, Luisa Biondi, Charles J. Newbold, Joseph P. Kerry and Alessandro Priolo (2018): Sustainability of feeding plant by-products: a review of the implications for ruminant meat production. *Animal Feed Science and Technology*.

1.1. INTRODUCTION

The global quest for livestock intensification has been driven rapidly by increasing demand for animal proteins due to a concomitant rise in population, urbanisation and household income (Herrero and Thornton, 2013). However, global livestock sector contributes 14.5% of total anthropogenic greenhouse gas (GHG) emissions and the ruminant sector comprises the largest share of approximately 81%, mainly as a result of enteric methane emissions (Gerber et al., 2013). The contribution of ruminants to total GHG emissions from livestock varies between ruminant sectors: cattle meat (35%) and milk (30%), buffalo (8.7%) and small ruminants (6.7%) (Opio et al., 2013). Ruminant meat production is associated with the largest environmental cost compared to other livestock products: beef (300 kg CO_2-eq/kg protein) and small ruminant meat (165 kg CO_2-eq/kg protein) exhibit the highest emission intensities when emissions of livestock commodities are expressed relative to their protein outputs (Gerber et al., 2013).

Feed production, transport, and utilisation play a major role in the environmental metrics of livestock production. Feed production and processing are linked to land-use changes and account for approximately 45% of GHG emissions from the livestock sector (Gerber et al., 2013). About 70% of the global agricultural land (Steinfeld et al., 2006) and 30 - 40% of human-edible feed crops (Erb et al., 2012) are currently utilised for livestock production. In addition, cultivation of conventional forages and fodder feed crops on non-marginal lands will deplete available land for arable crop production. The numerous environmental problems associated with livestock production is further complicated by a resultant increase in food-feed competition which poses a threat to global food security (Makkar, 2016).

Less food-competing feedstuffs (Schader et al., 2015) or *ecological leftovers* (Röös et al., 2016) include human-inedible biomass such as food wastes, crop residues and agro-

industrial by-products. The use of less food-competing feedstuffs in animal diets is a potential strategy which may reduce food-feed competition and mitigate the environmental impacts of livestock. This approach is particularly pertinent when coupled with other strategies such as improvements in livestock productivity and a reduction in the share of animal products utilised in human diets (Schader et al., 2015; Röös et al., 2016; van Zanten et al., 2016a). Large amounts of waste biomass are generated as agricultural and food industry by-products, accounting for approximately 30% of global agricultural production (Ajila et al., 2012). Plant by-products (PBP) include a wide range of secondary residues generated from industrial processing of plants into commercially valuable products (Santana-Méridas et al., 2012). These by-products are considered safe and widely accepted as animal feeds. However, utilisation of PBP in livestock nutrition is limited due to constraints such as variation in nutrient composition and technical requirements for preservation essential for product stabilization and attenuation of seasonal availability. Moreover, preservation techniques such as thermal processing may impose high cost and reduce the environmental sustainability of feeding PBP (Bremer et al., 2011).

Apart from the environmental impact of livestock feeds, animal nutrition is closely linked with other components of livestock production including food security and animal product quality and safety (Makkar, 2016). Nutritional strategies may alter the fatty acid (FA) composition, eating quality and technological characteristics of ruminant meat (Andersen et al., 2005). Thus, it is important to assess if dietary utilisation of PBP could enhance the environmental sustainability of ruminant meat production without compromising the quality attributes and consumer acceptability of meats. Interestingly, some PBP contain bioactive compounds such as vitamins, unsaturated fatty acids, and phytochemicals. Feeding bioactive-rich PBP offers potential to mitigate enteric methane and nitrogen emissions while improving meat quality attributes. However, these effects are addressed

individually in several studies. This review endeavours to investigate the dual-impact of dietary PBP on environmental sustainability and meat quality attributes in ruminant production.

1.2. GLOBAL LIVESTOCK SYSTEMS AND FEED BIOMASS

Global livestock production systems have been broadly divided into two categories based on resource use, agro-ecological distribution and input-output variables: *solely livestock systems* (SLS) and *mixed farming systems* (MFS) (Seré et al., 1996). In relation to feed input, SLS is defined as a production system in which feed resources obtained from pastures, rangelands, forages and commercial feeds represent >90% of the dry matter (DM) consumed by the animals. The SLS could be a *landless production system* (LPS) or *grassland-based system* (GBS) depending on the proportion of feed produced on the farm; the former produced <10% of the dietary DM on the farm while the latter produced >10% of the dietary DM. While GBS is mainly dominated by ruminant livestock, the LPS is an intensive system comprising of both ruminant and monogastric animals and the increased utilisation of PBP can be relevant to enhance the sustainability of this system. In contrast, MFS is a production system in which >10% of the DM consumed by the animals is derived from plant wastes and crop by-products. Mixed farming systems are distributed throughout the world and can be classified based on the proportion of farm production value obtained from rain-fed or irrigated land use (Steinfeld et al., 2006).

Ruminant meat (beef cattle and sheep) accounts for approximately 30% of the total meat output from global livestock production systems with proportional outputs of 5.5%, 25.3% and 69.1% from LPS, GBS and MFS, respectively (Steinfeld et al., 2006). Recent research by Opio et al. (2013) confirmed that MFS are the largest producer of ruminant meat, accounting for 79% beef, 96% buffalo meat and 68% small ruminant meat outputs. Demand

for feed crops, mainly grains and conserved forages, for ruminant production is largely concentrated in LPS and MFS (Erb et al., 2012). For instance, the landless sheep production systems are prevalent in countries in the dry environments of Northern Africa and Western Asia (Steinfeld and Mäki-Hokkonen, 1995). Consumption of ruminant meat exceeds production with ensuing ruminant meat deficit in many countries found in these regions (Nordblom and Shomo, 1995; Aw-Hassan et al., 2008). Small ruminant systems are less dependent on grazing in these regions but improved feeding technologies allowed for the inclusion of 40% crop by-products and 40% grains in animal diets (Nordblom and Shomo, 1995). However, most grains are imported due to deficits in the local production of cereals (Erb et al., 2012).

Feed crops, mostly human-edible, used for livestock feeding varies between different regions of the world (Table 1.1). Erb et al. (2012) revealed that North America and Europe utilised more feed crops while countries in Sub-Saharan Africa and Southern Asia utilized the least quantity of feed crops. Cereals and oil crops including oilseed cakes are the dominant feed crop categories used as animal feed in all regions of the world. The predominant use of cereals as animal feed is peculiar to the developed area in North America, Europe, Oceania, Latin America, Central Asia and Russia. Maize is the major cereal in most regions of the world while North America has the greatest prevailing quantity of cereals used as livestock feed (Erb et al., 2012). There is a local deficit of cereals in North Africa and Western Asia, Latin America, Sub-Saharan African and Asia. However, countries in North Africa and Western Asia, East Asia, South Asia and Western Europe regions lack sufficient local availability of oil crops. Strategies aimed to enhance the use of PBP as animal feeds would be useful to offset importation of feed crops in these regions.

1.3. RUMINANTS AS BIO-RECYCLERS OF PBP

Agricultural residues can be in form of crop residues or PBP. Crop residues represent primary biomass left on the farm following harvesting while PBP is secondary biomass obtained from post-harvest processing of crops into valuable products by the agro-food industry (Johnson and Linke-Hepp, 2007). Theoretically, PBP have better nutritional profiles than crop residues and can serve as viable sources of protein, energy and fibre to meet the nutritional requirements of animals. However, variation in the feeding value of PBP is dependent on the technical conditions employed during manufacture of the primary product. Moreover, the need for efficient preservation techniques is a major constraint limiting the use of PBP in animal nutrition because of their inherent instability which results in rapid quality deterioration and extreme variation in nutrient composition (Ajila et al., 2012; FAO, 2012; Wadhwa et al., 2013).

The ruminant forestomach or rumen contains a diverse microbial population conferring an exclusive physiological adaptation for the greater utilisation of PBP compared to monogastric livestock. The microbial ecosystem inhabiting the rumen comprises of bacteria, fungi, protozoa, archaea, and phages which engage in mutualistic interaction with the host animal. Microbial fermentation in the rumen unleashes nutrients from low-quality, high-fibre diets resulting in the production of volatile fatty acids and microbial

Table 1.1 Regional proportion of total feed crop supply (PFCS) used as livestock feeds and self-sufficiency ratio (SSR) as modelled for year 2000 (adapted from Erb et al., 2012)

Feed crop category	Variables	NA-WA	SSAfrica	CA-Russia	EAsia	SAsia	SEAsia	NAmerica	LAmerica	WEurope	EEurope	Oceania	Global
Cereals	PFCS (%)	33	15	46	30	11	20	78	44	67	62	67	38
	SSR	0.54	0.75	1.00	0.82	0.96	0.93	1.51	0.86	1.07	1.01	2.76	
Roots	PFCS (%)	2	16	14	41	0	11	2	29	35	38	5	25
	SSR	0.98	1.02	1.01	0.99	1.00	1.56	1.01	1.00	0.77	1.00	1.04	
Sugar crops	PFCS (%)	7	10	4	13	4	7	7	9	13	12	16	8
	SSR	0.53	0.94	0.34	0.72	1.09	1.24	0.34	1.87	0.84	0.76	2.68	
Pulses	PFCS (%)	16	9	68	41	8	13	27	1	72	57	84	24
	SSR	0.77	0.97	0.98	1.04	0.91	1.34	2.16	0.89	0.71	1.06	1.69	
[1]Oil crops	PFCS (%)	49	24	51	49	38	32	67	53	72	62	44	53
	SSR	0.42	0.99	1.00	0.7	0.89	1.01	1.61	2.17	0.33	0.96	1.99	
Vegetables and fruits	PFCS (%)	3	0	4	2	0	2	0	1	1	3	0	1
	SSR	1.07	1.12	0.81	0.99	1.01	1.10	0.79	1.47	1.03	1.04	1.39	

NA-WA: North Africa and Western Asia; SSAfrica: Sub-Saharan Africa; CA-Russia: Central Asia and Russia; EAsia: Eastern Asia; SAsia: Southern Asia; SEAsia: South-East Asia; NAmerica: North America; LAmerica: Latin America; WEurope: Western Europe; EEurope: Eastern Europe

[1]Oil seed cakes were included in the estimation of oil crops even though they are not directly human-edible products.

proteins providing the animal with sources of energy and highly digestible proteins, respectively (Loor et al., 2016). Indeed, diet has a significant impact on the composition and diversity of the rumen microbiome. Low or imbalanced nutritional profiles in PBP may contribute to the imbalanced supply of dietary nutrients to the rumen which can negatively influence the stability and function of the rumen microbiota (Loor et al., 2016). There are, however, effective nutritional options exist for improving the feeding value of PBP in ruminant nutrition. Many options focus on manipulating the rumen microbiome to optimise the efficiency of ruminal fermentation and microbial protein synthesis (Wadhwa et al., 2016). These strategies include: balancing the diet with energy, protein and minerals; the dietary addition of non-protein nitrogen; use of dietary supplements (botanical additives, feed enzymes and probiotics); and detoxification of phytotoxins (Wadhwa et al., 2016).

The feeding value and nutritional implications of dietary PBP on animal productivity have been examined in a large number of studies. Figure 1 illustrates PBP that can be derived from numerous industrial production sectors for further utilisation in animal feeding. A detailed description and information on the feeding value of several PBP highlighted in Figure 1 can be retrieved from online repositories of animal feeds; Feedipedia (http://www.feedipedia.org/) and Feed Tables (https://www.feedtables.com/).

By-products containing a high concentration of plant secondary metabolites (Table 1.2) and contaminants such as mycotoxins (Zhang and Caupert, 2012) may elicit anti-nutritional effects and impair ruminant productivity and health. However, the rumen microbiome has considerable potential to degrade contaminants (Upadhaya et al., 2010; Manubolu et al., 2014) and adapt to the anti-nutritional effect of plant bioactive compounds (Smith et al., 2005). Furthermore, by-products of oil extraction containing a high content of residual oil can increase dietary lipids, especially unsaturated fatty acids which may elicit an inhibitory

effect on rumen microbiota and impair rumen function (Maia et al., 2007). Negative effects on rumen function may result in decreased feed intake, nutrient digestibility, and ultimately, impair the performance of ruminant animals. However, the use of PBP in ruminant nutrition can be enhanced through technical options such as the manipulation of physical characteristics; chemical treatments and detoxification; use of feed blocks; ensiling; and processing into feed pellets (Ben Salem and Smith, 2008).

1.4. IMPACTS ON ENVIRONMENTAL SUSTAINABILITY

The environmental impacts of livestock production are related to emissions of GHG, nitrogen and phosphorus, land degradation, water pollution and insecurity, and biodiversity loss (Steinfeld et al., 2006). Feed production, transport, and utilisation play a major role in environmental issues attributed to livestock production (Gerber et al., 2013). The contributions of dietary PBP to environmental stewardship of ruminant production are evaluated in subsequent sections of this review.

1.4.1. Effect of dietary PBP on enteric methane and nitrogen emissions

Greenhouse gas (GHG) emissions from ruminants are composed of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Ruminants emit CH_4 into the environment as a by-product of ruminal fermentation of feed substrates and rumen methane accounts for 2 to 12% loss of ingested gross energy in ruminants (Johnson and Johnson, 1995). Enteric CH_4 emissions from the ruminant sector account for approximately 90% of the total CH_4 emission and 47% of the total GHG emissions from this sector (Opio et al., 2013). Furthermore, excessive microbial hydrolysis of dietary protein in the rumen produces

Table 1.2. Bioactive compounds in plant by-products (PBP) used in livestock feeding (adapted from Santana-Méridas et al., 2012)

Crop classification	PBP	Bioactive compounds
Cereals		
Rice	Bran	Tocotrienols-tocopherols, γ -oryzanol, β-sitosterol
Wheat	Bran	Vitamin E, carotenoids, quinones
Barley	Spent grains	Xylitol, ferulic and *p*-coumaric acids
Legumes		
Pea	Husks	Growth factors
	Pods	Polyphenols
Faba bean	Pods	Polyphenols
Peanut	Skins, seed coats	Polyphenols oligomeric proanthocyanidins, indole alkaloids, phenolic acids
Chicken pean, common bean and faba bean	Off-quality/Discarded grains	Peptides
Sugar crops		
Sugarcane	Bagasse	Pentosans
Beet	Stalk	Azelaic acid
Fruits and vegetables		
Mango	Peels, pits/ seeds	Tannins, vanillin, mangiferin
Apple	Pomace (peels, core, seeds, calyces, stems)	Pectin, catechins, hydroxycinnamates, phloretin glycosides, quercetin glycosides, procyanidins
Watermelon	Peels, Rinds	Lycopene, citrulline, phenolic compounds
Rambutans	Peels	Ellagitannins
Mangosteen	Pericarps	Proanthocyanidins
Guava	Bagasse	Epicatechin, quercetin, syringic acid, mirycetin
Banana	Peels	α-amilasa, laccasa, citric acid
Citrus fruits	Seeds, molasses	Limonoids
	Peels	Flavonoids (hesperidin, diosmin, narirutin, didymin, sinesetin), carotenoids (violaxanthin, β-crytoxanthin, β-carotene), vitamin C, essential oils (limonene)
Pineapple	Peels, core, crowns, stems	Bromelain
Pomegranate	Husk	Poly- and mono-meric phenols
Pawpaw	Peels, seeds	Phenolic compounds
Tomato	Seeds, pulps, skin	Lycopene, β-carotene, sterols, tocopherols, terpenes, glycoalkaloids
Coffee	Spent coffee grounds	Caffeine, chlorogenic acid
Grape	Pomace	Flavonols, flavonols glucosides, flavanols, gallate esters, anthocyanins, proanthocyanins
	Seeds	Epicatechin, caffeic and gallic acids
	Stems	Betulinic acid, stilbenoid transresveratrol, trans-3-viniferin, sitosterol 6'-*O*-acylglucosides
Root and tuber crops		
Potato	Peels	Glycoalkaloids

Table 1.2. Continued

Crop Classification	PBP	Bioactive compounds
Oilseed crops		
Almond	Hulls	Triterpenes (oleanoic, ursolic, betulinic acids), daucosterol
Olive	Olive mill waste water	Hydroxytyrosol, gallic acid, oleuropein, ligstroside isomers and derivatives, squalene, tocopherols, triterpenes, polyphenols
	Pomace	Hydroxytyrosol, tyrosol, caffeic protocatechuic, vanillic, *p*-coumaric and syringic acids, vanillin, oleuropein, apigenin
	Flesh, stones, seeds	Polyphenols, tocopherols
Rapeseed	Meals	Gallic and syringic acids, kaempferol, naringenin
	Defatted cakes	Glucosinolates, peptides
Turnip	Hulls, defatted cakes	Polyphenols
Cotton	Meals	Kaempferol, naringenin, rutin
Soybean	Meals	Caffeic acid, naringenin, daidzein
Sunflower	Defatted cakes	Peptides
Herbs, shrubs and trees		
Lavandin	Solid residues	Phenolic acids, flavonoids, hydroxycinnamoylquinic acid derivatives, glucosides of hydroxycinnamic acids
Rosemary	Solid residues	Phenolic acids (rosmarinic, carnosic, caffeic, chlorogenic acid and *p*-coumaric acids)
Sage	Solid residues	Coumarins, hydrocarbons, monoterpenes, phenolic compounds, sesquiterpenes, diterpenes, triterpenes, fatty acid ester, hydroxycinnamic acid, luteolin
Thyme	Solid residues	Rosmarinic acid, hydroxycinnamic acid, luteolin
Chestnut	Shells	Tannins, polyphenols, tocopherols
Pecan nut	Shells (endocarp)	Poly- and mono-meric phenols
Hazelnut	Skins, hard shells, leafy covers	Phenolic acids (gallic, caffeic, *p*-coumaric, ferulic, sinapic)
Artichoke	Bracts, receptacles, stems, juice, heads	Neochlorogenic acid, chlorogenic acid, caffeoylquinic acids
Pine	Vinegar	Aldehydes, ketones, esters, phenols (cresols)
	Bark	Polyphenols

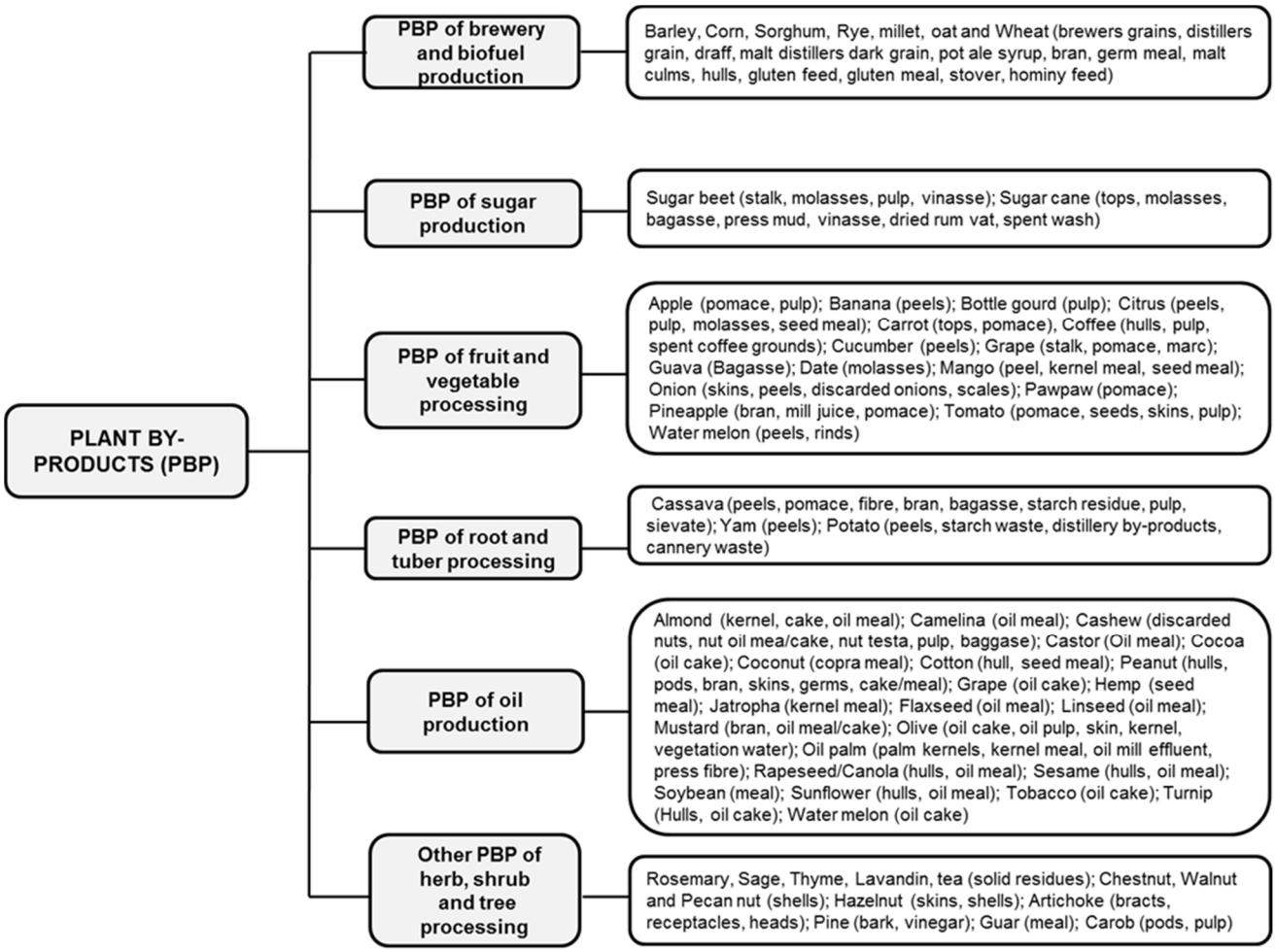


Figure 1.1. Schematic diagram of plant by-products from different agro-industrial processes.

ammonia (NH_3) used for urea synthesis, which contributes significantly to the pool of urinary and faecal nitrogen (N) losses in ruminants. Nitrogen losses are associated with serious environmental concerns such as nitrate leaching and accumulation in the soil, and N_2O emission resulting from the conversion of volatilized NH_3 (Steinfeld et al., 2006). Both CH_4 emissions and N losses are inefficient processes which undermine the productivity of ruminants. Similarly, CH_4 and N_2O are important GHG emissions contributing to climate change due to their high global warming potential of 25 and 298 times greater than that of CO_2, respectively (Solomon et al., 2007).

Feeding strategies are important abatement options to mitigate CH_4 and N_2O emissions in livestock production (Montes et al., 2013). Dietary lipids and phytochemicals such as tannins, saponins, and essential oils have the potential to influence rumen microbial diversity and fermentation and thus inhibit ruminal methanogenesis (Hart et al., 2008; Kamra et al., 2012). Moreover, phenolic groups in phytochemicals such as tannins could enhance their complexation with proteins and consequently decrease microbial degradation of proteins in the rumen (Patra et al., 2012). Accordingly, feed resources containing these phytochemicals might reduce N losses and improve the efficiency of dietary protein utilisation. Some PBP obtained from biofuel and brewery productions and vegetable oil production may contain high levels of residual unsaturated fats. Similarly, polyphenols, saponins and essential oils are abundant in by-products of oilseed extraction, and fruit and vegetable processing (Table 1.2). Data from *in vitro* and *in vivo* ruminal studies indicated that selective utilisation of PBP in ruminant rations can contribute to nutritional strategies aimed at reducing CH_4 emission and N losses. To date, most of the available literature is focused on the dietary effect of by-products of distillery and biofuel production, oilseed processing and fruit and vegetable processing.

1.4.1.1. By-products of distillery and biofuel production

Biofuel co-products obtained from wet or dry milling process of cereals may contain a considerable amount of fat (FAO, 2012). Crude glycerol, obtained as a by-product of biodiesel production, could serve as a significant energy source in ruminant diets (Vongsamphanh et al., 2017). Several *in vitro* ruminal studies have reported conflicting results on the inhibitory effect of glycerol on methane production. In a recent study, a meta-analysis of 13 experiments and 42 treatments quantified the effect of glycerol in *in vitro* ruminal studies (Syahniar et al., 2016). Results indicated that increasing levels of glycerol reduced the acetate/propionate ratio accompanied by a decrease in methane production without a detrimental effect on gas production and *in vitro* digestibility. An *in vivo* anti-methanogenic effect has also been confirmed in cattle fed supplementary glycerol (up to 10.8% DM diet) with concomitant increases in growth rates (Vongsamphanh et al., 2017).

Furthermore, distillers' grains only or distillers' grains with solubles (DGS) have increasingly become an important source of energy, protein and fibre in ruminant rations due to rapid expansion in the use of cereals in the biofuel industry (FAO, 2012). Dietary inclusion of 40% DM of high-fat corn-dried DGS significantly reduced CH_4 emission (as % of gross energy intake) by approximately 15% and 20% (compared to a barley-based control diet) in growing beef cattle (Hünerberg et al., 2013a) and finishing beef cattle (Hünerberg et al., 2013b), respectively. Decreased CH_4 production due to the inclusion of 60% corn-dried DGS was also reported in an *in vitro* fermentation study (Wu et al., 2015). The anti-methanogenic effect of dried DGS may be related to its lipid constituents and dietary inclusion level. The inclusion of 40% DM wheat-dried DGS did not affect CH_4 production whereas addition of corn oil to the wheat-dried DGS reduced CH_4 emission in cattle (Hünerberg et al., 2013a,b). Similarly, there was no effect on CH_4 emission when steers were fed up to 27% corn-dried DGS diets (Segers et al., 2015) and when 25% corn-

dried DGS diets with differential fat levels (low *vs.* moderate) were incubated *in vitro* (Keomanivong et al., 2015). Moreover, the replacement of soybean meal, alfalfa hay and soybean hulls with up to 47% DM wheat-dried DGS elevated *in vitro* CH_4 production and decreased lamb performance *in vivo* (Avila-Stagno et al., 2013).

Contrary to a possible reduction in CH_4 production, dried DGS increased *in vitro* NH_3 concentration (Khiaosa-Ard et al., 2015; Wu et al., 2015) and nitrogen excretion in growing and finishing cattle (Hünerberg et al., 2013a,b). Similarly, dietary inclusion of up to 60% corn-wet DGS for feedlot steers increased N and phosphorus (P) intakes, resulting in a linear increase in total N and P excretion (Spiehs and Varel, 2009). These observations signify that dietary utilisation of DGS may constitute important environmental concerns in terms of N and P losses, which may counterbalance the environmental benefits accrued from a reduction in CH_4 emission. In this regard, dietary inclusion of DGS in ruminant diets requires careful diet formulation not to exceed the N requirement of the animal (Hünerberg et al., 2014). Moreover, an appropriate manure management strategy should be considered for managing the excess N and P excretions that may result from feeding DGS (Yang and Li, 2017). Further research is necessary to evaluate the optimum fat level in DGS and its dietary inclusion level required to exert consistent anti-methanogenic effects without increasing N and P losses.

1.4.1.2. By-products of oilseed processing

By-products of vegetable oil extraction may contain a high concentration of unsaturated fat due to potential residual oil content. In an exploratory *in vitro* study comparing the CH_4 production potential of 26 feed ingredients, Lee et al. (2003) concluded that the CH_4 production potential of oilseed meals are generally lower than those of brans and hulls, and grains. Indeed, there is a clear discrepancy in the relative methane production potentials

(RMPP) of different oilseed meals in comparison to other conventional feedstuffs (Table 1.3). Estimation of RMPP showed that oilseed meals and cakes generally have lower CH_4 production potentials compared to cereals, with a greater reduction when compared to corn. However, coconut meal, Brazilian soybean meal and perilla meal had higher RMPP than barley, while the comparison between oilseed meals and cakes also showed apparent differences in RMPP (Table 1.3). In particular, cottonseed meal had a lower RMPP than corn and barley, while corn gluten meal displayed a consistently lower RMPP compared to corn, barley and soybean meal. Kim et al. (2013) demonstrated that cottonseed meal exhibited the lowest potential for *in vitro* CH_4 production in comparison to soybean meal, rapeseed meal, coconut meal, lupine seed, brewers' dried DGS, corn-dried DGS, corn gluten feed, perilla meal and whole soybean. The CH_4 production potential of oilseed meals was reported to be in an ascending order of canola meal < soybean meal < coconut meal < corn germ meal < rapeseed meal < sunflower meal < cottonseed meal < palm kernel meal < corn gluten meal (Lee et al., 2003).

Furthermore, mustard seed cakes (Kumar et al., 2007) and moringa seed press cake (Olivares-Palma et al., 2013) were highlighted to have considerable potential to inhibit *in vitro* CH_4 emission without compromising rumen fermentation. Potential negative effects of oilseed by-products on ruminal fermentation can be related to increased dietary fat levels (Benhissi et al., 2014). The potential of these feedstuffs to inhibit CH_4 production correlates with their increasing fibre, protein and fat contents (Lee et al., 2003). A further study associated low CH_4 production potential with a high amount of rumen undegradable protein in PBP such as cottonseed cake (Lamba et al., 2014). In summary, data from *in vitro* studies suggest the possibility of reducing methane emission with oilseed meals and cakes in ruminant rations.

However, there is limited information on the *in vivo* anti-methanogenic effect of oilseed meals, kernels, and cakes in ruminants. Increasing the fat level of a low-fat rapeseed meal basal diet from 3.5% DM to 5.5% DM with a partial replacement of 15.6% DM rapeseed cake decreased CH_4 production by 9.1% in Holstein dairy cows without detrimental effects on animal performance (Brask et al., 2013). Dietary inclusion of 27% hominy meal only (HM) and a combination of 12.3% cold-pressed canola meal and 14.4% hominy meal (CCHM) in total mixed rations increased total dietary fat to 6.5% and 5.2% DM, respectively compared to 2.6% DM in the basal diet (Moate et al., 2011). The HM and CCHM diets reduced CH_4 emission (as g/L milk) by approximately 13.6% and 13.1%, respectively, without impairing the performance of Holstein cows. However, *in vivo* anti-methanogenic effects reported in other bovine studies were accompanied by concomitant negative effects on animal performance. Partial replacement of cassava chip and soybean meal with 7.1% coconut kernel increased total fat levels in cattle diets to 5.7% DM compared to 1% DM in the control diet resulting in reduced levels of CH_4 emission by approximately 48% (as L/kg DMI) (Chuntrakort et al., 2014). Nonetheless, dry matter intake and digestibility decreased even though ruminal fermentation was not affected. Similarly, a 50:50 forage:concentrate feeding trial revealed that inclusion of 86% copra meal (otherwise called coconut meal) in a cattle concentrate diet increased total dietary fat levels from 1% to 8.4% DM (Jordan et al., 2006). The copra meal diet suppressed CH_4 emission by 14.4% (as kg/DMI) but decreased feed digestibility and weight gain of beef heifers (Jordan et al., 2006). However, the inclusion of coconut meal (up to 1.0% live weight per day) in a diet containing cassava pulp and elephant grass, decreased methane emission and improved growth rates and feed efficiency in growing cattle (Duy and Khang, 2016).

By-products including those from pistachio and olive oil extraction can contain a considerable amount of phenolic compounds particularly tannins. In a study reported by Denek et al. (2017), the addition of 2 – 10% pistachio by-products to corn silage reduced *in vitro* CH_4 production but negatively affected *in vitro* ruminal fermentation through decreased gas production and organic matter digestibility. Similarly, the inclusion of 30 - 40% pistachio by-products in sheep or goat diets improved *in vivo* nitrogen metabolism (Ghasemi et al., 2012a) due to decreased ruminal protein degradation (Ghasemi et al., 2012b; Ghaffari et al., 2014). Other studies showed that olive fruit skin and pulp reduced *in vitro* CH_4 production (as mL/g DM) up to 38.5% compared to substrates of oaten chaff and commercial concentrates (Shakeri et al., 2017). Moreover, the inclusion of 12.8% two-stage olive cake in a concentrate diet fed together with alfalfa hay decreased urinary N losses in goats and sheep (Yáñez-Ruiz and Molina-Alcaide, 2007). However, both experiments resulted in negative effects in terms of decreased gas production and volatile fatty acids (Shakeri et al., 2017) and depression of nutrient digestibility (Yáñez-Ruiz and Molina-Alcaide, 2007).

Table 1.3. Relative methane production potential (RMPP %) of oil seed meals and cakes in comparison to corn, barley, groundnut cake and soybean meal following 24 h *in vitro* incubation

	RMPP %			
	[1]Corn	[2]Barley	[3]Groundnut cake	[4]Soybean meal
Corn germ meal	-41.2	-	-	-
Sunflower meal	-48.5	-	-	-
Palm kernel meal	-62.0	-	-	-
Coconut meal	-35.8	+8.81	-	-
Cotton seed meal	-56.6	-58.01	-	-
Soybean meal	-30.9	-	-	-
Soybean meal (Brazil)	-	+70.62	-	-
Soybean meal (Korea)	-	-7.22	-	-
Canola meal	-27.9	-	-	-
Rapeseed meal	-44.9	-9.67	-	-
Perilla meal	-	+83.10	-	-
Soybean cake	-	-	+13.90	-
Mustard seed cake	-	-	-8.38	-31.42
Deoiled mustard cake	-	-	-	-34.63
Deoiled groundnut cake	-	-	-	-24.63
Maize oil cake	-	-	-	-1.99
Cotton seed cake	-	-	+3.11	-62.42
Karanj seed cake expeller extracted	-	-	-25.68	-
Karanj seed cake solvent extracted	-	-	-22.42	-
Caster bean cake expeller extracted	-	-	-20.08	-
Caster bean cake solvent extracted	-	-	-17.96	-

[1]Lee *et al.,* 2003 (absolute CH_4 value expressed as ml/0.2 g DM) ; [2]Kim *et al.,* 2013 (absolute CH_4 value expressed as ml/g DM); [3]Kumar *et al.,* 2007 (absolute CH_4 value expressed as ml/g DM); [4]Lamba *et al.,* 2014 (absolute CH_4 value expressed as ml/g DM)

1.4.1.3. By-products of fruit and vegetable processing

The anti-methanogenic potential of fruit and vegetable by-products can be mainly attributed to the presence of residual phenolic compounds including tannins. However, the efficacy of these compounds can vary with dietary concentration, structural features, diet type, and ruminant species. Recently, Kobayashi et al. (2016) provided a detailed review highlighting the anti-methanogenic potential of some PBP in the Asian continent with an emphasis on cashew nut shell liquid (CNSL), tea by-products and ginkgo fruit. These PBP were notable for their phenolic content which potentially modulates the rumen microbiota to reduce methane emissions and protein degradation in the rumen (Kobayashi et al., 2016). However, thermal processing of CNSL may impair its functional potency in inhibiting ruminal methane production (Kobayashi et al., 2016).

An *in vitro* study conducted using a rumen simulation technique indicated that fortification of dried DGS with 1 to 20% grape seed meal resulted in a linear decrease in CH_4 emission and an associated decrease in the proportion of *Methanobrevibacter spp.* as part of the rumen archaeal community (Khiaosa-Ard et al., 2015). Similarly, dried and ensiled grape pomace reduced methane emission up to 22.6% with an associated alteration in rumen bacteria and archaeal communities in dairy cows fed at an inclusion rate of 5 kg DM/d in partial replacement for alfalfa hay (Moate et al., 2014). The anti-methanogenic effect of grape by-products in these experiments was attributed to potentially high concentrations of dietary fat, total phenols, tannins, lignin and tartaric acid. Romero-Huelva and Molina-Alcaide (2013) noted that dietary condensed tannin content could be increased by substituting 50% cereal-based concentrate with a tomato waste- or cucumber waste-based feed block (FB) in a 50:50 alfalfa hay:concentrate feeding regime. This study demonstrated that tomato waste FB suppressed CH_4 emission by up to 28% in goats but both tomato waste- and cucumber waste-based FB reduced nitrogen retention which could elevate the

pool of nitrogen emission into the environment. In another study with a similar experimental design, Romero-Huelva et al. (2012) reported that a lower level of replacement of cereal-based concentrate with 35% tomato waste- and cucumber waste-based FB consistently reduced CH_4 emission and lowered urinary N excretion without hampering nutrient utilization. The potential of tomato wastes to reduce ruminal CH_4 production has been attributed to its high content of rumen undegradable protein (Lamba et al., 2014) that may have decreased the N supply for microbial growth and consequently, affected rumen fermentation. The anti-methanogenic effect of olive by-products and tomato wastes may not be related to rumen microbial changes but rather to an alteration of rumen fermentation favouring propionate formation and lower the acetate/propionate ratio (Romero-Huelva and Molina-Alcaide, 2013; Shakeri et al., 2017).

However, partial replacement of a concentrate diet with high-fibre fruit and vegetable by-products rich in phenolic compounds may not always be effective in reducing CH_4 emissions. This has been demonstrated *in vivo* in studies involving replacement of concentrates with beet pulp (Ibáñez et al., 2015), citrus pulp (López et al., 2014), almond hulls and citrus pulp (Durmic et al., 2014) and potato by-product (Pen et al., 2006). These observations could be associated with the high level of non-structural carbohydrates in high-fibre diets, which increased the acetate/propionate ratio and CH_4 emissions in ruminal fermentation (Johnson and Johnson, 1995). Thus, the anti-methanogenic effect of fibrous PBP rich in bioactive compounds could be dependent on dietary inclusion level. For instance: the inclusion of 50% almond hulls suppressed *in vitro* CH_4 production by 26% (Durmic et al., 2014) whereas there was no *in vivo* effect with 17% dry matter intake (DMI) of almond hulls in dairy cows (Durmic et al., 2014).

1.4.2. Life cycle assessment of feeding PBP for ruminant meat production

The production of ruminant meat results in greater GHG emissions compared to other livestock products – milk and meat from monogastric animals (Table 1.4). There are several global advocacies for the adoption of zero or reduced meat diets for humans with much emphasis on ruminant meat due to its high environmental impacts (Hedenus et al., 2014). However, it can be argued that the sustainability of livestock sectors should be shaped by the conversion of human-inedible inputs to human-edible animal proteins (Wilkinson, 2011). Feed efficiency is the major driver of resource use and environmental sustainability of livestock systems and ruminants are known to be less efficient than monogastric livestock (Capper and Bauman, 2013). The replacement of human-edible feeds by human-inedible feedstuffs could be a viable way to improve the feed efficiency use of ruminants relative to their edible outputs (Wilkinson, 2011). With reference to the livestock systems in the USA and South Korea, the efficiencies of energy and protein production from beef have been estimated to be better than those of pig and poultry meat when expressed relative to the human-edible inputs (Gill et al., 2010).

Recent life cycle assessment (LCA) studies demonstrated that sustainable animal diets based on human-inedible forages and PBP are potent complementary strategies for improving the environmental sustainability of livestock systems (Schader et al., 2015; Pardo et al., 2016; Röös et al., 2016). These environmental benefits include a reduction in GHG, N and P emissions; better soil conservation; a decrease in the use of pesticide, freshwater and non-renewable energy; and reduction in arable land use. Moreover, simulation of different feeding scenarios for 2050 indicated that strategies based on feeding human-inedible biomass to livestock could provide equal amounts of human-digestible energy and protein/calorie ratio compared to feeding human-edible concentrate feeds (Schader et al., 2015). Inedible feed biomasses include forages, food wastes, crop residues,

and agro-industrial by-products. However, there are constraints associated with the adoption of inedible feed biomasses in sustainable livestock diets. The use of crop residues as animal feed could diminish soil organic carbon and therefore, decomposition on the field has been proposed (Van Zanten et al., 2016b). Furthermore, the use of food wastes and animal by-products as animal feeds is usually subjected to strict regulatory control because of the high potential for transmitting infectious zoonotic pathogens into the food chain (Sapkota et al., 2007).

Ruminants are fed forages and fodder crops for most parts of the production cycle. The exploitation of forage development in marginal lands can provide ecosystem services and could be a viable strategy to reduce food-feed competition and improve global food security (Chaudhry, 2008). However, this strategy may conflict with the objective of reducing the environmental footprint of ruminant production especially when forages and fodder crops are cultivated in lands suitable for arable cultivation (Röös et al., 2016). Moreover, high-fibre feeds based on forages and crop residues result in higher enteric methane emission per kg of product compared to high grain rations (Johnson and Johnson, 1995). Low feed efficiency associated with forage feeding may further hamper the intensification of ruminant production to meet increased demand for animal proteins. Also, the cultivation of conventional feed roughages (grass and legume pasture) occupies a significant proportion of land and requires an appreciable amount of inputs (Flachowsky et al., 2017). Thus, the choice of inedible feedstuffs requires careful consideration for strategies aimed at improving the sustainability of ruminant production. The use of PBP as animal feed is generally subjected to a more relaxed regulatory appraisal and they are practical for developing concentrate feed mixture in ruminant production. In this regard, we postulated that increasing dietary replacement of conventional forages and fodders with PBP could further improve the environmental performance of ruminant meat production.

To test this hypothesis, a simulation study was conducted to assess the environmental impacts of increasing substitution of PBP for conventional roughages (forages and fodder) in the rations of meat-producing ruminants, using the Global Livestock Environmental Assessment Model (GLEAM, http://www.fao.org/gleam/en/). GLEAM was developed by the Food and Agriculture Organization of the United Nations (FAO) as an LCA simulation tool that allows for the quantification of multiple environmental impacts of livestock production. We designed three feeding intervention scenarios (FIS) which were assessed in the model by increasing the feeding levels of PBP as a replacement for fresh grass, hay, and silage in the rations of beef cattle, sheep meat, and goat meat herds (See appendix: Table S1.1). The FIS default, FIS1, FIS2 and FIS3 contain 20%, 30%, 40% and 50% PBP, respectively. It should be noted that the current version of GLEAM only allows for the selection of a broad category of PBP, such as dry by-products from grain industries, as indicated in Table S1.1. The US was selected as the reference country and default information relating to herd structure and manure management were kept constant in all scenarios assessed in the model. The model outcomes demonstrated that increasing levels of PBP resulted in a linear decrease in total GHG emissions from beef cattle, sheep meat, and goat meat herds (Figure 1.2). Indeed, the reduction of GHG losses suggests that dietary utilisation of PBP may enhance resource efficiency in ruminant meat production (Gerber et al., 2013). The decrease in GHG losses was largely related to a reduction in total N_2O emissions in all ruminant meat systems assessed. This was mainly attributed to decrease in N_2O emissions from fertilization and crop residues, manure application and deposition. This observation is consistent with the results from a different LCA model which demonstrated that dietary substitution of concentrates with olive or tomato by-products silages reduced GHG emissions attributed to N_2O emissions from feed production stages in dairy goat production of Spain (Pardo et al., 2016).

In comparison to FIS default, total N_2O emissions in beef cattle, sheep meat and goat meat systems were lower for FIS1 (-14.8%, -15.0%, -15.1%), FIS2 (-24.0%, -32.2%, -32.4%) and FIS3 (-38%, -45.4%, -45.6%), respectively. Total CH_4 emissions in beef cattle, sheep meat and goat meat systems linearly reduced for FIS1 (-2.4%, -4.0%, -4.1%), FIS2 (-6.8%, -6.1%, -6.3%) and FIS3 (-8.8%, -10.3%, -10.6%), respectively when compared to FIS default. However, in comparison to FIS default, there was only a slight decrease in total CO_2 emissions in beef cattle, sheep meat and goat meat herds for FIS1 (-1.2%, -1.1%, -1.1%), FIS2 (-2.2%, -5.6%, -5.2%) and FIS3 (-7.4%, -4.3%, -4.0%), respectively. The total CO_2 emissions in goat meat and sheep meat systems were higher in FIS3 than FIS2, suggesting a diminishing eco-benefit when PBP are substituted above a certain threshold in such systems. In this scenario, the increase was primarily due to elevated CO_2 emissions from feed production, transport and processing. Indeed, technological conversion of PBP to animal feed could undermine their environmental benefits, especially when subjected to an energy-demanding process such as thermal processing. In support of this, an LCA study conducted on the use of DGS in feedlot cattle indicated that wet DGS resulted in a greater reduction of GHG emissions compared to partially or completely dried DGS (Bremer et al., 2011). Thus, harnessing the environmental benefit of feeding PBP demands that eco-friendly and efficient preservation techniques should be used in their processing and dietary utilisation of PBP should be used locally to reduce GHG emissions from feed transport.

Although GLEAM provides empirical data on the potential environmental stewardship of livestock production under different feeding scenarios, it should be noted that the result is not definitive and should be interpreted with caution. This simulation tool is still under continuous refinement and may therefore not be a total reflection of the environmental impacts of PBP in ruminant feeding systems. Moreover, GLEAM does not account for the impact on other environmental categories such as biodiversity loss, acidification potential,

eutrophication potential, and water use. The adoption of feeding strategies based on increased levels of PBP in diet formulation also demands adequate consideration in terms of the nutritional effect on animal productivity.

Table 1.4. Greenhouse gas (GHG) emissions per unit of livestock product (adapted from Gill et al. 2010)

Product	GHG (kg CO_2e)		
	Per kg product	Per MJ human-edible product	Per kg human-edible protein
Milk	1.0	0.37	28.6
Ruminant meat			
Beef	13	1.4	93.5
Sheep	13	1.51	92.9
Non-ruminant meat			
Poultry	2.7	0.4	18.4
Pig	3.9	0.3	34.2

CO_2e: carbon dioxide equivalents

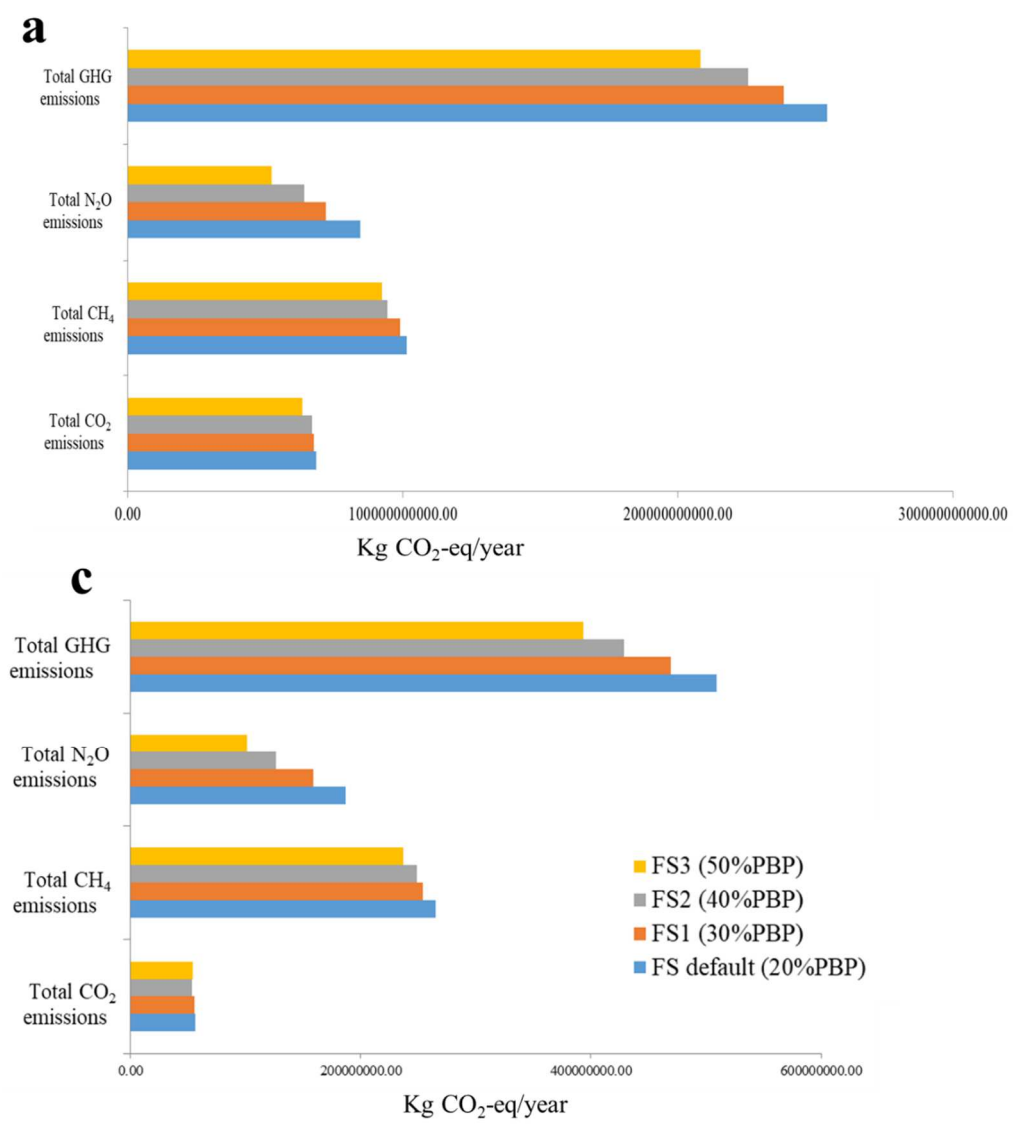


Figure 1.2. Impact of increased dietary inclusion of plant by-products (PBP) on emission intensities of (a) beef cattle herd (b) meat sheep herds (c) meat goat herds in the US, based on simulation results obtained with GLEAM. Feeding scenario (FS) default represents a diet with 20% inclusion level of PBP; FS1 represents a diet with 30% inclusion level of PBP; FS2 represents a diet with 40% inclusion level of PBP; FS3 represents a diet with 50% inclusion level of PBP.

GHG: Greenhouse gases

1.5. IMPACTS OF FEEDING PBP ON RUMINANT MEAT QUALITY

Ruminant meat can play a significant role in healthy human diets as a protein-rich food with a low glycemic index and a viable source of bioactive peptides, vitamins, minerals and conjugated linoleic acids (CLA) (Biesalski, 2005). It is well-established that nutrition plays a prime role in regulating physicochemical and metabolic traits of muscle development in farm animals, which contribute to the nutritional, organoleptic and shelf-life quality of meat (Geay et al., 2001). The nutritional value of meat is most relevant for human health while organoleptic traits such as colour, texture, and flavour strongly influence consumers' perception and purchasing decisions (Wood et al., 2008). Ruminant meat is low in health-promoting polyunsaturated fatty acids (PUFA) and has high levels of saturated fatty acids (SFA) and *trans* fatty acids (TFA) associated with cardiovascular disease and carcinogenic ailments in humans (Cross et al., 2007; Kontogianni et al., 2008). Interestingly, PBP containing residual oil (unsaturated fats) and phytochemicals could inhibit ruminal biohydrogenation and enhance endogenous synthesis of beneficial FA in muscle tissues (Vasta and Luciano, 2011). This could result in increased PUFA and CLA contents and a reduced SFA level and *n*-6/*n*-3 PUFA ratio in ruminant meat (Vasta and Luciano, 2011). Moreover, feeding phytochemical-rich PBP can enhance the deposition of bioactive antioxidant compounds in muscle tissues, thereby enhancing meat shelf-life and facilitating the development of functional meat products (Vasta et al., 2008).

1.5.1. Improving fatty acid composition of ruminant meat through modulation of ruminal biohydrogenation

Ruminant diets (forages and grains) contain unsaturated fatty acids (UFA) - C18:1 *n*-9 (oleic acid), C18:2 *n*-6 (linoleic acid) and C18:3 *n*-3 (linolenic acid). Metabolism (lipolysis and biohydrogenation) of dietary lipids in the rumen is the major factor contributing to the

low content of polyunsaturated FA (PUFA) and high saturated FA (SFA) and *trans* FA (TFA) levels in ruminant meat and milk (Bessa et al., 2015). This typical lipid profile in ruminant-derived foods is associated with an increased incidence of cardio-metabolic diseases and cancer in humans (Givens, 2005). Nonetheless, ruminal biohydrogenation (RBH) also produces functional FA intermediates, such as *trans*-11 18:1 (vaccenic acid) and *cis*-9, *trans*-11 conjugated linoleic acids (CLA, rumenic acid), with potential benefits on human health (Buccioni et al., 2012).

RBH is considered as a detoxifying mechanism to minimize the negative effect of unsaturated FA on the functional activity of rumen microbiota (Maia et al., 2007). RBH comprises of several steps, depending on UFA, and several pathways, depending on diet and ruminal environment (Jenkins et al., 2008; Bessa et al., 2015). Figure 1.3 showed the predominant RBH pathway (isomerization and hydrogenation) of oleic, linoleic and linolenic acids. Bacteria are thought to be the most active microbes responsible for RBH. Rumen bacteria that are capable of hydrogenating dietary PUFA have been previously isolated and studied (Jenkins et al., 2008). Earlier culture-dependent studies noted that bacteria belonging to *Butyrivibrio* genera are of principal importance in RBH (Jenkins et al., 2008). For instance, *Butyrivibrio fibrisolvens* is known for its ability to isomerize linoleic acid to rumenic acid, and for its further biohydrogenation to vaccenic acid (Wallace et al., 2007). However, current evidence has shown that there is limited knowledge on the different bacteria responsible for RBH pathways under different dietary conditions (Enjalbert et al., 2017). Recent advances in molecular techniques suggest that production of several RBH intermediates is associated with many as-yet uncultured bacterial groups such as *Prevotella*, *Lachnospiraceae incertae sedis*, *Ruminococcaceae*, *Bacteroidales*, *Clostridiales* and *Succinivibrionaceae* (Huws et al., 2011; Castro-Carrera et al., 2014; Toral et al., 2016).

Several biohydrogenation intermediates have been identified in ruminant edible fats including *trans*-18:1 isomers, CLA, CLnA, and non-conjugated diene isomers (Vahmani et al., 2015). There is a special research interest to increase the percentage of *cis*-9, *trans*-11 CLA in ruminant meat and milk because of its protective effects against cancers, cardiovascular diseases, obesity, bone density loss, and diabetes in humans (McGuire and McGuire, 2000). The availability of *cis*-9, *trans*-11 CLA in ruminant meat and milk is derived from its synthesis during ruminal biohydrogenation and endogenous desaturation of *t*-11 18:1 via Δ-9-desaturase activity in ruminant tissues (Bessa et al., 2015). However, endogenous synthesis is considered as the predominant source, accounting for up to 95% of *c*-9,*t*-11 CLA in ruminant meat and milk (Palmquist et al., 2005). Therefore, increasing ruminal synthesis of *trans*-11 18:1 could be a strategy to increase *de novo* synthesis and eventual concentration of *c*-9, *t*-11 CLA in ruminant meat.

Feeding forage based diets increase the concentration of beneficial PUFA and biohydrogenation intermediates such *trans*-11 18:1 and *cis*-9, *trans*-11 CLA in ruminant meat (Scollan et al., 2014). However, concentrate diets containing high amounts of rapidly-fermented starch increase the rate of volatile fatty acid production in the rumen, resulting in a decline in rumen pH which is accompanied by a shift from *trans*-11 18:1 to *trans*-10 18:1 containing biohydrogenation pathways. *Trans*-10 18:1 has been associated with up-regulation of fat and cholesterol synthesis, and cytotoxicity in cell culture (Vahmani et al., 2016, 2017). Replacing starchy-grains with highly-digestible, low-starch PBP in concentrate diets may be an effective strategy to prevent a RBH shift from *trans*-11 18:1 to *trans*-10 18:1 and increase the accumulation of UFA in meat (Oliveira et al., 2017).

Animal dietary strategies, including feeding PBP, that increase the ingestion of UFA and polyphenols potentially modulate the rumen microbiome and inhibit RBH, and consequently increase the accumulation of health-promoting unsaturated FA in meat or

milk (Buccioni et al., 2012; Vasta et al., 2008). Dietary fat sources, especially those rich in UFA, have been reported to reduce RBH by decreasing the abundance of hydrogenating bacteria such as *Butyrivibrio, Pseudobutyrivibrio,* and *Clostridium proteoclasticum* (Enjalbert et al., 2017). In addition, decreased abundances of cellulolytic species such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus* have been associated with high amount of dietary PUFA (Maia et al., 2007). In an extensive review of literature, Vasta and Bessa et al. (2012) noted that feeding phytochemicals (tannins, saponins and essential oils) can increase the concentration of PUFA and bioactive FA intermediates (vaccenic and rumenic acids) in ruminant meat and milk by manipulating RBH through an action on the rumen bacterial and protozoal community. However, this effect is inconsistent across studies and can be influenced by factors such as structural complexity of phytochemicals, dose-response effect, type of diets and animal species (Vasta and Bessa et al., 2012; Vasta et al., 2008).

1.5.2. Enhancing oxidative stability of ruminant meat

Lipid oxidation is a major cause of quality deterioration and reduced consumer acceptability during the shelf life of meat. Negative effects of lipid oxidation manifest as rancid flavour development and colour deterioration resulting from the conversion of oxymyoglobin (red) to metmyoglobin (brown) (Morrissey et al., 1998). The susceptibility of meat to oxidative deterioration can be influenced by factors such as the fatty acid composition (polyunsaturated lipids), storage (state and access of oxygen) and processing factors such as catalysts (such as trace metals and enzymes), light exposure and temperature (Bekhit et al., 2013). Figure 1.5 shows the schematic oxidative steps/pathways (initiation, propagation and termination) involved in the peroxidation of polyunsaturated lipids.

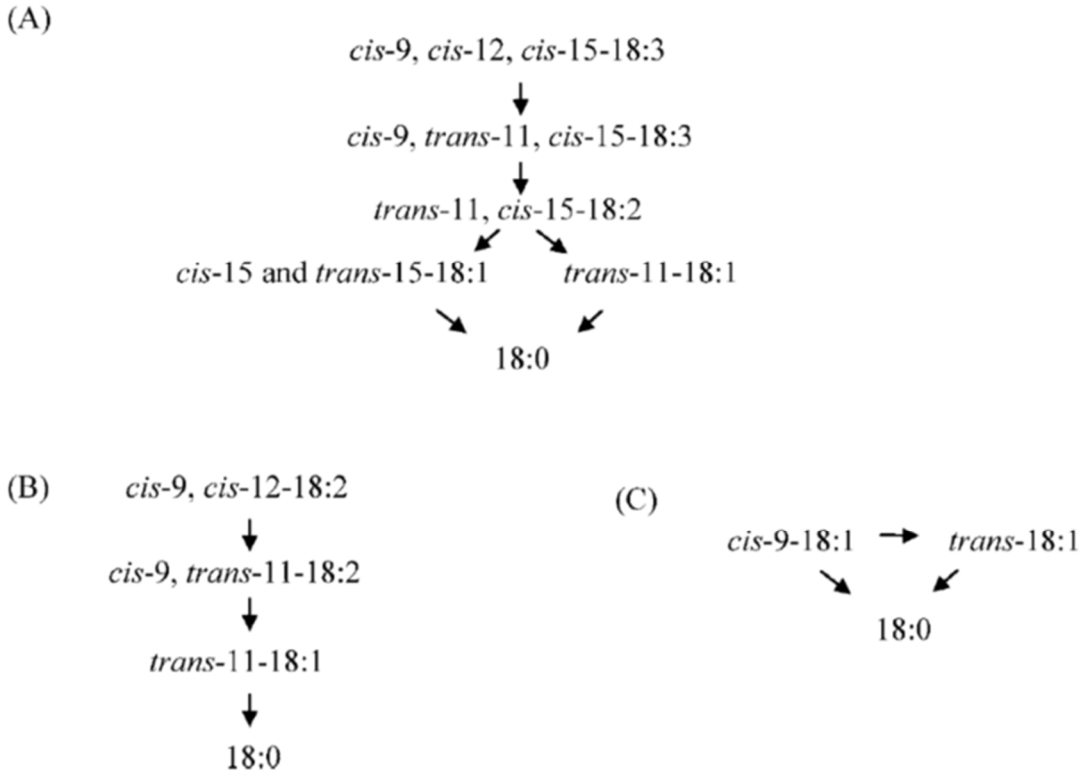


Figure 1.3. Ruminal biohydrogenation pathways of (A) α-linolenic, (B) linoleic, and (C) oleic acids (adapted from Harfoot and Hazlewood, 1988).

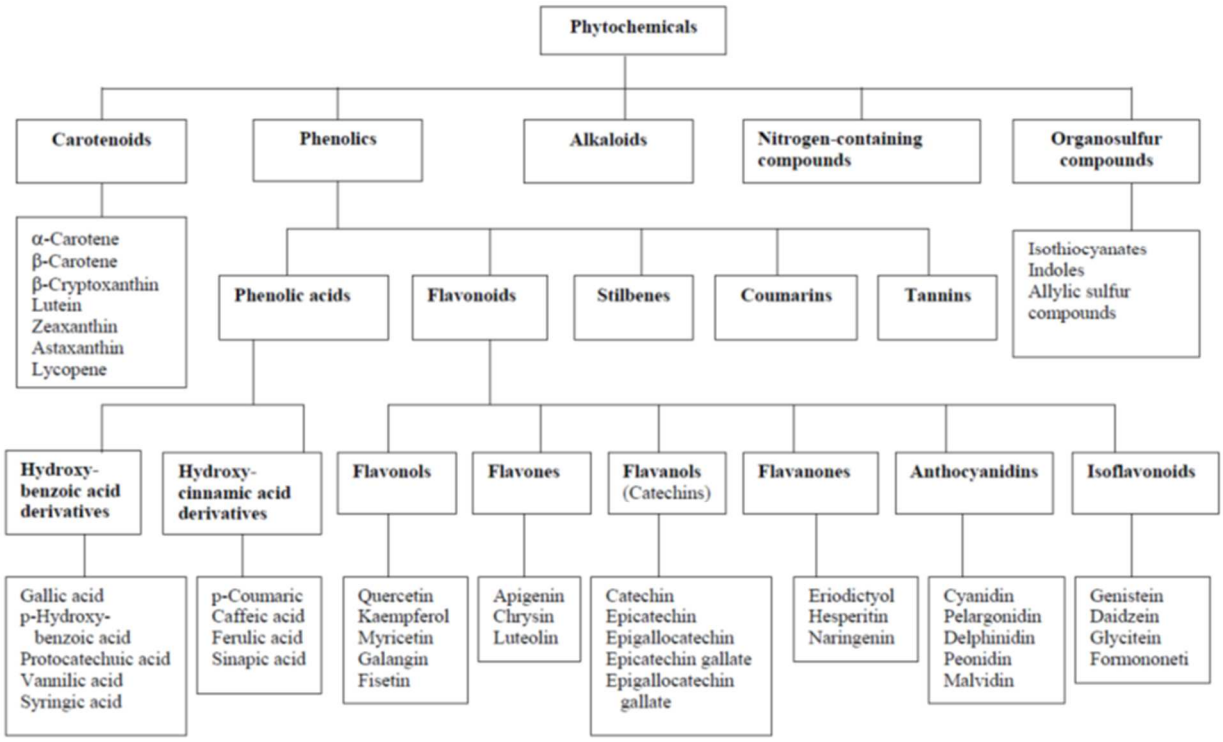


Figure 1.4. Overview of classification of dietary phytochemicals (Adapted from Liu et al., 2004).

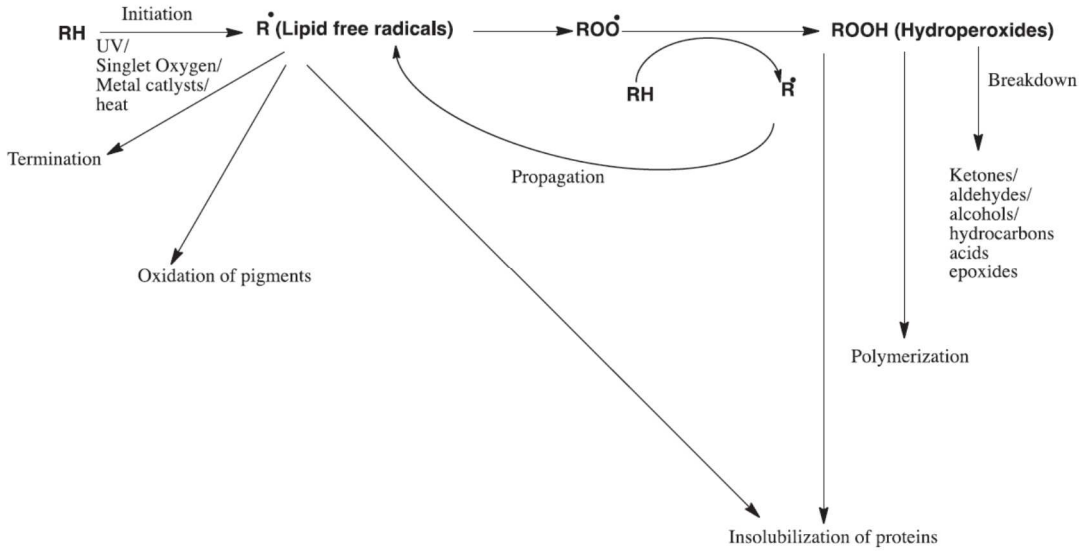


Figure 1.5. Schematics for autoxidation of lipids containing polyunsaturated fatty acids and their consequences (Adapted from Shahidi and Ambigaipalan, 2015)**.**

The antioxidant effect of phytochemicals present in PBP can be explored to inhibit oxidative deterioration of colour and flavour, and extend the shelf-life of meat products (Vasta and Luciano, 2011; Salami et al., 2016). Phenolic compounds can exhibit their antioxidant efficacy through radical scavenging activity, metal chelating activity, or antioxygen activity (Benavente-García et al., 1997). Shahidi and Ambigaipalan (2015) noted that phenolics act primarily as free radical scavengers that delay or inhibit the initiation step or interrupt the propagation step of lipid oxidation, thus decreasing the formation of volatile degradation products (e.g., aldehydes and ketones) that cause rancidity. The antioxidant efficacy of dietary phenolics in meat depends on the successful deposition in muscle tissues, which is influenced by the interaction of factors such as structural complexity, dosage, type of diets, animal species and physiological status, microbial metabolism in the gut, as well as characteristics inherent to the basal diet (Vasta and Luciano, 2011).

1.5.3. Effect of by-products of distillery and biofuel production on ruminant meat quality

Distillery and biofuel by-products are viable dietary sources of unsaturated fatty acids that can alter the lipid composition, oxidative stability, and sensory characteristics of ruminant meat. Dietary inclusion of ethanol co-products including DGS, gluten feed, gluten meal and crude glycerine have little or no effect on the proximate composition of ruminant meat in terms of moisture, protein, fat and ash contents (Jenschke et al., 2008; Aldai et al., 2010a; Segers et al., 2011; Mello et al., 2012a,b). However, by-products such as DGS and glycerin could potentially improve meat FA profiles due to their reduced susceptibility to ruminal biohydrogenation which increases the flow of desirable unsaturated fatty acids to the distal gut for subsequent incorporation into muscle tissues (Vander Pol et al., 2006; Krueger et al., 2010). Feeding DGS at 15 to 70% DM can improve the FA profiles of ruminant meat

by increasing the unsaturated fatty acids and CLA contents desirable for human health (Gill et al., 2008; Mello et al., 2012b; Veracini et al., 2013; Chao et al., 2017; Kawęcka et al., 2017). However, there is increasing evidence that feeding corn DGS may result in a greater concentration of linoleic acid in muscle tissues, with a consequent increase in *n*-6:*n*-3 PUFA ratio (Gill et al., 2008; Aldai et al., 2010b; Kawęcka et al., 2017). Notably, *n*-6:*n*-3 PUFA values (5.5 – 13.6) reported for the DGS-fed meat in these studies are greater than the maximum value (4.0) recommended by the UK Department of Health (HMSO, 1994).

Furthermore, the effect of dietary DGS on meat quality may vary with the type of grain used as a fermentation substrate. Feeding a similar level (15% DM) of corn DGS and sorghum DGS resulted in greater concentrations of linoleic acid and *trans*-vaccenic acid, and *n*-6:*n*-3 PUFA ratio in corn DGS-fed beef steaks compared to beef steaks from steers fed sorghum DGS (Gill et al., 2008). Moreover, consumer panellists indicated that corn DGS-fed beef steaks had greater tenderness compared to sorghum DGS-fed beef steaks (Gill et al., 2008). Similarly, Aldai et al. (2010a) demonstrated that meat from cattle fed 40% DM corn-dried DGS has improved tenderness and palatability compared to those from a barley-based control diet. However, beef from steers fed 40% wheat-dried DGS exhibited a healthier FA profile compared to those from animals fed 40% corn-dried DGS (Aldai et al., 2010b).

Enrichment of muscle lipid profiles with PUFA can increase the susceptibility of meat to oxidative deterioration with potential negative impact on nutritive properties, shelf-life, eating quality and overall consumer acceptability (Bekhit et al., 2013). Dietary inclusions of wet or dry corn DGS at ≥ 30% DM have been shown to increase lipid oxidation and reduce consumer acceptability of beef under retail display conditions even though the sensory attributes may not be affected (Roeber et al., 2005; Koger et al., 2010; Buttrey et al., 2013; de Mello et al., 2017). Thus, increasing dietary content of antioxidant agents (such

as vitamins C and E, carotenoids, flavonoids, phenolics) is recommended when DGS is fed at ≥ 30% DM in order to prevent a negative effect on meat shelf-life properties. Future studies are necessary to assess the complementary use of phytochemical-rich PBP in order to supply natural antioxidants, such as phenolics and flavonoids, in DGS-based diets.

Dietary inclusion of 6 – 30% glycerine favourably altered beef and lamb FA composition through decreased SFA and cholesterol levels and increased unsaturated fatty acids and CLA contents without detrimental effect on sensory traits (Eiras et al., 2014; Lage et al., 2014; Carvalho et al., 2015; van Cleef et al., 2017). However, Stelzleni et al. (2016) and Segers et al. (2011) indicated that finishing steers for 100 days on a diet containing corn gluten feed at 25% DM did not compromise meat quality and shelf-life. Barley brewers' grain, a by-product of the brewery industry, fed *ad libitum* or as partial replacement of compound feeds increased the PUFA concentrations in lean tissues of lambs (Vipond et al., 1995). However, the inclusion of 16.7% brewers' grains in a total mixed ration of beef steers did not affect meat FA profiles nor eating quality (Shand et al., 1998).

1.5.4. Effect of by-products of oilseed processing on ruminant meat quality

By-products of oil extraction are valuable alternative sources of energy and protein in ruminant diets. They potentially contain residual oil particularly when a mechanical extraction method is employed. Oilseed by-products exhibit different effects on meat FA profiles depending on their FA composition and subsequent effect on ruminal biohydrogenation. Soybean cake, sunflower cake and peanut cake can be used to replace conventional soybean meal at a level of 14% in diets of lambs without adversely affecting animal performance or the chemical or FA composition of meat (Santos et al., 2013). Similarly, sunflower cake did not affect the FA profiles nor sensory attributes of meat when substituted for corn and soybean meal at up to 16% DM in the diets of Boer goats (Oliveira

et al., 2015) and up to 52% DM in a concentrate supplement fed to finishing heifers (de Souza et al., 2017).

Other studies have shown that the inclusion of olive cake in different dietary regimens can enrich diets with oleic acids but have only a marginal effect on the FA profiles in lamb meat (Mele et al., 2014; Kotsampasi et al., 2017; Ozdogan et al., 2017). Moreover, olive cake had little effects on either volatile odour compounds (Gravador et al., 2015a) or eating quality of lamb meat (Hamdi et al., 2016). However, dietary inclusion of olive cake in conventional concentrate diets improved the oxidative stability and shelf-life of lamb and beef due to the natural antioxidant effect of residual α-tocopherol and phenolic compounds present in the olive cake (Luciano et al., 2013; Branciari et al., 2015). Moreover, olive cake has proven effective in enhancing the oxidative stability of meat when included in diets high in unsaturated fats, such as feeding linseeds, which increase the PUFA content of meat (Luciano et al., 2013). The content of bioactive compounds in olive cake can be enhanced when this by-product is obtained from a modern three-phase oil extraction technology (Servili et al., 2011).

Furthermore, elevated concentration of PUFA and CLA contents have been reported in beef from bulls fed concentrate mixtures containing 29% DM rapeseed cake (Pustkowiak, 2000) and 12% peanut cake (Correia et al., 2016) substituted for soybean meal. However, feeding peanut cake up to 21% dietary DM as a replacement for soybean meal did not positively affect the FA profiles in lamb and goat meat (Bezerra et al., 2016; Silva et al., 2016). This inconsistency could be partly due to differences in the FA composition of the peanut cake used in these studies. Bezerra et al. (2016) indicated that increasing inclusion levels of peanut cake decreased the levels of linoleic acid in the diet while in the study of Correia et al. (2016), peanut cake included at increasing levels maintained the dietary level of this FA.

However, both studies demonstrated that dietary peanut cake did not affect the sensory characteristics of fresh meat such as colour, water holding capacity, cook loss or tenderness.

The use of by-products from cottonseed extraction in ruminant diets can alter the FA profile of meat. Do Prado Paim et al. (2014) investigated the FA profiles of meat from lambs fed 19.5% DM of whole cottonseeds, solvent-extracted cottonseed meal (CSM) or pressure-extracted high oil CSM as a replacement for corn and soybean meal in a concentrate diet. Meat from lambs fed both CSM products had a greater content of beneficial CLA and high oil CSM also increased the vaccenic acid concentrations. Despite these positive effects, diets containing whole cottonseeds and CSM products reduced *n*-3 PUFA and elevated *n*-6:*n*-3 ratio in meat, which is less desirable from a human nutrition perspective (HMSO, 1994). Another study conducted in lambs found that increasing dietary replacement (0 – 20%) of CSM with corn-dried DGS resulted in a linear decrease in linoleic and arachidonic acids in meat (Whitney and Braden, 2010), suggesting that CSM might have a more beneficial impact on the FA profile of meat compared to corn-dried DGS. Nonetheless, a complete dietary replacement of 20% CSM with corn-dried DGS reduced cook-loss and improved the juiciness of meat (Whitney and Braden, 2010).

On the contrary, by-products of oil extraction that contain high levels of saturated fat and/or are highly susceptible to ruminal lipid hydrolysis and biohydrogenation could result in a meat FA profiles that are less desirable from a human nutrition perspective. Ribeiro et al. (2011) found that increasing inclusion levels (6.30 to 19.5%) of palm kernel meal (PKM) in concentrate lamb feeds increased dietary levels of SFA (lauric and myristic acids). Consequently, the PKM diets resulted in a linear increase in SFA (lauric, myristic and palmitic acids) in the meat with a greater atherogenicity index, a dietary risk index for cardiovascular disease. An additional study demonstrated that feeding diets containing 80% palm oil by-products (decanter cake, DC and palm kernel cake, PKC) resulted in a lower

linoleic acid and PUFA/SFA ratio, and a greater concentration of SFA in the *longissimus muscle* of goats (Abubakr et al., 2015). On the contrary, dietary DC and PKC resulted in a desirably lower *n*-6/*n*-3 PUFA ratio in the *biceps femoris* and *infraspinatus* muscles (Abubakr et al., 2015). Nonetheless, the inclusion of PKC at up to 21% dietary DM did not affect the sensory and acceptability of beef (Santana Filho et al., 2016). Moreover, the negative effect of PKC on meat FA profiles may be prevented when included in a concentrate supplement fed to grazing ruminants, possibly due to the complementary effect of the high content of unsaturated fatty acids inherent in forages (Freitas et al., 2017).

1.5.5. Effect of by-products of fruit and vegetable processing on ruminant meat quality

Fruit and vegetable by-products have been widely exploited in animal feeding (Wadhwa and Bakshi, 2013). Despite their rich phytochemical components and their potential effects on animal product quality (Kasapidou et al., 2015), there have been few studies on the impact of fruit and vegetable by-products on ruminant meat quality.

The effect of citrus pulp (CP) on ruminant meat quality has been investigated in lambs while limited data exist on its effect on the quality attributes of beef and goat meat production systems. Scerra et al. (2001) indicated that *ad libitum* feeding of 30% CP-silage (80% CP and 20% wheat straw) as a replacement of oat hay did not affect performance nor carcass or meat quality of lambs. However, recent studies found that 24 or 35% DM inclusion of dried CP in concentrate diets inhibited ruminal biohydrogenation of PUFA and increased desirable rumenic acid and long-chain PUFA, and lower PUFA/SFA ratios in the *longissimus muscle* of lambs (Lanza et al., 2015). Notably, the inclusion of 35% dried CP had the greatest improvement in the overall FA profile of the meat. Further reports relating to this experiment demonstrated that dried CP reduced lipid and protein oxidation in muscle tissue stored aerobically for up to 6 days at 4°C (Gravador et al., 2014; Inserra et al., 2014).

Recent findings showed that vitamin E content in CP, and not the presence of phenolic compounds, could be the main contributor to the antioxidant effect of dietary dried CP in lamb meat (Luciano et al., 2017).

There is an emerging interest in using polyphenol-rich extracts from fruits, such as grape and pomegranates, in animal nutrition in order to supply natural antioxidants that can be beneficial to animal health and productivity. Guerra-Rivas et al. (2016) demonstrated that dietary inclusion of 5% dried grape pomace or 50 mg/kg grape seed extract did not affect sensory acceptability and shelf-life of lamb meat stored in modified atmosphere packs. However, feeding pomegranate by-product (seed and peels) silage (Kotsampasi et al., 2014) or seed pulp (Emami et al., 2015a; Emami et al., 2015b) increased the concentration of healthy FA such as linoleic acid, α-linolenic acid and CLA in lamb and goat meat, respectively. Moreover, these studies indicated that pomegranate by-products could enhance the antioxidant potential and oxidative stability of lamb and goat meat. Interestingly, pomegranate by-products retained their antioxidant capacity when subjected to feed preservation methods such as drying or ensilage (Shabtay et al., 2008).

1.5.6. Effect of by-products of sugar production on ruminant meat quality

The potential of by-products from sugar production as animal feed ingredients has been widely explored in the Mediterranean and tropical countries. Dietary sugar beet pulp (SBP) exhibits a differential effect on meat quality attributes dependent on whether it was substituted for cereals or roughage sources in ruminant diets. It has been demonstrated that replacement of wheat straw with up to 8% SBP dietary DM in bull diets decreased drip loss and improved instrumental texture profiles and sensory panel acceptability without altering the fatty acid composition of beef (Yuksel et al., 2009; Yuksel et al., 2011). On the other hand, the substitution of SBP for cereals (mainly barley and corn) at a dietary inclusion

level of 50% increased cooking loss and meat tenderness measured as Warner-Bratzler shear force at 48 h *post-mortem* (Cuvelier et al., 2006). Dietary impact on meat tenderness may be a consequence of the effect of diet influence on meat ultimate pH and intramuscular fat. In a recent study, Asadollahi et al. (2017) found that the inclusion of 36% SBP as a partial replacement for barley in a concentrate diet increased intramuscular fat content and Warner-Bratzler shear force values, with minimal impacts on FA composition in lamb meat. However, it is unlikely that the dietary effects of SBP on physicochemical characteristics of beef or lamb would have a negative influence on the sensory evaluation or consumer acceptance properties (Olfaz et al., 2005; Asadollahi et al., 2017).

Vinasse or molasses distillers' soluble is a by-product obtained from the fermentation and distillation of sugarcane molasses. Dietary inclusion of 10 or 20% DM beet vinasse to concentrate diets of feedlot lambs reduced growth performance and increased meat toughness without affecting other physicochemical characteristics or the FA composition of meat (Lopez-Campos et al., 2011). However, dietary inclusion level of vinasse should be < 10% for feedlot lambs and veal calves to avoid detrimental effects on animal performance (Lopez-Campos et al., 2011; Zali et al., 2017). Other studies showed that animal performance and meat physicochemical and sensory attributes were not affected by feeding up to 30% sugarcane bagasse (Ahmed and Babiker, 2015) or 20% sugar cane press mud (Kumar et al., 2015) in concentrate diets of finishing bulls and lambs, respectively.

1.5.7. Effect of by-products of root and tuber processing on ruminant meat quality

Commercial processing of root and tuber crops for food and starch production generates an enormous quantity of by-products. In this category, only by-products of potato and cassava have been widely explored for their use as animal feeds with little evaluation of their influence on meat quality attributes. Potato is the third most important crop after rice and

wheat and this could account for the global relevance of potato by-products (usually a mixture of potato peelings, pulp, scrapings and gluten feed) in animal feeding regimes. The inclusion of 10 or 20% potato by-products in barley- or corn-based diets had a minimal impact on cattle growth performance or muscle pH, FA composition, sensory characteristics or the consumer acceptability of beef (Busboom et al., 2000; Nelson et al., 2000; Thornton et al., 2015). Moreover, potato by-products can be fed in ensiled form or as a portion of a concentrate diet up to 80% of diet DM without detrimental effects on animal performance, physicochemical or the sensory characteristics of beef (Radunz et al., 2003; Pen et al., 2005; Duynisveld and Charmley, 2016). However, Pen et al. (2005) indicated that feeding potato by-products-based silage can increase the PUFA content particularly the linoleic acid content in subcutaneous fat. The potential of dietary potato by-products to improve the FA profile and oxidative stability of ruminant meat may be attributed to the presence of phenolic compounds which may inhibit ruminal biohydrogenation. Approximately 50% of the bioactive compounds are located in potato peel (Friedman, 1997), suggesting that the effect of potato by-products on meat quality is dependent on the proportion of potato peel present in potato by-products used as feed. Indeed, existing studies have demonstrated that potato peel extract could protect dietary PUFA against ruminal biohydrogenation (Gadeyne et al., 2016) and exhibited antioxidant activity in inhibiting lipid peroxidation of radiation-processed lamb meat (Kanatt et al., 2005).

Cassava is another staple root crop commonly grown in Africa, Asia and Latin America with Nigeria, Brazil, Thailand, Indonesia and DR Congo accounting for 70% of the global cassava production (FAO, 2008). Cassava pulp, a by-product from the production of cassava starch, could be an important feed resource in many developing countries. The few available studies reported that inclusion of dried cassava pulp at levels up to 50% in a

concentrate diet did not affect the chemical composition, physicochemical or beef eating quality parameters (Yimmongkol et al., 2009; Laorodphan et al., 2012).

1.5.8. Effect of by-products of herb, spice and tree processing on ruminant meat quality

There is little information on the effects of feeding herb and spice by-products on meat quality despite increasing interest in exploring their phenolic extracts in food applications. Concentrate diets containing sage distillation by-product (SDB, 100g/kg feed) were fed to lambs obtained from ewes reared in different feeding systems (grazing ewes or stall-fed ewes) (Leticia et al., 2017). Lambs obtained from grazing ewes had increased *n*-3 PUFA and polyphenol intakes, with a subsequent increase in *n*-3 PUFA and CLA contents of lamb meat. However, feeding the SDB diet to lambs obtained from stall-fed ewes had no effect on the lipid profile of lamb meat. This highlights the interaction of ewe grazing and SDB addition to the diet fed to lambs. The polyphenol content of SDB did not provide antioxidant protection as the high–PUFA meat exhibited greater susceptibility to lipid oxidation during retail display (Leticia et al., 2017). Also, two separate experiments were conducted to evaluate the effect of pellets containing 50% barley and 50% distillates from rosemary leaves (DRL) and another pellet containing 50% barley and 50% distillates from thyme leaves (DTL) (Nieto et al., 2010; Nieto et al., 2011; Nieto et al., 2012; Nieto, 2013). In both experiments, pellets were partially substituted to supply 10-20% of DRL and DTL in the concentrate diet fed to ewes during pregnancy and lactation, and the subsequent effect on meat quality of lambs was assessed.

Nieto (2013) reported that dietary DRL and DTL, with the greatest effect with 20% DRL, improved the FA profile of lamb meat by increasing the levels of PUFA and unsaturated fatty acids and decreasing the SFA and indices of saturation, atherogenicity and

thrombogenicity. Moreover, dietary DRL and DTL reduced colour deterioration, lipid oxidation and microbial spoilage in lamb meat stored in modified-atmosphere packs for 21 d at 4°C under simulated retail display conditions (Nieto et al., 2010; Nieto et al., 2012). The diet containing DRL also inhibited lipid oxidation and deteriorative flavour development in cooked lamb stored under retail display conditions for 4 days (Nieto et al., 2011). Considering the strong aroma possessed by herb and spice by-products, it is important for future studies to evaluate their dietary effect on meat flavour and consumer acceptability factors.

By-products generated from the processing of tree leaves and pods have proven effective in improving meat quality due to their high phenolic content. In an early study reported by Priolo et al. (1998), substitution of 20% tannin-containing carob pulp (CBP) for barley increased the lightness of lamb meat but no effect on animal performance, meat tenderness and sensory acceptability. However, increasing the partial replacement of maize/barley grains with 24 or 35% CBP in concentrate diets increased the concentration of rumenic acid and PUFA, and lowered the SFA and *n*-6/*n*-3 PUFA ratio in lamb meat (Gravador et al., 2015b). Despite increased PUFA content in the muscle, CBP stabilised the oxidative stability of meat when stored in a commercial PVC film for up to 6 days at 4°C (Gravador et al., 2015b). Conversely, feeding a maize-based concentrate diet containing 45% CBP resulted in a less nutritive lamb meat by increasing the accumulation of SFA and decreasing CLA and *n*-3 PUFA contents (Vasta et al., 2007). This contrary observation may be a consequence of a lower DMI which may have reduced the intake of dietary unsaturated FA and polyphenols necessary for influencing ruminal biohydrogenation and meat FA profile. Additionally, tannin effects associated with feeding higher inclusion levels (56% *as-fed* basis) of CBP impaired lamb performance and increased meat lightness values without discriminatory effect on the sensory properties of meat (Priolo et al., 2000). Dietary

supplementation of 40 g polyethylene glycol (PEG)/kg diet seemed to eliminate the negative effect of 56% CBP diet on animal performance without affecting meat quality (Priolo et al., 2000; 2002).

Furthermore, dietary inclusion of 0.5 - 2.0% green tea by-products (GTB) resulted in a beneficial FA profile in goat meat by increasing the proportion of linoleic acid, linolenic acid, PUFA and PUFA/SFA ratio without affecting sensory attributes (Ahmed et al., 2015). The highest inclusion level of GTB (2.0%) exhibited the greatest effect in reducing the cholesterol content, increasing the *n*-3 PUFA levels and inhibiting lipid oxidation in meat samples stored at for up to 2 weeks at 4°C.

1.6. THESIS CONCEPT: Utilization of PBP in concentrate feeding strategies to improve ruminant meat quality

Concentrate feeds contain a high density of nutrients (protein and energy), usually low in fibre content and high in total digestible nutrients. Concentrate feeds are usually utilized as a complete feedlot ration or fed in combination with forages for fattening of ruminants in intensive or semi-intensive production systems. The replacement of cereals and processed forages (such as dehydrated alfalfa) with by-products is an important strategy for reducing the high-cost of concentrate feeds and increasing the profitability of farmers.

In this thesis, five PBP (cardoon meal, dried corn gluten feed (CGF), dried corn (CDGS) and wheat distillers' grains with solubles (WDGS), and dried citrus pulp (DCP)) were evaluated as substitutes to a processed forage or cereals in concentrate diets offered to ruminants (lambs and beef cattle). These by-products were selected with the hypothesis that they contain residual unsaturated oil and/or phytochemicals (mainly phenolic compounds) that could be used in concentrate feeding strategies in improving the fatty acid composition and shelf-life quality of ruminant meat. Moreover, lower starch and higher fibre content in

these PBP, compared to grains, could decrease the negative effects of starchy concentrate-based feeds on ruminal pH and fibre digestion in ruminants fed forage-based diets (Boddugari et al., 2001; Stock et al., 2000). Thus, an increase in forage digestion could increase the ruminal flow of beneficial unsaturated fatty acids and subsequent absorption into muscle tissues.

Brief descriptions of the evaluated PBP are highlighted below:

- **Cardoon meal:**

This is a by-product retained after the extraction of oil from the seeds of cultivated cardoon (*Cynara cardunculus* var. *altilis*), a perennial herb native to the Mediterranean region and widespread in parts of Europe, Americas and Oceania (CABI, 2017). Cardoon meal is a valuable source of protein with a rich amino acid profile, high in fibre and contains bioactive compounds such as unsaturated fatty acids (oleic and linoleic acids) and polyphenols (Genovese et al., 2015). Hydroxycinnamic acids (caffeoylquinic acid derivatives) and flavonoids (apigenin and luteolin derivatives) have been identified as the main polyphenols present in cardoon vegetal organs (Pandino et al., 2011; Ramos et al., 2014).

- **Dried corn gluten feed:**

This is a by-product of the wet-milling of maize grain for starch (or ethanol) production. CGF consists mainly of corn bran and corn steep liquor but may also contain distillers' solubles, germ meal, cracked maize screenings, as well as minor quantities of end-products from other microbial fermentations (Feedipedia, 2018). Wet corn gluten feed is often dried to enhance storage and transport. CGF may contain unsaturated fats (linoleic and oleic acids) if corn germ meal containing residual oil is added (Feedipedia, 2018). CGF is mostly

used as a dietary source of energy and protein in cattle diets. CGF consists of bran and steep liquor that are rich sources of phenolic antioxidants such as protocatechuic acid, vanillic acid, *p*-coumaric acid, ferulic acid, sinapic acid and quercetin (Inglett and Chen, 2011; Rodríguez-López et al., 2016).

- **Dried distillers' grains with solubles:**

This is a by-product obtained after drying a blend of wet grains and condensed distillers' solubles produced from the distillation of ethanol from grains (corn, wheat, sorghum etc.) (Feedipedia, 2018). Distillers' grains with solubles (DGS) can be obtained from dry-milling or wet-milling distillery process used to produce alcoholic beverages, industrial ethanol and ethanol biofuel. Depending on the manufacturing process, DGS may contain residual corn oil (Feedipedia, 2018), rich in unsaturated fatty acids (linoleic and oleic acids) (Duckett et al., 2002). DGS is a valuable dietary source of protein, moderately rich in fat and relatively low in fibre. DGS also contains phenolic compounds such as vallinic, caffeic, *p*-coumaric, ferulic and sinapic acids, with potent antioxidant activity (Luthria et al., 2012).

- **Dried citrus pulp:**

This is a whole by-product obtained after drying the residue following the extraction of juice from citrus fruits (orange, grapefruit, lemon) and it consists of peel, pulp and seed residues (Martinez-Pascual and Fernandez-Carmona, 1980). Citrus pulp accounts for 50-70% of the fresh weight of the original fruit and contains the peel (60-65%), internal tissues (30-35%) and seeds (0-10%) (Feedipedia, 2018). Citrus pulp is often dehydrated to enhance storage and transport. Dried citrus pulp is used as a replacement for concentrate cereals because of its high energy content and high digestibility in ruminant species (Bampidis and Robinson, 2006). Depending on the proportion of seeds present in citrus pulp, this by-product may contain considerable amount of unsaturated fatty acids (mainly oleic and

linoleic acids) associated with citrus seed oil (El-Adawy et al., 1999). Citrus fruits and their by-products contain a substantial amount of phenolic compounds, predominantly flavonoids such as naringin, hesperidin, quercetin, rutin and luteolin (Balasundram et al., 2006; Benavente-García et al., 1997).

Due to the negative effect of high concentration of dietary phenolic compounds on ruminal metabolism and the microbiome in ruminants, an experiment was conducted as a prelude to this thesis to investigate the threshold of dietary phenolic compounds that would not impair rumen function. This experiment characterized the rumen function response in lambs fed phenolic-rich diets where a concentrate-based diet was supplemented with different vegetal sources of tannin extracts. Tannin extracts were selected as a source of phenolic compounds because of their wide application in animal nutrition and significant impacts on rumen function. Considering the structural complexity of phenolic compounds, different tannin sources (chestnut, tara, mimosa and gambier) were evaluated so as to cover a wide scope of possible effects on rumen function.

1.7. THESIS OBJECTIVES

The overall objective of this thesis is to evaluate feeding strategies that would increase the utilization of plant wastes (containing residual unsaturated fatty acids and/or phenolic compounds) for improving the quality attributes of ruminant meat.

The studies conducted in this thesis fulfil the following specific objectives:

- To characterize the response of ruminal metabolism and the rumen microbiome in lambs fed phenolic-rich diets through supplementation of a concentrate-based diet with different tannin sources.

- To compare the effect of a concentrate diet containing 15% dehydrated alfalfa or cardoon meal on the ruminal metabolism and microbiome in lambs.
- To examine the effect of dietary dehydrated alfalfa or cardoon meal on the fatty acid profile and oxidative stability of lamb meat.
- To evaluate the quality indices and oxidative stability of beef from grass silage-fed steers supplemented with concentrate diets in which barley/soybean meal was replaced with different levels (25% - 75%) of dried corn gluten feed.
- To determine the quality attributes and oxidative stability beef from steers fed 80% dried corn or wheat distillers' grains with solubles as a substitute for barley/soybean meal in a concentrate supplement to grass-silage.
- To examine the quality indices and oxidative stability of beef from steers offered grass silage and concentrate supplements containing different levels (40% and 80%) of dried citrus pulp in replacement for barley.

EXPERIMENTAL CHAPTERS

CHAPTER 2 – Characterization of the ruminal fermentation and microbiome in lambs supplemented with hydrolysable and condensed tannins

Saheed A. Salami, Bernardo Valenti, Marco Bella, Michael N. O'Grady, Giuseppe Luciano, Joseph P. Kerry, Eleanor Jones, Alessandro Priolo and Charles J. Newbold

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ABSTRACT

This study characterised the response of ruminal fermentation and the rumen microbiome in lambs fed commercial vegetal sources of hydrolysable tannins (HT) and condensed tannins (CT). Forty-four lambs (19.56 ± 2.06 kg) were randomly assigned to either a concentrate diet (CON, n = 8) or CON supplemented with 4% of two HT [chestnut (*Castanea sativa*, HT-c) and tara (*Caesalpinia spinosa*, HT-t)] and CT [mimosa (*Acacia negra*, CT-m) and gambier (*Uncaria gambir*, CT-g)] extracts (all, n = 9) for 75 days pre-slaughter. Tannin supplementation did not influence ruminal fermentation traits. Quantitative PCR demonstrated that tannins did not affect the absolute abundance of ruminal bacteria or fungi. However, CT-m (-12.8%) and CT-g (-11.5%) significantly reduced the abundance of methanogens while HT-t (-20.7%) and CT-g (-20.8%) inhibited protozoal abundance. Ribosomal amplicon sequencing revealed that tannins caused changes in the phylogenetic structure of the bacterial and methanogen communities. Tannins inhibited the fibrolytic bacterium, *Fibrobacter* and tended to suppress the methanogen genus, *Methanosphaera*. Results demonstrated that both HT and CT sources could impact the ruminal microbiome when supplemented at 4% inclusion level. HT-t, CT-m and CT-g extracts displayed specific antimicrobial activity against methanogens and protozoa without compromising ruminal fermentation in a long-term feeding trial.

Keywords: Tannins, ruminal fermentation, microbiome, bacteria, methanogens, metagenomics.

2.1. INTRODUCTION

Structural composition and functional activity of the rumen microbiome influence the efficiency of ruminal metabolism, which in turn affects ruminant productivity, environmental emissions (methane and nitrogen) and the quality of ruminant-derived foods (McCann et al., 2014). Several chemical additives and in-feed antibiotics have been developed as rumen modifiers to favourably manipulate ruminal fermentation, methanogenesis, dietary protein degradation and biohydrogenation of dietary fatty acids (Adesogan, 2009). Currently, there is a renewed interest in the use of dietary phytochemicals as natural rumen modifiers due to increasing consumer demand for natural food products coupled with global concerns for antibiotic resistance (Patra and Saxena, 2009). Tannins, saponins and essential oils are the main classes of phytochemicals that have received attention for their potential to modify ruminal fermentation, methanogenesis, proteolysis and biohydrogenation of fatty acids (Hart et al., 2008; Patra et al., 2012).

Tannins are complex polyphenolic compounds that are ubiquitous in several plant species (Makkar, 2003) and are broadly divided into hydrolysable tannins (HT) and condensed tannins (CT) based on chemical structure. HT consist of gallic acids esterified to a core polyol and may be further complicated by additional esterification or oxidative crosslinking of the galloyl group whereas CT are flavonoid-based polymers commonly linked by C4-C8 and C4-C6 interflavan bonds (Hagerman, 2002). Both HT and CT have diverse and complex structural chemistry. The prevalence of HT and CT varies in different plant sources and a particular type of monomer may also dominate the HT or CT units. Different vegetal sources of tannins can exert beneficial or adverse effects on ruminants depending on factors such as structural complexity, dose-response effect, type of diets, animal species and physiological status (Makkar, 2003; Mueller-Harvey, 2006). The magnitude of

response of ruminal fermentation and feed digestibility can vary between different tannin sources supplemented at similar levels (Jayanegara et al., 2015; Makkar et al., 1995). Variations in the complex structural features of tannins largely account for the vast discrepancy in the biological potency of different tannin sources on ruminal metabolism and ruminant performance (Patra and Saxena, 2011; Waghorn, 2008).

The ability of tannins to modify the ruminal microbiome has been associated with decreased ruminal degradation of proteins, reduction of methanogenesis and inhibition of biohydrogenation of fatty acids (Patra et al., 2012; Patra and Saxena, 2011). Tannins can exhibit bacteriostatic effects, although the interaction of tannins with rumen microbes differs between tannin types given that HT are more susceptible to microbial hydrolysis than CT (Bhat et al., 1998; McSweeney et al., 2001). Few studies have compared the effect of CT and HT sources on ruminal microorganisms. A tannin-resistant bacterium isolated from the ruminal fluid of goats exhibited a greater tolerance to tannic acid concentration (up to 7%) compared to purified quebracho CT (up to 4%) (Nelson et al., 1995). O'donovan and Brooker (2001) indicated that both tannic acid (HT) and acacia CT inhibited the *in vitro* growth rate of *Streptococcus gallolyticus* (S. caprinus) and *S. bovis* but *Streptococcus gallolyticus* later maintained its growth through the initiation of adaptation mechanisms that differ between tannic acid and acacia CT. The resistance mechanisms of *S. gallolyticus* to tannic acid include induction of gallate decarboxylase activity and secretion of extracellular polysaccharide matrix while the resistance of *S. gallolyticus* to acacia CT involve an unknown mechanism. An *in vitro* ruminal study reported that purified HT (chestnut and sumach) and CT (mimosa and quebracho) decreased the population of methanogens and *Fibrobacter succinogenes* but sumach HT exhibited a greater inhibitory effect on *Ruminococcus flavefaciens* compared to other tannins (Jayanegara et al., 2015). Furthermore, dietary supplementation of chestnut HT and quebracho CT increased the

abundance of *Butyrivibrio fibrisolvens* in the rumen of dairy sheep by approximately 3-fold and 5-fold, respectively (Buccioni et al., 2015). On the other hand, chestnut HT and quebracho CT inhibited the population of *B. proteoclasticus* by 5-fold and 15-fold, respectively (Buccioni et al., 2015). These studies indicated that there could be a substantial variation in the response of the ruminal microbiome to dietary HT and CT sources.

The *in vivo* effect of different dietary tannins, especially when supplemented at a moderate dosage in a long-term experiment, on the rumen microbial ecosystem is still poorly understood. It was hypothesised that different types of tannins (HT *vs.* CT) would elicit differential impacts on the ruminal fermentation and microbiome. Thus, the present study characterised the ruminal fermentation and microbiome in lambs fed commercial extracts of HT: chestnut (*Castanea sativa*) and tara (*Caesalpinia spinosa*); and CT: mimosa (*Acacia negra*) and gambier (*Uncaria gambir*) for 75 d pre-slaughter. An inclusion rate of 4% tannin extracts was adopted in this experiment because the inclusion of 2.5 to 5% has been suggested as the moderate dosage threshold for the biological potency of tannins that would not impair nutrient intake and animal performance (Mueller-Harvey, 2006; Patra and Saxena, 2011).

2.2. MATERIALS AND METHODS

2.2.1. Animal, diets and experimental design

The feeding trial was carried out at the experimental farm in the University of Catania. The animals were handled according to the European Union legislation for the protection of animals used for scientific purposes (2010/63/EU Directive). Forty-four male cross-bred Sarda x Comisana lambs [initial body weight (BW) 19.56 ± 2.06 kg] were completely randomised to five experimental groups. Each animal was reared in an individual stall with straw bedding. The animals were adapted to the experimental diets for a period of 9 d by progressive substitution of the weaning feed with the experimental feeds until total replacement of the weaning diet was achieved. Subsequently, the animals were fed for 75 d pre-slaughter. The experimental diets consisted of a commercial concentrate diet (CON, n = 8) and four tannin treatments (n = 9) containing CON supplemented with 40 g/kg commercial extract sources of two hydrolysable tannins, HT [chestnut (HT-c) and tara (HT-t)] and condensed tannins, CT [mimosa (CT-m) and gambier (CT-g)]. The animals had *ad libitum* access to the concentrate feeds and water.

The concentrate diet consisted of the following ingredients (*as-fed* basis): barley (48.0%), dehydrated alfalfa (15.0%), wheat bran (23.0%), soybean (10.0%), molasses (2%) and mineral-vitamin premix (2%). The chemical composition of the experimental diets is indicated in Table 2.1. Commercial extracts of chestnut (Nutri-P®), tara (Tannino T80®) and mimosa (Mimosa OP®) were obtained from Silvateam (San Michele M.vì, Cuneo, Italy) and gambier (Retan FGC®) was obtained from Figli di Guido Lapi S.pA. (Castelfranco di Sotto, Pisa, Italy). These extracts were selected as the source of each specific type of tannins (HT or CT) based on published information on the chemical composition of their phenolic fractions (Apea-Bah et al., 2009; Kardel et al., 2013; Pizzi et

al., 2009). Furthermore, these tannins, except CT-g, have been previously evaluated in *in vitro* ruminal fermentation models (Jayanegara et al., 2015; Pellikaan et al., 2011). The concentrate diets were supplied in form of pellets and the tannin extracts were incorporated into the diets before pelleting at 40°C. The animals had *ad libitum* access to water, and feeds were supplied daily and orts were measured before morning feeding to calculate individual dry matter intake (DMI). Total phenols and tannins in the diets were analysed as previously described by Gravador, Luciano, Jongberg, *et al.* (2015).

Table 2.1. Chemical composition of experimental diets

Parameter	**CON**	**HT-c**	**HT-t**	**CT-m**	**CT-g**
Dry matter (%)	89.65	89.29	89.30	89.26	89.59
Crude protein (% DM)	15.67	14.20	14.49	14.36	15.15
Ether extract (% DM)	2.68	2.67	2.75	2.52	2.85
Ash (% DM)	7.01	6.43	6.55	6.19	6.49
NDF (% DM)	30.36	28.93	26.91	27.26	27.38
ADF (% DM)	15.97	14.07	13.85	13.85	14.49
ADL (% DM)	3.62	3.79	3.22	2.37	3.76
[1]Total phenol content	4.7	24.9	29.1	25.3	11.68
Total tannins (% of total phenols)	32.1	84.2	86.9	88.1	63.00

CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier Condensed tannin; DM: Dry matter; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin.
[1]Expressed as g of tannic acid (TA) equivalents / kg DM of diet

2.2.2. Rumen sampling and determination of fermentation variables

The lambs were slaughtered in a commercial abattoir using captive bolt stunning before exsanguination. The pH of the ruminal digesta was measured immediately after slaughter

using a pH-meter (Orion 9106, Orion Research Incorporated, Boston, MA). Two aliquots of approximately 80 g each of ruminal digesta were collected within 20 min of slaughter and immediately placed in dry ice prior to storage at -80°C for further analysis of rumen fermentation and microbial population. One aliquot of the ruminal digesta stored at -80°C was thawed overnight at 4°C and divided into two aliquots for volatile fatty acids (VFA) and ammonia analyses. The thawed aliquots were centrifuged at 1000 g for 20 min at 4°C and 4 ml of the supernatant (2 ml pooled from each of the two aliquots) was added to 1 ml of 25% trichloroacetic acid (TCA) containing 20 mM 2-ethylbutyric acid as an internal standard. The concentration of VFA was determined as previously described (de la Fuente et al., 2014). Ammonia analysis was performed by diluting the acidified rumen samples with 25% TCA in ratio 4:1, followed by centrifugation at 15,000 g for 15 min and the supernatant was analysed for ammonia concentration. Ammonia concentration was determined in a ChemWell®-T autoanalyser (Awareness Technology Inc., Palm City, FL, USA - Megazyme Cat. No. D-CHEMT) using the method described by Weatherburn (1967).

The protein content and enzymatic activities (carboxymethyl cellulase, xylanase and amylase) were determined in freeze-dried rumen samples (solid and liquid digesta) as described by Belanche et al. (2016b). Lipase activity was determined by a spectrophotometric assay with *p*-nitrophenyl butyrate (PNPB) as a substrate dissolved in acetonitrile at a concentration of 10mM (Lee et al., 1999). Briefly, freeze-dried rumen samples (~ 200 mg) were diluted with potassium phosphate buffer (3.5 mL) and sonicated for 10 min in ice water. The sample extracts were centrifuged at 5000 rpm for 10 min at 4°C. The cell-free supernatant (60 µl) was mixed with PNPB (180 µl) solution and incubated at 39°C for 30 minutes. Lipase activity was measured as the amount of p-nitrophenol (PNP) released by monitoring the change in absorbance on a spectrophotometer

(BioTek, Potton, UK) at 405 nm for 30 min. The blank containing sample supernatant (60 µl) and buffer (180 µl) was incubated to correct for non-enzymatic hydrolysis. All enzymatic activities were measured in triplicate. The specific activity of carboxymethyl cellulase, xylanase and amylase were expressed as mg of sugar released/g protein/min while lipase activity was expressed as mM PNP per g of protein per min.

2.2.3. DNA extraction and quantitative PCR analysis

The rumen samples were freeze-dried and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) detergent method (William and Feil, 2012) with slight modifications. Lysis of cells was achieved by incubating with sodium dodecyl sulphate (SDS) buffer for 10 min at 95°C and potassium acetate was substituted for phenol in removing proteins. DNA yield was assessed by spectrophotometry (Nanodrop ND-1000 spectrophotometer, Thermo Fisher Scientific, UK). The extracted DNA samples were stored at -80°C prior to further analysis. Quantitative PCR was performed in triplicate to determine the total concentration of bacteria, methanogens, anaerobic fungi and protozoa in the DNA samples using a LightCycler® 480 System (Roche, Mannheim, Germany) as previously described (Belanche et al., 2015b). Targeted primers used for qPCR analysis are indicated in Table S2.1 (See appendix).

2.2.4. Ion torrent next-generation sequencing (NGS) and bioinformatics

The extracted DNA samples were amplified at the V1-V2 and V2-V3 hypervariable regions of 16S rRNA for bacterial and methanogen profiling, respectively as detailed by Belanche et al. (2016a). For bacterial profiling, amplification of the V1-V2 hypervariable regions of the 16S rRNA was carried out using bacterial primers (27F and 357R) followed by ion torrent adaptors (Belanche, Jones, Parveen, *et al.* (2016), Table S2.1 – See appendix). For methanogens profiling, amplification of the V2-V3 hypervariable region of the 16S rRNA

was performed using archaeal primers (86F and 519R) also followed by ion torrent adaptors (Belanche, Jones, Parveen, *et al.* (2016), Table S2.1 – See appendix). The resultant PCR amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Inc., Fullerton, USA) and DNA concentration was quantified using an Epoch Microplate Spectrophotometer (BioTek, Potton, UK). The amplicon library was further purified using the E-Gel Safe Imager Trans-illuminator with E-Gel Size Select 2% Agarose gels (Life Technologies Ltd., Paisley, UK). The purified DNA libraries were then quantified for DNA yield and detection of artefacts post-PCR amplification using the Agilent 2100 Bioanalyzer with a high sensitivity DNA chip (Agilent Technologies, Ltd., Stockport, UK). The emulsion PCR of the DNA sample libraries was performed using the Ion PGM Template OT2 200 Kit (Life Technologies Ltd, Paisley, UK) following the appropriate manufacturer's guide for users. The bacterial and methanogen amplicon libraries were sequenced on the Ion Torrent PGM (Life Technologies Ltd, Paisley, UK) using the Ion PGM sequencing 316^{TM} Chip v2.

The resultant sequences were multiplexed for identification of barcodes corresponding to each sample and sequences were denoised to remove low-quality datasets and chimeras using MOTHUR software package as previously described (Belanche et al., 2016a). The sequences were further clustered into OTUs at 97% identity using the UPARSE pipeline (http://drive5.com/uparse/) (Edgar, 2013). The bacterial and methanogen OTU tables were normalised by random subsampling according to the sample with the minimum number of sequences. Taxonomic classification of bacteria and methanogens was carried out by comparison of the 16S rRNA gene sequences against RDP-II (Wang et al., 2007) and RIM-DB database (Seedorf et al., 2014). The methanogen sequences were further blasted to RDP-II to remove any OTU classified as bacteria from the OTU table and the taxonomy from the RIM-DB was identified at 80% confidence threshold. Raw sequence reads from

the bacterial and methanogen libraries were deposited at the EBI Short Read Archive (SRA) from the European Nucleotide Archive (ENA) and can be accessed under the study accession numbers: PRJEB26026 and PRJEB26027, respectively.

2.2.5. Statistical analysis

Data from ruminal fermentation and microbial abundance were analysed as a one-way ANOVA in SPSS (IBM Statistics version 22) using the following linear model:

$$Y_{ij} = \mu + t_i + \varepsilon_{ij}$$

where Y_{ij} = measured response, μ = overall mean effect, t_i = fixed effect of treatment (i = 1, …, 5) and ε_{ij} = experimental error term. Contrast tests were used to assess the overall effect of dietary tannins (control *vs.* HT-c + HT-t + CT-m + CT-g) and the effect of tannin type (HT *vs.* CT: HT-c + HT-t *vs.* CT-m + CT-g). Shapiro-Wilk normality test was applied on qPCR data and the $\log_{10}$ transformation was performed if unequal variances were found. Log-transformed data were subsequently analysed as highlighted for rumen fermentation variables. Significance was considered when $P \leq 0.05$ and a tendency for treatment effect was observed when $0.05 < P \leq 0.10$. When significance was detected, Tukey's HSD test was used for multiple comparisons of treatment means. Pearson's correlation analysis was performed to assess the relationship between the abundance of methanogens and protozoa.

The biodiversity indices of bacterial and methanogen microbiome were calculated using the normalised OTU datasets using PRIMER-v6 ecological software (PRIMER-E Ltd., Plymouth, UK). Permutational multivariate analysis of variance (PERMANOVA) was used to enumerate the structural differences in the bacterial and methanogen communities using Bray-Curtis similarity measurement of log-transformed OTU datasets. The pseudo *F*-statistics and *P*-values were obtained by performing 999 random permutations of residuals

under a reduced model using Monte Carlo test as described by Belanche et al. (2016a). Non-parametric multi-dimensional scaling plots and dendrogram plot of hierarchical cluster analysis were generated using PRIMER-v6. Heat map and rarefaction curve were constructed using the vegan package in R statistical software (version 3.2.5). Treatment effect on the relative abundances of bacterial and methanogen taxa was analysed as a one-way ANOVA as indicated for qPCR data. Tukey's HSD test was used for multiple comparisons of treatment means when significance was detected and multiple comparisons of relative abundances were adjusted for false positives using Bonferroni correction. The correlations between ruminal fermentation and the bacterial and methanogen population structure were elucidated on ordination plots using canonical correspondence analysis (CCA) generated in R software. *P*-values of the variables were computed using 999 random permutations.

2.3. RESULTS

2.3.1. Fermentation parameters and microbial abundance

In comparison to CON, only lambs fed HT-c had reduced ($P < 0.05$) DMI while diet tended to influence rumen pH and total VFA (Table 2.2). Multiple comparisons of treatments showed that HT-c elicited lower ($P = 0.044$) rumen pH and higher ($P = 0.049$) total VFA compared to CT-g. Diet did not affect ($P > 0.05$) ammonia concentration nor the molar proportion of acetate, propionate, valerate and acetate/propionate ratio, and enzymatic activities. However, CT-m lambs had an increased ($P < 0.05$) butyrate compared to CON and CT-g animals. HT-c lambs had a lower proportion of *iso*-butyrate compared to CT-m lambs and lower *iso*-valerate than lambs fed the CT-m or CT-g. No significant effect of tannin supplementation on ruminal fermentation traits except for a tendency ($P = 0.066$) for tannin treatments to increase the molar proportion of butyrate. HT extracts lowered ruminal pH and increased the concentration of total VFA (+31.6%) compared to CT extracts. Moreover, HT extracts tended ($P = 0.074$) to elevate molar proportion of propionate but decreased ($P < 0.05$) the molar proportion of *iso*-butyrate and *iso*-valerate compared to CT extracts. However, enzymatic assays indicated that there was no effect of dietary tannins on specific activities of carboxymethyl cellulase, xylanase, amylase or lipase.

Real-time PCR indicated that absolute abundance of bacteria and fungi were comparable between treatments but tannin treatments reduced the abundance of methanogens ($P = 0.002$) and protozoa ($P = 0.003$) (Table 2.3). In comparison to CON, the abundance of methanogens was significantly inhibited by CT-m (-12.8%) and CT-g (-11.5%) while the abundance of protozoa was reduced by HT-t (-20.7%) and CT-g (-20.8%). However, the inhibitory effect of tannins on methanogen and protozoa abundance was similar ($P > 0.05$) between HT and CT extracts.

Table 2.2. Effect of different tannin extracts on ruminal pH and fermentation characteristics in lambs

Parameter	Dietary treatment[1]							Contrast[2] (*P*-value)	
	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
DMI	1064.09[a]	845.73[b]	961.87[ab]	1061.24[a]	994.82[a]	18.143	<0.001	0.010	<0.001
Rumen pH	6.50	6.01	6.41	6.54	6.63	0.073	0.052	0.572	0.017
NH_3-N (mMol/L)	13.67	13.24	15.98	15.54	15.78	0.899	0.818	0.546	0.611
Total VFA (mMol/L)	55.15	77.45	55.30	46.74	44.09	3.982	0.055	0.939	0.015
Molar proportion of VFA									
Acetate	50.67	53.87	51.04	51.08	52.92	0.678	0.506	0.383	0.766
Propionate	32.73	30.85	32.29	26.12	29.53	0.949	0.179	0.212	0.074
Iso-butyrate	3.68[ab]	2.09[b]	3.33[ab]	3.43[ab]	4.18[a]	0.221	0.031	0.430	0.019
Butyrate	6.52[b]	9.24[ab]	7.77[ab]	11.80[a]	6.56[b]	0.543	0.005	0.066	0.523
Iso-valerate	2.65[ab]	1.29[b]	2.41[ab]	3.74[a]	3.42[a]	0.241	0.006	0.905	0.001
Valerate	3.76	2.67	3.16	3.82	3.39	0.210	0.414	0.368	0.144
Acetate/propionate ratio	1.59	2.08	1.59	2.11	1.88	0.118	0.475	0.302	0.543
[3]Specific enzymatic activity									
Carboxymethyl cellulase	34.92	45.10	42.86	43.61	47.13	2.780	0.733	0.194	0.826
Xylanase	129.15	130.71	166.90	128.09	161.26	13.315	0.812	0.623	0.892
Amylase	77.80	51.52	69.29	47.14	77.50	7.478	0.577	0.407	0.909
Lipase	2.46	2.69	2.72	2.41	4.04	0.291	0.376	0.505	0.425

DMI: Dry matter intake; VFA: Volatile fatty acids; SEM: Standard error of mean

[1]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.

[2]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).

[3]Specific activity of carboxymethyl cellulase, xylanase and amylase were expressed as mg of sugar released per g of protein per min while lipase activity was expressed as mM PNP per g of protein per min. Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.

Table 2.3. Effect of different tannin extracts on microbial numbers, and bacterial and methanogen biodiversity indices in the rumen of lambs

Parameter	Dietary treatments[2]							Contrast[3] (*P*-value)	
	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
Microbial numbers[1]									
Bacteria (log copy/mgDM)	8.83	8.67	8.90	8.69	8.86	0.040	0.251	0.593	0.906
Methanogens (log copy/mgDM)	6.63^{a}	5.97^{ab}	6.13^{ab}	5.78^{b}	5.87^{b}	0.088	0.020	0.002	0.211
Fungi (log copy/mgDM)	3.19	3.42	2.37	2.27	2.90	0.233	0.456	0.458	0.557
Protozoa (log copy/mgDM)	8.37^{a}	6.86^{ab}	6.64^{b}	7.11^{ab}	6.63^{b}	0.203	0.039	0.003	0.771
Bacterial diversity indices									
Number of OTU	441.25	516.44	464.33	552.22	472.56	16.093	0.195	0.147	0.529
Pielou's evenness index	0.58	0.58	0.55	0.58	0.54	0.009	0.299	0.374	0.803
Shannon's index	3.55	3.63	3.36	3.67	3.32	0.063	0.272	0.749	0.997
Simpson's index	0.91	0.91	0.89	0.91	0.88	0.006	0.154	0.267	0.747
Methanogen diversity indices									
Number of OTU	32.38	34.00	33.56	34.11	32.22	0.478	0.610	0.389	0.572
Pielou's evenness index	0.37	0.43	0.38	0.42	0.38	0.012	0.344	0.267	0.929
Shannon's index	1.29	1.53	1.33	1.49	1.34	0.042	0.279	0.229	0.875
Simpson's index	0.57	0.72	0.60	0.68	0.63	0.019	0.081	0.070	0.812

OTU: Operational taxonomic unit; SEM: Standard error of mean

[1]Data were log-transformed to achieve normality; DM, dry matter of freeze-dried rumen content

[2]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.

[3]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).

Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.

2.3.2. Bacterial microbiome

Ion torrent NGS of bacterial 16s rDNA genes yielded approximately 1.6 million raw sequences clustered into 2027 OTUs across 44 samples. Subsequently, the NGS data sets were normalised resulting into 936,176 high-quality sequences clustered into 2010 unique OTUs, with an average of 21,276 sequences per sample. The rarefaction curves plateaued, suggesting that sampling of the rumen environments have comparable sequencing depth across treatments (See appendix: Figure S2.1). Biodiversity indices of the rumen bacterial community were unaffected by dietary treatment (Table 2.3). Non-metric multi-dimensional scaling (NMDS) plot indicated a slight clustering separating HT-c and other dietary treatments (Figure 2.1a). PERMANOVA showed that dietary treatment altered the bacterial community structure and tannin supplementation tended to influence the bacterial community structure (Table 2.4). Pairwise comparisons revealed that bacterial community structure differs between CON and HT-c as well as between CT-m and CT-g. Moreover, there were tendencies for bacterial community differences between CON and CT-m and between HT-c and CT-g (Table 2.4).

Taxonomic classification indicated that *Bacterioidetes* (40.7%) was the predominant phylum followed by *Proteobacteria* (38.5%), *Firmicutes* (15.2%), *Fibrobacteres* (2.3%), *Spirochaetes* (1.2%), *Actinobacteria* (0.4%), *SR1* (0.3%) and *Tenericutes* (0.2%), with a limited number of unclassified sequences (0.9%) (Table 2.5). At the genus level, *Prevotella* (28.6%) and *Ruminobacter* (21.9%) were the predominant groups and heat map illustrated no distinct grouping of the genera across treatments (See appendix: Figure S2.2). The effect of dietary treatment on phylogenetic composition was only obvious on the relative abundance of *Bacterioidetes* and *Fibrobacteres* phyla. The abundance of *Bacterioidetes* was lower in CT-g compared to CT-m and this was mediated by a shift from genus *Prevotella* (*Bacterioidetes*) to *Ruminobacter* (*Proteobacteria*) (Table 2.5). Dietary tannins

significantly decreased *Fibrobacteres*, *Fibrobacteraceae* and *Fibrobacter* at the phylum, family and genus levels, respectively (Table 2.5). The inhibitory effect of tannins was more apparent in HT-c when compared to CON (Table 2.5). The ordination plot of the canonical correspondence analysis (CCA) illustrated the correlation between the rumen bacterial community and the fermentation and microbial parameters (Figure 2.2a). There was a clustering of samples from HT-c-fed animals. The CCA plot indicated that positive correlations of butyrate (P = 0.001), acetate (P = 0.058), protein content (P = 0.002), acetate/propionate ratio (P = 0.001) and bacterial diversity (P = 0.001) appeared to be associated with the bacterial community in HT-c samples (Figure 2.2a and Table S2.2, See appendix). However, the correlation of amylase activity tended (P = 0.075) to be associated with the rumen bacterial community in lambs fed CON, HT-t, CT-m and CT-g.

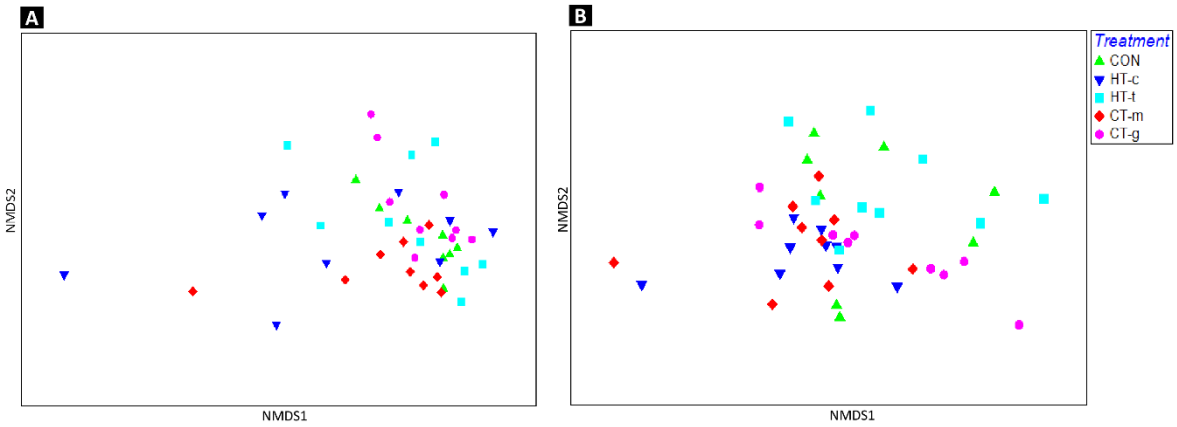


Figure 2.1. (A) Non-parametric multi-dimensional scaling (NMDS) plot (stress = 0.14) of rumen bacterial communities in lambs fed different tannin extracts; (B) NMDS plot (stress = 0.15) of rumen methanogen communities in lambs fed different tannin extracts. Dietary treatments are: CON, control; HT-c, chestnut hydrolysable tannin; HT-t, tara hydrolysable tannin; CT-m, mimosa condensed tannin; CT-g, gambier condensed tannin. NMDS plot was produced from a resemblance matrix created using Bray-Curtis similarity of log-transformed OTU data sets.

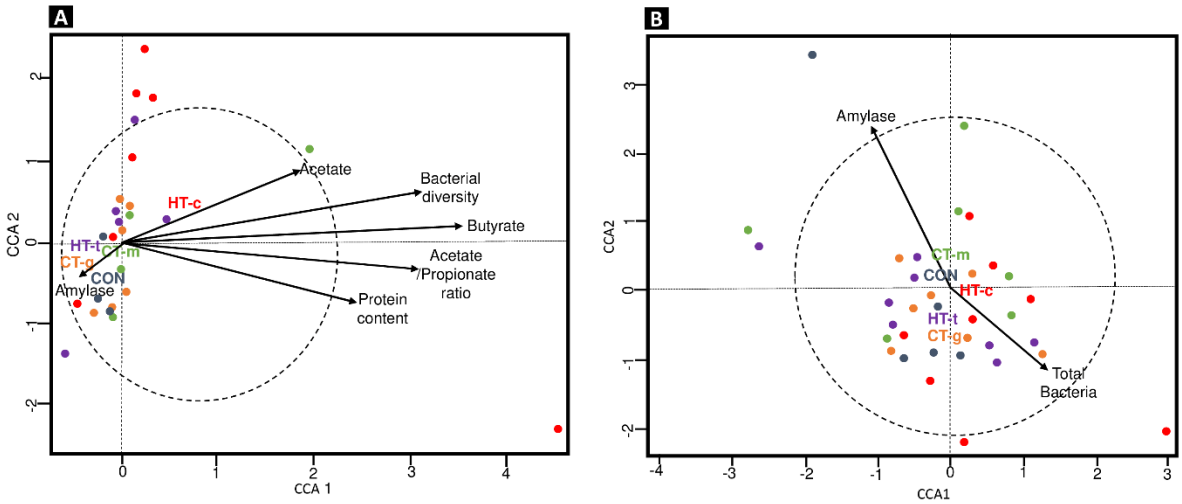


Figure 2.2. Canonical correspondence analysis (CCA) describing the correlations between: (A) rumen bacterial community structure and fermentation and microbial variables; (B) rumen methanogen community structure and fermentation and microbial variables. The arrows indicate the gradient direction and their length is relative to the proportion of correlation. Arrows longer than the dotted circle signify correlation significance ($P < 0.05$). Coloured dots represent distribution pattern of the animals fed each dietary treatments: CON, control; HT-c, chestnut hydrolysable tannin; HT-t, tara hydrolysable tannin; CT-m, mimosa condensed tannin; CT-g, gambier condensed tannin.

2.3.3. Methanogen microbiome

Sequencing of methanogenic 16s rDNA genes produced about 3 million sequences clustered into 57 OTUs across 44 samples after quality filtering. The NGS data sets were normalised resulting into 572,792 high-quality sequences clustered into 57 unique OTUs, with an average of 13,018 sequences per sample. Methanogen diversity indices were unaffected by dietary treatments (Table 2.3). However, Simpson's index suggests that dietary tannins tended to promote higher diversity in methanogen community. Visualisation of the methanogen community data sets on NMDS plot showed that there was no clear clustering between diets (Figure 2.1b). Dietary treatment elicited variable effects on the methanogen community structure but neither tannins nor tannin types (HT *vs.* CT) had a significant impact (Table 2.4). Pairwise comparison of the methanogen community

structure revealed that HT-c differed from HT-t and CT-g diets whereas CT-m differed from HT-t and CT-g diets. Additionally, there was a tendency for differences when CON was compared to CT-m methanogen community structure.

Euryarchaeota was the only identified phylum, accounting for 99.1% of methanogen abundance. The predominant families were *Methanobacteriaceae* (30.8%, *Methanobacteria*) and *Methanomassiliicoccaceae* (5.9%, class *Thermosplasmata*) while the abundances of *Methanocorpusculaceae* and *Methanosarcinaceae* (class *Methanomicrobia*) were less than 0.1% (Table 2.6). *Methanobrevibacter* (29.0%) dominate the genus level followed by *Methanomassiliicoccus* (5.9%) and *Methanosphaera* (1.8%). Neither diets, tannins nor tannin type influence ($P > 0.05$) the relative abundance of methanogen taxa (Table 2.6). However, dietary tannins tended ($P = 0.080$) to inhibit genus *Methanosphaera*. Methanogen microbiome significantly correlated with amylase activity ($P = 0.047$) and tended to correlate with bacteria abundance ($P = 0.062$) without apparent association to any dietary treatment (Figure 2.2b and Table S2.3, See appendix).

Table 2.4. Effect of different tannin extracts on rumen bacterial and methanogen community structure in lambs

	Similarity	Pseudo-F	*P*-value
Bacterial community[1]			
Treatment		1.60	0.005
Contrasts			
Tannin[2]		1.47	0.096
Tannin type[3]		1.28	0.127
Pair wise comparison of dietary treatment[4]			
CON *vs.* HT-c	37.92	1.49	0.038
CON *vs.* HT-t	44.45	1.08	0.330
CON *vs.* CT-m	44.87	1.37	0.051
CON *vs.* CT-g	46.71	1.21	0.133
HT-c *vs.* HT-t	36.60	1.16	0.195
HT-c *vs.* CT-m	38.11	1.24	0.142
HT-c *vs.* CT-g	38.28	1.34	0.073
HT-t *vs.* CT-m	41.48	1.20	0.156
HT-t *vs.* CT-g	43.16	1.06	0.327
CT-m *vs.* CT-g	42.89	1.44	0.039
Methanogen community[1]			
Treatment		1.91	0.005
Contrasts			
Tannin[2]		1.01	0.392
Tannin type[3]		1.02	0.411
Pair wise comparison of dietary treatment[4]			
CON *vs.* HT-c	73.26	1.26	0.131
CON *vs.* HT-t	72.42	0.85	0.634
CON *vs.* CT-m	71.52	1.34	0.093
CON *vs.* CT-g	70.90	1.28	0.134
HT-c *vs.* HT-t	71.84	1.65	0.025
HT-c *vs.* CT-m	75.64	0.92	0.535
HT-c *vs.* CT-g	71.70	1.71	0.019
HT-t *vs.* CT-m	71.16	1.50	0.035
HT-t *vs.* CT-g	70.90	1.31	0.122
CT-m *vs.* CT-g	70.42	1.68	0.023

[1]Higher Pseudo-F and lower similarities and *P*-values imply greater differences in the rumen bacterial community structure

[2]Tannin (contrast of overall effect of tannin: CON *vs.* HT-c + HT-t + CT-m + CT-g);

[3]Tannin type (contrast of effect of HT *vs.* CT: HT-c + HT-t *vs.* CT-m + CT-g);

[4]Dietary treatments: CON: control; HT-c: chestnut hydrolysable tannin; HT-t: tara hydrolysable tannin; CT-m: mimosa condensed tannin; CT-g: hambier condensed tannin.

Table 2.5. Effect of different tannin extracts on relative abundance (%) of bacteria taxa ≥ 0.2% of average abundance in the rumen of lambs

			Dietary treatment[1]							Contrast[2] (*P*-value)	
Phyla	**Family**	**Genus**	**CON**	**HT-c**	**HT-t**	**CT-m**	**CT-g**	**SEM**	***P*-value**	**Tannin**	**Tannin type**
Bacteroidetes			40.10^{ab}	39.12^{ab}	40.53^{ab}	49.04^{a}	34.72^{b}	1.470	0.028	0.830	0.495
	Porphyromonadaceae		3.14	3.53	3.21	2.71	4.55	0.369	0.602	0.713	0.755
		Barnesiella	2.75	1.46	2.50	1.65	3.82	0.364	0.242	0.673	0.348
	Prevotellaceae		31.38^{ab}	28.11^{b}	32.93^{ab}	39.81^{a}	23.71^{b}	1.436	0.003	0.939	0.652
		Prevotella	28.30^{ab}	25.50^{b}	30.27^{ab}	37.04^{a}	22.08^{b}	1.345	0.003	0.889	0.516
		Paraprevotella	0.51	0.55	0.30	0.40	0.22	0.055	0.306	0.328	0.360
		Hallella	0.45	0.35	0.28	0.60	0.22	0.056	0.219	0.550	0.449
		Solobacterium	0.55	0.36	0.25	0.39	0.43	0.079	0.849	0.367	0.573
	Rikenellaceae		1.99	0.12	0.72	0.04	2.94	0.527	0.331	0.448	0.359
		Rikenella	1.99	0.12	0.72	0.04	2.94	0.527	0.331	0.448	0.359
	Marinilabiliaceae		0.13	0.20	0.08	0.30	0.42	0.081	0.714	0.590	0.248
Proteobacteria			37.72	39.56	40.33	28.23	46.67	2.166	0.095	0.855	0.586
	Succinivibrionaceae		34.51	33.94	37.07	26.33	43.12	2.076	0.133	0.907	0.860
		Ruminobacter	20.84^{ab}	21.05^{ab}	23.27^{ab}	13.77^{b}	30.63^{a}	1.784	0.043	0.756	0.991
		Succinivibrio	0.16	2.26	0.40	3.65	0.19	0.795	0.559	0.488	0.742
		Anaerobiospirillum	6.20	3.37	5.14	4.47	2.60	1.106	0.875	0.442	0.775
Firmicutes			14.47	15.61	14.13	16.42	15.12	0.677	0.847	0.640	0.564
	Ruminococcaceae		4.11	5.35	4.55	5.01	5.11	0.286	0.704	0.247	0.869
		Ruminococcus	1.40	0.65	1.27	0.81	1.42	0.138	0.259	0.304	0.629
		Acetivibrio	0.27	0.42	0.82	0.51	0.82	0.113	0.464	0.211	0.867
	Lachnospiraceae		2.06	2.63	2.80	2.71	2.72	0.135	0.472	0.070	0.996
		Lachnospiracea incertae sedis	0.22	0.31	0.54	0.48	0.49	0.059	0.391	0.128	0.633
		Roseburia	0.89	0.83	1.06	0.47	0.84	0.094	0.387	0.726	0.176
		Butyrivibrio	0.39^{ab}	0.24^{b}	0.40^{ab}	0.74^{a}	0.28^{b}	0.052	0.014	0.835	0.078
	Veillonellaceae		1.81	1.41	1.68	1.72	2.65	0.199	0.345	0.910	0.152

Table 2.5. Continued.

Phylum	Family	Genus	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
		Dialister	0.93^{ab}	0.44^{b}	0.88^{ab}	0.76^{ab}	1.93^{a}	0.168	0.048	0.864	0.054
		Mitsuokella	0.26	0.07	0.28	0.12	0.31	0.050	0.464	0.597	0.742
	Erysipelotrichaceae		2.89	1.84	1.91	2.41	2.01	0.274	0.759	0.252	0.588
		Bulleidia	0.37	0.48	0.62	0.80	0.57	0.095	0.697	0.325	0.524
		Solobacterium	0.55	0.36	0.25	0.39	0.43	0.079	0.849	0.367	0.573
		Alloprevotella	0.35	0.04	0.10	0.41	0.21	0.062	0.270	0.319	0.085
		SR1 genera incertae sedis	0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275
	Acidaminococcaceae		2.88	2.79	1.98	2.56	1.92	0.222	0.529	0.339	0.772
		Succiniclasticum	2.39	2.65	1.62	2.10	1.54	0.204	0.350	0.430	0.483
		Acidaminococcus	0.34	0.09	0.34	0.38	0.34	0.047	0.303	0.689	0.178
Spirochaetes			1.48	1.06	0.76	1.72	0.86	0.210	0.562	0.492	0.422
	Spirochaetaceae		1.16	0.95	0.62	1.43	0.67	0.194	0.668	0.642	0.543
		Treponema	0.64	0.69	0.40	0.99	0.42	0.144	0.697	0.972	0.612
		Sphaerochaeta	0.49	0.14	0.17	0.43	0.24	0.064	0.333	0.149	0.208
Actinobacteria			0.49	0.24	0.63	0.44	0.29	0.063	0.295	0.579	0.624
	Coriobacteriaceae		0.48	0.21	0.61	0.33	0.29	0.060	0.223	0.431	0.445
		Olsenella	0.47	0.20	0.58	0.32	0.28	0.059	0.249	0.405	0.498
Fibrobacteres			4.93^{a}	0.75^{b}	1.97^{ab}	2.61^{ab}	1.41^{ab}	0.436	0.028	0.003	0.465
	Fibrobacteraceae		4.93^{a}	0.75^{b}	1.97^{ab}	2.61^{ab}	1.41^{ab}	0.436	0.028	0.003	0.465
		Fibrobacter	4.93^{a}	0.75^{b}	1.97^{ab}	2.61^{ab}	1.41^{ab}	0.436	0.028	0.003	0.465
Tenericutes			0.06	0.14	0.27	0.32	0.19	0.058	0.657	0.262	0.699
SR1			0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275

Table 2.5. Continued.

Phylum	Family	Genus	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
	SR1 genera incertae sedis		0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275
		SR1 genera incertae sedis	0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275
Unclassified			0.47^{b}	1.76^{a}	0.71^{b}	0.98^{ab}	0.61^{b}	0.127	0.006	0.067	0.083
	Unclassified		7.70	15.10	8.54	10.76	7.74	1.072	0.139	0.297	0.267
		Unclassified	20.19^{b}	31.94^{a}	22.40^{b}	21.90^{b}	23.05^{ab}	1.150	0.006	0.085	0.042

SEM: Standard error of mean

[1]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.

[2]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).

Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.

Table 2.6. Effect of different tannin extracts on relative abundance (%) of methanogen taxa in the rumen of lambs

			Dietary treatment[1]							Contrast[2] (*P*-value)	
Class	**Family**	**Genus**	**CON**	**HT-c**	**HT-t**	**CT-m**	**CT-g**	**SEM**	***P*-value**	**Tannin**	**Tannin type**
Methanobacteria			32.01	25.80	35.99	28.01	32.18	2.548	0.756	0.825	0.891
	Methanobacteriaceae		32.01	25.80	35.99	28.01	32.18	2.548	0.756	0.825	0.891
		Methanobrevibacter	29.39	24.77	33.87	26.40	30.65	2.385	0.781	0.942	0.884
		Methanosphaera	2.62	1.03	2.12	1.61	1.53	0.228	0.244	0.080	0.993
Methanomicrobia			0.03	0.03	0.06	0.03	0.02	0.007	0.306	0.880	0.156
	Methanocorpusculaceae		0.02	0.02	0.05	0.03	0.01	0.006	0.343	0.482	0.451
		Methanocorpusculum	0.02	0.02	0.05	0.03	0.01	0.006	0.343	0.482	0.451
	Methanosarcinaceae		0.02	0.01	0.02	0.00	0.00	0.003	0.510	0.371	0.138
Thermoplasmata			0.95	11.64	1.46	12.00	3.15	1.750	0.085	0.163	0.782
	Methanomassiliicoccaceae		0.94	11.63	1.46	12.00	3.15	1.750	0.085	0.163	0.781
		Methanomassiliicoccus	0.94	11.63	1.46	12.00	3.15	1.750	0.085	0.163	0.781

Table 2.6. Continued

Phylum	Family	Genus	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
Unclassified			67.01	62.53	62.48	59.96	64.65	2.757	0.955	0.540	0.975
	Unclassified		67.02	62.54	62.49	59.96	64.65	2.757	0.955	0.539	0.974
		Unclassified	67.03	62.55	62.50	59.96	64.66	2.757	0.955	0.539	0.973

SEM: Standard error of mean
[1]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.
[2]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).
Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.

2.4. DISCUSSION

Dietary tannins may have potential as natural rumen modifiers but their effect on ruminal metabolism varies with different tannin sources (Patra et al., 2012; Patra and Saxena, 2011). HT are more susceptible to microbial hydrolysis than CT (Bhat et al., 1998) and the products of tannin degradation may influence ruminal metabolism (McSweeney et al., 2001). The current study, therefore, hypothesised that different types of tannins (HT *vs.* CT) would elicit differential impacts on the ruminal fermentation and microbiome.

2.4.1. Ruminal fermentation

Tannins can react with proteins to form complexes that are resistant to ruminal degradation and/or inhibit the growth and activity of proteolytic bacteria, thereby increasing the post-ruminal flow of non-ammonia N and improving N utilisation efficiency in ruminants (Makkar, 2003; Waghorn, 2008). In the current study, none of the dietary tannins affected NH_3-N concentration in contrast with several *in vivo* studies supporting the effect of tannins in reducing protein degradation in the rumen (Al-Dobaib, 2009; Broderick et al., 2017; Puchala et al., 2005). The formation of stable and insoluble tannin-protein complexes is induced at pH 3.5 – 7.0 (Jones and Mangan, 1977), which is consistent with the rumen pH of lambs measured in this study. Similarly, the tannin/protein (w/w) intake ratios for lambs fed HT-c (1:7.1), HT-t (1:5.8) and CT-m (1:6.4) are within the range (≥1:10-12, w/w) of tannin concentration required to decrease ruminal proteolysis (Jones and Mangan, 1977; Tanner et al., 1994). However, the effect of tannins in inhibiting ruminal proteolysis is inconsistent. The inclusion of 20 – 40 g/kg DM extracts of quebracho-CT (Piñeiro-Vázquez et al., 2017) or chestnut-HT and valonea-HT (Wischer et al., 2014) did not affect ruminal NH_3-N nor protein utilisation efficiency in cattle and sheep, respectively. The protein precipitating capacity of tannins may be affected by their structural features (Kraus et al.,

2003), and the degree of tannin affinity to proteins can be influenced by molecular weight, isoelectric point, compatibility of binding sites and protein tertiary structure (Patra and Saxena, 2011; Reed, 1995). Thus, the interaction between tannin source and the type of feed protein could influence protein precipitation in the rumen (Giner-Chavez et al., 1997; Zeller et al., 2015). Lorenz et al. (2014) indicated that protein type influences the concentration of tannins that could induce precipitation, suggesting that higher inclusion level of the tannin extracts may be required to decrease rumen protein degradation in our study.

Furthermore, the comparison of tannin effect across *in vivo* studies may be complicated by differences in the digestion kinetics of the basal diet (concentrate *vs.* forage-based diet) and ruminant species. Concentrate diets are rapidly digested in the rumen with a shorter transit time compared to forage-based diets and ruminal digestion kinetics also varies among ruminant species (Colucci et al., 1984; Huhtanen et al., 2006). These variations result in a temporal factor limiting the formation of tannin-protein complex in the rumen especially when tannins are included in concentrate diets. For instance, HT-c did not affect ruminal proteolysis when supplemented in a concentrate diet of lambs and sheep in the present study and that of Wischer et al. (2014), respectively. In contrast, HT-c reduced ruminal protein degradation when included in a forage-based diet of cattle (Tabacco et al., 2006).

Typically, ruminants derive approximately 70% of their energy requirement from VFA (mainly acetate, propionate and butyrate) produced from microbial fermentation of feeds in the rumen (Bergman, 1990). Tannin concentration is often considered the most influential factor affecting ruminal fermentation (Patra and Saxena, 2011). However, it is evident that the response could also vary between different tannin sources supplemented at a similar dosage (Getachew et al., 2008; Makkar et al., 1995). High concentration of tannins can inhibit feed digestibility and ruminal fermentation by forming complexes with

lignocellulose or by directly inhibiting rumen micro-organisms and microbial enzymes (Bae et al., 1993; Chung et al., 1998; Nsahlai et al., 2011). In the present study, 4% inclusion of the HT and CT extracts modified the rumen microbiota without compromising overall ruminal fermentation. Indeed, greater total VFA concentration was measured in HT-fed lambs compared to lambs fed CT sources or the control diet. Min et al. (2014a) also reported that dietary addition of 100 g/d chestnut-HT resulted in a higher total VFA than quebracho-CT and the control diet in grazing goats. However, Jayanegara et al. (2015) showed that increasing dosage (0.5, 0.75 and 1.0 mg/ml) of either purified HT (chestnut and sumach) or CT (mimosa and quebracho) resulted in a quadratic decrease of *in vitro* dry matter digestibility, with a tendency for a more pronounced decrease in CT sources. Nonetheless, the increase in total VFA concentration elicited by HT extracts in this study may be related to their possible hydrolysis into derivates that are metabolised into fermentation products. Chestnut and tara tannins have been shown to be susceptible to biodegradation induced by bacterial tannase activity (Deschamps and Lebeault, 1984; Deschamps et al., 1983). The biodegradation of HT and its monomers, such as gallic acid, involves decarboxylation into pyrogallol that is further transformed to several intermediates in step-wise enzymatic reactions, to form acetate and butyrate (Bhat et al., 1998). However, the metabolism of polyphenols could limit their bioavailability and bioactivity for modulating gut microbiota (Ozdal et al., 2016). This may probably explain the limited effect of HT-c in terms of response of the rumen microbiota. Further investigation is required to elucidate the biochemical mechanism involved in the interaction between rumen microbes and HT sources, and the consequent effect on ruminal fermentation.

Molecular quantification of rumen microbial populations demonstrated that tannins did not influence the abundance of bacteria and fungi but inhibited methanogen and protozoal numbers. Tannins could exert anti-microbial effect through multiple mechanisms including

induction of membrane disruption; eliciting morphological changes in cell wall via interaction with extracellular enzymes and cell wall; decreasing availability of cations to microbes via chelation; direct effect on the metabolism of microbes and causing deficit of substrates for microbial proliferation (Patra and Saxena, 2011; Scalbert, 1991). Methanogenic archaea are responsible for producing methane as a by-product of ruminal fermentation and rumen methane is a potent greenhouse gas that contributes significantly to global warming (Morgavi et al., 2010). Moreover, methane losses constitute 2 to 12% loss of gross energy intake in ruminants and could undermine animal productivity (Johnson and Johnson, 1995). Methanogens may exist either as free-living or associated with protozoa in a symbiotic relationship (Janssen and Kirs, 2008). The roles of protozoa in methanogenesis include the supply of H_2 as a substrate for CH_4 formation; serving as hosts for methanogens and protecting methanogens from oxygen toxicity (Newbold et al., 2015). Indeed, there is evidence that suggests a positive relationship between ruminal methanogenesis and the abundance of methanogens and protozoa (Belanche et al., 2015b; Wallace et al., 2014). In agreement with the present study, Pearson's correlation analysis indicated that there was a positive correlation ($r = 0.423$; $P < 0.01$) between the abundance of methanogens and protozoa. Thus, strategies that could reduce the abundance of methanogens and protozoa could be useful to mitigate enteric methane emission in ruminants (Morgavi et al. 2010). The anti-methanogenic potential of tannins largely depend on sources and concentration (Goel and Makkar, 2012; Jayanegara et al., 2012) but there is a limited information on their long-term persistence effect. The anti-methanogenic effect of different tannins has been attributed to their inhibitory effect on methanogen and protozoa population (Bhatta et al., 2009; Tan et al., 2011).

Given that the tannins were fed for 75 d in the present study, the antimicrobial effect of HT-t, CT-m and CT-g highlight these tannins as potential anti-methanogenic agents that could

exhibit long-term persistence. Interestingly, CT-g had the most consistent effect on both methanogen and protozoa abundances but there is no available study assessing its anti-methanogenic effect in ruminants. The inability of HT-c to inhibit methanogen and protozoa abundance may explain the transient anti-methanogenic effect of chestnut extract when supplemented (1.7 g/kg BW0.75) in the diet of sheep for 85 d (Wischer et al., 2014). The authors postulated that the rumen microbes could have adapted to HT extracts from chestnut and valonea after 2 weeks of supplementation in two separate experiments conducted over a duration of 85 d and 190 d (Wischer et al., 2014). However, this argument contradicts the observation that dietary addition of 30 g/kg of chestnut extract fed over a duration of 60 d decreased methane emission with a concomitant reduction of methanogen and protozoa populations in sheep (Liu et al., 2011).

Goel and Makkar (2012) suggested that HT sources decreased methane emissions through a direct inhibitory effect on growth and/or activity of methanogens- and/or hydrogen-producing microbes while CT exhibit an indirect effect by reducing hydrogen availability via depression of fibre digestion. Our results suggest that a wide range of tannin sources could inhibit methanogens and protozoa regardless of their chemical type. Methane emission was not quantified in the present study, however, our results highlight the need for future studies to particularly evaluate the *in vivo* anti-methanogenic activity of CT-g. Moreover, it would be useful to investigate the combination of these tannins given that vegetal sources containing both HT and CT could exhibit more potent anti-methanogenic activity than sources containing a single tannin type (Bhatta et al., 2009). A possible reason could be related to the synergistic effect of different tannins to inhibit either methanogen or protozoa abundance.

2.4.2. Bacterial microbiome

As confirmed by our sequence-based data, *Bacteroidetes*, *Proteobacteria* and *Firmicutes* are the main bacteria phyla in the rumen even though their proportion can vary with different diets (McCann et al., 2014). Tannins can modify bacterial structure and phylogeny without affecting the abundance of ruminal bacteria (Saminathan et al., 2016b). In this study, supplemental tannins altered the phylogenetic structure and composition of the bacteria community without impairing ruminal fermentation. HT-c was the only tannin that had a large effect on the bacterial community when compared to CON treatment. Moreover, the CCA ordination plot (Figure 2.2a) indicated a subtle difference between HT-c and other dietary treatments, with respect to the interplay between the rumen bacterial community and fermentation variables.

The disparity in the bacterial community structure between CT-g and CT-m was associated with a shift from *Prevotella* and *Paraprevotella* to *Ruminobacter*. Despite the bacterial shifts, CT-g and CT-m displayed similar ruminal fermentation traits which could be attributed to the fermentative redundancy between microbial groups (Weimer, 2015). Indeed, the rumen ecosystem can maintain similar metabolic function despite differences in microbial communities (Taxis et al., 2015). The decrease in the abundance of *Fibrobacter* in tannin-supplemented lambs further confirmed the profound inhibitory effect of dietary tannins on fibrolytic bacteria and fibre digestibility (Bae et al., 1993; Jayanegara et al., 2015; Sivakumaran et al., 2004). This suggests that the current inclusion level of tannin extracts may impair ruminal digestion of high-fibre diets in contrast to the concentrate diet fed in the present study. Tannins may have a selective effect on fibrolytic bacteria depending on dosage and type of tannins (Patra et al., 2012). In the present study, HT-c specifically inhibited *Fibrobacter* in comparison to CON. However, a similar commercial chestnut extract did not affect the abundance of *Fibrobacter succinogenes*

when 30 g/kg was supplemented in sheep diets for 60 d (Liu et al., 2011). The longer duration of supplementation (75 d *vs.* 60 d) and greater dosage (40 g/kg *vs.* 30 g/kg) could account for the discrepancy in the effect of HT-c on *Fibrobacter* between the present study and that of Liu et al. (2011). Additionally, the structural features of tannins could play a dominant role in the effect of different tannins on fibrolytic bacteria. Saminathan et al. (2016b) suggested that the abundance of *Fibrobacter* increased with increasing molecular weight (469.6 – 1265.8 Da) of CT fractions from *Leucaena leucocephala* hybrid.

2.4.3. Methanogen microbiome

The authors believe that this is the first study to characterise the *in vivo* effect of HT-c, HT-t, CT-m and CT-g on the rumen methanogen communities. It has been shown that potential anti-methanogenic effect of tannins could be related to a decrease in the abundance of methanogens and/or alteration in methanogen population structure (Longo et al., 2013; Tan et al., 2011). However, the present study indicated that dietary tannins did not exert a significant effect on methanogen diversity or community structure in comparison to CON. Saminathan et al. (2016a) recently utilised different CT fractions from *Leucaena leucocephala* hybrid to demonstrate that the molecular weights (469.6 – 1265.8 Da) of tannins could play a role in their modulatory effect on *in vitro* methanogen community structure. Accordingly, CT fractions with higher molecular weights resulted in a greater impact on methane production and methanogen community structure (Saminathan et al., 2015; Saminathan et al., 2016a). This highlights the need for future studies to explore the diversity in structural chemistry of tannins with respect to their bioactivity to modulate rumen microbiota.

As confirmed in the present study, *Methanobrevibacter* may be the most dominant methanogen genus in the rumen (Janssen and Kirs, 2008) but tannins did not affect the

relative abundance of this methanogen phylotype. Notably, tannins exhibited a specific tendency to reduce the abundance of *Methanosphaera* by approximately 40% while HT-c supplementation, in particular, resulted in a decrease of approximately 61% compared to CON. *Methanosphaera* spp. are important methanogens that can utilise hydrogen and methyl groups as precursors for methane formation in the rumen (Tapio et al., 2017). Min et al. (2014b) reported that dietary inclusion of pine bark (3.2% CT diet DM) reduced the relative abundance of *Methanosphaera* by 37% in faecal samples from goats. It is possible that tannins elicit apparent effects on methanogens at the species level particularly on the predominant genera, *Methanobrevibacter* and *Methanosphaera* (Li et al., 2013). Nonetheless, this is currently constrained by the limited genomic database on methanogen diversity. Overall, our data suggest that the potential anti-methanogenic effect of these supplemental tannins could be more related to their inhibitory effect on methanogen abundance rather than modulation of methanogen population structure.

2.5. CONCLUSIONS

This study demonstrates that feeding concentrate diets containing phenolic compounds of up to 29 g TAE/kg DM diet (equivalent to approximately 32 g of gallic acid equivalent/kg DM diet) did not impair rumen function. HT-t, CT-m and CT-g extracts displayed specific antimicrobial activity against methanogens and protozoa without compromising ruminal fermentation and animal productivity in a long-term feeding trial. Further *in vivo* studies are necessary to examine the effect of these tannins, particularly CT-g, in relation to their long-term persistence in reducing enteric methane emission in ruminants. Ruminal degradation of HT sources, such as HT-c, should be further investigated as a factor influencing their modulatory effect on rumen microbiota and fermentation. Moreover, both HT and CT extracts could impact the ruminal microbiome when supplemented at moderate levels but their negative effect on fibrolytic bacteria should be considered when fed with high-fibre diets.

CHAPTER 3 – Effects of dietary dehydrated alfalfa or cardoon meal on the modulation of ruminal biohydrogenation and microbial community in lambs

Saheed A. Salami, Bernardo Valenti, Giuseppe Luciano, Massimiliano Lanza, Joseph P. Kerry, Michael N. O'Grady, Charles J. Newbold, and Alessandro Priolo

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ABSTRACT

Alfalfa (*Medicago sativa*) is a forage legume widely used as a protein source in ruminant diets whereas cardoon meal is a novel by-product from oil extraction of cardoon seeds (*Cynara cardunculus* var. *altilis*) and is rich in protein, unsaturated fatty acids (FA) and polyphenols. The effect of substituting dehydrated alfalfa with cardoon meal was examined on the ruminal fermentation, biohydrogenation of FA (RBH) and the rumen microbial community of lambs fed a concentrate diet containing either 15% dehydrated alfalfa (CON, n = 8) or cardoon meal (CMD, n = 7) for 75 days pre-slaughter. In comparison to CON, lambs fed CMD had lower dietary intakes ($P < 0.05$) of C18:3 *n*-3 (-26.7%) but greater intakes of C18:0 (+34.7%), C18:1 *n*-9 (+20.2%), C18:2 *n*-6 (+19.6%) and polyphenols (+117.1%). Dietary treatment did not influence ruminal fermentation characteristics and the absolute abundance of rumen bacteria, methanogens, fungi or protozoa. Feeding CMD promoted RBH resulting in a greater concentration ($P < 0.05$) of ruminal C18:0 (+71.7%) and total saturated FA (+41.3%) and lower C18:1 *n*-9 (-21.8%) and monounsaturated FA (-46.0%) compared to CON-fed lambs. In addition, the inhibition of RBH in CON-fed lambs resulted in a higher concentration of *trans*-10 C18:1, *trans*-11 C18:1, total odd- and branched-chain FA and total *trans*-18:1 isomers in the ruminal digesta. Dietary effect on RBH was accompanied by changes in the rumen bacterial community with lower population diversity indices observed in lambs fed CMD compared to CON. Lambs fed CMD displayed a greater relative abundance of *Proteobacteria* and a lower abundance of *Bacteroidetes* and *Fibrobacteres* compared to CON-fed lambs. At the genus level, CMD mediated a specific shift from *Prevotella, Alloprevotella, Solobacterium* and *Fibrobacter* to *Ruminobacter*, suggesting that these bacterial genera may play an important role in RBH. Canonical correspondence analysis indicated that the rumen bacterial community in CON-fed lambs was positively correlated with *cis*-16 C18:1, *trans*-10 C18:1 and C18:3 *n*-3.

Overall, results demonstrated that dietary replacement of dehydrated alfalfa with cardoon meal promoted RBH through a modulation of the bacterial community, which may consequently reduce the accumulation of health-promoting unsaturated FA in ruminant meat or milk.

Keywords: Diet, rumen, biohydrogenation, fatty acids, bacteria, high-throughput sequencing

3.1. INTRODUCTION

Alfalfa (*Medicago sativa*), otherwise called alfalfa, is a forage legume widely used as a conventional protein source in the diet of ruminants. However, agro-industrial by-products (AIBP) can be explored as an economical substitute for conventional feedstuffs such as dehydrated alfalfa. This alternate feeding strategy can also enhance the resource efficiency and sustainability of ruminant production while reducing the environmental burden associated with the industrial disposal of these by-products (Makkar, 2016). Cardoon meal is a by-product obtained after the extraction of oil from the seeds of cultivated cardoon (*Cynara cardunculus* var. *altilis*), a perennial plant native to the Mediterranean region and widely distributed as a naturalized or invasive species in parts of Europe, Americas and Oceania (CABI, 2017). The global biomass of cardoon meal is increasing rapidly due to a renewed interest in the use of cardoon oil as a cheap source of biodiesel (De Domenico et al., 2016; Fernández and Curt, 2004). Cardoon meal is a valuable source of protein with a rich amino acid profile, high in fibre and contains bioactive compounds such as unsaturated fatty acids (oleic and linoleic acids) and polyphenols (Genovese et al., 2015). Hydroxycinnamic acids (caffeoylquinic acid derivatives) and flavonoids (apigenin and luteolin derivatives) have been identified as the main polyphenols present in cardoon vegetal organs (Pandino et al., 2011; Ramos et al., 2014). To date, the feeding potential of cardoon meal has not been explored in ruminant nutrition.

Evaluating the effect of novel feeds on rumen function is crucial considering the significant impact of diets on the rumen microbiome and metabolism, which in turn affect ruminant performance and the quality of meat and milk (Loor et al., 2016). The functional association between rumen microbiota (bacteria, archaea, fungi and protozoa) aid the fermentation of feed substrates to produce volatile fatty acids (VFA) and microbial protein that provide

ruminants with significant sources of energy and digestible proteins, respectively. However, this ruminal process is accompanied by microbial lipolysis and subsequent biohydrogenation (RBH) of dietary unsaturated fatty acids (FA) resulting in the formation of saturated FA (SFA), *trans* FA (TFA) and several FA intermediates (Jenkins et al., 2008). Ruminal lipid metabolism is a major factor contributing to the low content of polyunsaturated FA (PUFA) and high SFA and TFA levels in ruminant meat and milk. This typical lipid profile in ruminant-derived foods is associated with an increased incidence of cardio-metabolic diseases and cancer in humans (Givens, 2005). Nonetheless, RBH also produces functional FA intermediates, such as *trans*-11 18:1 and conjugated linoleic acids (CLA), with potential benefits on human health (Buccioni et al., 2012). Bacteria are thought to be the most active microbes involved in RBH and recent advances in high-throughput sequencing could elucidate the diverse bacteria species involved in the complex biochemical pathways related to RBH intermediates (Enjalbert et al., 2017).

Nutritional strategies, including dietary AIBP, that increase the ingestion of unsaturated FA and polyphenols potentially modulate the rumen microbiome and inhibit RBH, and consequently increase the accumulation of health-promoting unsaturated FA in meat or milk (Buccioni et al., 2012; Vasta et al., 2008). Moreover, dietary phenolic compounds can reduce ruminal proteolysis by inhibiting the growth and activity of proteolytic bacteria and/or by reacting with dietary proteins to form complexes that are resistant to ruminal degradation (Hart et al., 2008; Makkar, 2003). Therefore, it was hypothesized that feeding cardoon meal may exert a functional effect on the rumen microbiota and metabolism due to its content of bioactive compounds (polyphenols and unsaturated FA). The objective of this study was to investigate the effect of replacing dietary dehydrated alfalfa with cardoon meal on ruminal fermentation and RBH, and to utilize next-generation sequencing to characterize changes in the rumen bacterial community.

3.2. MATERIALS AND METHODS

3.2.1. Animals, diets, slaughter and rumen sampling

The experimental protocol was approved by the ethics committee of the University of Catania and the feeding trial was conducted indoor at the experimental farm of the University. The animals were handled by specialized personnel according to the European Union legislation for the protection of animals used for scientific purposes (2010/63/ EU Directive). Fifteen male Sarda x Comisana lambs (average age 75 d and initial BW 19.58 ± 2.01 kg) were randomly assigned to two experimental groups. Each animal was reared in an individual pen and adapted to the experimental diets for a period of 9 d by progressive substitution of the weaning feed with the experimental feeds until a total replacement of the weaning diet was achieved. The control group (CON, n = 8), was raised on a commercial concentrate-based diet containing the following ingredients (*as-fed* basis): barley (48.0%), dehydrated alfalfa (15.0%), wheat bran (23.0%), soybean meal (10.0%), molasses (2.0%) and vitamin premix (2.0%). The control group used in the present study was the same as the experiment reported by Salami et al. (2018). The cardoon meal group (CMD, n = 7), received the same diet as the CON lambs except that the 15% dehydrated alfalfa was completely replaced by cardoon meal. The chemical composition of the cardoon meal and experimental diets are outlined in Table 3.1. The CON and CMD diets were supplied in form of pellets and lambs had *ad libitum* access to feeds and water for 75 days pre-slaughter. Diets were supplied daily and the amount of refusal was measured before morning (09:00h) feeding to calculate nutrient intakes from the total dry matter intake.

The lambs were slaughtered (stunned by captive bolt before exsanguination) in a commercial abattoir, where they had free access to the experimental diets and water until approximately 3 h before slaughter. The pH of the ruminal digesta was measured

immediately post-slaughter using a pH-meter (Orion 9106, Orion Research Incorporated, Boston, MA). Two aliquots of the ruminal digesta (70 – 80 g) were collected from each lamb within 20 min of slaughter and immediately placed in dry ice prior to storage at -80 °C for analysis of rumen fermentation, fatty acids and microbial population.

3.2.2. Feed analysis

Cardoon meal and the experimental diet samples were analysed for chemical composition. Crude protein, ether extract and ash content were determined following the method of AOAC (1995). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed as described by Van Soest et al. (1991). Phenolic compounds were extracted from the cardoon meal and experimental diets using aqueous methanol (50:50, vol/vol) and acetone (70:30, vol/vol) solvents (Jiménez-Escrig et al., 2001). Polyphenol-rich extracts were analysed for total phenol content (TPC) using the Folin-Ciocalteu reagent. The TPC was expressed as g of gallic acid equivalent/g of dry matter (DM). Protein partitioning of cardoon meal and experimental diets into nitrogen fractions was carried out according to the Cornell Net Carbohydrate and Protein System (CNCPS) as described by Licitra et al. (1996). The FA composition in cardoon meal and the experimental diets was determined by a one-step extraction–transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003). Gas chromatographic analysis of fatty acid methyl esters (FAME) was performed as described later for FA profile in the ruminal digesta. Individual FA were expressed as mg/g of DM.

Table 3.1. Chemical composition of cardoon meal (CM) and experimental diets.

	Ingredient	Diets[1]	
Item	**CM**	**CON**	**CMD**
Dry matter, % as fed	92.71	89.65	89.63
Nutrient content			
Crude protein, % DM	18.17	15.67	16.45
Ether extract, % DM	7.99	2.68	3.84
Ash, % DM	5.52	7.01	6.31
NDF, % DM	45.46	30.36	27.32
ADF, % DM	39.54	15.97	12.39
ADL, % DM	12.74	3.62	4.15
Total phenolic content[2]	60.39	5.21	13.08
Protein fractions (% CP)			
A[3]	6.05	10.34	5.65
B1[3]	49.92	8.87	20.97
A + B1[3]	55.97	19.21	26.63
B2[3]	29.22	70.07	62.86
B3[3]	5.72	7.02	7.23
C[3]	9.08	3.70	3.28
Fatty acids (mg/g DM)			
C14:0	0.06	0.03	0.03
C16:0	5.82	4.36	5.06
cis-9 C16:1	0.06	0.035	0.03
C18:0 SA[3]	1.51	0.45	0.71
C18:1 *n*-9 OA[3]	8.94	3.86	5.36
cis-11 C18:1	0.25	0.21	0.21
C18:2 *n*-6 LA[3]	28.03	12.19	16.85
C18:3 *n*-3 ALA[3]	0.07	1.26	1.07
C20:0	0.16	0.09	0.09

[1]CON: control diet; CMD: cardoon meal diet

[2] Expressed as grams gallic acid equivalents/kg DM

[3]A: non-protein nitrogen; B1: true protein soluble in buffer; A + B1: total soluble nitrogen; B2: neutral detergent soluble protein; B3: neutral detergent insoluble protein but soluble in acid detergent; C: acid detergent insoluble protein; SA: stearic acid; OA: oleic acid; LA: linoleic acid; ALA: α-linolenic acid; ND: not detected

3.2.3. Analysis of ruminal fermentation characteristics

An aliquot of ruminal digesta from each lambs stored at -80 °C were thawed overnight at 4 °C and used for VFA and ammonia analyses. The thawed aliquots were centrifuged at 1000 g for 20 min at 4 °C and 4 mL of the supernatant was added to 1 mL of 25% trichloroacetic acid (TCA) containing 20 mM 2-ethylbutyric acid as an internal standard. The VFA

concentration was determined by gas liquid chromatography as outlined by de la Fuente et al. (2014). Ammonia analysis was carried out by diluting the acidified rumen samples with 25% TCA in ratio 4:1, followed by centrifugation at 15,000 g for 15 min and the supernatant was analysed for ammonia concentration. The method described by Weatherburn (1967) was used to determine the ammonia concentration using a ChemWell®-T autoanalyser (Awareness Technology Inc., Palm City, FL, USA - Megazyme Cat. No. D-CHEMT). Microbial enzymatic activities (carboxymethyl cellulase, xylanase and amylase) of freeze-dried rumen samples were determined as described by Belanche et al. (2016b). Lipase activity was determined by a spectrophotometric assay with *p*-nitrophenyl (PNP) butyrate as a substrate dissolved in acetonitrile at a concentration of 10 mM (Lee et al., 1999). The specific activity of carboxymethyl cellulase, xylanase and amylase were expressed as milligram of sugar released per gram of protein per minute while lipase activity was expressed as millimolar PNP per gram of protein per minute.

3.2.4. Analysis of ruminal fatty acids

Lipid was extracted in duplicate from 200 mg finely-ground freeze-dried ruminal digesta using a mixture of hexane and 2-propanol (3:2, vol/vol) as described by Shingfield et al. (2003). The extracted lipid was dissolved in 2 mL hexane and converted into fatty acid methyl esters (FAME) using a base-acid catalyzed transesterification procedure (Toral et al., 2010). The methyl esters were quantified on a gas chromatograph Trace Thermo Finningam GC equipped with a flame ionization detector (FID; ThermoQuest, Milan, Italy) and 100 m high polar fused silica capillary column (0.25 mm i.d., 0.25 μm, film thickness; SP 2560, Supelco, Bellefonte, PA). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Total FAME profile in a 2 μL sample volume at a split ratio of 1:50 was determined using the following GC conditions: oven temperature was programmed at 50 °C and held for 4 min, then increased to 120 °C at 10 °C/min, held for 1 min, increased up

to 180 °C at 5 °C/min, held for 18 min, increased up to 200 °C at 2 °C/min, held for 15 min, and increased up to 230 °C at 2 °C/min, and held for 19 min. The injector and detector temperatures were at 270 °C and 300 °C, respectively. The identification of FAME was based on a standard mixture of 52 Component FAME Mix (Nu-Chek Prep Inc., Elysian, MN, USA) and 77 individual FAME standards (Larodan Fine Chemicals, Malmo, Sweden). The 18:1 and 18:2 isomers were identified based on commercial standard mixtures (Larodan Fine Chemicals) and on chromatograms published by Kramer et al. (2008) and Alves and Bessa (2007). For individual FA, response factors to FID and inter- and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Bureau of Reference, Brussels, Belgium). Fatty acids were expressed as g/100 g of total methylated fatty acids.

3.2.5. DNA extraction and quantitative PCR assay

The extraction of DNA from freeze-dried ruminal digesta was performed using the cetyltrimethylammonium bromide (CTAB) detergent method (William et al., 2012) except that cell lysis was achieved by incubation with sodium dodecyl sulphate (SDS) buffer for 10 min at 95 °C and potassium acetate was used for protein removal. The quantification of DNA yield was carried out by spectrophotometry (Nanodrop ND-1000 spectrophotometer). Extracted DNA samples were stored at -80 °C prior to further analysis. DNA samples were quantified for total concentration of bacteria, anaerobic fungi, methanogens and protozoa using a LightCycler® 480 System (Roche, Mannheim, Germany) as described by Belanche et al. (2015a). Targeted primers used for quantitative PCR (qPCR) analysis of the microbes are indicated in Table S2.1 (See appendix).

3.2.6. Ion-torrent sequencing and data processing

The V1-V2 hypervariable region of 16S rRNA was amplified in extracted DNA samples for analysis of the bacterial community as described by Belanche et al. (2016a). PCR amplification was carried out using targeted primers shown in Table S2.1 (See appendix). The PCR amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Inc., Fullerton, USA) and DNA concentration was assessed using an Epoch Microplate Spectrophotometer (BioTek, Potton, UK). Further purification of amplicon library was carried out using the E-Gel Safe Imager Trans-illuminator with E-Gel Size Select 2% Agarose gels (Life Technologies Ltd., Paisley, UK). The purified DNA libraries were quantified for DNA yield and detection of artefacts post-PCR amplification using an Agilent 2100 Bioanalyzer with a high sensitivity DNA chip (Agilent Technologies, Ltd., Stockport, UK). The emulsion PCR of the DNA sample libraries was performed using the Ion PGM Template OT2 200 Kit (Life Technologies Ltd, Paisley, UK) following the appropriate manufacturer's guide for users. Sequencing of the bacterial amplicon library was performed on the Ion Torrent PGM (Life Technologies Ltd, Paisley, UK) using the Ion PGM sequencing 316TM Chip v2.

The barcodes corresponding to individual samples were identified by multiplexing the sequences and low-quality datasets and chimeras were removed by denoising the sequences using MOTHUR software package (Belanche et al., 2016a). The UPARSE pipeline (http://drive5.com/uparse/) was used to cluster the sequences into operational taxonomic units (OTU) at 97% identity (Edgar, 2013). The bacterial OTU table was normalized by random subsampling according to the sample with the minimum number of the sequence. The taxonomic classification of bacteria was carried out by comparison of the 16S rRNA gene sequences against Ribosomal Database Project (RDP-II).

3.2.7. Statistical analysis

Data on nutrient intakes, ruminal fermentation and FA profiles, and rumen microbial abundance were analyzed as a one-way ANOVA in SPSS (IBM Statistics version 22). Shapiro-Wilk normality test was applied to qPCR data and a $\log_{10}$ transformation was performed if unequal variances were found. Log-transformed data were subsequently analyzed by one-way ANOVA. Significance was considered when $P < 0.05$ and a tendency for treatment effect were observed when $0.05 \leq P \leq 0.10$. The biodiversity indices of rumen bacterial community were calculated using the normalized OTU datasets using PRIMER-v6 software (PRIMER-E Ltd., Plymouth, UK). The dendrogram plot of hierarchical cluster analysis was generated using PRIMER-v6. The Vegan package in R statistical software (version 3.2.5) was used to perform principal coordinate analysis (PCoA) on the log-transformed data using the mean Bray-Curtis distances and the multivariate analysis of variance (MANOVA) was used to assess the treatment significance. Heat maps and rarefaction curves were also constructed using the Vegan package in R statistical software. Treatment effect on the relative abundances of bacteria was analyzed using a one-way ANOVA as indicated for qPCR data. Correlations between the rumen bacterial community and ruminal FA profiles were elucidated on ordination plots using canonical correspondence analysis (CCA) generated in R software. The significance level of the variables was computed using 999 random permutations.

3.3. RESULTS

3.3.1. Diet composition and nutrient intakes

Chemical composition of experimental diets (CON and CMD) are outlined in Table 3.1. Dietary inclusion of cardoon meal enriched CMD with approximately 2.5-fold greater concentration of phenolic compounds compared to CON containing dehydrated alfalfa. The proportion of true soluble protein and total soluble nitrogen were 136.4% and 38.6% greater in CMD compared to CON. The inclusion of cardoon meal in CMD increased the concentration of oleic (+39.1%) and linoleic acids (+38.2%) compared to CON.

Feeding CMD reduced the intake ($P < 0.001$) of neutral detergent fibre (NDF, -22.2%) and tended to decrease protein intake (-9.3% , $P = 0.078$) compared to CON (Table 3.2). However, lambs fed CMD exhibited greater intakes ($P < 0.05$) of total fat and phenolic compounds compared to CON-fed lambs (Table 3.2). Dietary intake of individual fatty acids differed between the two animal groups. The CMD-fed lambs consumed greater amount of dietary 18:0 ($P < 0.001$), 18:1 *n*-9 ($P = 0.005$) and 18:2 *n*-6 ($P = 0.006$), and lesser amount of 18:3 *n*-3 ($P < 0.001$) than CON-fed lambs (Table 3.2).

3.3.2. Ruminal fermentation

Table 3.3 indicated that lambs fed CON and CMD diets displayed similar ruminal pH, total VFA concentration, and molar proportion of acetate, propionate, valerate and acetate/propionate ratio. However, the concentration of NH_3-N tended to be greater ($P = 0.086$) in CMD-fed lambs. Dietary treatment had no effect on the activities of carboxymethyl cellulose, xylanase, amylase and lipase measured in the rumen of lambs fed CON and CMD (Table 3.3).

Table 3.2. Effect of supplementing cardoon meal (CMD) for dehydrated alfalfa (CON) in a concentrate diet on the nutrient intakes in lambs.

	Dietary treatment[1]			
Nutrient intakes	**CON**	**CMD**	**SEM**	***P*-value**
Total NDF intake, g/d	327.4	254.7	11.90	<0.001
Total protein intake, g/d	168.97	153.34	4.450	0.078
Total phenol intake, g/d	5.62	12.19	0.917	<0.001
Total fat intake, g/d	28.90	35.80	1.250	0.002
C16:0, mg/d	47.0	47.2	1.17	0.934
C18:0 intake, mg/d	4.9	6.6	0.27	<0.001
C18:1 *n*-9 intake, mg/d	41.6	50.0	1.64	0.005
C18:2 *n*-6 intake, mg/d	131.4	157.1	5.07	0.006
C18:3 *n*-3 intake, mg/d	13.5	9.9	0.55	<0.001

[1]CON: control diet; CMD: cardoon meal diet
NDF: Neutral detergent fibre

Table 3.3. Effect of supplementing cardoon meal (CMD) for dehydrated alfalfa (CON) in a concentrate diet on rumen pH, fermentation traits and microbial enzyme activities in lambs.

	Dietary treatment[1]			
Item	**CON**	**CMD**	**SEM**	***P*-value**
Rumen pH	6.50	6.54	0.125	0.869
NH_3-N, mMol/L	13.67	19.82	1.790	0.086
Total VFA, mM	55.15	52.56	7.956	0.878
Molar proportion of VFA				
Acetate	50.67	49.16	0.921	0.434
Propionate	32.73	32.76	1.180	0.991
Iso-butyrate	3.68	4.47	0.376	0.307
Butyrate	6.52	6.84	0.321	0.644
Iso-valerate	2.65	3.62	0.384	0.220
Valerate	3.75	3.16	0.317	0.364
Acetate/propionate ratio	1.59	1.52	0.074	0.662
Microbial enzyme activities[2]				
Carboxymethyl cellulase	34.92	30.85	4.151	0.643
Xylanase	129.15	119.61	16.599	0.786
Amylase	77.80	95.88	18.170	0.638
Lipase	2.46	2.13	0.253	0.545

[1]CON: control diet; CMD: cardoon meal diet
[2]Specific activity of carboxymethyl cellulase, xylanase and amylase were expressed as mg of sugar released per g of protein per min while lipase activity was expressed as mM PNP per g of protein per min.

3.3.3. Rumen fatty acid composition

Fatty acid profile in the rumen digesta differed between dietary treatments when visualized on a PCoA plot (Figure 3.1a). The fatty acid profiles of the ruminal digesta differed between lambs fed CON and CMD (Table 3.4). The CMD-fed lambs exhibited greater concentration of total SFA (+41.3%; $P < 0.001$) than CON-fed lambs. The SFA concentration was dominated by C18:0 (stearic acid, SA) which was greater ($P < 0.001$) in CMD-fed lambs. Also, the proportion of C16:0 was high in all lambs but there was no effect of diets on this FA. For differences in SFA with minor abundance, lambs fed CMD had greater proportion of C20:0 ($P < 0.05$) compared to CON-fed lambs.

Furthermore, dietary treatment affected the formation of odd- and branched-chain fatty acids (OBCFA) in the rumen (Table 3.4). Lambs fed CON accumulated greater concentration of total OBCFA (+39.0%; $P < 0.001$) compared to CMD-fed lambs. This alteration was mainly due to differences in the branched-chain FA. Lambs fed CON showed a greater ($P < 0.05$) abundance of *iso* C14:0, *iso* C15:0, *anteiso* C15:0, *iso* C16:0 and *iso* C17:0. Among the branched-chain FA, only *anteiso* C17:0 was not affected by diet. Regarding the odd-chain FA, there was a tendency to a greater accumulation of C15:0 ($P = 0.069$), C21:0 ($P = 0.063$) and C23:0 ($P = 0.057$) in CON-fed lambs.

Feeding CMD reduced the proportion of total MUFA (-46.0%; $P < 0.001$), largely attributed to changes in total *trans* 18:1 isomers (-65.6%, $P < 0.001$) (Table 3.4). Lambs fed CON displayed greater ($P < 0.05$) proportion of *trans*-6+8 C18:1, *trans*-9 C18:1, *trans*-10 C18:1 and *trans*-11 C18:1 compared to CON-fed lambs. Diet exhibited a minor effect on *cis*-MUFA; CMD decreased *cis*-6 C18:1 ($P = 0.035$) and *cis*-13 C22:1 ($P = 0.001$) and tended to lower *cis*-9 C17:1 ($P = 0.054$) and *cis*-9 C18:1 ($P = 0.053$).

There was no effect of diet on the concentration of total PUFA and long-chain PUFA such as C18:2 *n*-6, C18:3 *n*-3, C20:5 *n*-3, C22:5 *n*-3 and C22:6 *n*-3 (Table 3.4). However, the proportion of C20:4 *n*-6 ($P = 0.045$) was greater in animals fed CMD. Lambs fed CON and CMD had similar proportion of CLA isomers (*cis*-9, *trans*-11 C18:2; *trans*-8, *cis*-10 C18:2 and *trans*-10, *trans*-12 C18:2). However, diet caused some differences in the proportion of atypical dienoic FA. Lambs fed CON showed greater percentage of *cis*-9, *trans*-12 C18:2 ($P = 0.003$) and *trans*-11, *cis*-15 C18:2 ($P = 0.013$) but tended to increase *trans*-9, *cis*-13 C18:2 ($P = 0.056$).

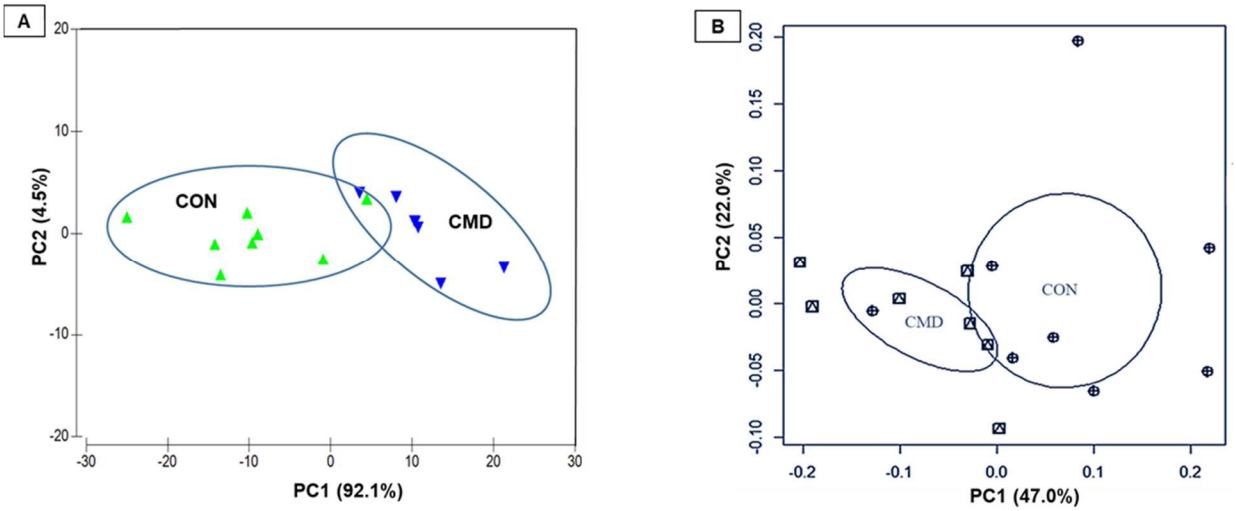


Figure 3.1. Principal coordinate analysis (PCoA) plot of the (A) fatty acid profile in rumen digesta (B) bacterial community structure in lambs fed dehydrated alfalfa diet (CON) and cardoon meal diet (CMD).

Table 3.4. Effect of supplementing cardoon meal (CMD) for dehydrated alfalfa (CON) in a concentrate diet on fattty acid composition of rumen digesta in lambs.

	Dietary treatment[1]			
Item	**CON**	**CMD**	**SEM**	***P*-value**
Fatty acids, g/100 g of total fatty acids				
Σ *SFA*				
C4:0	0.49	0.36	0.060	0.279
C6:0	0.25	0.14	0.034	0.107
C8:0	0.16	0.11	0.016	0.122
C10:0	0.26	0.22	0.024	0.442
C12:0	0.40	0.35	0.047	0.592
C14:0	1.33	1.22	0.093	0.574
C16:0	12.90	12.62	0.275	0.622
C18:0	27.13	46.57	3.140	<0.001
C20:0	0.52	0.66	0.031	0.023
C22:0	0.64	0.70	0.102	0.795
C24:0	0.53	0.46	0.038	0.398
Σ*OBCFA*				
C5:0	0.79	0.59	0.114	0.349
C9:0	0.28	0.24	0.023	0.448
C11:0	0.10	0.09	0.013	0.671
C13:0	0.20	0.15	0.024	0.259
iso C14:0	0.20	0.11	0.021	0.022
C15:0	0.90	0.67	0.064	0.069
iso C15:0	0.33	0.13	0.042	0.011
anteiso C15:0	1.29	0.60	0.107	<0.001
iso C16:0	0.16	0.06	0.018	0.001
C17:0	0.51	0.37	0.046	0.148
iso C17:0	0.22	0.11	0.026	0.029
anteiso C17:0	0.40	0.20	0.060	0.111
C21:0	0.01	0.00	0.003	0.063
C23:0	0.59	0.32	0.070	0.057
Σ *MUFA*				
cis-9 C12:1	0.21	0.14	0.021	0.102
cis-9 C14:1	0.18	0.16	0.017	0.494
cis-7 C16:1	0.13	0.13	0.018	0.980
cis-9 C16:1	0.47	0.40	0.067	0.644
cis-9 C17:1	0.08	0.03	0.013	0.054
cis-6 C18:1	1.22	0.87	0.085	0.035
cis-9 C18:1	5.45	4.26	0.313	0.053
cis-11 C18:1	0.94	0.78	0.067	0.251
cis-12 C18:1	1.12	0.92	0.084	0.226
cis-13 C18:1	0.13	0.11	0.021	0.694
cis-14 C18:1	0.28	0.29	0.051	0.964
cis-16 C18:1	0.41	0.39	0.061	0.932
cis-11 C20:1	0.28	0.20	0.027	0.149
cis-13 C22:1	0.21	0.10	0.020	0.001

Table 4. Continued	CON	CMD	SEM	*P*-value
trans-9 C14:1	0.08	0.01	0.019	0.076
trans-7 C16:1	0.04	0.03	0.007	0.438
trans-5 C18:1	0.30	0.22	0.039	0.330
trans-6+8 C18:1	1.68	0.75	0.173	0.003
trans-9 C18:1	1.02	0.70	0.067	0.009
trans-10 C18:1	9.43	1.94	1.290	0.001
trans-11 C18:1	1.59	0.98	0.154	0.046
trans-12 C18:1	0.42	0.38	0.033	0.506
trans-11 C20:1	0.10	0.12	0.018	0.614
Σ *PUFA*				
cis-9 trans-11 C18:2	0.63	0.44	0.065	0.153
trans-8 *cis*-10 C18:2	0.05	0.06	0.010	0.560
trans-10 *trans*-12 C18:2	0.03	0.05	0.009	0.208
cis-9 trans-12 C18:2	0.20	0.06	0.028	0.003
trans-8 *cis*-13 C18:2	0.08	0.06	0.018	0.560
trans-9 *cis*-12 C18:2	0.30	0.39	0.036	0.236
trans-9 *cis*-13 C18:2	0.13	0.08	0.013	0.056
trans-11 *cis*-15 C18:2	0.23	0.08	0.030	0.013
C18:2 *n*-6	5.10	5.06	0.539	0.976
C18:3 *n*-6	0.08	0.09	0.013	0.895
C18:3 *n*-3	0.39	0.25	0.043	0.104
C20:4 *n*-6	0.11	0.20	0.023	0.045
C20:5 *n*-3	0.32	0.23	0.037	0.264
C22:2 *n*-6	0.09	0.11	0.036	0.809
C22:4 *n*-6	0.08	0.06	0.013	0.590
C22:5 *n*-6	0.24	0.20	0.072	0.771
C22:5 *n*-3	0.03	0.03	0.007	0.688
C22:6 *n*-3	0.12	0.10	0.016	0.690
Summary				
Σ SFA	44.77	63.28	3.120	<0.001
Σ OBCFA	5.98	3.65	0.390	<0.001
Σ *trans*-18:1	14.44	4.97	1.600	<0.001
Σ MUFA	25.78	13.91	1.920	<0.001
Σ PUFA	8.21	7.56	0.651	0.635

[1]CON: control diet; CMD: cardoon meal diet

SFA: Saturated fatty acids; OBCFA: Odd-and branched-chain fatty acids; MUFA: Monounsaturated fatty acids; CLA: Conjugated linoleic acids; AD: Atypical dienoic fatty acids; PUFA: Polyunsaturated fatty acids; LC-PUFA: Long-chain polyunsaturated fatty acids.

3.3.4. Rumen microbiome

Quantification of rumen microbial populations showed that there was no effect of dietary treatment on the abundance of bacteria, methanogens, fungi and protozoa in the rumen (Table 3.5). High-throughput sequencing of bacterial 16S rRNA genes generated approximately 600,000 raw sequences clustered into 2027 OTUs across 15 rumen samples. Normalization of the sequence datasets resulted in 318,857 high-quality sequences clustered into 2010 unique OTUs, averaging 21,257 sequences per sample. The plateau attained by the rarefaction curves (See appendix: Figure S3.1) indicated that sampling of the rumen environment resulted in similar bacterial sequencing depth in lambs fed CON and CMD. Diet altered (MANOVA, $P = 0.013$) the rumen bacterial population structure when visualized on a PCoA plot (Figure 3.1b). This observation was supported by the separate clustering of rumen samples from lambs fed CON and CMD on the dendrogram plot (See appendix: Figure S3.2). The CMD-fed lambs exhibited a less even distribution of highly abundant and minor bacterial species ($P < 0.05$) and lower bacterial diversity values (Shannon's index and Simpson's index, $P < 0.05$) compared to CON-fed lambs.

The bacterial community in all samples was dominated by the phyla *Proteobacteria* (44.5%), *Bacteriodetes* (35.7%) and *Firmicutes* (14.3%) together with minor proportions of *Spirochaetes* (1.5%), *Actinobacteria* (0.5%), *Fibrobacteres* (2.6%) and unclassified sequences (0.6%) (Table 3.6). Furthermore, diet modulated the abundance of bacterial phylogenetic taxa. At the phylum level, lambs fed CMD had greater abundance of *Proteobacteria* ($P < 0.01$) and lower *Bacteriodetes* ($P < 0.01$) and *Fibrobacteres* ($P < 0.05$). This bacterial shift was obvious at the family taxa as CMD-fed lambs exhibited a greater abundance of *Succinivibrionaceae* ($P < 0.05$) and a decreased abundance ($P < 0.05$) of *Prevotellaceae* and *Fibrobacteraceae*. The distribution of bacterial genera in rumen

samples presented few differences between diets as observed on the heat map (See appendix: Figure S3.3). *Prevotella* and *Ruminobacter* are the most dominant genera in all animals and *Fibrobacter* clearly appeared to be less abundant in CMD-fed lambs. The significance of the effect of diet on bacterial genera showed that CMD specifically increased the abundance of *Ruminobacter* ($P < 0.01$) and decreased *Fibrobacter* ($P < 0.05$). Moreover, CMD tended to decrease *Prevotella* ($P = 0.070$) and *Alloprevotella* ($P = 0.096$) in phylum *Bacteriodetes* and *Solobacterium* ($P = 0.054$) in phylum *Firmicutes*.

The correlation between the rumen bacterial community and ruminal FA profiles was elucidated on the CCA ordination plot (Figure 3.2). There was a clear clustering between rumen samples from lambs fed CON and CMD. The bacterial community in CON-fed lambs positively correlated with *cis*-16 C18:1 ($P = 0.009$), *trans*-10 C18:1 ($P = 0.038$) and C18:3 *n*-3 ($P = 0.009$), with a tendency to correlate with C18:2 *n*-6 ($P = 0.083$) (Figure 3.2 and Table S3.1 – See appendix). In contrast, C9:0 ($P = 0.085$) and *trans*-8, *cis*-10 C18:2 ($P = 0.081$) tended to correlate with the bacterial community in CMD-fed lambs. Moreover, *cis*-11 C20:1 tended ($P = 0.075$) to correlate with the bacterial community but not clearly related to either CON or CMD.

Table 3.5. Effect of supplementing cardoon meal (CMD) for dehydrated alfalfa (CON) in a concentrate diet on rumen microbial numbers and bacterial biodiversity indices in lambs.

Item	Dietary treatment[1]		SEM	*P*-value
	CON	CMD		
Microbial numbers[2]				
Bacteria	8.83	8.82	0.034	0.869
Methanogens	6.63	6.18	0.205	0.276
Fungi	3.19	2.78	0.460	0.668
Protozoa	8.37	7.36	0.376	0.189
Bacterial diversity indices				
Number of OTU[3]	441.25	436.57	32.316	0.946
Pielou's evenness index	0.58	0.53	0.010	0.007
Shannon's index	3.55	3.20	0.084	0.031
Simpson's index	0.91	0.86	0.009	0.002

[1]CON: control diet; CMD: cardoon meal diet;
[2]Data were log-transformed to achieve normality and results expressed as log copy/mg DM of freeze-dried rumen content;
[3]OTU: Operational taxonomic unit

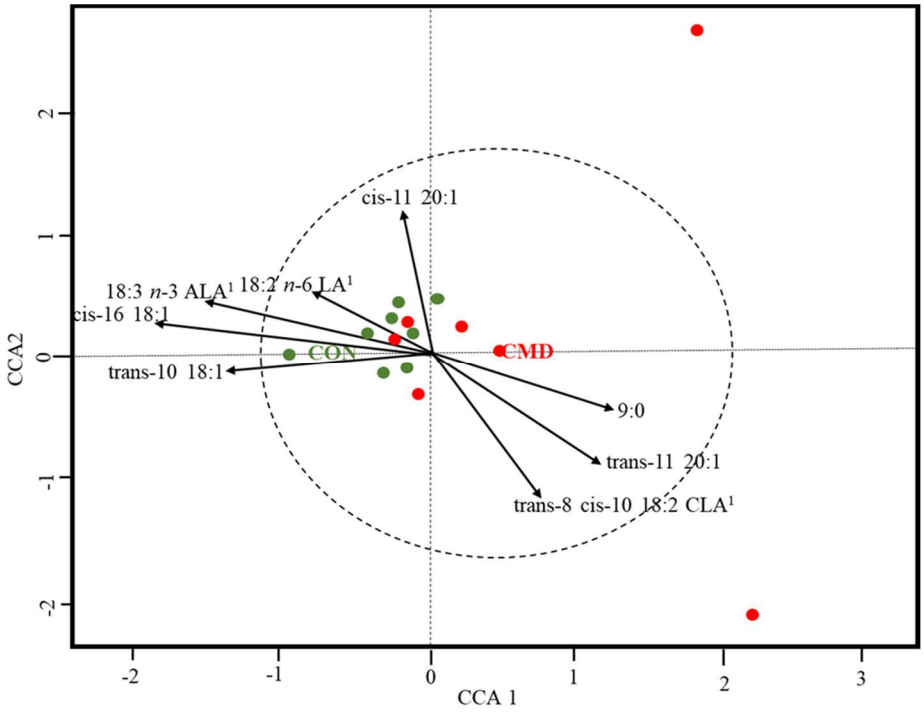


Figure 3.2. Canonical correspondence analysis (CCA) describing the correlations between the rumen bacterial community structure and the fatty acid profiles in rumen digesta. The arrows indicate the gradient direction and their length is relative to the proportion of correlation. Arrows longer than the dotted circle signify correlation significance (P < 0.05). Coloured dots represent distribution pattern of the lambs fed control diet (CON) and cardoon meal diet (CMD).

1LA: Linoleic acid; ALA: α-linolenic acid; CLA: Conjugated linoleic acid.

Table 3.6. Effect of supplementing cardoon meal (CMD) for dehydrated alfalfa (CON) in a concentrate diet on relative abundance (%) of rumen bacteria taxa (≥ 0.2% of average abundance) in lambs

				Dietary treatment[1]			
Phylum	**Class**	**Family**	**Genus**	**CON**	**CMD**	**SEM**	***P*-value**
Proteobacteria				37.72	51.25	2.775	0.009
	Gammaproteobacteria			37.02	50.80	2.879	0.011
		Succinivibrionaceae		34.51	46.76	2.567	0.011
			Ruminobacter	20.84	33.05	2.254	0.002
			Succinivibrio	0.16	0.50	0.198	0.416
			Anaerobiospirillum	6.20	2.01	1.596	0.201
	Alphaproteobacteria			0.50	0.17	0.185	0.404
Bacteroidetes				40.10	31.26	1.810	0.009
	Bacteroidia			39.02	30.31	1.822	0.011
		Porphyromonadaceae		3.14	3.56	0.408	0.625
			Barnesiella	2.75	3.04	0.421	0.746
		Prevotellaceae		31.38	22.57	2.150	0.035
			Prevotella	28.30	20.46	2.177	0.070
			Paraprevotella	0.51	0.30	0.108	0.350
			Hallella	0.45	0.26	0.105	0.405
			Alloprevotella	0.35	0.09	0.077	0.096
		Rikenellaceae		1.99	2.60	1.068	0.788
			Rikenella	1.99	2.60	1.068	0.788
Firmicutes				14.47	14.14	0.659	0.811
	Clostridia			6.74	6.49	0.459	0.791
		Ruminococcaceae		4.11	3.81	0.383	0.707
			Ruminococcus	1.40	0.99	0.228	0.386
			Acetivibrio	0.27	0.29	0.123	0.934
			Clostridium IV	0.15	0.29	0.115	0.547
		Lachnospiraceae		2.06	1.95	0.162	0.747
			Lachnospiracea incertae sedis	0.22	0.30	0.065	0.532
			Roseburia	0.89	0.54	0.139	0.221
			Butyrivibrio	0.39	0.27	0.082	0.509
	Negativicutes			4.69	5.42	0.299	0.237

Table 3.6. Continued

Phylum	Class	Family	Genus	CON	CMD	SEM	*P*-value
		Veillonellaceae		1.81	2.34	0.326	0.433
			Dialister	0.93	0.69	0.256	0.647
			Mitsuokella	0.26	0.13	0.071	0.379
			Selenomonas	0.13	0.30	0.074	0.247
	Erysipelotrichia			2.89	1.76	0.452	0.226
		Erysipelotrichaceae		2.89	1.76	0.452	0.226
			Bulleidia	0.37	0.31	0.052	0.593
			Sharpea	0.71	0.86	0.265	0.798
			Solobacterium	0.55	0.08	0.124	0.054
			Kandleria	1.05	0.45	0.331	0.381
		Acidaminococcaceae		2.88	3.03	0.398	0.859
			Succiniclasticum	2.39	2.61	0.355	0.774
			Acidaminococcus	0.34	0.34	0.064	0.988
Spirochaetes				1.48	1.54	0.368	0.942
	Spirochaetia			1.48	1.54	0.368	0.942
		Spirochaetaceae		1.16	1.33	0.321	0.795
			Treponema	0.64	0.81	0.226	0.711
			Sphaerochaeta	0.49	0.33	0.136	0.584
Actinobacteria				0.49	0.44	0.118	0.823
	Actinobacteria			0.49	0.44	0.118	0.823
		Coriobacteriaceae		0.48	0.43	0.119	0.830
			Olsenella	0.47	0.42	0.120	0.839
Fibrobacteres				4.93	0.36	1.027	0.020
	Fibrobacteria			4.93	0.36	1.027	0.020
		Fibrobacteraceae		4.93	0.36	1.027	0.020
			Fibrobacter	4.93	0.36	1.027	0.020
Unclassified				0.47	0.71	0.101	0.238
	Unclassified			1.62	2.13	0.331	0.460
		Unclassified		7.70	8.56	0.999	0.685
			Unclassified	20.19	25.39	1.834	0.165

[1]CON: control diet; CMD: cardoon meal diet

3.4. DISCUSSION

To our knowledge, the present study is the first to investigate the rumen function response in lambs fed a concentrate diet supplemented with cardoon meal as a substitute for dehydrated alfalfa. It was hypothesized that residual phenolic compounds and unsaturated FA in cardoon meal might modulate the rumen microbiota and ruminal metabolism including inhibition of RBH. In agreement with previous data reported by Genovese et al. (2015), the nutritive value of cardoon meal is characterized by a high content of protein, fibre, phenolic compounds and unsaturated FA profiles. The predominance of oleic and linoleic acids in the FA profile of cardoon meal is consistent with the relative proportion of these FA in cardoon seed (Cajarville et al., 2000) and oil (Curt et al., 2002). Moreover, cardoon meal has a nutritional composition comparable to sunflower meal (Heuzé et al., 2015), which has been widely used as a dietary source of protein in animal diets.

3.4.1. Ruminal fermentation and microbial population

Microbial fermentation of feed substrates in the rumen produces VFA and microbial proteins that supply ruminant animals with energy and highly digestible proteins, respectively for maintenance and production purposes (Loor et al., 2016). In the present study, no effect of diet on rumen pH, total VFA, the molar proportion of individual VFA, microbial enzymatic activities nor abundance of rumen bacteria, methanogens, fungi and protozoa was observed. Similarly, it has been shown that feeding up to 25% whole cardoon seeds did not impair rumen fermentation, nutrient digestibility and fibrolytic activity in sheep (Cajarville et al., 2000). However, diet modified the structure of the bacterial community without affecting ruminal fermentation. A possible explanation could be related to the fermentative redundancy of the rumen microbiome (Weimer, 2015), which allow the

rumen ecosystem to maintain similar digestive function despite differences in microbial communities (Taxis et al., 2015).

Ruminal proteolysis influences the nitrogen utilization efficiency in ruminants with possible consequences for decreased animal performance and increased emission of nitrogen into the environment (Calsamiglia et al., 2010). Despite higher protein intake in lambs fed CON, the tendency for a greater concentration of ruminal NH_3-N in CMD-fed lambs suggests that cardoon meal protein may be more susceptible to ruminal proteolysis compared to dehydrated alfalfa protein. This observation is contrary to the expectation that the high phenolic content in cardoon meal would reduce protein degradation in the rumen. Phenolic compounds can inhibit the growth and activity of proteolytic bacteria or react with dietary proteins in the rumen to form complexes that are resistant to ruminal degradation (Makkar, 2003). However, this effect can be inconsistent as demonstrated by the lack of effect on ruminal protein degradation in cattle and sheep supplemented with dietary tannin extracts (Piñeiro-Vázquez et al., 2017; Wischer et al., 2014). The extent and rate of ruminal proteolysis vary with different dietary protein sources. Dehydrated alfalfa protein has been shown to exhibit a slower rate of *in vitro* ruminal proteolysis compared to soybean meal protein (Loerch et al., 1983). On the contrary, an elevated level of ruminal NH_3-N was found in sheep fed up to 25% whole cardoon seeds, suggesting that cardoon seeds may contain a high amount of rumen degradable protein (Cajarville et al., 2000). In agreement with this hypothesis, we found a greater amount of total soluble nitrogen fraction in CMD compared to CON (Table 3.1), which may explain the substantial ruminal proteolysis resulting in higher NH_3-N concentration in CMD-fed lambs. Moreover, it is unclear if the modulatory effect of diet on the rumen bacterial community could contribute to the difference in NH_3-N concentration. In particular, feeding CMD mediated a shift from *Prevotella, Alloprevotella* and *Fibrobacter* to *Ruminobacter*. However, dietary amino acids

are degraded by extensive bacterial interactions including proteolytic bacteria strains that belong to *Prevotella* and *Ruminobacter* groups (Wallace, 1996).

Differences in diversity indices indicate that diet altered the taxonomic composition of the rumen bacterial community. Considering the crucial role of bacteria in ruminal digestion, the effect of diet on the bacterial community may influence other ruminal fermentation activities not measured in this study. Antimicrobial property of cardoon polyphenols (Kukić et al., 2008) could account for the inhibitory effect of CMD on the fibrolytic bacterium (*Fibrobacter*), which may negatively affect fibre digestion in the rumen (Makkar, 2003). Furthermore, the higher abundance of *Proteobacteria*, predominantly *Succinivibrionaceae*, in CMD-fed lambs may be linked to reduced CH_4 production in the rumen. Previous studies have consistently shown that the dominance of *Proteobacteria* belonging to *Succinivibrionaceae* is closely associated with low CH_4 emission in dairy cows (Danielsson et al., 2017), beef cattle (Wallace et al., 2015) and Tammar wallabies (Pope et al., 2011). Decreased CH_4 production may be related to the functional activity of *Succinivibrionaceae* to synthesize succinate which serves as a metabolic precursor for propionate, a fermentation product that does not produce H_2 for CH_4 formation (Danielsson et al., 2017). Thus, it would be interesting for future studies to investigate the effect of dietary cardoon meal on other ruminal fermentation activities such as nutrient digestibility and methanogenesis.

3.4.2. Ruminal biohydrogenation and bacterial community

Ruminal biohydrogenation (RBH) of unsaturated FA to SFA reduce the ruminal outflow of health-promoting unsaturated FA for subsequent absorption and incorporation into ruminant meat or milk. However, RBH also produces several intermediates (mainly C18:1 and C18:2 isomers) including *trans*-11 C18:1 and *cis*-9, *trans*-11 CLA that exhibit potential health benefits in humans when incorporated in ruminant foods (Lourenço et al., 2010). As

expected, dietary cardoon meal increased the intake of unsaturated fatty acids (oleic and linoleic acids) and phenolic compounds. However, the extent of RBH was greater in CMD-fed lambs as evidenced with greater accumulations of stearic acid, SFA, and lower concentrations of oleic acid, *trans*-11 C18:1 and MUFA. This observation contradicts our hypothesis that high phenolic content in cardoon meal could reduce RBH compared to dehydrated alfalfa. However, the inhibition of RBH by dietary phenolic compounds is inconsistent across studies due to the interaction of factors such as structural complexity, dosage, type of diets, animal species and physiological status, as well as characteristics inherent to the basal diet (Vasta and Bessa, 2012).

The main phenolic compounds in cultivated cardoon are flavonoids (luteolin, apigenin, quercetin etc.) and caffeoylquinic acid derivatives, of which chlorogenic acid and *trans*-cinnamic acid are abundant (Falleh et al., 2008; Pandino et al., 2011; Ramos et al., 2014). Indeed, there is limited information on the efficacy of these compounds in inhibiting RBH. It has been shown that hydroxycinnamic acids including caffeic and chlorogenic acids can be metabolised by the gut microflora (Gonthier et al., 2006). This suggests that the phenolic compounds present in CMD might have been metabolised by the rumen microbiota, resulting in a lack of efficacy of CMD in inhibiting RBH. A previous study showed that dietary addition of quercetin or triterpene saponin extracts did not inhibit RBH assessed in dual-flow continuous culture fermenters (Lourenço et al., 2008). Moreover, extract from flower leaves of artichoke (*Cynara scolymus* L.), a botanical relative of cultivated cardoon, exhibited high polyphenol oxidase activity but was not effective in inhibiting *in vitro* RBH of α-linolenic acid (Gadeyne et al., 2016). In contrast, the presence of saponins and flavonoids in alfalfa may partly explain its inhibitory effect on *in vitro* RBH compared to phenolic-rich plant species such as birdsfoot trefoil, chicory and English plantain (Petersen and Jensen, 2014). Moreover, heat treatment of feedstuffs, as applied in the production of

dehydrated alfalfa, causes changes in the protein matrix surrounding the fat droplets resulting in a simultaneous reduction in ruminal proteolysis and RBH (Kennelly, 1996; Lashkari et al., 2017). This theory is consistent with a tendency for reduced ruminal proteolysis and RBH inhibition found in lambs fed CON compared to CMD-fed lambs. The inhibitory effect of dietary alfalfa on RBH may account for increased PUFA and CLA contents in ruminant milk and meat (Benchaar et al., 2007; Realini et al., 2017). Thus, the application of feed processing methods such as heat treatment can be useful to protect proteins and unsaturated fats in cardoon meal against ruminal proteolysis and RBH, respectively.

Bacteria are thought to be the main microbes responsible for RBH but there is limited knowledge on the complexity of bacterial species related to the production of several RBH intermediates. In this study, high-throughput sequencing of 16S rRNA genes revealed that changes in the rumen bacterial community could be the underlying mechanism responsible for observed differences in RBH. The bacterial shift towards *Proteobacteria*, mainly *Succinivibrionaceae*, could account for the substantial RBH in CMD-fed lambs. This is consistent with previous studies that linked the abundance of *Succinivibrionaceae* to increased RBH in cattle, sheep and goat fed with dietary lipid supplements (Castro-Carrera et al., 2014; Toral et al., 2016). However, it appears that the present study is the first to classify that increased abundance of family *Succinivibrionaceae* specifically occurs in genus *Ruminobacter*. Moreover, increased RBH in CMD-fed lambs was associated with a lower abundance of *Prevotella*, *Alloprevotella*, *Solobacterium* and *Fibrobacter*. In agreement with our observation, Huws et al. (2015) indicated that decreased abundance of *Prevotella* and *Fibrobacter* correspond to substantially increased RBH in the rumen of cattle fed a grass silage diet supplemented with flax oil. This suggests that these bacterial

genera may be preferentially suppressed at the expense of bacteria (e.g. *Succinivibrionaceae*) involved in complete biohydrogenation of FA.

Feeding CON inhibited the terminal step of RBH as indicated by a lower concentration of C18:0 and a greater proportion of total *trans* 18:1 isomers including *trans*-11 C18:1 and *trans*-10 C18:1. *Trans*-11 C18:1 is the main precursor desaturated into *cis*-9, *trans*-11 CLA in the muscle tissues or mammary gland, known for its potential human health benefits when incorporated into meat or milk (Palmquist et al., 2005). On the other hand, increased concentration of ruminal *trans*-10 C18:1 has been implicated in the modification of mammary lipogenesis, causing a reduction of milk fat synthesis in dairy ruminants (Shingfield and Griinari, 2007). Feeding high concentrate diets induce the accumulation of *trans*-10 C18:1 in the rumen (Shingfield and Griinari, 2007) but it is unclear if the formation of *trans*-10 C18:1 occur as a competing intermediate to *trans*-11 C18:1 (Bessa et al., 2015). In the present study, simultaneous accumulation of *trans*-11 18:1 and *trans*-10 18:1 in CON-fed lambs suggested that the production of both intermediates may have occurred via different RBH pathways (Vlaeminck et al., 2015). A possible explanation may relate to the fact that ruminal digesta in CON-fed lambs contained a greater proportion of *trans*-11, *cis*-15 18:2, a major non-conjugated 18:2 isomer from which *trans*-11 18:1 could be formed via an alternative pathway for α-linolenic acid hydrogenation (Honkanen et al., 2016). On the other hand, *trans*-10 18:1 could be formed via isomerisation of oleic acid or reduction of *trans*-10, *cis*-12 18:2 (Shingfield and Griinari, 2007). The reduction pathway is unlikely in the present study because *trans*-10, *cis*-12 18:2 was not identified in the rumen of lambs. However, a greater concentration of oleic acid in CON-fed lambs could enable an isomerisation pathway for the production of *trans*-10 18:1.

Bacterial strains belonging to *Butyrivibrio* spp. are believed to be mainly responsible for the hydrogenation of unsaturated FA (linoleic and α-linolenic acids) via C18:2

intermediates to *trans*-11 C18:1 and eventually to C18:0 (Paillard et al., 2007). However, the abundance of *Butyrivibrio* spp. was not affected in this study, in agreement to recent molecular studies that linked other several as-yet-uncultured bacteria to RBH (Castro-Carrera et al., 2014; Toral et al., 2016). The CCA ordination plot indicated that the rumen bacterial community in CON-fed lambs favoured the accumulation of α-linolenic acid and *trans*-10 C18:1, and tended to promote oleic acid concentration, suggesting a possible link of the bacterial community with incomplete RBH. In comparison to CMD-fed lambs, the rumen of lambs fed CON was characterized by a lower abundance of *Succinivibrionaceae* (*Ruminobacter*) and greater abundance of *Prevotellaceae* (*Prevotella*, and *Alloprevotella*), *Solobacterium* and *Fibrobacteraceae* (*Fibrobacter*). However, there is limited knowledge on the metabolic role of these bacterial groups in relation to RBH.

3.5. CONCLUSIONS

The inclusion of 15% cardoon meal to replace dehydrated alfalfa in a concentrate diet resulted in differences in nutrient intakes without affecting ruminal fermentation characteristics in lambs. However, dietary inclusion of cardoon meal increased RBH which may reduce the accumulation of health-promoting unsaturated FA in ruminant meat or milk. The dietary effect on RBH was accompanied by a modification of the rumen bacterial community. Feeding CMD mediated a specific shift from *Prevotella*, *Alloprevotella*, *Solobacterium* and *Fibrobacter* to *Ruminobacter*. Future studies should assess the role of these bacteria genera in RBH so as to enhance the understanding of strategies required for manipulating specific bacteria groups related to the formation of important RBH intermediates such as *trans*-11 C18:1 and *trans*-10 C18:1. Moreover, further investigation is required to understand if the effect of dietary cardoon meal on the rumen bacterial ecology could affect other ruminal activities including nutrient digestibility and methanogenesis.

CHAPTER 4 – Influence of dietary cardoon meal and cardoon extract on selected quality parameters in lamb meat

Saheed A. Salami, Bernardo Valenti, Michael N. O'Grady, Joseph P. Kerry, Giuseppe Luciano, Simona Mattioli, Giuseppe Licitra, and Alessandro Priolo

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ABSTRACT

The fatty acid composition and oxidative stability of meat (*longissimus lumborum et thoracis*, LTL) was determined in lambs fed a concentrate diet containing 15% dehydrated alfalfa (CON, $n = 8$) or cardoon meal (CMD, $n = 7$). Furthermore, the antioxidant activity of a phenolic-rich cardoon meal extract (1.32 GAE mg/ml) in 25% LTL homogenates (0, 0.5, 1, 5% v/w) subjected to iron/ascorbate-induced lipid oxidation following incubation for 4 h at 4 ºC was examined. Feeding CON increased the proportion ($P < 0.05$) of intramuscular vaccenic and rumenic acids and lowered stearic acid compared to CMD. Dietary treatment did not affect the oxidative stability parameters of raw and cooked lamb meat stored at 4 ºC for 7 and 4 days, respectively. The addition of 5% cardoon extract increased (+114.3%; $P = 0.03$) the total phenolic content and reduced (-77.6%; $P < 0.01$) lipid oxidation in LTL homogenates relative to the control, demonstrating the antioxidant potential of compounds present in cardoon meal.

Keywords: *Lamb meat, Growth performance, Fatty acids, Lipid oxidation, Cardoon, Polyphenol*

4.1. INTRODUCTION

Meat from ruminant animals (cattle, sheep, goat and buffalo) can be a viable dietary source of bioactive lipids including conjugated linoleic acids (CLA), monounsaturated fatty acids (MUFA) and *n*-3 long-chain polyunsaturated fatty acids (PUFA; Bessa et al., 2015). However, ruminant meat also contains high levels of saturated fatty acids (SFA) and *trans*-fatty acids (TFA) linked to increased risk of cardio-metabolic diseases in humans (McAfee et al., 2010). The fatty acid profile of ruminant meat is significantly influenced by extensive conversion of dietary PUFA to SFA during biohydrogenation of lipids that occur in the fore-stomach (rumen) (Shingfield et al., 2013). However, feeding strategies that inhibit ruminal biohydrogenation might increase the deposition of PUFA and CLA and reduce SFA levels in ruminant meat (Bessa et al., 2015).

Lipid oxidation is the main chemical process associated with oxidative deterioration of meat, resulting in undesirable rancid off-flavour and colour deterioration that limit shelf-life and negatively impact consumer acceptability of meat (Morrissey et al., 1998). The balance between antioxidant and pro-oxidant components in muscle tissues mainly influences lipid stability. Animal diets could influence the biochemical components of muscle tissues through enrichment with antioxidant compounds, such as vitamin E, that enhance the oxidative stability of meat (Luciano et al., 2013; Salami et al., 2016).

Phenolic compounds are secondary metabolites that are ubiquitous in several plant species and may be found in considerable amounts in agro-industrial by-products (AIBP; Balasundram et al., 2006). The AIBP can be a valuable and economical resource for animal feeding or for the extraction of bioactive phenolic compounds used in functional food applications. Feeding phenolic-rich AIBP improves the fatty acid composition of ruminant meat through inhibition of ruminal biohydrogenation (Lanza et al., 2015) and enhances the

oxidative stability of meat by increasing the deposition of antioxidant compounds in the muscle (Gravador et al., 2014; Luciano et al., 2013). Moreover, phenolic-rich extracts obtained from AIBP have been directly incorporated into meat products during processing to inhibit oxidative deterioration and extend shelf-life (Balzan et al., 2017; Kanatt et al., 2005; Rodríguez-Carpena et al., 2011).

Cardoon meal is a by-product retained after the extraction of oil from the seeds of cultivated cardoon (*Cynara cardunculus* var. *altilis*), a perennial herb native to the Mediterranean region and widespread in parts of Europe, Americas and Oceania. Cardoon meal has been proposed as an alternative feed resource because of its potential as a valuable source of fibre, protein, amino acids and bioactive compounds such as polyphenols and unsaturated fatty acids (Genovese et al., 2015). Moreover, extracts obtained from different morphological parts (leaf, seed, stem and flower) of cardoon have demonstrated *in vitro* antioxidant and antimicrobial activities attributed to the phenolic constituents present (Falleh et al., 2008; Pandino et al., 2011; Ramos et al., 2014). To our knowledge, no information has been published on the effect of dietary cardoon meal or cardoon extract on meat quality. Therefore, the first objective of this study was to determine the effect of dietary cardoon meal on the fatty acid composition and oxidative stability of lamb meat. In addition, the antioxidant potential of a phenolic-rich cardoon meal extract on lipid oxidation in an ovine muscle model system was investigated.

4.2. MATERIALS AND METHODS

4.2.1. Animals, diets and experimental design

The experimental protocol was approved by the ethics committee of the University of Catania and the feeding trial was conducted indoors in the experimental farm of the University. The animals were handled by trained personnel according to the European Union legislation for the protection of animals used for scientific purposes (2010/63/ EU Directive). Fifteen male Sarda x Comisana lambs (age 75 d and average weight 19.58 ± 2.01 kg) were randomly assigned to two experimental groups. Each animal was reared in an individual pen and adapted to the experimental diets for a period of 9 d by progressive substitution of the weaning feed with the experimental feeds until a total replacement of the weaning diet was achieved. The control group (CON, n = 8), was raised on a commercial concentrate-based diet containing the following ingredients (*as-fed* basis): barley (48.0%), dehydrated alfalfa (15.0%), wheat bran (23.0%), soybean meal (10.0%), molasses (2.0%) and vitamin premix (2.0%). The cardoon meal group (CMD, n = 7), received the same diet as the CON lambs except that the 15% dehydrated alfalfa was completely replaced by cardoon meal. The chemical composition of the experimental diets is outlined in Table 4.1. The CON and CMD diets were supplied in form of pellets and lambs had *ad libitum* access to feeds and water for 75 days pre-slaughter. Experimental feed samples were collected at two-week intervals during the feeding trial and stored in vacuum packs at -30 °C prior to chemical analysis. Diets were supplied daily and the amount of refusal was measured before morning feeding to calculate dry matter intake (DMI). The body weight (BW) of the lambs was measured at the start of the experiment and recorded weekly (at 09:00 h before providing fresh feed) to calculate average daily gain (ADG).

4.2.2. Slaughter, and carcass sampling and measurement

The lambs were slaughtered (stunned by captive bolt before exsanguination) in a commercial abattoir, where they had free access to the experimental diets and water until approximately 3 h before slaughter. Carcass weight was recorded following removal of the visceral organs and dressing percentage was calculated as the percentage of carcass weight to final BW. Visual appraisal of hot hanging carcasses was performed by a certified meat grader to determine the conformation and fat cover scores according to the European Union's EUROP carcass classification system (Commission Regulation (EC) No 823/98, 1998) . Carcass conformation and fatness scores were based on a five-point scale and each score class was further classified into high, medium and low to obtain a 15-point score for a more precise description of carcass traits. Carcasses were stored at 4°C for 24 h *post-mortem* and ultimate pH (pH24) was measured on the *longissimus thoracis* muscle using a pH-meter (Orion 9106). Colour descriptors lightness (L*), redness (a*), yellowness (b*), Chroma (C*), and Hue angle (H*) were recorded on the *longissimus thoracis* muscle, using Minolta CM-2022 spectrophotometer (d/8° geometry; Minolta Co., Ltd. Osaka, Japan) on specular components excluded (SCE) mode, illuminant A and a 10° standard observer. After 24 h of storage at 4 °C, the entire *longissimus lumborum et thoracis* (LTL) from the right half-carcass was removed, packed under vacuum and stored at -80 °C for analyses of intramuscular fatty acids and tocopherols. The entire LTL from the left half-carcass was vacuum-packed and stored at 4 °C for 3 days, pending oxidative stability measurements.

4.2.3. Feed analysis

4.2.3.1. Analysis of chemical composition, fatty acids and vitamin E

Dry matter (DM), crude protein, ether extract and ash content were determined in the experimental diets following the method of AOAC (1995). The neutral detergent fibre, acid

detergent fibre and acid detergent lignin were analysed according to Van Soest et al. (1991). The fatty acid composition of the experimental diets was determined by a one-step extraction–transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003). Gas chromatographic (GC) analysis of fatty acid methyl esters (FAME) was performed as described later (section 4.2.4) for fatty acid profile of lamb meat. Individual fatty acids of experimental diets were expressed as mg/g of DM.

Vitamin E (α-, γ- and δ-tocopherols) was analysed in the experimental diets as described by Cherif et al. (2018). Briefly, freeze-dried samples were homogenized and saponified with ethanolic KOH stabilized with BHT. Tocopherols were extracted using hexane/ethyl acetate (9/1, v/v), dried under N_2 and dissolved in acetonitrile. Vitamin E was analysed by HPLC (Perkin Elmer series 200), equipped with an autosampler (model AS 950-10, Tokyo, Japan) and a Synergy Hydro-RP column (4 μm, 4.6 × 100 mm; Phenomenex, Bologna, Italy). Tocopherols were eluted at a flow rate of 2 ml/min and identified using a fluorescence detector (model Jasco, FP-1525) set at an excitation and emission λ of 295 nm and 328 nm, respectively. Quantification was based on external calibration curves of pure standard compounds (Sigma-Aldrich, Bornem, Belgium) solubilised in ethanol.

Table 4.1. Chemical composition of experimental diets

	Diets[1]	
Parameter	**CON**	**CMD**
Dry matter, % *as-fed*	89.65	89.63
Crude protein, % DM	15.67	16.45
Ether extract, % DM	2.68	3.84
Ash, % DM	7.01	6.31
NDF, % DM	30.36	27.32
ADF, % DM	15.97	12.39
ADL, % DM	3.62	4.15
Total phenolic content[2]	5.21	13.08
Fatty acids (mg/g DM)		
C14:0	0.034	0.034
C16:0	4.357	5.062
cis-9 C16:1	0.035	0.034
C18:0	0.454	0.705
C18:1 *n*-9	3.855	5.362
cis-11 C18:1	0.209	0.206
C18:2 *n*-6	12.190	16.852
C18:3 *n*-3	1.255	1.065
C20:0	0.088	0.086
Vitamins (µg/g DM)		
α-Tocopherol	5.267	20.877
γ-Tocopherol	0.155	0.119
δ-Tocopherol	0.012	0.005

[1]CON: control diet; CMD: cardoon meal diet
[2]Expressed as grams gallic acid equivalents/kg DM
NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin

4.2.3.2. Analysis of total phenol content and in vitro antioxidant activity

Phenolic compounds were extracted from cardoon meal, dehydrated alfalfa and experimental diets using aqueous methanol (50:50, v/v) and acetone (70:30, v/v) solvents (Jiménez-Escrig et al., 2001). Polyphenol-rich extracts were analysed for total phenol content (TPC) using the Folin-Ciocalteu method (Singleton et al., 1999) with minor modifications. Briefly, extracts (0.5 ml) were mixed with Folin-Ciocalteu reagent (2.5 ml, 20% in distilled water) and sodium carbonate (2 ml, 7.5% in distilled water) was added after 5 min. The mixture was stored in the dark for 2 h at room temperature and absorbance

measurements were recorded at 750 nm using a UV-vis spectrophotometer (Cary 300 Bio, UV-vis spectrophotometer, Varian Instruments, CA, USA) against a blank containing all reagents and distilled water. A calibration curve using standard solutions of aqueous gallic acid (20 – 100 μg/ml) was plotted and results were expressed as g of gallic acid equivalents (GAE)/kg of DM feed.

In vitro antioxidant activity of polyphenol-rich extracts was measured using 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging (Yen and Wu, 1999) and ferric reducing antioxidant power (FRAP) total antioxidant assays (Benzie and Strain, 1999), with minor modifications. For the DPPH assay, extract (0.6 ml) and distilled water (2.4 ml) was mixed with 0.2 mM DPPH in methanol (3 ml) and stored in the dark for 1 h at room temperature. Absorbance measurements were recorded at 517 nm using a UV-vis spectrophotometer (Cary 300 Bio) against a methanol blank. An assay blank containing distilled water (3 ml) and 0.2 mM DPPH in methanol (3 ml) was used for calculation purposes. A calibration curve using standard solutions of methanolic Trolox (10 – 50 μg/ml) was plotted and results were expressed as g of Trolox equivalents (TE)/kg of DM feed.

For the FRAP assay, extract (0.45 ml) was mixed with 8.55 ml FRAP reagent (a mixture of 30 mM acetate buffer (pH 3.6), 10 mM 2,4,6–Tris (2-pyridyl)-s-triazine in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in distilled water in the ratio 10:1:1, respectively incubated at 37 °C for 10 min prior to use). The mixture was stored in the dark for 30 min at room temperature and absorbance measurements were recorded at 593 nm using a UV-vis spectrophotometer (Cary 300 Bio) against a blank containing all reagents. A calibration curve using standard solutions of methanolic Trolox (0.033 – 0.1 mg/ml) was plotted and results were expressed as g of Trolox equivalents (TE)/kg of DM feed.

4.2.4. Analysis of fatty acids and vitamin E in lamb meat

Intramuscular fat was extracted from 10 g of minced LTL with a mixture of methanol and chloroform (2:1, v/v) according to the method of Folch et al. (1957). Lipids (30 mg) were converted to FAME by base-catalysed transesterification (Christie, 1982) using 0.5 mL of sodium methoxide in methanol 0.5 N and 1 mL of hexane containing 1mg /mL nonadecanoic acid (C19:0) as an internal standard. Gas chromatographic analysis was conducted as described by Valenti et al. (2018) using a GC 8000 Top ThermoQuest (Milan, Italy) gas-chromatograph equipped with a flame ionization detector and a high polar column (WCOT-fused silica CP-Select CB for FAME Varian, Middelburg, the Netherlands; 100m×0.25mm i.d.; film thickness 0.25 μm). Helium was the carrier gas at a constant flow of 1 ml/min. Total FAME profile in a 1 μL sample volume (2 μL for feed samples) at a split ratio of 1:80 was determined using the following conditions: the oven temperature was programmed at 40 °C and held for 4 min, then increased to 120 °C at 10 °C/min, held for 1 min, then increased up to 180 °C at 5 °C/min, held for 18 min, then increased up to 200 °C at 2 °C/min, held for 15 min, and then increased up to 230 °C at 2 °C/min, held for 19 min. The injector and detector temperatures were set at 270 °C and 300 °C, respectively. FAME identification was based on a standard mixture of 52 Component FAME Mix (Nu-Chek Prep Inc., Elysian, MN, USA) and individual FAME standards (Larodan Fine Chemicals, Malmo, Sweden). Fatty acids were expressed as g/100 g of total methylated fatty acids. The dietary risk for cardiovascular diseases was assessed by calculating the atherogenic index (the relationship between FA with pro-atherogenic and anti-atherogenic properties) and thrombogenic index (the relationship between FA with pro-thrombogenic and anti-thrombogenic properties). The atherogenic index (AI) and thrombogenic index (TI) were calculated as outlined by Ulbricht and Southgate (1991):

$$AI = \frac{C12:0 + (4 \times C14:0) + C16:0}{\text{n-6 PUFA + n-3 PUFA + MUFA}}$$

$$TI = \frac{C14:0 + C16:0 + C18:0}{(0.5 \times \text{MUFA}) + (0.5 \times \text{n-6 PUFA}) + (3 \times \text{n-3 PUFA}) + (\frac{\text{n-3 PUFA}}{\text{n-6 PUFA}})}$$

Vitamin E (α-, γ- and δ-tocopherols) and retinol were extracted from lamb muscle according to the method of Schüep and Rettenmaier (1994). Chromatographic analysis of vitamin E was performed as described in section 4.2.3.1 for feeds. Retinol was analyzed in the same chromatographic run and identified using the UV-VIS detector set at λ 325 nm (Cherif et al., 2018). Identification and quantification were achieved using external calibration curves of standard compounds (Sigma-Aldrich) solubilised in ethanol.

4.2.5. Measurement of lamb meat oxidative stability

Oxidative stability parameters were measured in raw and cooked lamb meat, as well as in LTL homogenates incubated with pro-oxidant catalysts, as described by Valenti et al. (2018). Briefly, for analyses on raw and cooked meat, 6 slices (2 cm thickness) were cut from the LTL which was previously stored under vacuum at 4 °C as described in section 4.2.2. Three slices were placed in polystyrene trays, covered with PVC film and stored at 4 °C. Each slice was used for measuring lipid oxidation and colour stability at one of three-time points: day 0 (after 2 hours of blooming), and days 4 and 7. The remaining 3 slices were packed under vacuum and cooked for 30 min at 70°C in a water bath. After cooling in a cold-water bath, one slice was used immediately for measurement of lipid oxidation, while the other 2 slices were stored at 4°C in the same conditions described for the raw meat samples, and lipid oxidation was measured after 2 and 4 days. For both raw and cooked meat, lipid oxidation was measured as thiobarbituric acid reactive substances (TBARS) values according to the procedure of Siu and Draper (1978) and results were

expressed as μg malonaldehyde (MDA)/g of meat. Colour was measured in raw meat using a Minolta CM-2022 spectrophotometer (d/8° geometry; Minolta Co., Ltd. Osaka, Japan) set in the specular components excluded (SCE) mode, illuminant A and a 10° standard observer. The colour descriptors lightness (L^*), redness (a^*), yellowness (b^*), Chroma (C^*), and Hue angle (H^*) were recorded, as well as the reflectance spectra from 400 to 700 nm. The ratio $(K/S)_{572} \div (K/S)_{525}$ was calculated to monitor the accumulation of metmyoglobin (MetMb) on the meat surface over time, with values of the ratio decreasing with increasing proportion of MetMb. The ratio (K/S) between the absorption (K) and the scattering (S) coefficients at the selected wavelengths was calculated as: $(K/S)_\lambda = (1 - R_\lambda)^2 / 2R_\lambda$.

The resistance of meat to lipid and myoglobin oxidation was also assessed in LTL homogenates incubated in the presence of Fe^{3+} and ascorbate (Fe/Asc) as catalysts of oxidative reactions. Briefly, the minced LTL (7.5 g) was homogenized with 37.5 g of MES buffer (pH 5.6). Homogenates were equilibrated to 37 °C and two aliquots (3 ml and 4 ml) were collected for measuring the initial extent of lipid and myoglobin oxidation (0 minutes). Ferric chloride hexahydrate and L-sodium ascorbate were added at equimolar concentration to reach the final concentration of 45 μM. The homogenates were incubated under continuous stirring in a temperature controlled IKA KS-4000 orbital shaker (IKA-Werke GmbH & Co. KG, Staufen, Germany) set at 37°C and 190 rpm. After 30 and 60 minutes of incubation, two aliquots (3 ml and 4 ml) were collected for lipid and myoglobin oxidation analyses. Lipid oxidation was measured in the 3 ml aliquots using the method of Siu and Draper (1978). The 4 ml aliquots were centrifuged at 6800 × g at 4 °C, filtered through Whatman 541 filter paper and directly scanned in a UV/VIS spectrophotometer (UV-1601, Shimadzu Co., Milan, Italy). The absorbances at 503, 525, 557, 582, and 730 nm were used to calculate the proportion of metmyoglobin (MetMb %; Tang et al., 2004).

4.2.6. Determination of antioxidant potential of cardoon extract in a muscle model system

4.2.6.1. Preparation of cardoon extract

Phenolic compounds were extracted in triplicate by suspending finely-ground cardoon meal (0.5 g) in 100% methanol (20 ml) and incubated at room temperature in an orbital shaker (Max Q 6000 Shaker Thermo Fisher Scientific, Ireland) at 200 rpm for 4 h. After 4 h, the mixture was filtered through Whatman No. 1 filter paper. Pooled solvent extracts were concentrated by placing in a 50 ml round-bottomed flask and solvent (methanol) was removed by rotary evaporation (Labo-Rota C-311, Resona Technics, Switzerland) for 1 h at 55 °C. The dried extract was re-suspended in 20 ml methanol and analysed for TPC using the Folin-Ciocalteu reagent as described for experimental feeds (section 4.2.3.2.). Result was expressed as mg GAE/ml extract.

4.2.6.2. Preparation of muscle homogenates

Fresh lamb LTL (n = 3) was obtained from a meat retail outlet (Cork, Ireland) and stored at 4 °C prior to analysis. Muscle homogenates (25%) were prepared in triplicate following a minor modification of the method described by O'Grady et al. (2001). Briefly, LTL (15 g) was chopped and homogenised in 0.12 M KCL 5 mM histidine (45 ml), pH 5.5, using an Ultra-turrax T25 homogeniser (Janke & Kunkel GmbH, IKA® Labortechnik, Staufen, Germany) at 24,000 rpm for 5 min. The muscle tissue and buffer were surrounded by crushed ice to control the temperature during homogenisation. Cardoon extract (stock concentration of 1.32 GAE mg/ml) was added to the homogenates (39.2 g) at 0, 0.5, 1 and 5% v/w of the final volume (40 ml). Lipid oxidation in muscle homogenate samples was initiated by the addition of equimolar $FeCl_3$/sodium ascorbate (45 μM) pro-oxidants. The TPC and lipid oxidation in muscle homogenate were measured after 4 h of storage at 4 °C.

4.2.6.3. Measurement of TPC and lipid oxidation in muscle homogenate

The total phenolic content in muscle homogenates was measured in triplicate by mixing 10% TCA (0.5 ml), 0.05 M phosphate buffer (3 ml) and muscle homogenate (2 g). The mixture was centrifuged at 7800 ×g for 10 min at 4 °C using an Avanti® J-E Centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA). The supernatant was filtered through Whatman No. 1 paper and the filtrate was analysed for TPC using the Folin-Ciocalteu reagent as described for experimental feeds (section 4.2.3.2). Results were expressed as g GAE/g muscle. Lipid oxidation was measured in triplicate following the TBARS assay described by Siu and Draper (1978) and results were expressed as µg MDA/g meat.

4.2.7. Statistical analysis

One-way ANOVA test was used to analyse the effect of dietary treatment on growth performance parameters, carcass traits, intramuscular fatty acids and fat-soluble vitamins, using individual lambs as the experimental units. Data on the oxidative stability parameters (raw, cooked and muscle homogenates) were analysed with a full-repeated measures ANOVA. Effects of diet represented the 'between-subjects' factor and the effect of storage time/incubation was measured using the 'within-subjects' factor and the interaction between diet and storage time/incubation was tested. Individual lambs were considered as the experimental units in all the statistical analyses performed for the effect of dietary cardoon meal on growth performance and meat quality. Analyses relating to the effect of cardoon extract addition on TPC and lipid oxidation (TBARS) in LTL homogenates was performed in triplicate and mean sample values ($n = 3$) for each of the four treatment groups (CON, CE0.5, CE1.0 and CE5.0) were subjected to a one-way ANOVA. Pearson's correlation analysis was performed to assess the relationship between the TPC and TBARS in LTL homogenates. Significance was declared when $P \leq 0.05$, while a tendency for effects

were considered when $0.05 < P \leq 0.10$. All statistical analyses were performed using the SPSS software (IBM Statistics version 22).

4.3. RESULTS AND DISCUSSION

The functional effect of bioactive-rich agricultural by-products can be exploited in meat production through direct feeding to livestock or by further processing to obtain phenolic-rich extracts that can be added directly to fresh or processed meat products. Cardoon meal is a by-product obtained after the extraction of oil from the seeds of cultivated cardoon and is rich in phenolic compounds. Recent investigations have shown that feeding animals with diets high in phenolic contents (e.g. tannins) may improve the fatty acid composition of ruminant meat and enhance the antioxidant status of meat against *post-mortem* oxidative deterioration (Vasta and Luciano, 2011). Therefore, in the present study, it was hypothesized that high concentration of phenolic compounds in dietary cardoon meal may improve the fatty acid composition and oxidative stability of lamb meat. Furthermore, the antioxidant effect of phenolic-rich extract obtained from cardoon meal was examined in an ovine muscle model system in order to highlight the potential of cardoon extract as a natural antioxidant for use in the development of functional meat products. To our knowledge, this is the first study to report the effect of cardoon meal or cardoon extract on lamb meat quality.

4.3.1. Antioxidant activity of experimental feedstuffs and diets

The TPC of cardoon meal was 4-fold higher ($P < 0.01$; 60.4 *vs* 15.5 g GAE/kg DM) than dehydrated alfalfa, which may explain the 2.5-fold increase in TPC when dehydrated alfalfa was replaced by cardoon meal in CMD, compared to CON (Table 4.1). The phenolic concentration of cardoon meal was 5-fold higher than previously reported for cardoon press cake (Genovese et al., 2015), a similar by-product obtained from the mechanical extraction of oil from cardoon seeds. Variation in the TPC of cardoon extracts may be related to factors such as plant geographical origin, varieties and maturity stage, agricultural methodologies,

and phenol extraction procedures (Ramos et al., 2014). As expected, assessment of the *in vitro* antioxidant activities showed that cardoon meal exhibited a higher ($P < 0.01$) DPPH free radical scavenging activity and FRAP total antioxidant activity compared to dehydrated alfalfa (Figure 4.1a). Similarly, replacement of dehydrated alfalfa with cardoon meal in the experimental diets resulted in higher ($P < 0.01$) antioxidant activities in CMD compared to CON (Figure 4.1b).

Flavonoids and hydroxycinnamic acids are the main classes of phenolic compounds, which contribute to the antioxidant effect of extracts obtained from cardoon leaf, seed, stem and flower (Falleh et al., 2008; Pandino et al., 2011; Pinelli et al., 2007; Ramos et al., 2014). It has been shown that the TPC of cardoon extracts strongly correlated with DPPH antiradical activity (Falleh et al., 2008; Ramos et al., 2014) and FRAP total antioxidant capacity (Pandino et al., 2011). In addition, α-tocopherol is another potent antioxidant which can be found in high residual levels in cardoon meal as cardoon oil has been shown to contain considerable amounts of vitamin E (Maccarone et al., 1999). This is consistent with the 4-fold increase in α-tocopherol content found in CMD compared to CON (Table 4.1).

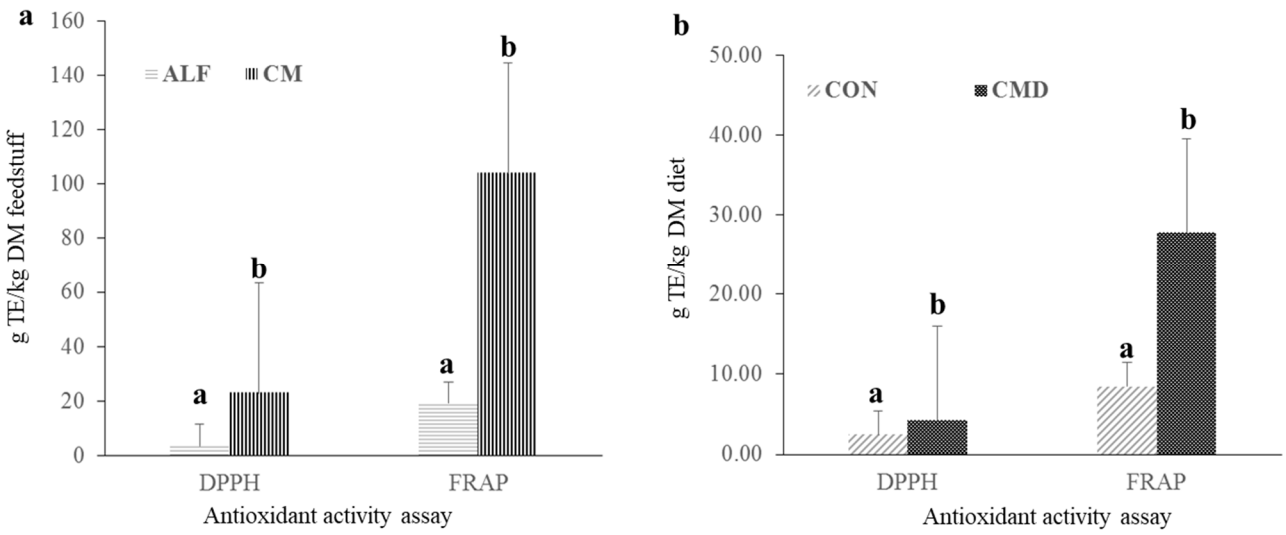


Figure 4.1. Antioxidant activity of **(a)** dietary test ingredients (feedstuff): dehydrated alfalfa (ALF) and cardoon meal (CM) **(b)** experimental diets: control (CON) and cardoon meal diet (CMD). Values are presented as means with standard error bars.
[a,b]For antioxidant activity assay, bars with different letters are significantly different ($P < 0.05$).
DPPH: 2,2-diphenyl-1-picrylhydrazyl assay (free radical scavenging activity); FRAP: Ferric reducing antioxidant power assay (total antioxidant activity); TE: trolox equivalent.

4.3.2. Growth performance and carcass traits

Lambs fed CMD exhibited lower ($P < 0.05$) DMI compared to CON-fed lambs. Lower DMI in lambs fed CMD may be related to low feed palatability due to the high content of dietary phenolic compounds. Cajarville et al. (1999) reported a similar decrease in the voluntary intake of sheep fed *ad libitum* green forage of cardoon possibly due to the presence of high concentration of phenolic compounds in cardoon leaves (Kukić et al., 2008). Phenolic compounds, such as tannins, may confer unpleasant taste or bind to salivary proteins forming a polyphenol-protein complex that induce astringency sensations and trigger low feed intake in animals (Makkar, 2003). However, dietary treatment did not affect ($P > 0.05$) growth performance parameters in terms of final BW, ADG and feed efficiency (Table 4.2). Similarly, there was no effect of dietary treatment on carcass characteristics such as carcass weight, dressing percentage, carcass conformation, fatness scores, muscle ultimate pH and

colour characteristics (Table 4.2). The values of colour variables are within the satisfactory range for average consumer acceptance of fresh meat, particularly in terms of lightness (L^*) and redness (a^*) (Khliji et al., 2010).

Table 4.2. Effect of dietary treatment on the growth performance and carcass characteristics of lambs

	Dietary treatment[1]			
Item	**CON**	**CMD**	**SEM**	***P*-value**
Growth performance				
Dry matter intake, g/d	1078.3	932.2	31.15	0.013
Initial body weight, kg	20.1	20.1	0.54	0.974
Final body weight, kg	35.5	33.9	0.74	0.303
Average daily gain, g/d	204.5	184.0	8.04	0.215
Feed efficiency	190.8	198.7	8.24	0.649
Carcass traits				
Carcass weight, kg	17.1	16.1	0.41	0.264
Dressing percent, %	48.1	47.5	0.29	0.333
Conformation score[2]	2.3	1.9	0.13	0.102
Fatness score[3]	2.5	2.4	0.04	0.738
Ultimate pH	5.91	5.83	0.04	0.310
Lightness, L^*	37.95	38.72	0.58	0.528
Redness, a^*	11.90	12.53	0.43	0.481
Yellowness, b^*	6.93	7.32	0.34	0.588
Saturation, C^*	13.78	14.51	0.53	0.512
Hue angle, H^*	29.96	30.23	0.56	0.817

[1]CON: control diet; CMD: cardoon meal diet
[2]Calculated as: g BW gain/kg DMI
[3]Conformation score: E = 5, excellent shape and muscularity; U = 4; R = 3; O = 2; P = 1, poor shape and muscularity.
[4]Fatness score: 1 = low, 5 = very high

Table 4.3. Effect of dietary treatment on the intramuscular fat content, fatty acid composition and fat soluble vitamins in the *longissimus thoracis et lumborum* muscle of lambs

	Dietary treatment[1]			
Parameter	**CON**	**CMD**	**SEM**	***P*-value**
IMF (g/100g of muscle)	2.07	2.06	0.117	0.946
Fatty acids (g/100 g of total fatty acids)				
C12:0	0.13	0.13	0.007	0.920
C14:0	2.90	2.81	0.121	0.727
cis-9 C14:1	0.11	0.10	0.007	0.367
C15:0	0.36	0.36	0.011	0.848
iso C15:0	0.07	0.06	0.002	0.146
anteiso C15:0	0.11	0.11	0.005	0.907
C16:0	23.60	22.81	0.245	0.110
cis-9 C16:1	1.77	1.57	0.075	0.174
C17:0	1.15	1.22	0.052	0.544
iso C17:0	0.37	0.33	0.010	0.065
anteiso C17:0	0.49	0.45	0.011	0.060
C18:0	12.13	13.80	0.267	<0.001
cis-9 C18:1	38.78	39.56	0.447	0.403
cis-11 C18:1	1.58	1.58	0.047	0.994
trans-9 C18:1	0.26	0.22	0.011	0.066
trans-10 C18:1	1.58	0.72	0.149	0.001
trans-11 C18:1	0.75	0.48	0.055	0.006
cis-9 *cis*-12 C18:2	6.85	6.89	0.323	0.944
cis-9 *trans*-11 C18:2	0.43	0.30	0.024	0.002
trans-8 *cis*-10 C18:2	0.01	0.01	0.002	0.792
cis-11 *trans*-13 C18:2	0.02	0.01	0.002	0.064
cis-9 *cis*-12 *cis*-15 C18:3	0.53	0.47	0.015	0.070
C20:0	0.09	0.10	0.004	0.183
C20:3 *n*-6	0.15	0.15	0.013	0.992
C20:4 *n*-6	1.40	1.39	0.136	0.963
C20:5 *n*-3	0.08	0.07	0.010	0.589
C22:4 *n*-6	0.13	0.15	0.014	0.624
C22:5 *n*-6	0.04	0.04	0.005	0.541
C22:5 *n*-3	0.22	0.21	0.024	0.878
C22:6 *n*-3	0.05	0.06	0.006	0.577
Summary				
Σ SFA	41.80	42.59	0.255	0.128
Σ MUFA	47.21	46.35	0.444	0.357
Σ PUFA	10.71	10.67	0.524	0.975
Σ OBCFA	2.80	2.80	0.060	0.978
Σ *n*-6 /Σ *n*-3 PUFA	8.16	8.92	0.197	0.052
Atherogenic index	0.62	0.61	0.014	0.716
Thrombogenic index	1.24	1.29	0.019	0.196
Fat soluble vitamins, ng/g muscle				
Retinol	229.05	311.21	21.131	0.048
α-Tocopherol	276.94	365.51	34.200	0.207
γ-Tocopherol	1.99	1.48	0.191	0.191
δ-Tocopherol	19.90	26.54	1.970	0.093

[1]CON: control diet; CMD: cardoon meal diet
IMF: intramuscular fat; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; OBCFA: Odd-and branched-chain fatty acids

4.3.3. Effect of dietary cardoon meal on lamb meat fatty acid composition and fat-soluble vitamin levels

The effects of dietary cardoon meal on ruminal biohydrogenation of fatty acids were reported in Chapter 3. In summary, results showed that feeding CMD significantly increased daily dietary intakes of stearic acid (+35.7%), oleic acid (+20.2%), linoleic acid (+19.6%) and polyphenols (+116.9%) and reduced intake of α-linolenic acid (-26.7%) compared to CON. Despite the higher intake of unsaturated FA and polyphenols in CMD-fed lambs, CMD promoted ruminal biohydrogenation of fatty acids due a modification of rumen bacterial community. In comparison to CON-fed lambs, the ruminal digesta of lambs fed CMD was characterized by higher levels of stearic acid and total SFA, and lower concentrations of oleic acid, *trans*-10 C18:1, *trans*-11 C18:1 and total MUFA. The observed effects of diet on ruminal biohydrogenation are helpful in explaining intramuscular fatty acid results reported in the present study.

Intramuscular fat, fatty acid profile and fat-soluble vitamin contents in LTL muscle from lambs fed CON and CMD diets are presented in Table 4.3. In general, dietary treatment did not influence ($P > 0.05$) intramuscular fat content or the total composition of SFA, MUFA, PUFA, odd- and branched- chain fatty acids (OBCFA). Dietary treatment did not influence ($P > 0.05$) the concentration of individual SFA (C12:0, C14:0 and C16:0) that results in hypercholesterolemic effects in humans (Mensink, 2005). This could in part explain why meat from lambs fed CON and CMD had similar ($P > 0.05$) nutritional indices (atherogenic index and thrombogenic index) for risk of cardiovascular diseases. However, increased ruminal biohydrogenation in lambs fed CMD was consistent with the observed higher concentration ($P < 0.05$) of intramuscular C18:0 (stearic acid), and lower proportions ($P < 0.05$) of *trans*-10 C18:1, *trans*-11 C18:1 (vaccenic acid) and *cis*-9 *trans*-11 C18:2 CLA

(rumenic acid) compared to CON-fed lambs. In addition, the proportion of C18:3 *n*-3 (α-linolenic acid) tended ($P = 0.070$) to be greater in the muscle of lambs fed CON compared to CMD-fed lambs. Consequently, the *n*-6:*n*-3 PUFA ratio tended to be lower ($P = 0.052$) in CON-fed lambs compared to CMD-fed lambs. Regarding fat-soluble vitamins in LTL, the concentration of retinol ($P = 0.048$) was greater in muscle from lambs fed CMD relative to CON but diet did not affect ($P > 0.05$) vitamin E (α-, γ- and δ-tocopherols) concentration (Table 4.3).

The present study showed that dietary treatment significantly affected the intramuscular concentration of individual fatty acids (stearic, *trans*-10 C18:1, vaccenic and rumenic acids) with potential nutritional implications. Stearic acid is the predominant saturated fatty acid found in ruminant meat or milk as the main end product of ruminal biohydrogenation (Bessa et al., 2015). It has been suggested that dietary consumption of stearic acid does not increase plasma low-density lipoprotein and cholesterol levels in humans in contrast to the negative effect of other SFA that are risk factors for cardio-metabolic diseases (Mensink, 2005). Thus, the greater amount of stearic acid in lambs fed CMD may not have detrimental effects on human health. On the other hand, a lower concentration of *trans*-10 C18:1, a biohydrogenation intermediate, in CMD-fed lamb meat may be viewed as a positive outcome from a nutritional perspective due to the potential cytotoxic effect of this fatty acid at higher concentrations (Vahmani et al., 2016). Muscle from lambs fed CON exhibited a higher proportion of vaccenic and rumenic acids that are known for their potential health benefits in humans (Bessa et al., 2015). Both vaccenic and rumenic acids are intermediate FA synthesized during ruminal biohydrogenation but rumenic acid can be further synthesized in muscle tissues through endogenous desaturation of vaccenic acid by the enzyme Δ-9-desaturase (Corl et al., 2001). Notably, our previous results on ruminal biohydrogenation showed that the concentration of vaccenic acid was higher in CON-fed

lambs but there was no dietary effect on the concentration of rumenic acid. Thus, it could be explained that higher concentration of intramuscular rumenic acid in CON-fed lambs was related to greater ruminal outflow and absorption of vaccenic acid that serves as the main precursor for the endogenous synthesis of rumenic acid in muscle tissues. Rumenic acid is the major naturally occurring CLA isomer found in ruminant meat and milk (Bessa et al., 2015) and dietary consumption of this FA potentially prevent human diseases including cancers, cardiovascular diseases, obesity, bone density loss, and diabetes (McGuire and McGuire, 2000).

Indeed, the current results contradict our hypothesis that a high concentration of phenolic compounds in dietary cardoon meal would inhibit ruminal biohydrogenation and enhance the incorporation of health-promoting fatty acids (PUFA, MUFA and CLA) in lamb meat. A number of factors may account for the lack of positive effect of CMD on ruminal biohydrogenation and intramuscular fatty acids. It is possible that hydroxycinnamic acids, the major cardoon phenolic compounds, were metabolised by the consortium of microbes residing in the fore-stomach (rumen) of lambs as shown with human faecal microbiota (Gonthier et al., 2006). In support of this speculation, it was observed that the rumen bacterial community significantly differed between lambs fed CON and CMD (data not shown), suggesting that possible differences in microbial metabolism may exist between the two diets. Furthermore, positive effects of dietary phenolics on the fatty composition of ruminant meat have been largely reported for diets containing a high concentration of tannins (Vasta et al., 2008). Though the tannin content of diets was not measured in the present study, it has been shown that cardoon extracts contain a very low concentration of tannins (Falleh et al., 2008). Thus, results from the present study emphasized the variation in the effect of diets containing a high concentration of bioactive compounds, which may be dependent on the type and/or concentration of phenolic compounds present.

In contrast, alfalfa fed in various processed forms (fresh, hay, dehydrated, silage) has been documented to enrich lamb muscle and offal with lower SFA and higher content of PUFA and rumenic acid (Cerci et al., 2011; Ciftci et al., 2010; Realini et al., 2017). The presence of saponins and flavonoids in alfalfa may explain why feeding CON inhibited ruminal biohydrogenation and enhanced the fatty acid profile of lamb meat in the present study. These results support the hypothesis that positive effects of dietary alfalfa on the fatty acid profile of ruminant meat may be more attributed to its inhibitory effect on ruminal biohydrogenation (Petersen and Jensen, 2014) and less influenced by its effect on lipid metabolism in muscle tissues (González-Calvo et al., 2015).

4.3.4. Effect of dietary cardoon meal on meat oxidative stability

The results of oxidative stability of raw meat, cooked meat and muscle homogenates are presented in Table 4.4. There was no effect of diet on lipid oxidation of raw and cooked lamb meat stored aerobically for up to 7 and 4 days, respectively (Table 4.4). As expected, lipid oxidation significantly increased ($P < 0.05$) in raw and cooked meat as a function of storage time. A significant interaction between dietary treatment and storage time was observed for lipid oxidation in raw meat. The interaction indicated that meat from lambs fed CMD exhibited higher lipid oxidation on day 7 compared to CON. Although meat from lambs fed CON and CMD had similar total PUFA content, a higher level of CLA (*cis*-9 *trans*-11 18:2) in the meat from CON-fed lambs may be a plausible reason of the lower TBARS value as CLA is an oxidative-stable component of muscle PUFA. The CLA present in meat does not participate in oxidation processes and thus reduce the formation of oxidative fatty acid free radicals resulting in reduced lipid oxidation (Hur et al., 2004). In addition, dietary treatment did not influence the colour stability of raw lamb meat measured using instrumental colour descriptors (lightness L^*, redness a^*, saturation C^* and hue angle H^*) and an index for metmyoglobin accumulation ($(K/S)_{572} \div (K/S)_{525}$). Over the storage

period of raw lamb meat, redness and colour saturation declined ($P < 0.05$) while colour hue increased ($P < 0.05$). However, colour stability parameters were not affected by the interaction between diet and storage time. In general, feeding CMD did not affect the oxidative stability of aerobically stored fresh and cooked meat despite the higher antioxidant activities exhibited by CMD compared to CON (Figure 4.1b). The antioxidant effect of dietary phenolics in meat depends on its effective absorption and deposition into muscle tissues, which in turn is influenced by the size of the phenolic molecules and/or potential metabolism in the gastrointestinal tract. As previously highlighted in this study, possible microbial metabolism of cardoon phenolics in the gut of lambs may account for the lack of positive effect of CMD on meat fatty acid composition and oxidative stability.

Post-mortem oxidative changes occur naturally in meat and can be accelerated by several factors during processing, storage or retail display (Bekhit et al., 2013). In the current experiment, intensed lipid oxidation was induced by incubating muscle homogenates with $FeCl_3$/sodium ascorbate pro-oxidants. There was no significant interaction ($P > 0.05$) of treatment × time on TBARS and metmyoglobin concentration (indicative of formation of brownish pigment) values measured up to 60 min of incubation.

Table 4.4. Effect of dietary treatment and time of storage or incubation on oxidative stability parameters of lamb meat

Item	Diet (D)[1]	Storage /incubation time (T)[2]			SEM	Effect (*P*-value)		
		1	2	3		D	T	D x T
Raw meat								
	CON	0.18[a]	0.87[a]	1.70[a]				
TBARS[3]	CMD	0.18[a]	0.78[a]	2.42[b]	0.136	0.129	<0.001	0.036
	CON	44.23	43.93	42.95				
Lightness, L^*	CMD	43.46	42.82	42.61	0.299	0.229	0.364	0.872
	CON	14.49	12.40	10.44				
Redness, a^*	CMD	15.77	12.97	10.82	0.340	0.075	<0.001	0.635
	CON	12.03	12.17	11.27				
Yellowness, b^*	CMD	12.87	12.11	11.61	0.194	0.033	0.098	0.626
	CON	18.84	17.38	15.39				
Saturation, C^*	CMD	20.36	17.75	15.88	0.356	0.146	<0.001	0.630
	CON	39.49	44.53	47.32				
Hue angle, H^*	CMD	39.15	42.99	47.05	0.550	0.183	<0.001	0.549
	CON	1.00	0.91	0.88				
$(K/S)_{572} \div (K/S)_{525}$	CMD	0.99	0.91	0.87	0.009	0.392	<0.001	0.880
Cooked meat								
	CON	1.36	3.73	5.64				
TBARS[3]	CMD	1.48	3.97	5.89	0.281	0.337	<0.001	0.930
Muscle homogenates								
	CON	0.09	3.48	3.73				
TBARS[3]	CMD	0.15	3.81	4.22	0.271	0.037	<0.001	0.437
	CON	12.84	64.60	88.78				
Metmyoglobin	CMD	11.29	65.67	89.71	4.911	0.925	<0.001	0.747

[1]CON: control diet; CMD: cardoon meal diet
[a,b] Treatment effect: Mean values bearing different superscripts are significantly different ($P < 0.05$)
[2]Times 1, 2, 3 correspond to: 0, 4, 7 days (raw meat slices stored at 4°C under aerobic conditions); 0, 2, 4 days (cooked meat slices stored at 4°C under aerobic conditions); 0, 30 and 60 minutes (muscle homogenates incubated with Fe/Asc at 37°C under continuous stirring)
[3]Expressed as μg MDA/g meat

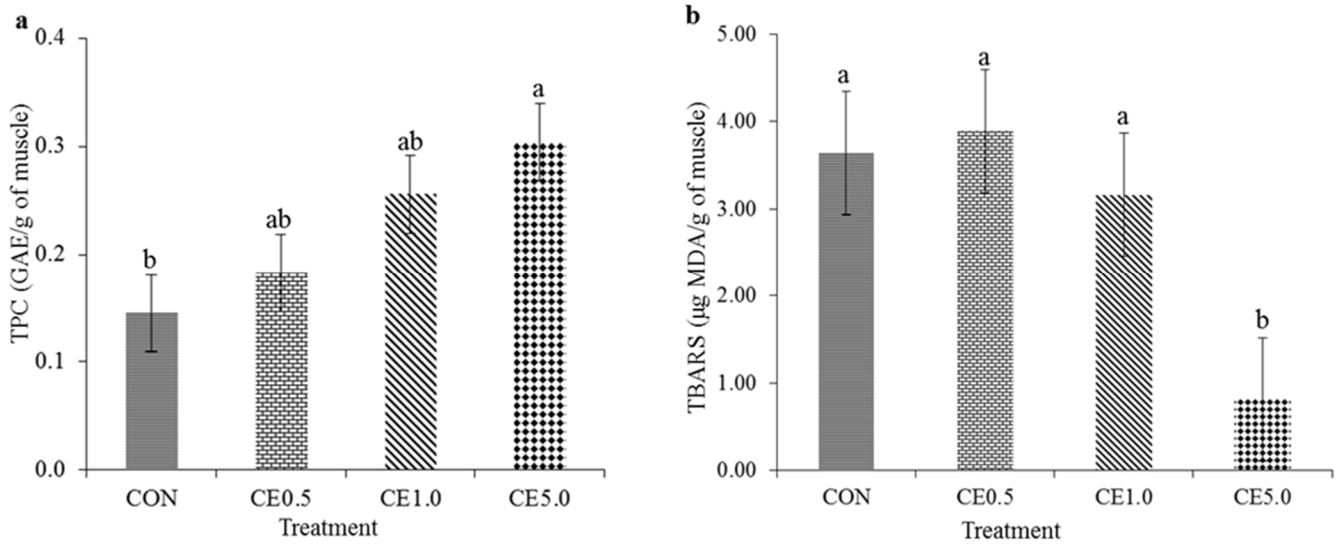


Figure 4.2. Effect of cardoon extract (CE) addition on **(a)** total phenolic contents (TPC, GAE/g of muscle) **(b)** lipid oxidation (TBARS, µg MDA/g of muscle) of ovine *longissimus thoracis et lumborum* (LTL) muscle homogenates after 4 h of incubation with a $FeCl_3$/sodium ascorbate pro-oxidant system. Treatments are as follows: CON, CE0.5, CE1.0 and CE5.0 represent addition of 0, 0.5%, 1.0%, and 5.0% of cardoon extract to muscle homogenates, respectively. Values are presented as means with standard error bars. [a,b]For each treatment, bars with different letters are significantly different ($P < 0.05$).
GAE: gallic acid equivalents; TBARS: thiobarbituric acid reactive substances.

4.3.5. Effect of cardoon extract on lipid oxidation in a muscle-based system

Antioxidant effect of the phenolic-rich extract obtained from cardoon meal was tested in LTL homogenates subjected to $FeCl_3$/sodium ascorbate-induced oxidation by incubating with iron/ascorbate pro-oxidants for 4 h. Addition of 5% cardoon extract significantly increased (+114.3%; $P < 0.05$) the total phenolic content (TPC) in LTL homogenates compared to the control (Figure 4.2a). Similarly, addition of 5% extract inhibited (-77.6%; $P < 0.001$) lipid oxidation (TBARS values) relative to the control (Figure 4.2b). Pearson's correlation analysis showed that TPC negatively correlated ($r = -0.724$; $P = 0.008$) with TBARS values, suggesting that cardoon phenolics contributed to the inhibition of lipid oxidation in LTL homogenates. The present study suggests that the antioxidant effect of

cardoon extract in an ovine muscle system is dose-dependent as only the addition of 5% cardoon extract significantly inhibited lipid oxidation relative to the control. Similarly, Falleh et al. (2008) demonstrated that cardoon extracts exhibited *in vitro* antioxidant activities (DPPH radical and superoxide anion scavenging activities) in a concentration-dependent manner.

Several plant extracts have been shown to exhibit antioxidant efficacy with potential application as natural alternatives to potentially-toxic synthetic antioxidants in meat products (Jiang and Xiong, 2016). Indeed, the present study is the first to demonstrate the antioxidant potential of cardoon extract in muscle model systems. Moreover, the addition of a cardoon extract can enhance the healthiness of meat products as cardoon phenolics may exert functional therapeutic properties such as antimicrobial, antimutagenic, hepatoprotective, choleretic and anti-cholestatic actions in humans (Adzet et al., 1987; Falleh et al., 2008). Therefore, further research is required to examine the use of cardoon extract as a natural additive for developing functional meat products with extended shelf life and health-promoting properties.

4.4. CONCLUSIONS

The present study demonstrated that cardoon meal is a rich source of phenolic compounds with potent antioxidant activity. The inclusion of 15% cardoon meal replaced dehydrated alfalfa in a concentrate diet without adverse effect on lamb performance and carcass traits. Dietary cardoon meal did not influence the intramuscular composition of SFA, MUFA, PUFA and nutritional indices (atherogenic index and thrombogenic index) but presented a lower concentration of potentially health-promoting fatty acids (vaccenic and rumenic acids) in lamb meat. Moreover, dietary inclusion of cardoon meal had no negative effects on the oxidative stability of raw and cooked meat stored aerobically at 4 ºC for up to 7 and 4 days, respectively. In addition, a phenolic-rich extract obtained from cardoon meal exhibited potent antioxidant activity against lipid oxidation in an ovine muscle model system. Further research is required to evaluate the antioxidant effect of cardoon extract on the shelf-life and quality parameters of meat products.

CHAPTER 5 – Concentrate supplementation with dried corn gluten feed improves the fatty acid profile of beef from steers offered grass silage

Saheed A. Salami, Michael N. O'Grady, Giuseppe Luciano, Alessandro Priolo, Mark McGee, Aidan P. Moloney and Joseph P. Kerry

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ABSTRACT

This study examined the chemical composition, fatty acid profile, oxidative stability and sensory attributes of beef (*longissimus thoracis* muscle) from steers offered grass silage and concentrate supplements containing varying levels (0%, 25%, 50%, 75%) of dried corn gluten feed (CGF) substituted for barley/soybean meal. Feeding 50%CGF decreased the protein content and increased intramuscular fat compared to 25%CGF. Total phenol content and iron-reducing antioxidant power followed the order: 0%CGF > 50%CGF and 25%CGF > 0%CGF = 50%CGF, respectively. Compared to 0%CGF, 25%CGF and 75%CGF decreased C14:0 and increased C20:5*n*-3, C22:5*n*-3and total *n*-3 polyunsaturated fatty acids whereas 75%CGF increased conjugated linoleic acids (*c*-9, *t*-11+*t*-9, *c*-11 18:2) and C18:3*n*-3. Diet did not affect the oxidative stability and sensory attributes of beef patties. The inclusion of up to 75%CGF in a supplementary concentrate for steers increased the proportion of health-promoting unsaturated fatty acids without negatively influencing the shelf-life and eating quality of beef.

Keywords: corn gluten feed, beef antioxidant potential, fatty acid, eating quality

5.1. INTRODUCTION

Concentrate supplementation of a grass silage-based diet is a typical practice employed for indoor winter finishing of beef cattle in many temperate countries including Ireland (Keane et al., 2006). Replacement of conventional feedstuffs (such as corn, barley and soybean meal) in concentrate diets with less-expensive agro-industrial by-products, is one strategy for reducing feed cost in this type of production system. Corn gluten feed (CGF) is a by-product of a wet milling process containing bran and steep liquor obtained after the removal of germ, gluten and starch from corn kernel (Stock et al., 2000). The rapid expansion of the corn milling industry has increased the use of CGF as a dietary source of energy and protein for beef cattle without negatively influencing animal performance (Domby et al., 2014).

Animal diet has a significant impact on meat quality traits including those related to nutritional value, shelf-life, and sensory characteristics, which influence consumer preference and acceptability (Hocquette et al., 2012). Related to these traits, research into the fatty acid composition of beef has attracted considerable attention in recent years due to its nutritional significance and relationship to other meat quality attributes (e.g. flavour and oxidative stability) which in turn influence retail value and eating quality (Wood et al., 2008). Microbial transformation of dietary unsaturated fatty acids to saturated fatty acids (SFA) in the rumen is a major factor limiting the accumulation of polyunsaturated fatty acids (PUFA) in ruminant meat and dietary strategies designed to alter this process are of interest to animal/food scientists and the meat industry (Bessa et al., 2015).

Grass silage-based diets, compared to concentrate-based diets, have been particularly effective in reducing ruminal biohydrogenation and improving beef nutritional value by increasing the content of PUFA and conjugated linoleic acid (CLA) while decreasing SFA levels in beef (Scollan et al., 2014). In addition, grass silage-based diets may enhance the

antioxidant capacity and extend the shelf-life stability of beef by increasing the deposition of antioxidant compounds, such as vitamin E, in the muscle (Scollan et al., 2014). However, supplementation of grass-based diets with concentrate diets has a varied effect on meat quality indices depending on several factors which include the nutrient composition of concentrate ingredients that influence the metabolic response of animals and the ultimate composition of the meat (Luciano et al., 2011; Pesonen et al., 2013). The lower starch and higher fibre content in CGF, compared to grains, decreases the negative effects of starchy concentrate-based feeds on ruminal pH and fibre digestion in cattle fed forage-based diets (Boddugari et al., 2001; Stock et al., 2000). It is proposed that feeding CGF, compared to high-starch grains, in combination with grass silage could increase ruminal outflow of PUFA for absorption into the muscle tissue and improve the fatty acid profile of meat. Moreover, CGF consists of bran and steep liquor that are rich sources of phenolic antioxidants such as protocatechuic acid, vanillic acid, *p*-coumaric acid, ferulic acid, sinapic acid and quercetin (Inglett and Chen, 2011; Rodríguez-López et al., 2016). Ingestion and deposition of phenolic compounds in muscle tissues may ultimately enhance the oxidative stability of meat (Salami et al., 2016).

Previous studies have shown that replacement of corn/soybean meal with CGF (25% of dry matter, DM) in a concentrate feedlot diet had a minimal effect on the fatty acid profile, retail shelf-life, sensory attributes and acceptability of beef from steers fed corn silage (Segers et al., 2011; Stelzleni et al., 2016). To our knowledge, there is no information available to date on the quality of meat from beef cattle fed a grass silage-based diet supplemented with concentrate diets containing CGF. Therefore, the objective of this study was to examine the chemical composition, fatty acid profile, oxidative stability and sensory attributes of beef from steers fed *ad libitum* grass silage and a concentrate supplement in which barley/soybean meal was replaced by varying levels (0, 25, 50, 75%) of dried CGF.

5.2. MATERIALS AND METHODS

5.2.1. Animals, diets and experimental design

The experimental procedures used in this study were approved by the Teagasc animal ethics committee and conducted under license from the Irish Government Department of Health and Children. The animals were managed by trained personnel according to the European Union legislation for the protection of animals used for scientific purposes (2010/63/EU Directive). Forty-eight weaned, spring-born Charolais and Limousin-sired suckler bulls were purchased directly from suckler farms at ~7 months of age and assembled at Teagasc Animal & Grassland Research and Innovation Centre, Grange, Ireland. Following assembly, the bulls were castrated and offered grass silage *ad libitum* plus 2 kg of a barley-based concentrate and 60 g of a mineral-vitamin supplement per head daily for a 187-d back-grounding period. All animals had *ad libitum* access to clean water.

Steers were subsequently blocked by breed and live weight (424.0 ± 39.0 kg) and, from within each block, randomly assigned to one of four concentrate diets (n = 12 steers/treatment) offered separately as a supplement to *ad libitum* grass (*Lolium perenne*) silage. Concentrate diets contained either 0% CGF (control, barley/soybean meal-based diet), or 25% CGF, 50% CGF or 75% CGF as a replacement for the barley/soybean meal (*as-fed* basis). The ingredient and chemical composition of the experimental concentrate diets are outlined in Table 5.1. The concentrates were prepared as coarse mixtures. Representative samples of the concentrate diets were obtained twice weekly and stored at -20 °C prior to chemical analysis. The steers were housed in a slatted-floor building in groups of five or six animals per pen with a Calan gate feeding system (American Calan Inc., Northwood, NH, USA) allowing individual feed intake of steers to be recorded. Due to individual animal-feeding facility constraints, 11 steers were later assigned to the 50% CGF treatment, rather than 12 steers assigned to the other dietary treatments. Steers were individually offered 4.0 kg DM daily (2 kg in the morning and afternoon feeding sessions) of their respective supplementary concentrates for 124 days pre-slaughter.

Animals were slaughtered in a commercial abattoir on two consecutive weeks (balanced for treatment) to facilitate sample collection and measurements. Samples of *longissimus thoracis* muscle (LT) were removed from the left side of the carcass at 48 h post-mortem, vacuum-packed and aged for 14 days at 4 °C, and subsequently stored at -20 °C prior to further analysis. Information pertaining to animal intake, growth, carcass traits, LT drip loss, and colour (lightness L^*, redness a^* and yellowness b^*) of subcutaneous fat and LT is presented in Kelly et al. (2018)

5.2.2. Chemical analysis of feed

Representative samples of concentrate diets were analyzed for dry matter (DM), crude protein, ash, neutral detergent fibre, acid detergent fibre and starch concentrations as described by O'Kiely (2011). Total fat concentration or Oil-B (acid hydrolysis/ether extract) was measured using a Soxtec instrument (Tecator, Höganäs, Sweden).

Phenolic compounds were extracted from samples of concentrate diets using aqueous methanol (50:50, v/v) and acetone (70:30, v/v) solvents (Jiménez-Escrig et al., 2001). Polyphenol-rich extracts were analysed for total phenol content (TPC) using the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999) and absorbance measurements were recorded at 750 nm using a UV-vis spectrophotometer (Cary 300 Bio, UV-vis spectrophotometer, Varian Instruments, Palo Alto, CA, USA). Results were expressed as g of gallic acid equivalents (GAE)/kg of DM feed.

The fatty acid composition was determined in freeze-dried samples of concentrate diets by a one-step extraction–transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003), with 19:0 nonadecanoate (Larodan, Solna, Sweden) added as an internal standard. Gas

chromatographic analysis of fatty acid methyl esters (FAME) was performed as described by Cherif et al. (2018). Individual fatty acids were expressed as g/kg of DM feed.

Table 5.1. Ingredient and chemical composition of experimental concentrate diets

Item	**0% CGF**	**25% CGF**	**50% CGF**	**75% CGF**
Ingredient (as-fed basis, g/kg)				
Rolled barley	862	673	424	175
Soybean meal	60	-	-	-
Dried corn gluten feed (CGF)	-	250	500	750
Cane molasses	50	50	50	50
Minerals and vitamins	28	27	26	25
Chemical composition				
Crude protein[1]	131	136	162	191
Ash[1]	58	67	80	82
Total fat[1]	28	30	26	35
Neutral detergent fibre[1]	201	184	245	314
Acid detergent fibre[1]	62	76	88	100
Starch[1]	502	422	341	211
Total phenol content[2]	6.55	10.16	13.60	14.69
Fatty acid (g/kg dry matter (DM))				
C12:0	0.10	-	0.20	0.10
C14:0	0.10	0.10	0.10	0.10
C16:0	3.30	4.60	5.70	6.70
C18:0	0.20	0.40	0.50	0.80
c-9 C18:1	1.90	3.30	4.90	7.10
c-9,12 C18:2	6.50	10.40	13.7	16.9
c-9,12,15 C18:3	0.60	0.80	0.90	0.80

[1]Expressed as g/kg DM
[2]Expressed as gram gallic acid equivalents/kg DM

5.2.3. Determination of muscle pH and proximate composition

The LT muscle was thawed and trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). Raw minced

LT samples (5 g) were homogenised for 3 min at 24,000 rpm in 45 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany). The pH of the beef homogenates was measured at 20 °C using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). Minced LT samples were analysed for moisture and fat contents using a SMART Trac rapid moisture/fat analyser (CEM Corporation, Matthews, NC, USA). The ash content was determined using a muffle furnace (550 °C for 3 h) and protein content was determined by the Kjeldahl method (AOAC, 1996).

5.2.4. Analysis of vitamin E in LT muscle

The α-tocopherol (vitamin E) content in minced LT samples was determined by high-performance liquid chromatography (HPLC) following the extraction procedure described by Buttriss and Diplock (1984). HPLC analysis was carried out on a ProStar liquid chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA) equipped with a ProStar autosampler (Model 410, Varian Instruments). Sample injection volume (partial loop fill) was 20 µl. The α-tocopherol was separated on a 250 x 4.6 mm Polaris C18-A 5u column (Metachem, Ansys® Technologies, CA, USA) and detected using a ProStar UV/Vis detector (Varian Instruments) at 292 nm. The mobile phase was methanol/water (97:3) and isocratic elution took place at 2ml/min for a total run time of 10 min. A personal computer and Star LC workstation software (version 6.20, Varian Inc.) was used for calculation of peak areas. A standard curve was generated using a range of α-tocopherol concentrations (7, 14, 21 µg/ml) and the concentration of α-tocopherol in beef was expressed in µg/g of beef muscle. The percentage recovery of vitamin E from beef samples, through the extraction procedure, was determined by including vitamin E (0.2 ml of 22.8 µg/ml) as an internal standard. The percentage recovery (92.8%) was calculated by comparison of peak

areas of vitamin E recovered through the extraction procedure with those obtained by direct injection of the vitamin E standard (22.8 µg/ml) onto the column.

5.2.5. Analysis of fatty acids in LT muscle

The lipid fraction in minced LT samples was extracted following the procedure of Bligh and Dyer (1959). The lipid fraction was transesterified to fatty acid methyl esters (FAME) using BF_3 in methanol as a catalyst following the method described by Park and Goins (1994). FAMEs were dissolved in isooctane, dried over anhydrous sodium sulphate (0.3 g) for 15 min and stored at -20 °C prior to gas chromatography analysis. FAMEs were separated using a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) using a WCOT fused silica capillary column (Varian CP-SIL 88 Tailor Made FAME, 60 m x 0.25 mm i.d. x 0.20 µm film thickness) and a flame ionisation detector. The column oven temperature was held at 150 °C for 25 min and programmed to increase from 150 °C to 240 °C at 4 °C/min and held for 2 min. The injector and detector temperatures were 270 ºC and 260 °C respectively. Helium was used as the carrier gas at a pressure of 30 psi. The injection was carried out using a Combi PAL (CTC Analytics AG, Zwingen, Switzerland) auto-injector. The injection volumes and split ratios for FAMEs were 1 µl and 1:2 split, respectively. Individual fatty acids were identified by comparing relative retention times with pure FAME standards (a mixture of Supleco 37 component FAME mix, *trans*-11 vaccenic acid methyl ester and conjugated linoleic acid methyl ester; Sigma-Aldrich Ireland Ltd., Vale Road, Arklow, Wicklow, Ireland). Results were reported as individual FA expressed as a percentage of the total fatty acids ((peak area of individual FAME/total peak area of FAME examined) x 100). The atherogenic index (AI) and thrombogenic index (TI) were calculated according to Ulbricht and Southgate (1991) as follows:

$$AI = \frac{C12{:}0 + (4 \times C14{:}0) + C16{:}0}{n\text{-}6\ PUFA + n\text{-}3\ PUFA + MUFA}$$

$$TI = \frac{C14:0 + C16:0 + C18:0}{(0.5 \times MUFA) + (0.5 \times n\text{-}6\ PUFA) + (3 \times n\text{-}3\ PUFA) + (\frac{n\text{-}3\ PUFA}{n\text{-}6\ PUFA})}$$

where MUFA is monounsaturated fatty acids.

5.2.6. Determination of total phenol content and in vitro antioxidant activity

5.2.6.1. Preparation of muscle homogenates

Beef homogenates (10% w/v) were prepared as described by Qwele et al. (2013) for the determination of *in vitro* antioxidant activities. In brief, minced LT (5 g) was homogenised in 0.05 M phosphate buffer (45 ml) using an Ultra-turrax T25 homogeniser at 24,000 rpm for 3 min. Muscle homogenates were centrifuged at 7800 g for 10 min at 4 °C using an Avanti® J-E Centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA). The supernatant was filtered through Whatman No. 1 paper and the filtrate was analysed for ferric reducing antioxidant power (FRAP) and ferric ion chelating activity (FICA). For the determination of TPC and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assays, 10% trichloroacetic acid (5 ml) was added to muscle homogenates and the mixture was centrifuged at 7800 g for 10 min at 4 °C. The supernatant was filtered through Whatman No. 1 paper and analysed for TPC and DPPH activity.

5.2.6.2. Measurement of the total phenol content

Muscle extracts were analysed for TPC using the Folin-Ciocalteu method (Singleton et al., 1999) with minor modifications. Briefly, extracts (0.5 ml) were mixed with Folin-Ciocalteu reagent (2.5 ml, 20% in distilled water) and sodium carbonate (2 ml, 7.5% in distilled water) was added after 5 min. The mixture was stored in the dark for 2 h at room temperature and absorbance measurements were recorded at 750 nm on a UV-vis spectrophotometer (Cary 300 Bio, UV-vis spectrophotometer, Varian Instruments, CA, USA) against a blank

containing all reagents and distilled water. A calibration curve using standard solutions of aqueous gallic acid (20 – 100 μg/ml) was constructed and results are expressed as mg of gallic acid equivalents (GAE)/g of muscle.

5.2.6.3. Measurement of in vitro antioxidant activities

Radical scavenging activity in muscle was measured using the DPPH assay following a minor modification of the method described by Yen and Wu (1999). Muscle extract (0.6 ml) and distilled water (2.4 ml) were mixed with 0.2 mM DPPH in methanol (3 ml) and incubated in the dark for 1 h at room temperature. Absorbance measurements were recorded at 517 nm on a UV-vis spectrophotometer (Cary 300 Bio) against a methanol blank. An assay blank containing distilled water (3 ml) and 0.2 mM DPPH in methanol (3 ml) was used for calculation purposes. A calibration curve using standard solutions of methanolic Trolox (10 – 50 μg/ml) was constructed and results were expressed as mg of Trolox equivalents (TE)/g of muscle.

Total antioxidant activity in muscle was determined using FRAP assay following a minor modification of the method described by Benzie and Strain (1999). Briefly, muscle extract (0.45 ml) was mixed with 8.55 ml FRAP reagent (a mixture of 30 mM acetate buffer (pH 3.6), 10 mM 2,4,6–Tris (2-pyridyl)-s-triazine in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in distilled water in the ratio 10:1:1, respectively incubated at 37 °C for 10 min prior to use). The mixture was incubated for 30 min in the dark and absorbance was recorded at 593 nm on a UV-vis spectrophotometer (Cary 300 Bio) against a blank containing all reagents. A calibration curve using solutions of methanolic Trolox (0.033 – 0.1 mg/ml) was constructed and results are expressed as mg TE/g of muscle.

The iron chelating activity of muscle was measured using a minor modification of FICA assay described by Yen and Wu (1999). Muscle extract (0.5 ml) was mixed with $FeCl_2$ (2

mM in distilled water, 0.1 ml), ferrozine solution (5 mM in distilled water, 0.2 ml) and distilled water (4.2 ml). The assay control contained $FeCl_2$ (0.1 m), ferrozine solution (0.2 ml) and distilled water (4.7 ml). The mixture was incubated for 1 h in the dark at room temperature and absorbance measurements were recorded at 562 nm against a water blank on a UV-vis spectrophotometer (Cary 300 Bio). Chelating activity was calculated as follows:

Chelating activity (%) = [1 − (absorbance of sample)/(absorbance of control)] × 100.

5.2.7. Determination of the oxidative stability and sensory properties of beef

5.2.7.1. Measurement of lipid oxidation and oxymyoglobin in muscle homogenates

Muscle homogenates (25%) were prepared by homogenising 15 g of LT in buffer (0.12 M KCL 5 mM histidine, pH 5.5) surrounded by crushed ice using an Ultra Turrax T25 homogeniser. Lipid oxidation in muscle homogenates (39.2 g) was initiated by the addition of pro-oxidants (45 μM $FeCl_3$/sodium ascorbate, 1:1) (O'Grady et al., 2001). Lipid oxidation (2-thiobarbituric acid reactive substances, TBARS) and oxymyoglobin (OxyMb) content in muscle homogenate were measured at 1 and 4 h of storage at 4 °C as described by Hayes et al. (2009).

5.2.7.2. Beef processing and packaging

The LT muscles were thawed overnight at 4 °C, trimmed of visible fat and connective tissue, and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). The minced muscle was formed into beef patties (100 g portions) using a meat

former (Ministeak burger maker, O.L Smith Co. Ltd., Italy). For the fresh beef study, patties were individually placed in a low oxygen permeable (<1 cm^3/m^2/24 h at STP) polystyrene/ethyl vinyl alcohol/polyethylene (PE) trays and flushed with 80% O_2:20% CO_2 (modified atmosphere packs, MAP) using a vacuum-sealing unit (VS 100, Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat-sealable layer. Fresh beef patties in MAP were stored for up to 14 days under fluorescent lighting (660 lx) at 4 °C. The gas atmosphere (% O_2 and % CO_2) in MAP was measured using a CheckMate 9900 (PBI-DanSensor, Denmark). The average gas composition in MAP was 79.53 ± 0.39% O_2 and 20.77 ± 0.23% CO_2 on day 1 of storage and 74.03 ± 0.77% O_2 and 25.85 ± 0.73% CO_2 on day 14 of storage.

For the cooked beef study, minced patties were individually placed on an aluminium foil-lined trays and cooked at 180 °C for 20 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72 °C was reached. Cooked beef patties were placed in PE trays over-wrapped with oxygen permeable film and stored for up to 6 days at 4 °C.

5.2.7.3. Measurement of lipid oxidation and surface colour of beef patties

Lipid oxidation was measured in fresh beef patties on days 1, 4, 7, 11, and 14 of storage and on days 1, 3, and 6 in cooked beef patties. Lipid oxidation measurements were carried out following the method described by Siu and Draper (1978). Results were expressed as TBARS in mg malondialdehyde (MDA)/kg meat.

The surface colour of fresh beef patties on days 1, 4, 7, 11, and 14 of storage was measured using a Konica Minolta CR-400 Chroma-Meter (Minolta Camera Co., Osaka, Japan). The

Chroma-Meter consisted of a measuring head (CR-400), with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-400). The Chrom-Meter was calibrated on the CIE LAB color space system using a white tile (D_c: L = 97.79, a = -0.11, b = 2.69). The 'L^*', 'a^*' and 'b^*' value represents lightness, redness and yellowness, respectively. Colour measurements were averaged for readings taken from four different locations on the surface of beef patties.

5.2.7.4. Measurement of textural properties of beef patties

The texture profile analysis (TPA) of fresh beef patties stored in MAP was measured on days 2 and 7 of storage. The TPA parameters (hardness (N), springiness (mm), cohesiveness (dimensionless), gumminess (N), chewiness (N × mm), adhesiveness (N)) were measured using a 30 kg load cell texture analyser (TA.XT2i Texture Analyser, Stable Micro Systems, UK) as described by Moroney et al. (2013).

5.2.7.5. Sensory analysis of beef patties

Sensory acceptance testing of fresh beef patties (n = 8/treatment) stored in MAP was carried out by 40 untrained panellists in 2 sessions (20 panellists/session) on days 2 and 7 of storage as described by O'Sullivan et al. (2003). Beef patties were cooked for sensory analysis in a Zanussi oven at 180 °C for 20 min until an internal temperature of 72 °C was reached. Following cooking, patties were cooled to room temperature and cut into 2 cm × 2 cm cubes, identified with random three-digit codes. On each day of evaluation, beef samples were served to panellists in two separate sessions (morning and afternoon sessions). Prior to serving to panellists, beef samples were re-heated in a microwave for 10 s to release the meat odour and flavour. Sensory evaluation was performed in the panel booths of the University's sensory laboratory according to international standard regulations (ISO, 2007).

Panellists were provided with water to cleanse their palates between samples. Each panellist received beef samples presented in a randomised order to prevent any flavour carryover effects (MacFie et al., 1989). Panellists were asked to indicate their degree of liking for appearance, odour, texture, juiciness, flavour and overall acceptability on a 10 cm line scale ranging from 0 (extremely dislike) to 10 (extremely like).

5.2.8. Statistical analysis

All analyses were performed in duplicate. Data relating to proximate composition, antioxidant capacity and fatty acid profile analyses were analysed using a general linear model including dietary treatment as a fixed factor and block as a random factor. The intramuscular fat content was included as a covariate in the model used for the analysis of fatty acid profiles in the muscle. The orthogonal polynomial contrast was used to determine the linear, quadratic, and cubic responses for dietary inclusion levels of CGF. Linear discriminant analysis was employed to determine the muscle FA variables that best differentiate the dietary treatments. The statistical significance of the discriminant model was assessed using Wilks' lambda test and the accuracy of the model in assigning individual animals to their respective dietary group was cross-validated using the "leave-one-out" classification method.

Data on the oxidative stability and sensory attributes were analysed using a full-repeated measures ANOVA. Effects of dietary treatment represented the 'between-subjects' factor and the effect of storage/incubation time was measured using the 'within-subjects' factor and the interaction between diet and storage/incubation time was tested. The effects of panellist and session were included as random terms in the model used for the analysis of sensory eating attributes. Tukey's HSD test was used for multiple comparisons of treatment means when significance was detected at $P \leq 0.05$ and a tendency for treatment effect was

observed when $0.05 < P \leq 0.10$. All data analysis was performed using SPSS statistical software (IBM Statistics version 22).

5.3. RESULTS AND DISCUSSION

The nutrition of animals plays a significant role in regulating physicochemical and metabolic traits of muscles which in turn influences meat quality. Corn gluten feed (CGF) is a co-product of wet milling of corn that is increasingly used in concentrate diets for finishing beef cattle (Stock et al., 2000). There is no information on the impact of dietary CGF on beef quality when included in a concentrate supplement for finishing beef cattle fed grass silage. In this regard, the present study investigated quality indices of beef from steers fed grass silage and a concentrate supplement in which barley/soybean meal was replaced with different levels (0%, 25%, 50% and 75%) of CGF. The inclusion of 25%, 50% and 75% CGF in concentrate supplement correspond to 10.9%, 21.5% and 33.7% of the total dietary DM intake (grass silage + concentrate supplement), respectively.

5.3.1. pH and proximate composition of muscle

The pH and proximate composition of LT muscle is presented in Table 5.2. Values of muscle pH in all treatments (5.47 – 5.53) were within the normal range (5.4 – 5.8) for beef (Faustman and Cassens, 1990). The moisture, IMF and protein content of LT muscles from the CGF treatments was not significantly different from the control (0% CGF). Similarly, it has been reported that proximate composition of different muscles obtained from steers fed CGF (25% dietary DM) was not different from those fed corn/soybean meal (Segers et al., 2011; Stelzleni et al., 2016). In comparison to 50% CGF, muscles from steers fed 25% CGF had greater protein content and lower IMF content (Table 5.2). The observed differences in IMF may not be related to the level of feed intake as steers had a similar DM intake (grass silage and concentrate) and growth rate across the dietary treatments (Kelly et al., 2018). Considering that the diet containing 50% CGF had a lower starch level compared to 25% CGF (Table 5.1), this observation contradicts the theory that high starch diets

increase the supply of glucose for tissue lipogenesis which enhances IMF deposition (Vasconcelos et al., 2009). However, it is possible that variation in IMF content is related to differences in the ruminal fermentation pattern, producing more of a glucogenic precursor (propionate), and/or extent of starch digestion in the small intestine (Pethick et al., 2004). In addition, the ash content in LT muscle decreased quadratically ($P = 0.015$) as CGF inclusion increased, with 25% CGF being lower ($P < 0.05$) than 0% CGF. Overall, the present results showed that inclusion of 75% CGF in supplementary concentrate diet did not affect the muscle protein level, IMF, moisture, and ash values compared to the control and other CGF treatments.

5.3.2. Antioxidant capacity of muscle

The antioxidant status of muscle was assessed by measuring the concentration of vitamin E and TPC. Dietary treatment did not affect the concentration of vitamin E in LT muscle as shown in Table 5.2. Muscle vitamin E levels are similar to those reported for beef cattle grazed entirely on pasture (Luciano et al., 2011), due to the inclusion of grass silage in the diet fed in the present study. The inclusion levels of CGF decreased ($P < 0.05$) the TPC in muscle in a quadratic manner, with a significantly lower TPC in steers fed 50% CGF compared to those fed 0% CGF. This observation was contrary to the higher amount of TPC found in CGF diets (Table 5.1), suggesting that CGF phenolics were not deposited in the muscle. This may be due to a low bioavailability of CGF phenolics as shown in rats fed phenolic compounds from corn bran, a major constituent of CGF (Zhao et al., 2005). Moreover, the deposition of dietary phenolics in ruminant meat is influenced by the interaction of polyphenols with other dietary components, microbial metabolism in the digestive tract or limitations for the absorption of phenolic compounds into muscle tissues (Vasta and Luciano, 2011)

Regardless of the differences in muscle TPC, dietary treatment did not influence ($P > 0.05$) the DPPH-radical scavenging activity and chelating activity of the LT muscle (Table 5.2). However, the FRAP value was greater in cattle fed 25% CGF compared to those fed 0% and 50% CGF, which was inconsistent with the pattern of dietary effects observed for muscle TPC. The FRAP is an indicator of total antioxidant capacity estimated via the reduction of Fe^{3+} to Fe^{2+} (Benzie and Strain, 1999). Beef muscle is a complex biological system comprising of enzymatic (glutathione peroxidase, catalase, superoxide dismutase etc.) and non-enzymatic (selenium, vitamins E, C and A, etc.) factors contributing to the overall total antioxidant activity of meat (Salami et al., 2016). Thus, the discrepancy between the pattern of dietary effect on TPC and FRAP values may be related to the contribution of other compounds, apart from polyphenols, to the overall antioxidant capacity of muscle. This speculation may be supported by a lack of significant correlation (r = -0.16; P = 0.288) between TPC and FRAP values. Nonetheless, the current results demonstrated that dietary inclusion of up to 75% CGF in the concentrate supplement did not compromise the antioxidant capacity of muscle.

Table 5.2. Effect of feeding steers with grass silage and a supplementary concentrate containing varying levels of dried corn gluten feed (CGF) on the pH, proximate composition, antioxidant status and antioxidant activity of *longissimus thoracis* muscle

	Dietary treatment (% CGF)						Contrast effect		
Item	**0**	**25**	**50**	**75**	**SEM**	***P*- value**	**Linear**	**Quadratic**	**Cubic**
Muscle pH	5.47[b]	5.53[a]	5.50[ab]	5.51[a]	0.007	0.002	0.052	0.045	0.006
Proximate composition (g/100 g wet weight)									
Protein	23.67[ab]	23.87[a]	22.75[b]	23.64[ab]	0.139	0.020	0.297	0.191	0.006
Intramuscular fat	2.57[ab]	2.11[b]	2.84[a]	2.26[ab]	0.089	0.016	0.786	0.709	0.002
Moisture	73.12	73.19	72.59	73.04	0.102	0.170	0.361	0.348	0.062
Ash	1.11[a]	1.03[b]	1.05[ab]	1.05[ab]	0.009	0.006	0.040	0.017	0.082
Antioxidant status									
α-tocopherol (μg/g muscle)	2.38	2.59	2.66	2.66	0.065	0.397	0.129	0.433	0.877
TPC (mg GAE/g muscle)	0.94[a]	0.88[ab]	0.72[b]	0.85[ab]	0.025	0.015	0.049	0.037	0.069
Antioxidant activity									
DPPH (mg TE/g muscle)	0.25	0.25	0.25	0.25	0.002	0.621	0.584	0.228	0.997
FRAP (mg TE/g muscle)	0.38[b]	0.44[a]	0.38[b]	0.41[ab]	0.007	<0.001	0.446	0.070	<0.001
Iron-chelating activity (%)	55.22	57.87	56.01	62.34	1.284	0.199	0.088	0.469	0.270

[a,b]Means within the same row bearing different superscripts are significantly different ($P < 0.05$).
SEM: Standard error of mean
TPC: Total phenol content; DPPH: 1,1-diphenyl-2- picrylhydrazyl; FRAP: Ferric reducing antioxidant power;
GAE: gallic acid equivalent; TE: trolox equivalent

Table 5.3. Effect of feeding steers with grass silage and a supplementary concentrate containing varying levels of dried corn gluten feed (CGF) on the fatty acid (FA) composition of *longissimus thoracis* muscle

Fatty acid (% of total fatty acids)	Dietary treatment (% CGF)				SEM	*P*-value	Contrast effect[1]		
	0	25	50	75			L	Q	C
C12:0	0.07	0.04	0.06	0.07	0.007	0.241	0.669	0.179	0.158
C14:0	2.51^{a}	1.59^{b}	2.21^{a}	1.66^{b}	0.076	<0.001	<0.001	0.095	<0.001
c-9 C14:1	0.44	0.37	0.35	0.36	0.026	0.483	0.223	0.506	0.484
C15:0	0.39	0.94	0.45	0.56	0.077	0.073	0.960	0.143	0.032
C16:0	22.99	14.44	20.23	17.12	1.124	0.074	0.222	0.202	0.052
C16:1	1.85^{a}	0.39^{b}	0.54^{b}	0.42^{b}	0.107	<0.001	<0.001	<0.001	0.004
C17:0	0.86^{a}	0.54^{b}	0.88^{a}	0.63^{b}	0.031	<0.001	0.076	0.471	<0.001
c-9 C17:1	0.50^{a}	0.05^{b}	0.44^{a}	0.04^{b}	0.035	<0.001	<0.001	0.499	<0.001
C18:0	13.32	14.13	13.95	13.13	0.268	0.379	0.805	0.156	0.362
t-9 18:1	2.07^{a}	1.25^{ab}	1.72^{ab}	0.99^{b}	0.132	0.037	0.014	0.811	0.113
t-11 C18:1	0.95	0.77	0.97	0.98	0.036	0.080	0.363	0.190	0.045
c-9 C18:1	29.11	29.64	31.77	29.83	0.710	0.507	0.530	0.375	0.268
t-9,12 C18:2	0.28	0.11	0.29	0.37	0.060	0.475	0.431	0.326	0.376
c-9,12 C18:2	2.29	1.83	1.47	2.50	0.208	0.328	0.886	0.081	0.507
C20:0	0.02^{b}	0.11^{a}	0.10^{ab}	0.08^{ab}	0.012	0.024	0.111	0.008	0.916
c-11 C20:1	0.46	0.55	0.40	0.51	0.386	0.371	0.169	0.318	0.645
c-9,12,15 C18:3	0.34^{b}	0.35^{ab}	0.35^{ab}	0.45^{a}	0.014	0.032	0.013	0.115	0.554
CLA[2]	0.14^{b}	0.33^{ab}	0.17^{ab}	0.37^{a}	0.030	0.015	0.038	0.955	0.010
C22:0	0.52	0.42	0.41	0.61	0.034	0.116	0.340	0.026	0.838
C20:4 *n*-6	1.10	0.98	1.01	0.89	0.038	0.176	0.140	0.238	<0.201
C20:5 *n*-3	0.18^{a}	0.46^{b}	0.38^{b}	0.44^{b}	0.031	0.002	0.003	0.054	0.043
C22:5 *n*-3	0.44^{b}	0.71^{ab}	0.45^{b}	0.79^{a}	0.045	0.021	0.041	0.679	0.015
Summary									
∑SFA	41.15	32.98	38.51	34.34	1.200	0.128	0.157	0.369	0.091
∑MUFA	36.98	33.02	36.19	33.12	0.750	0.138	0.235	0.786	0.045
∑PUFA	4.97	5.59	4.32	5.61	0.254	0.067	0.157	0.161	0.059
Total *trans*	3.02^{a}	2.02^{b}	2.69^{ab}	1.97^{b}	0.129	0.015	0.020	0.518	0.025
∑*n*-6 PUFA	3.87	3.74	2.97	4.35	0.245	0.177	0.623	0.086	0.182
∑*n*-3 PUFA	0.96^{a}	1.52^{b}	1.18^{b}	1.68^{b}	0.033	<0.001	<0.001	0.229	0.021
n-6:*n*-3	4.03	2.46	2.52	2.59	0.487	0.091	0.221	0.042	0.753
PUFA:SFA	0.12	0.17	0.11	0.16	0.012	0.245	0.565	0.283	0.098
AI	0.82	0.57	0.76	0.63	0.035	0.098	0.212	0.334	0.053
TI	1.78	1.45	1.70	1.47	0.061	0.346	0.217	0.615	0.222

[a,b]Means in the same row bearing different superscripts are significantly different ($P \leq 0.05$).
SEM: Standard error of mean.
Contrast effect: L: Linear; Q: Quadratic; C: Cubic.
[1]CLA: *c*-9 *t*-11 18:2 + *t*-9 *c*-11 18:2
CLA: conjugated linoleic acid; SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: poly-unsaturated fatty acids.
AI: Atherogenicity index; TI: Thrombogenicity index.

5.3.3. Fatty acid composition of muscle

5.3.3.1. Effect of diet on the fatty acid profile of LT muscle

Results of FA composition in LT muscle are presented in Table 5.3. Dietary treatment did not affect ($P > 0.05$) the relative amount of total SFA in LT muscle but influenced the proportion of individual SFA with nutritional significance. Muscle from steers fed 25% and 75% CGF had decreased ($P < 0.05$) percentages of C14:0 and C17:0 compared to those fed 0% and 50% CGF. In addition, CGF diets tended ($P = 0.074$) to decrease C16:0 in a cubic manner, with 25% CGF displaying the greatest decrease (-37.2%) compared to 0% CGF. Notably, the consumption of elevated levels of dietary C14:0 and C16:0 increases low-density lipoprotein cholesterol, linked to increased risk of cardiovascular diseases (CVD), obesity and insulin resistance in humans (Calder, 2015b). Thus, it can be deduced that feeding 25% and 75% CGF decreased the proportion of hypercholesterolemic SFA in beef.

The proportion of total MUFA was unaffected ($P > 0.05$) by feeding CGF and no dietary effect was observed ($P > 0.05$) on the relative amount of *c*-9 C18:1, the predominant MUFA in beef (Dinh et al., 2010). Compared to the CGF treatments, steers fed 0% CGF had higher ($P < 0.05$) C16:1 and *c*-9 C17:1, monounsaturated derivatives of C16:0 and C17:0, respectively. The accumulation of C16:1 and *c*-9 C17:1 suggests that *de novo* lipid synthesis may play a role in the alteration of LT muscle fatty acid profile in this study. A linear decrease in the percentage of total *trans* fatty acids (TFA) was mainly influenced by a linear decrease ($P < 0.05$) of *t*-9 18:1. However, the proportion of *t*-11 18:1 (*trans*-vaccenic acid) was not affected ($P > 0.05$) by dietary treatment. The observed changes in muscle TFA may be of minimal implication because current evidence suggests that dietary consumption of ruminant TFA may have a limited health impact in contrast to the increased CVD risks associated with industrial TFA (Calder, 2015b; Scollan et al., 2014).

Dietary treatment tended (P = 0.063) to influence the percentage of total PUFA in a cubic response, with the highest increase (+37.6%) observed in the 75% CGF compared to those fed 0% CGF. Feeding CGF diets increased (P < 0.05) C20:5*n*-3 in cubic manner with correspondingly higher (P < 0.01) total *n*-3 PUFA. In comparison to 0% CGF, feeding 75% CGF increased CLA (*c*-9,*t*-11+*t*-9,*c*-11 18:2) and α-linolenic acid (C18:3*n*-3) and docosapentaenoic acid (C22:5 *n*-3). Ruminant meat and milk represent the main dietary source of *c*-9,*t*-11 CLA in the human diet, where CLA is the major isomer synthesized during ruminal biohydrogenation and from endogenous desaturation of *trans*-vaccenic acid in muscle or mammary tissues (Bessa et al., 2015). It is well-documented that consumption of dietary PUFA and CLA exhibit biological activities that prevent CVD, cancer and metabolic syndromes in humans (Calder, 2015b). Thus, the effect of dietary CGF in increasing the proportion of these fatty acids in LT muscle can be considered as a promising feeding strategy for enhancing the proportion of healthy fat in beef.

Different FA indices were used to evaluate the nutritional importance of beef fat and the contribution to healthy human diets. Dietary treatment did not influence (P > 0.05) PUFA to SFA ratio and the thrombogenic index. However, the *n*-6:*n*-3 ratio tended to be lower (P = 0.093) in the CGF treatments, with values of 25% and 50% CGF desirably lower than the maximum nutritional recommendation of 4.0 (HMSO, 1994). The inclusion levels of CGF tended to favourably decrease (P = 0.098) muscle atherogenicity index in a cubic response, due to a lower proportion of C14:0 and C16:0, and higher *n*-3 PUFA.

Overall, it is noteworthy that dietary CGF favourably altered the FA composition of beef in the present study contrary to a previous study which reported that CGF had a minimal impact on the fatty acid profile of *longissimus lumborum* muscle (Segers et al., 2011). Differences in feed fatty acid composition could be one of the reasons for the discrepancies between the results presented and those of Segers et al. (2011). The present study showed

that increasing the inclusion levels of CGF resulted in corresponding increases in dietary PUFA (oleic acid and linoleic acid) in experimental diets (Table 5.1) whereas information on feed fatty acid composition was not reported by Segers et al. (2011). In addition, inclusion of CGF in the present study decreased dietary starch levels which may mitigate the impairment of ruminal digestion that can occur from supplementation of forage with cereals (Stock et al., 2000). Thus, it is possible that CGF exhibited a complementary effect in protecting dietary unsaturated fatty acids against ruminal biohydrogenation.

5.3.3.2. Multivariate analysis of muscle fatty acid profile

The application of multivariate statistical analysis to chemical constituents (such as fatty acids) in muscle tissues can be used to discriminate meat from different feeding provenance, geographical origins or production systems (Monahan et al., 2018). This approach may be relevant to authenticate meat produced from sustainable feeding systems that promote the substitution of by-products for cereals in animal diets. In the present study, linear discriminant function analysis was performed to identify the fatty acids that better contribute to the differentiation of beef from animals fed diets in which barley/soybean meal was replaced by CGF in concentrate diets. Six fatty acids (C15:0, C16:1, C17:0, *c*-9 C17:1, *t*-11 C18:1 and C22:0) were retained as the best quantitative predictor variables after a stepwise selection procedure was applied to the muscle fatty acid dataset. The predictor variables follow a similar observation that a combination of fatty acids derived from ruminal biohydrogenation intermediates and *de novo* fatty acid synthesis contribute to the discrimination of the feeding provenance of beef (Alfaia et al., 2009). The predictor variables were linearly combined to obtain three canonical discriminant functions (CAN). The first two CAN described 97.1% (CAN 1 = 89.1% and CAN 2 = 8.0%) of the total variance associated with the multivariate structure on the CAN plot (Figure 5.1).

Discriminant power of the canonical model was significant ($P < 0.01$) based on Wilks' lambda test of significance.

The scattered distribution on the CAN plot indicated that muscles from animals fed 25% and 75% CGF (located on the left side of the quadrant) were not clearly differentiated from each other but were discriminated by CAN 1 from those fed 0% and 50% CGF (located on the right side of the quadrant). The discriminant power of CAN 1 was maximized by *c*-9 C17:1, C17:0, *t*-11 C18:1 and C22:0 as shown with higher values of standardized coefficients of variables (Table 5.4). However, CAN 2 distinguished beef from animals fed 0% CGF and 50% CGF, located on the upper and lower right side of the quadrant, respectively. The discriminant power of CAN 2 was highly influenced by C16:1 and *c*-9 C17:1 (Table 5.4). Cross-validation of the discriminant model revealed that 10 steers were correctly classified to each of 0%, 25% or 50% CGF while 9 steers were correctly assigned to 75% CGF. The model inaccuracy showed that steers belonging to 0% ($n = 2$), 25% ($n = 2$), 50% ($n = 1$) and 75% ($n = 3$) CGF were wrongly assigned to 50%, 75%, 75% and 25% CGF, respectively, indicating that none of the CGF-fed steers was wrongly assigned to the control (0% CGF) group. Therefore, this data suggests that muscle fatty acid profile may be a potential chemical marker for discriminating beef from cattle finished on a forage-based diet and concentrate supplement containing CGF. Also, among the entire set of the identified fatty acids, the discriminant analysis allows for highlighting fatty acids which are more linked to feeding CGF.

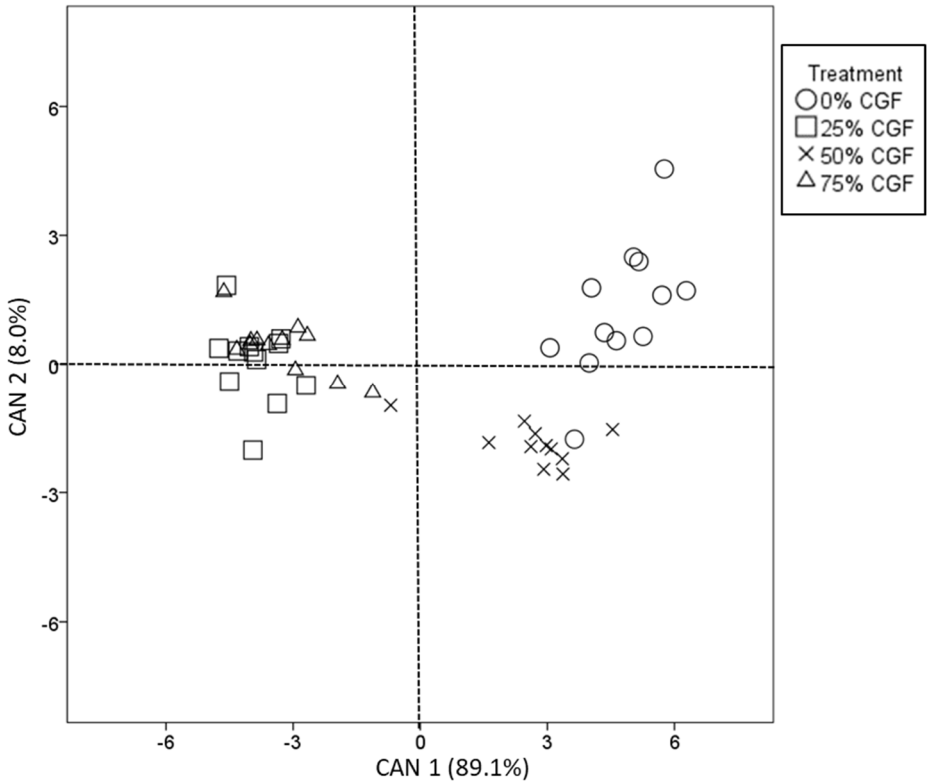


Figure 5.1. Plot showing the discrimination of dietary treatments following canonical discriminant function (CAN) analysis of the fatty acid profiles of *longissimus thoracis* muscles from steers offered grass silage and a supplementary concentrate containing varying levels (0%, 25%, 50% and 75%) of dried corn gluten feed (CGF).

Table 5.4. Summary of standardized coefficient of variables and variance structure described by the canonical discriminant function (CAN)

Variables	**CAN 1**	**CAN 2**	**CAN 3**
C15:0	-.505	-.043	-.456
C16:1	.297	1.028	-.111
C17:0	.804	-.127	.441
c-9 C17:1	1.310	-.564	-.260
t-11 C18:1	-.710	.018	.710
C22:0	.709	.223	.692
Statistics			
Eigen value	15.242	1.370	0.498
Variance (%)	89.1	8.0	2.9
Cumulative variance (%)	89.1	97.1	100
Canonical correlation	0.969	0.760	0.577

5.3.4. Oxidative stability of beef

Feeding CGF diets did not affect ($P > 0.05$) lipid oxidation (TBARS) and colour (lightness L^*, redness a^*, yellowness b^*) stability of fresh beef patties stored in high-oxygen modified atmosphere packs (MAP) for up to 14 days at 4 °C (Table 5.5). Significant effects ($P < 0.01$) of storage time was observed on measured parameters for lipid and colour stability. However, the interaction between treatment and storage time did not affect ($P > 0.05$) the lipid and colour stability parameters. In agreement with the current results, previous research has shown that substitution of corn/soybean meal with CGF (25% dietary DM) did not affect lipid oxidation and subjective colour acceptance of aerobically-stored fresh beef steaks subjected to refrigerated retail display over a 9-d period (Segers et al., 2011).

Furthermore, dietary treatment did not affect ($P > 0.05$) the levels of lipid oxidation in cooked beef patties stored in aerobic packs for up to 6 days at 4 °C (Table 5.5). The effect of storage time on the level of lipid oxidation was only significant ($P < 0.05$) in 75% CGF beef patties, with an increase occurring between days 1 and 6 of storage (Table 5.5). However, there was no diet × storage time effect on the lipid oxidation of cooked beef patties. As observed with higher TBARS values in this study, the combination of cooking and aerobic storage increased the susceptibility of pork patties to lipid oxidation compared to MAP storage of fresh meat patties (Moroney et al., 2015). Therefore, the current results demonstrate that feeding CGF did not negatively influence the stability of beef patties evaluated under different oxidative conditions (MAP and aerobic packs).

Indeed, dietary CGF did not negatively influence the oxidative stability of beef patties stored in MAP and aerobic packs despite the increased amount of highly-peroxidizable PUFA in meat from steers fed 25% and 75% CGF. Furthermore, the extent of lipid oxidation and OxyMb oxidation were similar ($P > 0.05$) among dietary treatments when

muscle homogenates were subjected to a more vigorous oxidative condition by incubating with iron/ascorbate pro-oxidants (Table 5.5). This observation further confirms that dietary CGF did not enhance the oxidative stability of beef possibly due to the lack of deposition of antioxidant phenolics in the muscle of CGF-fed steers. Similarly, it has been shown that dietary supplementation of ferulic acid, a major phenolic compound in CGF, did not enhance the oxidative stability of beef (Torres et al., 2016).

5.3.5. Instrumental texture properties and sensory eating attributes of beef

Dietary treatment did not influence ($P > 0.05$) texture profile analysis (TPA) parameters (hardness, springiness, cohesiveness, gumminess, chewiness and adhesiveness) of beef patties on days 2 and 7 of storage in MAP (Table 5.6). A significant effect of storage time ($P < 0.05$) was observed on hardness, gumminess and chewiness while springiness, cohesiveness and adhesiveness did not differ ($P > 0.05$) over the storage time. No significant interaction ($P > 0.05$) was found between diet × storage time. Previous studies have demonstrated that inclusion of CGF in a concentrate finishing diet of steers did not affect the instrumental texture (Warner-Bratzler shear force) of beef (Segers et al., 2011; Stelzleni et al., 2016).

Sensory naïve panellists did not detect differences ($P > 0.05$) between dietary treatments in the eating quality characteristics (appearance, odour, texture, juiciness, flavour, and overall acceptability) of beef patties at days 2 and 7 of storage in MAP (Table 5.6). Storage time did not influence ($P > 0.05$) eating quality characteristics except the liking of texture which was decreased ($P < 0.05$) in 50% CGF beef patties between days 2 and 7 of storage. Moreover, no significant effect ($P > 0.05$) of diet × storage time was observed on all the eating quality attributes rated by the panellists. It has been shown that TPA parameters correlate with the assessment of subjective sensory tenderness of beef (Caine et al., 2003),

which may explain why the lack of dietary effect on TPA parameters was consistent with the similarity in consumer liking for beef texture. Overall, the current results indicate that dietary inclusion of CGF did not negatively influence the consumer acceptance of beef in accordance with a similar result reported with trained sensory panellists (Stelzleni et al., 2016).

Table 5.5. Effect of feeding steers with grass silage and a supplementary concentrate containing varying levels of dried corn gluten feed (CGF) on the oxidative stability of beef patties (*longissimus thoracis* muscle)

Parameter	Diet (% CGF)	Storage/incubation time[1]				
		1	**2**	**3**	**4**	**5**
Fresh beef patties						
TBARS[2]	0	0.40^{av}	0.66^{avw}	1.46^{aw}	1.60^{aw}	6.25^{az}
	25	0.25^{av}	0.77^{av}	1.36^{av}	2.00^{avw}	3.94^{aw}
	50	0.44^{av}	0.75^{av}	1.64^{av}	1.99^{av}	5.93^{aw}
	75	0.33^{av}	0.86^{av}	1.79^{av}	2.32^{av}	7.24^{aw}
	SEM	0.027	0.053	0.133	0.143	0.559
Lightness L^*	0	48.02^{av}	48.86^{av}	48.46^{av}	49.24^{av}	51.65^{av}
	25	47.80^{av}	48.20^{av}	48.64^{av}	48.22^{av}	52.15^{aw}
	50	46.62^{av}	48.23^{av}	49.17^{av}	47.86^{av}	52.97^{aw}
	75	48.13^{av}	48.73^{av}	49.27^{av}	49.10^{av}	51.89^{aw}
	SEM	0.401	0.299	0.317	0.435	0.349
Redness a^*	0	22.42^{av}	20.86^{aw}	18.98^{ax}	15.44^{ay}	5.90^{az}
	25	22.86^{av}	20.87^{avw}	18.21^{awx}	15.39^{ax}	6.81^{ay}
	50	22.49^{av}	20.89^{avw}	18.74^{aw}	14.98^{ax}	5.75^{ay}
	75	23.66^{av}	21.11^{avw}	18.42^{aw}	14.09^{ax}	5.73^{ay}
	SEM	0.181	0.152	0.186	0.515	0.244
Yellowness b^*	0	16.46^{av}	16.00^{av}	15.12^{avw}	14.15^{aw}	14.45^{aw}
	25	16.75^{av}	15.93^{avw}	14.88^{awx}	14.08^{ax}	14.00^{ax}
	50	16.31^{av}	15.75^{avw}	15.09^{awx}	13.92^{axy}	14.50^{ay}
	75	16.98^{av}	15.87^{avw}	15.00^{awx}	13.67^{axy}	14.56^{ay}
	SEM	0.142	0.110	0.089	0.168	0.146
Cooked beef patties						
TBARS[2]	0	1.93^{av}	2.38^{av}	2.55^{av}		
	25	1.65^{av}	2.04^{av}	2.61^{av}		
	50	1.63^{av}	1.97^{av}	2.57^{av}		
	75	1.15^{av}	1.45^{avw}	2.00^{aw}		
	SEM	0.111	0.129	0.173		
Muscle homogenates						
TBARS[2]	0	2.23^{av}	6.19^{aw}			
	25	1.95^{av}	5.62^{aw}			
	50	2.36^{av}	6.26^{aw}			
	75	2.55^{av}	6.46^{aw}			
	SEM	0.108	0.152			
OxyMb[3] (%)	0	85.51^{av}	45.12^{aw}			
	25	85.24^{av}	51.75^{aw}			
	50	87.01^{av}	50.79^{aw}			
	75	86.42^{av}	47.59^{aw}			
	SEM	0.547	1.991			

[a]Effect of dietary treatment: within each parameter, values in the same column are not significantly different ($P > 0.05$).
[v,w,x,y,z]Effect of storage time: values in the same row bearing different superscripts are significantly different ($P \leq 0.05$).
SEM: Standard error of mean
[1]Times 1, 2, 3, 4, 5 correspond to: 1, 4, 7, 10 and 14 days (fresh beef patties stored at 4°C in modified atmosphere packs); 1, 3, 6 days (cooked beef patties stored at 4°C under aerobic conditions); 1 and 4 hours (muscle homogenates incubated with Fe/Ascorbate at 4°C)
[2]TBARS: Thiobarbituric acid reactive substances expressed as mg malondialdehyde/kg meat
[3]OxyMb: Oxymyoglobin, % of total myoglobin

Table 5.6. Effect of feeding steers with grass silage and a supplementary concentrate containing varying levels of dried corn gluten feed (CGF) on texture profile parameters and the eating quality of beef patties (*longissimus thoracis* muscle) stored in modified atmosphere packs at 4 °C for up to 7 days

	Storage time (day)/dietary treatment (%CGF)									
	Day 2					**Day 7**				
Parameters	**0% CGF**	**25% CGF**	**50% CGF**	**75% CGF**	**SEM**	**0% CGF**	**25% CGF**	**50% CGF**	**75% CGF**	**SEM**
Textural attributes										
Hardness	15.07^{ax}	18.24^{ax}	17.64^{ax}	18.41^{ax}	0.614	25.45^{ay}	24.55^{ax}	26.31^{ay}	23.72^{ay}	0.842
Springiness	0.83^{ax}	0.85^{ax}	0.86^{ax}	0.86^{ax}	0.006	0.86^{ax}	0.85^{ax}	0.86^{ax}	0.87^{ax}	0.003
Cohesiveness	0.59^{ax}	0.59^{ax}	0.57^{ax}	0.58^{ax}	0.009	0.61^{ax}	0.58^{ax}	0.60^{ax}	0.59^{ax}	0.008
Gumminess	8.88^{ax}	10.86^{ax}	9.98^{ax}	10.77^{ax}	0.449	15.51^{ay}	14.36^{ax}	15.82^{ay}	13.97^{ax}	0.641
Chewiness	7.32^{ax}	9.22^{ax}	8.57^{ax}	9.21^{ax}	0.401	13.35^{ay}	12.24^{ax}	13.67^{ay}	12.12^{ax}	0.548
Adhesiveness	-1.59^{ax}	-0.64^{ax}	-1.52^{ax}	-0.94^{ax}	0.172	-1.36^{ax}	-1.18^{ax}	-1.22^{ax}	-1.15^{ax}	0.12
Eating quality										
Appearance	5.38^{ax}	6.27^{ax}	5.97^{ax}	6.10^{ax}	0.182	6.06^{ax}	6.41^{ax}	6.28^{ax}	6.26^{ax}	0.165
Odour	5.87^{ax}	6.01^{ax}	6.10^{ax}	6.10^{ax}	0.155	6.14^{ax}	6.13^{ax}	6.04^{ax}	6.19^{ax}	0.142
Texture	4.58^{ax}	4.98^{ax}	5.08^{ax}	5.08^{ax}	0.182	4.33^{ax}	4.90^{ax}	4.32^{ay}	4.77^{ax}	0.162
Juiciness	3.47^{ax}	4.07^{ax}	3.95^{ax}	3.50^{ax}	0.181	3.14^{ax}	3.89^{ax}	3.57^{ax}	3.60^{ax}	0.155
Flavour	5.81^{ax}	5.85^{ax}	6.14^{ax}	5.62^{ax}	0.168	5.73^{ax}	6.09^{ax}	5.81^{ax}	5.71^{a}	0.146
Overall acceptability	5.10^{ax}	5.60^{ax}	5.52^{ax}	5.17^{ax}	0.160	5.04^{ax}	5.63^{ax}	5.23^{ax}	5.32^{ax}	0.135

[a]Effect of dietary treatment: within each parameter and storage day, values in the same row are not significantly different ($P > 0.05$).
[x,y]Effect of storage time: within each dietary treatment and between storage time (day 2 *vs.* day 7), values in the same row bearing different superscripts are significantly different ($P \leq 0.05$); SEM: Standard error of mean

5.4. CONCLUSIONS

The replacement of barley/soybean meal with CGF in concentrate supplement had a minimal effect on the chemical composition and antioxidant capacity of beef from steers offered grass silage. The inclusion of up to 75% CGF in supplementary concentrate diet improved the fatty acid profile of beef by decreasing the proportion of undesirable hypercholesterolemic SFA (C14:0) and increasing health-promoting PUFA (CLA, C18:3 *n*-3 and C20:5*n*-3). Moreover, the muscle fatty acid profile has the potential to discriminate beef from cattle finished on a grass silage-based diet and a concentrate supplement in which barley/soybean meal was replaced with CGF. The improved fatty acid profile of LT muscle did not negatively influence the oxidative stability, textural attributes, and sensory eating quality of beef patties.

CHAPTER 6 – Fatty acid composition, shelf-life and eating quality of beef from steers fed corn or wheat dried distillers' grains with solubles in a concentrate supplement to grass silage

Saheed A. Salami, Michael N. O'Grady, Giuseppe Luciano, Alessandro Priolo, Mark McGee, Aidan P. Moloney and Joseph P. Kerry

This chapter is in the form of a manuscript that is in preparation for submission to *Meat Science.*

ABSTRACT

Thirty-six steers were randomly assigned to one of three dietary treatments fed *ad libitum* grass silage and concentrate supplements containing either barley/soybean meal (control), 80% corn (CDGS)- or 80% wheat (WDGS)-dried distillers' grains with solubles (*as-fed* basis) for 124 days pre-slaughter. Chemical and fatty acid composition, shelf-life, and eating quality of *longissimus thoracis* muscle was determined. Dietary CDGS and WDGS increased the proportion of conjugated linoleic acids ($P < 0.05$) and tended to increase C18:3*n*-3 ($P = 0.075$) and total polyunsaturated fatty acids ($P = 0.060$) relative to the control. Feeding distillers' grains decreased the lipid and colour stability of beef patties stored in modified atmosphere packs (MAP), with CDGS exhibiting an intermediate effect between CON and WDGS. Diet did not negatively influence the texture profile parameters and eating quality attributes of beef stored in MAP. The inclusion of CDGS or WDGS in supplementary concentrates may improve the fatty acid profile but decreased the shelf-life of beef.

Keywords: *Distillers' grains, beef, oxidation, fatty acids, sensory attributes*

6.1. INTRODUCTION

Rapid expansion of the global bioethanol industry has resulted in an increased biomass availability of co-products such as distillers' grains with solubles (DGS, a blend of wet grains and condensed distillers' solubles), obtained after the removal of starch from the grains. DGS are often dehydrated to enhance storage and handling characteristics for utilization in livestock diets. Dried DGS has been extensively studied as a valuable source of energy, protein and fibre in cattle feed (Klopfenstein et al., 2008). Typically, winter-finishing of beef cattle in temperate countries like Ireland is accomplished by feeding medium to high quality grass silage supplemented with concentration diets (McGee, 2005). The inclusion of starch-rich grains in concentrate diets fed with forages increases the susceptibility of diets to rapid ruminal fermentation that may impair fibre digestion and negatively affect animal performance (Dixon and Stockdale, 1999). In addition to the low-cost of DGS, its lower starch and higher fibre content represent an ideal replacement for high-starch grains in concentrate diets fed with forages (Schoonmaker et al., 2010).

Previous studies have examined the effect of DGS in concentrate-based feedlot diets on quality indices in beef (Buttrey et al., 2013; De Mello et al., 2018; Domenech-Pérez et al., 2017). The interaction of dietary DGS with forages may exert a contrasting digestion pattern and carcass composition (Schoonmaker et al., 2010); however, limited information exists on subsequent effects on beef quality. Schoonmaker et al. (2010) demonstrated that replacing corn/soybean meal with up to 40% DM of wet distillers' grains in a low-forage (12% DM hay) diet increased the polyunsaturated fatty acids (PUFA) content in the *longissimus* muscle of steers but not in those fed a high-forage (50% DM hay) diet. Therefore, it is proposed that the impact of DGS and grass silage on nutrient metabolism

might contribute to variation in the physicochemical and metabolic traits of muscle that could influence beef quality attributes.

Furthermore, dietary wet or dried DGS may exhibit varied effects on meat quality attributes, particularly the fatty acid composition, depending on the fermentation substrate. Corn and wheat are the major grains used as fermentation substrates for bioethanol production depending on their relative availability in different countries (Klopfenstein et al., 2008; Yang and Li, 2017). Few studies have examined a direct comparison of the effect of corn (CDGS)- and wheat (WDGS)-distillers' grains with solubles on ruminant meat quality. Aldai et al. (2010b) reported that beef from steers fed 40% DM of WDGS exhibited a healthier *trans*-fatty acid profile compared to animals fed 40% CDGS. However, there was no difference in the fatty acid profile of lamb meat when 20% DM of CDGS or WDGS was substituted for barley grain and canola meal in a total mixed ration (McKeown et al., 2010). Additionally, feeding up to 40% DM of CDGS may improve the tenderness and palatability of beef compared to a barley-based diet while meat from steers fed WDGS possessed intermediate sensory characteristics (Aldai et al., 2010a). Therefore, the objective of the current study was to examine the chemical composition, fatty acid profile, shelf-life stability, and sensory eating quality of beef from steers fed grass silage and supplementary concentrates containing CDGS or WDGS compared to a barley/soybean meal-based concentrate.

6.2. MATERIALS AND METHODS

6.2.1. Animals, diets and experimental design

The experimental procedures used in this study were approved by the Teagasc animal ethics committee and conducted under license from the Irish Government Department of Health and Children. The animals were managed by trained personnel according to the European Union legislation for the protection of animals used for scientific purposes (2010/63/EU Directive). Thirty-six weaned, spring-born Charolais and Limousin-sired suckler bulls were purchased directly from suckler farms at ~7 months of age and assembled at Teagasc Animal & Grassland Research and Innovation Centre, Grange, Ireland. Following assembly, the bulls were castrated and offered grass silage *ad libitum* plus 2 kg of a barley-based concentrate and 60 g of a mineral-vitamin supplement per head daily for a 187-d back-grounding period. All animals had *ad libitum* access to clean water.

Steers were subsequently blocked by breed and live weight (421.9 ± 38.9 kg) and, from within each block, randomly assigned to one of three concentrate diets (n = 12 steers/treatment) offered separately as a supplement to *ad libitum* grass (*Lolium perenne*) silage. Concentrate diets contained either barley/soybean meal (control, CON), 80% CDGS or 80% WDGS (*as-fed* basis) as a replacement for the barley/soybean meal (*as-fed* basis). The ingredient and chemical composition of the experimental concentrate diets are presented in Table 6.1. The concentrates were prepared as coarse mixtures. Representative samples of the concentrate diets were obtained twice weekly and stored at -20 °C prior to chemical analysis. The steers were housed in a slatted-floor building in groups of five or six animals per pen with a Calan gate feeding system (American Calan Inc., Northwood, NH, USA). Steers were individually offered 4.0 kg DM daily (2 kg in the morning and

afternoon feeding sessions) of their respective supplementary concentrates for 124 days pre-slaughter.

Animals were slaughtered in a commercial abattoir on two consecutive weeks (balanced for treatment) to facilitate sample collection and measurements. Samples of *longissimus thoracis* muscle (LT) were removed from the left side of the carcass at 48 h post-mortem, vacuum-packed and aged for 14 days at 4 °C, and subsequently stored at -20 °C prior to further analysis.

6.2.2. Feed analysis

Representative samples of concentrate diets were analyzed for dry matter (DM), crude protein, ash, neutral detergent fibre, acid detergent fibre and starch concentrations as described by O'Kiely (2011). Total fat concentration or Oil-B (acid hydrolysis/ether extract) was measured using a Soxtec instrument (Tecator, Höganäs, Sweden).

Phenolic compounds were extracted from samples of concentrate diets using aqueous methanol (50:50, v/v) and acetone (70:30, v/v) solvents (Jiménez-Escrig et al., 2001). Polyphenol-rich extracts were analysed for total phenol content (TPC) using the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999) and absorbance measurements were recorded at 750 nm using a UV-vis spectrophotometer (Cary 300 Bio, UV-vis spectrophotometer, Varian Instruments, Palo Alto, CA, USA). Results were expressed as g of gallic acid equivalents (GAE)/kg of DM feed.

The fatty acid profile was determined in freeze-dried samples of concentrate diets by a one-step extraction–transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003), with C19:0 (Larodan, Solna, Sweden) added as an internal standard. Gas chromatographic analysis of fatty acid

methyl esters (FAME) was performed as described by Cherif et al. (2018). Individual fatty acids were expressed as g/kg of DM feed.

Table 6.1. Ingredient and chemical composition of experimental concentrate diets containing corn or wheat dried distillers' grains with solubles

Item	CON	CDGS	WDGS
Ingredient (as-fed basis, g/kg)			
Rolled barley	862	127	127
Soybean meal	60	-	-
CDGS	-	800	-
WDGS	-	-	800
Cane molasses	50	50	50
Minerals and vitamins	28	23	23
Chemical composition			
Crude protein[1]	131	241	295
Ash[1]	58	77	82
Total fat	28	81	75
Neutral detergent fibre[1]	201	328	352
Acid detergent fibre[1]	62	112	125
Starch	502	127	100
Total phenol content[2]	6.55	10.61	9.52
Fatty acid (g/kg dry matter (DM))			
C12:0	0.10	0.20	0.10
C14:0	0.10	0.10	0.10
C16:0	3.30	1.90	8.40
C18:0	0.20	1.40	0.80
c-9 C18:1	1.90	13.90	7.40
c-9,12 C18:2	6.50	31.5	21.0
c-9,12,15 C18:3	0.60	1.10	1.10

[1]Expressed as g/kg DM
[2]Expressed as gram gallic acid equivalents/kg DM
CON: control; CDGS: corn distillers' grains with solubles; WDGS: wheat distillers' grains with solubles

6.2.3. Measurement of muscle pH and proximate composition

The LT muscle was thawed and trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). Raw minced LT samples (5 g) were homogenised for 3 min at 24,000 rpm in 45 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany). The pH of the beef homogenates was measured at 20 °C using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). Minced LT samples were analysed for moisture and fat contents using a SMART Trac rapid moisture/fat analyser (CEM Corporation, Matthews, NC, USA). Ash content was determined using a muffle furnace (550 °C for 3 h) and protein content was determined by the Kjeldahl method (AOAC, 1996).

6.2.4. Determination of vitamin E in muscle

The α-tocopherol (vitamin E) content in minced LT samples was determined by high-performance liquid chromatography (HPLC) following the extraction procedure described by Buttriss and Diplock (1984). HPLC analysis was carried out on a ProStar liquid chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA) equipped with a ProStar autosampler (Model 410, Varian Instruments). Sample injection volume (partial loop fill) was 20 µl. The α-tocopherol was separated on a 250 x 4.6 mm Polaris C18-A 5u column (Metachem, Ansys® Technologies, CA, USA) and detected using a ProStar UV/Vis detector (Varian Instruments) at 292 nm. The mobile phase was methanol/water (97:3) and isocratic elution took place at 2ml/min for a total run time of 10 min. A personal computer and Star LC workstation software (version 6.20, Varian Inc.) was used for calculation of peak areas. The percentage recovery of vitamin E, from beef samples, through the extraction procedure, was determined by including vitamin E (0.2 ml of 22.8 µg/ml) as an

internal standard. The percentage recovery was calculated by comparison of peak areas of vitamin E recovered through the extraction procedure with those obtained by direct injection of the vitamin E standard (22.8 μg/ml) onto the column. A standard curve was generated using a range of α-tocopherol concentrations (7, 14, 21 μg/ml) and the concentration of α-tocopherol in beef was expressed in μg/g of beef muscle.

6.2.5. Determination of fatty acids in muscle

The Lipid fraction in minced LT samples was extracted following the procedure of Bligh and Dyer (1959). The lipid fraction was transesterified to fatty acid methyl esters (FAME) using BF_3 in methanol as a catalyst following the method described by Park and Goins (1994). FAMEs were dissolved in isooctane, dried over anhydrous sodium sulphate (0.3 g) for 15 min and stored at -20 °C prior to gas chromatography analysis. FAMEs were separated using a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) using a WCOT fused silica capillary column (Varian CP-SIL 88 Tailor Made FAME, 60 m x 0.25 mm i.d. x 0.20 μm film thickness) and a flame ionisation detector. The column oven temperature was held at 150 °C for 25 min and programmed to increase from 150 °C to 240 °C at 4 °C/min and held for 2 min. The injector and detector temperatures were 270 ºC and 260 °C respectively. Helium was used as the carrier gas at a pressure of 30 psi. The injection was carried out using a Combi PAL (CTC Analytics AG, Zwingen, Switzerland) auto-injector. The injection volumes and split ratios for FAMEs were 1 μl and 1:2 split, respectively. Individual fatty acids were identified by comparing relative retention times with pure FAME standards (a mixture of Supleco 37 component FAME mix, *trans*-11 vaccenic acid methyl ester and conjugated linoleic acid methyl ester; Sigma-Aldrich Ireland Ltd., Vale Road, Arklow, Wicklow, Ireland). Results were reported as individual fatty acid expressed as a percentage of the total fatty acids ((peak area of individual FAME/total peak

area of FAME examined) x 100). Indices of atherogenicity and thrombogenicity were calculated according to Ulbricht and Southgate (1991).

6.2.6. Measurement of total phenol content and *in vitro* antioxidant activity

Beef homogenates (10% w/v) were prepared as described by Qwele et al. (2013) for the determination of *in vitro* antioxidant activities. In brief, raw minced LT (5 g) was homogenised in 0.05 M phosphate buffer (45 ml) using an Ultra-turrax T25 homogeniser at 24,000 rpm for 3 min. Muscle homogenates were centrifuged at 7800 g for 10 min at 4 °C using an Avanti® J-E Centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA). The supernatant was filtered through Whatman No. 1 paper and the filtrate was analysed for ferric reducing antioxidant power (FRAP) and ferric ion chelating activity (FICA). For the determination of TPC and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assays, 10% trichloroacetic acid (5 ml) was added to muscle homogenates and the mixture was centrifuged at 7800 g for 10 min at 4 °C. The supernatant was filtered through Whatman No. 1 paper and the filtrate was analysed for used for these assays.

Muscle extracts were analysed for TPC using the Folin-Ciocalteu method (Singleton et al., 1999) with minor modifications. Briefly, extracts (0.5 ml) were mixed with Folin-Ciocalteu reagent (2.5 ml, 20% in distilled water) and sodium carbonate (2 ml, 7.5% in distilled water) was added after 5 min. The mixture was stored in the dark for 2 h at room temperature and absorbance measurements were recorded at 750 nm on a UV-vis spectrophotometer (Cary 300 Bio, UV-vis spectrophotometer, Varian Instruments, CA, USA) against a blank containing all reagents and distilled water. A calibration curve using solutions of aqueous gallic acid (20 – 100 μg/ml) was constructed and results are expressed as mg of gallic acid equivalents (GAE)/g of muscle.

Radical scavenging activity in muscle was measured using the DPPH assay following a minor modification of the method described by Yen and Wu (1999). Muscle extract (0.6 ml) and distilled water (2.4 ml) were mixed with 0.2 mM DPPH in methanol (3 ml) and incubated in the dark for 1 h at room temperature. Absorbance measurements were recorded at 517 nm on a UV-vis spectrophotometer (Cary 300 Bio) against a methanol blank. An assay blank containing distilled water (3 ml) and 0.2 mM DPPH in methanol (3 ml) was used for calculation purposes. A calibration curve using standard solutions of methanolic Trolox (10 – 50 μg/ml) was plotted and results were expressed as mg of Trolox equivalents (TE)/g of muscle.

Total antioxidant activity in muscle was determined using FRAP assay following a minor modification of the method described by Benzie and Strain (1999). Briefly, muscle extract (0.45 ml) was mixed with 8.55 ml FRAP reagent (a mixture of 30 mM acetate buffer (pH 3.6), 10 mM 2,4,6–Tris (2-pyridyl)-s-triazine in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in distilled water in the ratio 10:1:1, respectively incubated at 37 °C for 10 min prior to use). The mixture was incubated for 30 min in the dark and absorbance was recorded at 593 nm on a UV-vis spectrophotometer (Cary 300 Bio) against a blank containing all reagents. A calibration curve using solutions of methanolic Trolox (0.033 – 0.1 mg/ml) was constructed and results are expressed as mg TE/g of muscle.

The iron-chelating activity of muscle was measured using a minor modification of FICA assay described by Yen and Wu (1999). Muscle extract (0.5 ml) was mixed with $FeCl_2$ (2 mM in distilled water, 0.1 ml), ferrozine solution (5 mM in distilled water, 0.2 ml) and distilled water (4.2 ml). The assay control contained $FeCl_2$ (0.1 m), ferrozine solution (0.2 ml) and distilled water (4.7 ml). The mixture was incubated for 1 h in the dark at room temperature and absorbance measurements were recorded at 562 nm against a water blank

on a UV-vis spectrophotometer (Cary 300 Bio). Chelating activity was calculated as follows:

Chelating activity (%) = [1 − (absorbance of sample)/(absorbance of control)] × 100.

6.2.7. Analysis of shelf-life and sensory attributes of beef

6.2.7.1. Determination of lipid oxidation and oxymyoglobin in muscle homogenates

Muscle homogenates (25%) were prepared by homogenising 15 g of LT in buffer (0.12 M KCL 5 mM histidine, pH 5.5) surrounded by crushed ice using an Ultra Turrax T25 homogeniser. Lipid oxidation in muscle homogenates (39.2 g) was initiated by the addition of pro-oxidants (45 μM $FeCl_3$/sodium ascorbate, 1:1) (O'Grady et al., 2001). Lipid oxidation (2-thiobarbituric acid reactive substances, TBARS) and oxymyoglobin (OxyMb) content in muscle homogenate were measured at 1 and 4 h of storage at 4 °C as described by Hayes et al. (2009).

6.2.7.2. Preparation and packaging of beef patties

The LT muscles were thawed overnight at 4 °C, trimmed of visible fat and connective tissue, and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). The minced muscle was formed into beef patties (100 g portions) using a meat former (Ministeak burger maker, O.L Smith Co. Ltd., Italy). For the fresh beef study, beef patties individually placed in low oxygen permeable (<1 cm^3/m^2/24 h at STP) polystyrene/ethyl vinyl alcohol/polyethylene (PE) trays and flushed with 80% O_2:20% CO_2 (modified atmosphere packs, MAP) using a vacuum-sealing unit (VS 100, Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat-sealable

layer. Fresh beef patties in MAP were stored for up to 14 days under fluorescent lighting (660 lx) at 4 °C. The gas atmosphere (% O_2 and % CO_2) in MAP was measured using a CheckMate 9900 (PBI-DanSensor, Denmark). The average gas composition in MAP was 79.94 ± 0.97% O_2 and 20.67 ± 0.19% CO_2 on the first day of storage and 73.41 ± 1.33% O_2 and 26.53 ± 1.27% CO_2 on day 14 of storage.

For the cooked beef study, minced patties were individually placed on an aluminium foil-lined trays and cooked at 180 °C for 20 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72 °C was reached. Cooked beef patties were placed in PE trays over-wrapped with oxygen permeable film and stored for up to 6 days at 4 °C.

6.2.7.3. Measurement of lipid oxidation and surface colour of beef patties

Lipid oxidation was measured in fresh beef patties (n = 12/treatment) on days 1, 4, 7, 11, and 14 of storage and on days 1, 3, and 6 in cooked beef patties. Lipid oxidation measurements were carried out following the method described by Siu and Draper (1978). Results were expressed as TBARS in mg malondialdehyde (MDA)/kg meat.

The surface colour of fresh beef patties (n = 12/treatment) was measured on days 1, 4, 7, 11, and 14 of storage using a Konica Minolta CR-400 Chroma-Meter (Minolta Camera Co., Osaka, Japan). The Chroma-Meter consisted of a measuring head (CR-400), with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-400). The Chrom-Meter was calibrated on the CIE LAB color space system using a white tile (D_c: L = 97.79, a = -0.11, b = 2.69). The 'L^*', 'a^*' and 'b^*' value represents lightness, redness and yellowness, respectively. Colour measurements were averaged for readings taken from four different locations on the surface of beef patties. Chroma (C^*) and hue angle (H^*) were calculated as $[(a^{*2} + b^{*2})^{1/2}]$ and $[\tan^{-1}b^*/a^*]$, respectively.

6.2.7.4. Sensory analysis of beef patties

On storage days 2 and 7, texture profile analysis (TPA) was measured in fresh beef patties (n = 12/treatment) stored in MAP. The TPA parameters (hardness (N), springiness (mm), cohesiveness (dimensionless), gumminess (N), chewiness (N × mm), adhesiveness (N)) were measured using a 30 kg load cell texture analyser (TA.XT2i Texture Analyser, Stable Micro Systems, UK) as described by Moroney et al. (2013).

Fresh beef patties (n = 8/treatment) stored in MAP were randomly selected for sensory evaluation, carried out by 40 naïve panellists in 2 sessions (20 panellists/session) on days 2 and 7 of storage following the method described by O'Sullivan et al. (2003). Beef patties were cooked for sensory analysis in a Zanussi oven at 180 °C for 20 min until an internal temperature of 72 °C was reached. Following cooking, patties were cooled to room temperature and cut into 2 cm × 2 cm cubes, identified with random three-digit codes. On each day of sensory analysis, beef patties were served to panellists over two 2 separate sessions (morning and afternoon sessions). Prior to serving to panellists, beef samples were re-heated in a microwave for 10 s to release the meat odour and flavour. Sensory analysis was performed in the panel booths of the University's sensory laboratory according to international standard regulations (ISO, 2007). Panellists were provided with water to cleanse their palates between samples. Each panellist received beef samples presented in a randomised order to prevent any flavour carryover effects (MacFie et al., 1989). Panellists were requested to indicate their degree of liking for appearance, odour, texture, juiciness, flavour and overall acceptability on a 10 cm line scale ranging from 0 (extremely dislike) to 10 (extremely like).

6.2.8. Statistical analysis

All analyses were performed in duplicate. Proximate composition, antioxidant potential and fatty acid profile data were analysed using a general linear model including dietary treatment as a fixed factor and block as a random factor. A full repeated measures ANOVA was used to analysed the data relating to shelf-life (lipid and colour stability) and sensory attributes (TPA parameters and eating quality attributes). Effects of dietary treatment represented the 'between-subjects' factor and the effect of storage /incubation time was measured using the 'within-subjects' factor and the interaction between diet and storage/incubation time was tested. For eating quality attributes, grouped effect (panellist number and session number) of "sensory panel" was also included in the model as a random term, and its interaction with treatment and storage days was tested. Tukey's HSD test was used for multiple comparisons of treatment means when significance was detected at $P \leq 0.05$ and a tendency for treatment effect was observed when $0.05 < P \leq 0.10$. All data analysis was performed using SPSS statistical software (IBM Statistics version 22).

6.3. RESULTS AND DISCUSSION

In the scientific literature, limited information exists on the quality of meat from cattle finished on a grass silage-based diet in a combination with supplementary concentrate containing distillers' grains. Feeding distillers' grains with a forage-based diet may influence ruminal and post-ruminal digestion due to a lower starch level and, higher PUFA and fibre contents, compared to grains (Schoonmaker et al., 2010). The variation in nutrient metabolism profile might contribute to differences in the physicochemical and metabolic traits of muscle that could influence meat quality attributes. Current evidence suggests that dietary CDGS and WDGS markedly influence beef quality parameters, in particular fatty acid profiles (Aldai et al., 2010a). Thus, the current study examined the quality traits of beef from steers offered grass silage and concentrate supplements containing 80% CDGS or WDGS as substitutes for barley/soybean meal. The inclusion of 80% CDGS or WDGS in concentrate supplement represents 35% of the total dietary DM intake (grass silage + concentrate), respectively.

6.3.1. Chemical composition and antioxidant potential of LT muscle

The pH and proximate composition of LT muscle are shown in Table 6.2. Beef pH values ranged from 5.47 to 5.54 and were not influenced by dietary CDGS or WDGS relative to the control. The beef pH values are in agreement with the normal pH values (5.4 – 5.8) for *post-mortem* muscle (Faustman and Cassens, 1990). In comparison to WDGS, dietary CDGS increased ($P < 0.05$) the protein content and decreased the ash content in LT. A similar decrease in protein content was found in the LT of steers fed WDGS compared to a barley-based control diet while that of CDGS was intermediate (Aldai et al., 2010a). However, the moisture and intramuscular fat contents were not influenced ($P > 0.05$) by dietary treatments. In general, previous studies demonstrated that inclusion of CDGS or

WDGS in concentrate-based feedlot diets had little or no impact on the pH and proximate composition of *longissimus* muscle (Aldai et al., 2010a; De Mello et al., 2018; Domenech-Pérez et al., 2017; Koger et al., 2010).

The concentration of α-tocopherol (vitamin E) and phenolic compounds in muscle tissues is dependent on dietary intakes (Salami et al., 2016). Dried distillers' grains, compared to the corresponding cereal, contains a considerably higher amount of α-tocopherol (Nade et al., 2013) and phenolic compounds such as vallinic, caffeic, *p*-coumaric, ferulic and sinapic acids, with potent antioxidant activity (Luthria et al., 2012). However, there is limited data on the effect of feeding DGS on the concentration of these compounds in beef. Despite higher TPC levels in CDGS and WDGS diets compared to CON (Table 6.1), feeding DGS did not influence ($P > 0.05$) the TPC in LT muscle (Table 6.2). This observation suggests that phenolic compounds from dietary DGS were not deposited in the muscle possibly due to factors such as molecular complexity and microbial metabolism in the rumen, limiting their bioavailability and absorption into muscle tissues (Vasta and Luciano, 2011). Similarly, dietary treatment did not influence ($P > 0.05$) the α-tocopherol concentration in LT muscle. Previous research has also shown that feeding 15 – 30% wet or dried corn distillers grains to steers did not significantly influence the α-tocopherol concentration in plasma (Nade et al., 2013) and *longissimus* muscle (Chao et al., 2017c). Moreover, the antioxidant capacity of LT muscle (DPPH, FRAP and FICA assays) was not influenced ($P > 0.05$) by dietary treatment. Similarly, the inclusion of up to 25% CDGS as a replacement for corn/soybean meal in broiler diets did not enhance antioxidant enzyme activities and the total antioxidant capacity of breast muscle and liver tissues (Min et al., 2012). Results from the present study suggest that feeding CDGS or WDGS does not enhance the antioxidant potential of beef.

Table 6.2. Effect of including corn or wheat dried distillers' grains with solubles in supplementary concentrate diets of steers on the pH, proximate composition, antioxidant status and antioxidant activity of *longissimus thoracis* muscle

Item	Dietary treatment			SEM	*P*-value
	CON	CDGS	WDGS		
Muscle pH	5.47[b]	5.54[a]	5.52[ab]	0.011	0.009
Proximate composition (g/100 g wet weight)					
Protein	23.67[ab]	24.34[a]	22.60[b]	0.234	0.006
Intramuscular fat	2.57	2.76	2.76	0.142	0.828
Moisture	73.12	72.52	72.42	0.150	0.122
Ash	1.11[a]	1.05[b]	1.13[a]	0.009	0.001
Antioxidant status					
α-tocopherol (μg/g muscle)	2.38	2.62	2.70	0.072	0.180
TPC (mg GAE/g muscle)	0.94	0.85	0.76	0.032	0.074
Antioxidant activity					
DPPH (mg TE/g muscle)	0.25	0.25	0.25	0.002	0.720
FRAP (mg TE/g muscle)	0.38	0.41	0.41	0.007	0.110
FICA (%)	55.22	60.26	61.23	1.410	0.176

[a,b]Means within the same row bearing different superscripts are significantly different ($P < 0.05$).

SEM: Standard error of mean.

CON: Control; CDGS: Corn distillers' grains with solubles; WDGS: Wheat distillers' grains with solubles;

TPC: Total phenol content DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; FICA: Ferric ion chelating activity; GAE: Gallic acid equivalent; TE: Trolox equivalent.

6.3.2. Fatty acid profiles of LT muscle

The protection of dietary PUFA from ruminal biohydrogenation is a significant strategy for improving the fatty acid composition of ruminant meat and milk (Bessa et al., 2015). There is evidence that dietary DGS may increase dietary fat digestibility and increase the amount of unsaturated fatty acids reaching the distal gut, indicative of decreased susceptibility of dietary PUFA to ruminal biohydrogenation (Vander Pol et al., 2007; Xu et al., 2014). Previous studies have examined the fatty acid profile of beef from cattle fed DGS included

in concentrate-based feedlot diets whereas limited data exists on the inclusion of DGS in concentrate diets fed in combination with forages.

The inclusion of CDGS or WDGS in supplementary concentrates did not affect ($P > 0.05$) the percentage of total saturated fatty acids (SFA); however, few changes were observed for individual SFA (Table 6.3). Feeding WDGS decreased ($P < 0.05$) the proportion of C14:0 (myristic acid) and increased C20:0 (arachidic acid) compared to CON. Notably, the effect of WDGS may be nutritionally desirable because dietary myristic acid increases low-density lipoprotein cholesterol associated with increased incidence of cardiovascular diseases (Mensink, 2005). However, feeding distillers grains did not influence ($P > 0.05$) the proportion of two major SFA (C16:0 and C18:0) associated with beef. Previous studies have also shown that C16:0 and C18:0 were unaffected by DGS fed in a concentrate-based diet (De Mello et al., 2018; Depenbusch et al., 2009) or forage-based diets (Schoonmaker et al., 2010). Stearic acid (C18:0) is the main end-product of microbial hydrogenation of dietary PUFA in the rumen (Bessa et al., 2015), and results (non-significance of C18:0) suggest that complete inhibition of ruminal biohydrogenation was not altered by diet.

The proportion of total monounsaturated fatty acids (MUFA) and *trans* fatty acids did not differ ($P > 0.05$) between dietary treatments. Accordingly, the percentage of the most abundant MUFA in beef, oleic acid (*c*-9 C18:1), was not affected ($P > 0.05$) by diet even though this fatty acid was predominant in the DGS diets (Table 6.1). This observation may be attributed to the susceptibility of oleic acid to rapid hydrogenation to form stearic acid usually via a *trans*-vaccenic acid (*t*-11 C8:1) pathway (Jenkins et al., 2008). Similarly, the proportion of *t*-11 C8:1 was not different ($P > 0.05$) across dietary treatments, suggesting that changes in muscle fatty acid profile associated with feeding DGS may have occurred through a modulation of the overall process of lipid metabolism in the rumen, rather than a specific inhibition of the terminal step. However, a higher proportion ($P < 0.05$) of C16:1

Table 6.3. Effect of including corn or wheat dried distillers' grains with solubles in supplementary concentrate diets of steers on the fatty acid composition of *longissimus thoracis* muscle

Fatty acids (% of total fatty acids)	Dietary treatment			SEM	*P*-value
	CON	CDGS	WDGS		
C12:0	0.07	0.05	0.06	0.008	0.670
C14:0	2.51[a]	2.09[ab]	2.02[b]	0.085	0.036
c-9 C14:1	0.44	0.37	0.39	0.032	0.680
C15:0	0.39[b]	0.36[b]	0.71[a]	0.053	0.009
C16:0	22.99	21.35	22.15	1.321	0.886
C16:1	1.85[a]	0.50[b]	0.47[b]	0.133	<0.001
C17:0	0.86	0.67	0.80	0.034	0.068
c-9 C17:1	0.50[b]	0.30[a]	0.30[a]	0.036	0.031
C18:0	13.32	13.77	12.67	0.320	0.381
t-9 18:1	2.07	1.57	1.58	0.136	0.233
t-11 C18:1	0.95	0.90	0.91	0.035	0.836
c-9 C18:1	29.11	27.83	28.98	0.970	0.847
t-9,12 C18:2	0.28	0.35	0.30	0.033	0.710
c-9,12 C18:2	2.29	3.95	3.69	0.384	0.167
C20:0	0.02[b]	0.08[ab]	0.12[a]	0.013	0.006
c-11 C20:1	0.46	0.39	0.45	0.504	0.319
c-9,12,15 C18:3	0.35	0.43	0.48	0.025	0.075
[1]CLA	0.14[a]	0.27[b]	0.27[b]	0.026	0.042
C22:0	0.52	0.69	0.61	0.034	0.130
C20:4 *n*-6	1.10	1.02	0.97	0.027	0.152
C20:5 *n*-3	0.18	0.24	0.24	0.032	0.640
C22:5 *n*-3	0.44	0.52	0.53	0.046	0.713
Summary					
∑SFA	41.15	39.33	39.39	1.448	0.850
∑MUFA	36.98	31.87	33.08	1.015	0.117
∑PUFA	4.97	7.18	6.89	0.474	0.062
Total *trans*	3.02	2.47	2.49	0.127	0.132
∑n-6 PUFA	3.87	5.71	5.36	0.468	0.126
∑n-3 PUFA	0.96	1.19	1.26	0.053	0.149
n-6:*n*-3	4.03	4.80	4.25	0.746	0.572
PUFA:SFA	0.12	0.18	0.17	0.013	0.418
[2]Atherogenicity index	0.82	0.83	0.82	0.049	0.992
[3]Thrombogenicity index	1.78	1.87	1.78	0.089	0.897

[a,b]Means in the same row bearing different superscripts are significantly different ($P \leq 0.05$).

SEM: Standard error of mean

CON: control; CDGS: corn distillers' grains with solubles; WDGS: wheat distillers' grains with solubles.

[1]CLA: *c*-9 *t*-11 18:2 + *t*-9 *c*-11 18:2.

[2]Atherogenic index: (C12:0 + [4 × C14:0] + C16:0)/(*n*-3 PUFA + *n*-6 PUFA + MUFA).

[3]Thrombogenic index: (C14:0 + C16:0 + C18:0)/([0.5 × MUFA] + [0.5 × *n*-6 PUFA] + [3 × *n*-3 PUFA] + [*n*-3/*n*-6 PUFA]).

CLA: conjugated linoleic acid; SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: poly-unsaturated fatty acids.

and *c*-10 17:1 were found in the muscle of CON-fed steers compared to those fed CDGS or WDGS. The substitution of grains with DGS in a forage-based diet may results in a lower amount of ruminal volatile fatty acids (Schoonmaker et al., 2010) which could influence *de novo* fatty acid synthesis (Smith et al., 2009) which might explain the lower proportion of C16:1 and *c*-10 17:1 produced from the desaturation of their SFA equivalents (C16:0 and C17:0).

Furthermore, total PUFA in the LT muscle tended to be higher ($P = 0.06$) in steers fed CDGS or WDGS compared to CON. The increase in muscle PUFA of steers fed CDGS or WDGS was primarily driven by a greater ($P < 0.05$) proportion of CLA (*c*-9, *t*-11 18:2 + *t*-9, *c*-11 18:2) and a tendency for higher α-linolenic acid (C18:3 *n*-3; $P = 0.08$). It is well-established that dietary consumption of PUFA is associated with numerous health benefits in humans and strategies that enhance PUFA accumulation in the muscle can improve the nutritional image of beef (Bessa et al., 2015). In particular, *c*-9, *t*-11 CLA is a bioactive fatty acid with physiological effects that may prevent cardiovascular diseases, obesity, cancers, bone density loss, and diabetes in humans (Dilzer and Park, 2012). In the present study, it is noteworthy that *c*-9, *t*-11 CLA co-eluted with *t*-9, *c*-11 CLA as a single GC peak. Indeed, *c*-9, *t*-11 18:2 is the major CLA isomer present in ruminant edible fat and the quantitative ratio of *c*-9, *t*-11 18:2 to *t*-9, *c*-11 18:2 was approximately 32:1 in beef fat from steers (Fritsche and Fritsche, 1998). Thus, the CLA peak identified in our study was predominantly the *c*-9, *t*-11 CLA isomer.

In agreement with our study, dietary CDGS or WDGS (up to 60% DM) as substitutes for corn or barley in concentrate-based diets have been shown to increase the concentration of *c*-9, *t*-11 CLA in beef (Dugan et al., 2010; Mello et al., 2012b). In contrast, other studies have shown no changes in the *c*-9, *t*-11 CLA content in beef (Depenbusch et al., 2009). The ability of DGS to increase the content of *c*-9, *t*-11 CLA in beef may be attributed to its

higher fibre content, compared to grains, which could modify the growth and activity of the bacteria (*Butyrivibrio fibrisolvens*) linked to the synthesis of this fatty acid in the rumen (Mello et al., 2012b). Moreover, a higher proportion of intramuscular CLA in this study may be attributed to the abundance (3 to 5-fold) of linoleic acid (C18:2 *n*-6) in the CDGS and WDGS diets. This could be related to the fact that *c*-9, *t*-11 CLA is the major fatty acid intermediate synthesised from the isomerisation of linoleic acid during ruminal biohydrogenation (Jenkins et al., 2008). Although *c*-9, *t*-11 CLA may also be synthesised endogenously via desaturation of *t*-11 18:1 (Corl et al., 2001), the lack of dietary effect on *t*-11 18:1 suggests that *de novo* FA synthesis might not play a role in the accumulation of muscle CLA in this study. Finally, measures of nutritional indices of muscle fatty acid profiles indicated that feeding CDGS or WDGS did not influence ($P > 0.05$) the ratios of PUFA:SFA, *n*-6:*n*-3, and the indices of atherogenicity and thrombogenicity.

The present study demonstrated that feeding DGS with grass silage resulted in changes that may improve the fatty acid composition of beef. However, this effect is limited when compared to previous studies that reported a significant increase in the content of health-promoting fatty acid (linoleic and α-linoleic acids, and CLA and total PUFA) in beef from cattle fed CDGS or WDGS included in concentrate-based feedlot diets (Buttrey et al., 2013; De Mello et al., 2018; Domenech-Pérez et al., 2017; He et al., 2012). Moreover, the current study showed that dietary CDGS and WDGS had a comparable effect on muscle fatty acid composition. On the contrary, the inclusion of CDGS or WDGS in concentrate-based diets results in differences in the *trans*-18:1 isomers and *n*-3 PUFA profiles of backfat tissue in steers (Aldai et al., 2010b). Indeed, a dietary effect of DGS on muscle fatty acid profiles may depend on the proportion of forages fed in the diet. Schoonmaker et al. (2010) reported that feeding up to 40% DM of wet DGS in a low-forage (12% DM bromegrass hay) diet increased the PUFA content in the *longissimus* muscle of steers but not in those fed a high-

forage diet (50% DM bromegrass hay). A high-forage diet (50% DM bromegrass hay) may increase fibre intake similar to the *ad libitum* grass silage offered in the present study, which could also explain why CDGS and WDGS did not significantly increase the total PUFA of muscle in our study. Though feed fatty acid profile was not reported by Schoonmaker et al. (2010), the present study indicated that inclusion of CDGS and WDGS increased dietary PUFA (oleic and linoleic acids) which might have contributed to the alteration of ruminal biohydrogenation as observed with a significantly higher percentage of CLA in the muscle. This result emphasised the need for future studies to report the feed fatty acid composition when examining the effect of DGS on meat fatty acid profiles.

6.3.3. Shelf-life of beef patties

6.3.3.1. Lipid and colour stability in fresh beef patties

Lipid oxidation, measured as TBARS, increased ($P < 0.001$) in fresh LT beef patties stored in MAP (O_2:CO_2; 80%:20%) as a function of storage time (Table 6.4). On day 4, lipid oxidation was lower ($P < 0.05$) in CON beef patties compared to CDGS and WDGS. However, the extent of lipid oxidation was similar ($P > 0.05$) between CDGS and CON from days 7 to 14 of storage. WDGS had significantly higher level of ($P < 0.05$) lipid oxidation compared to CON on days 7, 11 and 14 of storage. The increase in lipid oxidation may be partly attributed to the tendency for a greater amount of PUFA in LT muscle from steers fed CDGS or WDGS. An elevated level of PUFA content increases the susceptibility of meat to oxidation, resulting in the development of rancid off-flavours over time (Faustman et al., 2010). A TBARS value of 2.28 MDA mg/kg of meat has been reported as the threshold for perceivable rancidity that may negatively influence consumer acceptability of oxidized beef (Campo et al., 2006). Thus, the retail shelf-life of beef patties

Table 6.4. Effect of including corn or wheat dried distillers' grains with solubles in supplementary concentrate diets of steers on the shelf-life of beef

Parameter	Dietary treatment	Storage/incubation time[1] 1	2	3	4	5
Fresh beef patties						
TBARS[2]	CON	0.40^{av}	0.66^{avw}	1.46^{bw}	1.60^{bw}	6.25^{bx}
	CDGS	0.45^{av}	1.17^{bv}	2.25^{abvw}	3.49^{abw}	7.32^{abx}
	WDGS	0.45^{av}	1.36^{bv}	2.90^{av}	3.82^{aw}	8.95^{ax}
	SEM	0.017	0.116	0.248	0.428	0.455
Lightness L^*	CON	48.02^{av}	48.86^{av}	48.46^{av}	49.24^{av}	51.65^{av}
	CDGS	48.20^{av}	48.61^{av}	49.37^{av}	49.72^{av}	53.53^{aw}
	WDGS	47.88^{av}	48.19^{av}	50.40^{av}	49.14^{avw}	53.38^{aw}
	SEM	0.434	0.457	0.604	0.542	0.533
Redness a^*	CON	22.42^{bv}	20.86^{aw}	18.98^{ax}	15.44^{ay}	5.90^{az}
	CDGS	23.58^{av}	21.03^{aw}	18.98^{aw}	12.81^{bx}	5.55^{aby}
	WDGS	22.56^{abv}	21.01^{av}	17.14^{bw}	11.64^{bx}	4.68^{by}
	SEM	0.220	0.249	0.352	0.625	0.229
Yellowness b^*	CON	16.46^{av}	16.00^{av}	15.12^{avw}	14.15^{aw}	14.45^{aw}
	CDGS	17.17^{av}	15.95^{aw}	15.55^{aw}	14.00^{aw}	15.25^{ax}
	WDGS	16.42^{a}	15.73^{a}	14.75^{a}	13.58^{a}	15.36^{a}
	SEM	0.192	0.157	0.149	0.161	0.159
Chroma[3] C^*	CON	27.82^{av}	26.30^{av}	24.27^{aw}	20.96^{ax}	15.61^{ay}
	CDGS	29.17^{av}	26.40^{aw}	24.54^{aw}	19.00^{bx}	16.23^{ay}
	WDGS	27.90^{av}	26.25^{av}	22.63^{bw}	17.89^{bx}	16.07^{ay}
	SEM	0.272	0.264	0.349	0.490	0.131
Hue angle[4] H^*	CON	36.28^{av}	37.50^{av}	38.53^{bv}	42.53^{bw}	67.77^{bx}
	CDGS	36.06^{av}	37.19^{av}	39.36^{abv}	47.67^{abw}	70.03^{abz}
	WDGS	36.09^{av}	36.84^{av}	40.84^{aw}	49.40^{ax}	73.09^{ay}
	SEM	0.211	0.284	0.391	1.246	0.889
Cooked beef patties						
TBARS[2]	CON	1.93^{av}	2.38^{av}	2.55^{av}		
	CDGS	1.72^{av}	2.63^{avw}	3.62^{aw}		
	WDGS	1.11^{av}	1.80^{aw}	2.32^{aw}		
	SEM	0.370	0.599	0.774		
Muscle homogenates						
TBARS[2]	CON	2.23^{av}	6.19^{aw}			
	CDGS	2.14^{av}	5.96^{aw}			
	WDGS	2.35^{av}	6.27^{aw}			
	SEM	0.130	0.166			
OxyMb[5] (%)	CON	85.51^{av}	45.12^{aw}			
	CDGS	86.44^{av}	49.82^{aw}			
	WDGS	87.90^{av}	43.23^{aw}			
	SEM	0.900	0.028			

^{a,b}Effect of dietary treatment: within each parameter, values in the same column bearing different superscripts are significantly different ($P \leq 0.05$).

^{v,w,x,y,z}Effect of storage time: values in the same row bearing different superscripts are significantly different ($P \leq 0.05$).

CON: control; CDG: corn distillers' grain with solubles; WDG: wheat distillers' grains with solubles; SEM: Standard error of mean.

[1]Times 1, 2, 3, 4, 5 correspond to: 1, 4, 7, 10 and 14 days (fresh beef patties stored at 4°C in modified atmosphere packs); 1, 3, 6 days (cooked beef patties stored at 4°C in aerobic packs); 1 and 4 hours (muscle homogenates incubated with Fe/Ascorbate at 4°C)

[2]TBARS: 2-thiobarbituric acid reactive substances expressed as mg malondialdehyde/kg meat.

[3]Chroma (colour vividness), higher values indicate greater saturation of red.

[4]Hue angle (trueness of red), lower values indicate a redder colour.

[5]OxyMb: Oxymyoglobin, % of total myoglobin.

from CDGS or WDGS may be limited to 7 days compared to CON that may extend to 10 days under the experimental conditions employed in the present study.

Meat colour is an important sensory attribute that contributes to quality perception and the purchasing decision of consumers during the shelf-life of fresh meat. Oxidative deterioration of meat colour is associated with the conversion of oxymyoglobin to metmyoglobin resulting in a change from a bright red colour to a brownish appearance (Faustman et al., 2010). As expected, storage time influenced ($P < 0.01$) all the instrumental colour parameters (L^*, a^*, b^*, C^*, H^*) measured in fresh beef patties stored in MAP for up to 14 days (Table 6.4). Lightness (L^*) and yellowness (b^*) of beef patties were similar ($P > 0.05$) between dietary treatments over the 14-day storage period. On day 1 of storage, beef patties from CDGS were more red (a^*) relative to those fed CON. Discoloration of beef patties occurred as a function of storage time with CON exhibiting higher a^* values compared to WDGS between days 7 and 14, while CON was more red than CDGS only at day 10. The decrease in chroma (C^*, colour vividness) over the storage period showed that the redness of beef from WDGS-fed steers was lower relative to those of CON and CDGS on day 7, whereas CON was more red than CDGS and WDGS on day 10. The hue angle values (H^*, trueness of red) also showed that beef patties from steers fed WDGS had a decreased red appearance compared to CON from storage days 7 to 14. It is noteworthy that on day 10 of storage, only the C^*value measured in beef from WDGS was less than 18, which represents the critical threshold for consumer rejection of discoloured fresh beef during retail purchase (Hood and Riordan, 1973). Overall, instrumental colour profiles indicated that feeding DGS increased the extent of discolouration of beef patties stored in MAP, with CDGS exhibiting an intermediate effect between CON and WDGS.

Lipid peroxidation and myoglobin oxidation are interactive processes that could influence each other in a reciprocal manner through chemical reactivity of their primary and

secondary products (Faustman et al., 2010). This could explain the consistency in lipid oxidation and discolouration of fresh beef from steers fed DGS, particularly WDGS. However, the mechanistic effect of dietary DGS on beef oxidation has been controversial. In agreement our study, it has been shown that extended retail display (≤ 7 days) increased lipid oxidation and discolouration of fresh beef from cattle fed ≥ 30% DM of DGS in concentrate-based diets (Buttrey et al., 2013; De Mello et al., 2018). However, other studies reported that increased colour deterioration of beef was not accompanied by changes in lipid oxidation (Depenbusch et al., 2009; Segers et al., 2011).

Feeding DGS preferentially increases the PUFA content in the sarcoplasmic membrane which may enhance its instability and increases the susceptibility of muscle tissues to rapid oxidation (Chao et al., 2017b). Therefore, the mincing of beef in the present study contributed to the disruption of the muscle cell structure exposing labile lipid components to oxygen (O'Grady et al., 2000), resulting in rapid lipid oxidation in LT beef patties from cattle fed DGS. Another contributing factor may be the peroxidizable nature of the PUFA content relative to the antioxidant potential of the muscle. In this study, it was observed that dietary DGS did not enhance the antioxidant potential of muscle which may partly explain why a tendency for increased PUFA content resulted in greater lipid oxidation and discolouration in fresh beef stored in MAP. In particular, vitamin E is a very important membrane-bound antioxidant and a concentration of 3.0 - 3.5 μg/g of muscle may be required to maintain cellular integrity and enhance the oxidative stability of beef (Liu et al., 1996). Lower vitamin E concentrations (2.38 - 2.70 μg/g muscle) were reported in the present study (Table 6.2) and dietary supplementation of vitamin E may be an effective strategy to improve the lipid and colour stability of fresh beef from cattle fed DGS (Chao et al., 2017c).

6.3.3.2. Oxidative stability of cooked beef patties and muscle homogenates

The susceptibility of meat to oxidative deterioration can be increased by storage and processing factors such as catalysts (such as trace metals and enzymes), lighting condition and temperature (Bekhit et al., 2013). The effect of feeding CDGS or WDGS on the oxidative stability of beef was further examined under intense oxidative conditions such as cooking or subjecting fresh LT muscle homogenates to iron/ascorbate-induced lipid oxidation. Lipid oxidation increased in aerobically-stored cooked beef patties over the 6-day storage period but no effect ($P > 0.05$) of dietary treatment on TBARS values were observed (Table 6.4). Furthermore, lipid and myoglobin oxidation was examined in muscle homogenates subjected to induced oxidation by incubating with iron/ascorbate pro-oxidants for 4 h at 4 ºC. Results indicated that both lipid and oxymyoglobin oxidation increased as a function of time but there was no effect ($P > 0.05$) of dietary treatments (Table 6.4). These results suggest that effect of feeding DGS on the susceptibility of beef to lipid peroxidation was more pronounced under moderate oxidative conditions as shown with the extended storage of fresh beef patties stored in MAP at 4 °C. Feeding DGS does not enhance the antioxidant capacity and oxidative stability of LT muscle possibly due to the lack of deposition of phenolic compounds in the muscle. Ferulic acid is the most abundant phenolics in CDGS (Luthria et al., 2012) and it has been shown that dietary supplementation of ferulic acid did not enhance the oxidative stability of beef (Torres et al., 2016).

6.3.4. Instrumental texture and sensory quality characteristics of beef

The perception of texture is an important quality attribute that influences the palatability of meat and consumer satisfaction. Animal diet can impact meat texture by directly influencing muscle structure and physicochemical composition (Andersen et al., 2005) or indirectly influencing oxidative processes that affect lipid and protein degradation in

muscle tissues (Bekhit et al., 2013). Instrumental TPA gives a detailed assessment of different textural attributes (such as hardness, springiness, and adhesiveness) that better define meat tenderness (De Huidobro et al., 2005). In the present study, hardness, gumminess and chewiness of beef patties increased ($P < 0.05$) over the 7-day storage period while springiness, cohesiveness and adhesiveness were unaffected ($P > 0.05$) by storage time (Table 6.5). Hardness is the most important singular TPA parameter and increased values as a function of storage time indicates decreased tenderness of beef patties, reducing palatability and consumer acceptance (Caine et al., 2003; De Huidobro et al., 2005). However, dietary treatment and dietary treatment x storage time did not affect ($P > 0.05$) any of the TPA parameters despite observed increase in lipid oxidation in beef patties from steers fed CDGS or WDGS during the 7-day storage period. Previous studies have also shown that feeding CDGS or WDGS (up to 40% DM) did not influence instrumental textural properties (Warner-Bratzler shear force) in fresh beef aged for up to 42 days (Aldai et al., 2010a; De Mello et al., 2018; Koger et al., 2010).

Furthermore, naïve panellists did not detect storage time or treatment differences ($P > 0.05$) in the sensory attributes (appearance, odour, texture, juiciness, flavour and overall acceptability) of beef patties stored in MAP over a 7-day storage period (Table 6.5). This outcome is particularly interesting given that increased lipid oxidation in beef patties from CDGS or WDGS did not result in perceivable rancidity that could negatively influence consumer acceptability of beef. Previous sensory evaluation studies have reported inconsistent effects of dietary DGS on eating attributes of beef. However, none of these studies has evaluated the sensory attributes of beef over the shelf-life period as examined in the present study.

Using trained or untrained sensory panellists, it has been shown that the sensory characteristics of beef were not affected by substituting corn with wet or dried CDGS (up

Table 6.5. Effect of including corn or wheat dried distillers' grains with solubles in supplementary concentrates of steers on instrumental texture parameters and sensory eating quality of beef patties stored in modified atmosphere packs at 4 °C for up to 7 days

	Storage time (day)/dietary treatment				**Storage time (day)/dietary treatment**			
	Day 2				**Day 7**			
Parameter	**CON**	**CDGS**	**WDGS**	**SEM**	**CON**	**CDGS**	**WDGS**	**SEM**
Textural attributes								
Hardness	15.07^{ax}	17.41^{ax}	17.25^{ax}	0.808	25.45^{ay}	26.66^{ay}	23.01^{ay}	1.042
Springiness	0.83^{ax}	0.85^{ax}	0.83^{ax}	0.008	0.86^{ax}	0.86^{ax}	0.85^{ax}	0.004
Cohesiveness	0.59^{ax}	0.58^{ax}	0.57^{ax}	0.008	0.61^{ax}	0.66^{ax}	0.62^{ax}	0.019
Gumminess	8.88^{ax}	10.12^{ax}	9.79^{ax}	0.539	15.51^{ay}	17.9^{ay}	14.16^{ay}	1.086
Chewiness	7.32^{ax}	8.61^{ax}	8.12^{ax}	0.492	13.35^{ay}	15.46^{ay}	12.10^{ay}	0.952
Adhesiveness	-1.59^{ax}	-1.46^{ax}	-0.88^{ax}	0.210	-1.36^{ax}	-1.02^{ax}	-1.24^{ax}	0.109
Eating quality								
Appearance	5.38^{ax}	6.10^{ax}	6.10^{ax}	0.213	6.06^{ax}	6.10^{ax}	6.18^{ax}	0.188
Odour	5.87^{ax}	5.72^{ax}	6.27^{ax}	0.197	6.14^{ax}	6.09^{ax}	6.06^{ax}	0.172
Texture	4.58^{ax}	5.14^{ax}	5.08^{ax}	0.218	4.33^{ax}	4.95^{ax}	4.98^{ax}	0.196
Juiciness	3.47^{ax}	4.02^{ax}	3.84^{ax}	0.212	3.14^{ax}	3.43^{ax}	3.89^{ax}	0.161
Flavour	5.81^{ax}	5.83^{ax}	5.82^{ax}	0.192	5.73^{ax}	5.48^{ax}	5.25^{ax}	0.177

[a]Effect of dietary treatment: within each parameter and storage day, values in the same row are not significantly different ($P > 0.05$).

[x,y]Effect of storage time: within each dietary treatment and between storage time (day 2 *vs.* day 7), values in the same row bearing different superscripts are significantly different ($P \leq 0.05$)

CON: control; CDGS: corn distillers' grain with solubles; WDGS: wheat distillers' grains with solubles; SEM: Standard error of mean.

to 35% DM) in concentrate feedlot diets (Buttrey et al., 2013; De Mello et al., 2018; Gill et al., 2008). In contrast, other studies using trained panellists reported that replacement of corn with dried CDGS (up to 75% DM) improved beef tenderness (Depenbusch et al., 2009) while inclusion of CDGS or WDGS (up to 40% DM) in barley-based finishing diets enhanced the flavour desirability of beef (Aldai et al., 2010a). Moreover, Aldai et al. (2010a) indicated that panellists rated beef steaks from steers fed CDGS as more tender and palatable compared to the control (barley-based diet) while beef steaks from WDGS had intermediate scores for such attributes. The ability of CDGS to improve sensory characteristics have been attributed to changes in muscle physicochemical properties such as the amount of connective tissues and fat content (Aldai et al., 2010a; Depenbusch et al., 2009). On the contrary, the current study indicated that dietary CDGS or WDGS did not affect the IMF content and instrumental texture parameters which may explain the similarity in beef sensory attributes such as the liking of flavour and texture.

6.4. CONCLUSIONS

The inclusion of CDGS or WDGS in supplementary concentrates offered to steers fed grass silage may increase the proportion of health-promoting fatty acids (CLA and PUFA) in beef. However, feeding DGS increased susceptibility to lipid oxidation and colour deterioration in fresh beef patties stored in MAP, with CDGS exhibiting an intermediate effect between CON and WDGS. The retail shelf-life of beef patties from steers fed CDGS and WDGS may be limited to 7 days while the CON treatment may extend shelf-life up to 10 days. Nonetheless, feeding CDGS or WDGS did not negatively influence the eating attributes of beef patties stored in MAP for up to 7 days.

CHAPTER 7 – Quality indices and sensory attributes of beef from steers offered grass silage and a concentrate supplemented with dried citrus pulp

Saheed A. Salami, Michael N. O'Grady, Giuseppe Luciano, Alessandro Priolo, Mark McGee, Aidan P. Moloney and Joseph P. Kerry

This chapter is in the form of a manuscript that is in preparation for submission to *Meat Science.*

ABSTRACT

This study investigated the quality composition, oxidative stability and sensory attributes of beef (*longissimus thoracis,* LT) from steers offered grass silage and a concentrate supplement in which rolled barley was replaced by 40% and 80% (*as-fed* basis) dried citrus pulp (DCP). Dietary treatment did not influence the antioxidant status (α-tocopherol and total phenolic contents) and activities of LT. Feeding DCP significantly increased the proportion of conjugated linoleic acids and polyunsaturated fatty acids in beef. Lipid and colour stability of fresh beef patties stored in modified atmosphere packs (MAP) were unaffected by dietary treatment but feeding 40% DCP reduced ($P < 0.05$) lipid oxidation in aerobically-stored cooked beef patties. Sensory panellists did not detect differences between treatments in eating quality and overall acceptability sensory descriptors of beef patties stored in MAP for up to 7 days. Results indicated that substitution of barley with DCP improved the fatty acid profiles of beef without negatively influencing the eating quality of beef.

Keywords: *Beef, citrus pulp, oxidative stability, sensory analysis, fatty acids*

7.1. INTRODUCTION

Concentrate feeding in combination with grass silage is a strategy typically used for winter-finishing of beef cattle in temperate countries like Ireland (McGee, 2005). The substitution of cereals with agro-industrial by-products in concentrate-based diets reduces feed cost, mitigates food-feed-fuel competition and enhances resource efficiency of livestock production (Makkar, 2016). Dried citrus pulp (DCP) is a whole by-product obtained after drying the residue following extraction of juice from citrus fruits and it consists of peel, pulp and seed residues (Martinez-Pascual and Fernandez-Carmona, 1980). Due to the high amount of digestible fibre in DCP, this by-product can be used as a high-energy feedstuff, compared to starch-rich cereals, to avoid a decline in ruminal pH and negative effects on forage digestibility in ruminants (Bampidis and Robinson, 2006). A recent study indicated that DCP can be used to replace rolled barley in a barley-soybean meal concentrate supplement without negative effects on intake and performance of young growing cattle offered to grass silage (Lenehan et al., 2017). Despite the significant impact of animal diet on meat quality traits (Geay et al., 2001), the effect of dietary DCP on beef quality traits has not been reported on to date.

Fatty acid composition in ruminant-derived foods (meat and milk) is characterized by a low content of health-promoting polyunsaturated fatty acid (PUFA) and high proportion of undesirable saturated fatty acids (SFA) and *trans* fatty acids (TFA), mainly due to the biohydrogenation of dietary PUFA in the rumen (Bessa et al., 2015). Concentrate-based diets in which cereals (corn and barley) are replaced with DCP may alter ruminal biohydrogenation possibly due to a reduced dietary starch content (Santos-Silva et al., 2016) or a high concentration of phenolic compounds in DCP (Lanza et al., 2015). This alteration in ruminal biohydrogenation might explain the effect of dietary DCP in

improving the fatty acid profile of ovine meat and milk by increasing the concentration of *t*-11 18:1 (vaccenic acid), *c*-9, *t*-11 conjugated linoleic acid (CLA) and PUFA while decreasing *trans* 18:1 isomers and the *n*-6:*n*-3 PUFA ratio (Lanza et al., 2015; Oliveira et al., 2017; Santos-Silva et al., 2016).

Lipid oxidation is the primary factor responsible for discolouration and rancid off-flavour development during the shelf-life of meat, resulting in quality deterioration and reduced acceptability (Morrissey et al., 1998). Increased PUFA content potentially increases the susceptibility of meat to lipid oxidation and dietary supplementation with antioxidant compounds enhance the oxidative stability of meat (Bekhit et al., 2013). Citrus fruits and their by-products are known to contain a substantial amount of phenolic compounds, predominantly flavonoids (such as naringin, hesperidin, quercetin, rutin and luteolin), that exhibit potent antioxidant activities (Balasundram et al., 2006; Benavente-García et al., 1997). Previous studies have shown that feeding DCP as a source of dietary phenolic antioxidants improved the oxidative stability of lamb meat (Gravador et al., 2014; Inserra et al., 2014) and enhanced the antioxidant capacity of bovine milk (Santos et al., 2014). Moreover, dietary supplementation of the major citrus flavonoid compounds (naringin and hesperidin) reduced lipid oxidation in aerobically-stored broiler meat (Goliomytis et al., 2015) and lamb meat (Simitzis et al., 2018).

Replacing cereals with DCP in a concentrate supplement offered to grass silage-fed cattle may improve beef quality by increasing the proportion of beneficial unsaturated fatty acids without negatively influencing the oxidative stability and acceptability of beef. The objective of this study was to examine the chemical composition, fatty acid profile, oxidative stability, texture characteristics and consumer acceptability of beef from steers offered grass silage and a concentrate supplement in which barley was substituted with increasing levels of DCP.

7.2. MATERIALS AND METHODS

7.2.1. Animals, diets and experimental design

Animal experimental procedures were approved by the Teagasc animal ethics committee and conducted under license from the Irish Government Department of Health and Children. The animals were managed by trained personnel according to the European Union legislation for the protection of animals used for scientific purposes (2010/63/EU Directive). Thirty-six weaned, spring-born Charolais and Limousin-sired suckler bulls were purchased directly from suckler farms at ~7 months of age and assembled at Teagasc Animal & Grassland Research and Innovation Centre, Grange, Ireland. Following assembly, the bulls were castrated and offered grass silage *ad libitum* plus 2 kg of a barley-based concentrate and 60 g of a mineral-vitamin supplement per head daily for a 187-d back-grounding period. All animals had *ad libitum* access to clean water.

Steers were subsequently blocked by breed and live weight (427.2 ± 37.2 kg) and, from within each block, randomly assigned to one of three concentrate diets (n = 12 steers/treatment) offered separately as a supplement to *ad libitum* grass (*Lolium perenne*) silage. Concentrate diets contained either 0% DCP (control, barley-based diet), or 40% DCP or 80% DCP as a replacement for rolled barley (*as-fed* basis). The concentrates were prepared as coarse mixtures. Table 7.1 indicates the ingredient and chemical composition of the experimental concentrate diets. Representative samples of the concentrate diets were obtained twice weekly and stored at -20 °C prior to chemical analysis. The steers were housed in a slatted-floor building in groups of five or six animals per pen with a Calan gate feeding system (American Calan Inc., Northwood, NH, USA) allowing individual feed intake of steers to be recorded. It should be noted that 11 steers were later assigned to the 40% DCP treatment due to individual animal-feeding facility constraints, rather than 12

steers assigned to other dietary treatments. Steers were individually offered 4.0 kg DM daily (2 kg in the morning and afternoon feeding sessions) of their respective supplementary concentrates for 124 days pre-slaughter.

Animals were slaughtered in a commercial abattoir on two consecutive weeks (balanced for treatment) to facilitate sample collection and measurements. Samples of *longissimus thoracis* muscle (LT) were removed from the left side of the carcass at 48 h post-mortem, vacuum-packed and aged for 14 days at 4 °C, and subsequently stored at -20 °C prior to further analysis. Information relating to production performance and carcass characteristics is presented in Kelly et al. (2017).

7.2.2. Feed analysis

Concentrate diets were analyzed for dry matter (DM), crude protein, ash, neutral detergent fibre, acid detergent fibre and starch concentrations as described by O'Kiely (2011). Total fat concentration or Oil-B (acid hydrolysis/ether extract) was measured using a Soxtec instrument (Tecator, Höganäs, Sweden).

Phenolic compounds were extracted from samples of concentrate diets using aqueous methanol (50:50, v/v) and acetone (70:30, v/v) solvents (Jiménez-Escrig et al., 2001). Polyphenol-rich extracts were analysed for total phenol content (TPC) using the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999) and absorbance measurements were recorded at 750 nm using a UV-vis spectrophotometer (Cary 300 Bio, UV-vis spectrophotometer, Varian Instruments, Palo Alto, CA, USA). Results were expressed as g of gallic acid equivalents (GAE)/kg of DM feed.

The fatty acid composition was determined in freeze-dried samples of concentrate diets by a one-step extraction–transesterification procedure using chloroform (Sukhija and

Palmquist, 1988) and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003), with 19:0 nonadecanoate (Larodan, Solna, Sweden) added as an internal standard. Gas chromatographic analysis of fatty acid methyl esters (FAME) was performed as described by Cherif et al. (2018). Individual fatty acid was expressed as g/kg of DM feed.

Table 7.1. Ingredient and chemical composition of experimental concentrate diets containing increasing levels of dried citrus pulp (DCP)

Ingredient (as-fed basis, g/kg)	**0% DCP**	**40% DCP**	**80% DCP**
Rolled barley	862	467	58
Soybean meal	60	70	80
Dried citrus pulp (DCP)	-	400	800
Cane molasses	50	50	50
Minerals and vitamins	28	13	12
Chemical composition			
Dry matter (DM, g/kg)	801	828	854
Crude protein [1]	131	130	113
Ash[1]	58	67	87
Total fat[1]	28	28	21
Neutral detergent fibre[1]	201	200	234
Acid detergent fibre[1]	62	132	237
Starch[1]	502	315	63
Total phenol content[2]	6.55	12.41	19.04
Fatty acid (g/kg dry matter (DM))			
C12:0	0.10	0.30	0.20
C14:0	0.10	0.10	0.10
C16:0	3.30	4.30	3.60
C18:0	0.20	0.50	0.60
c-9 C18:1	1.90	3.00	2.70
c-9,12 C18:2	6.50	7.60	4.50
c-9,12,15 C18:3	0.60	0.80	0.70

[1]Expressed as g/kg DM
[2]Expressed as g gallic acid equivalents/kg DM

7.2.3. Determination of muscle pH and proximate composition

The LT muscle was thawed and trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). Minced LT samples (5 g) were homogenised for 3 min at 24,000 rpm in 45 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany). The pH of the beef homogenates was measured at 20 °C using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). Raw minced LT samples were analysed for moisture and fat contents using a SMART Trac rapid moisture/fat analyser (CEM Corporation, Matthews, NC, USA). The ash content was determined using a muffle furnace (550 °C for 3 h) and protein content was determined by the Kjeldahl method.

7.2.4. Determination of vitamin E in LT muscle

The α-tocopherol (vitamin E) content in raw minced LT samples was determined by high-performance liquid chromatography (HPLC) following the extraction procedure described by Buttriss and Diplock (1984). HPLC analysis was carried out on a ProStar liquid chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA) equipped with a ProStar autosampler (Model 410, Varian Instruments). Sample injection volume (partial loop fill) was 20 µl. The α-tocopherol was separated on a 250 x 4.6 mm Polaris C18-A 5u column (Metachem, Ansys® Technologies, CA, USA) and detected using a ProStar UV/Vis detector (Varian Instruments) at 292 nm. The mobile phase was methanol/water (97:3) and isocratic elution took place at 2ml/min for a total run time of 10 min. A personal computer and Star LC workstation software (version 6.20, Varian Inc.) was used for calculation of peak areas. A standard curve was generated using a range of α-tocopherol concentrations (7, 14, 21 µg/ml) and the concentration of α-tocopherol in beef was expressed in µg/g of

beef muscle. The percentage recovery of vitamin E from beef samples, through the extraction procedure, was determined by including vitamin E (0.2 ml of 22.8 µg/ml) as an internal standard. The percentage recovery was calculated by comparison of peak areas of standard amount of vitamin E recovered through the extraction procedure with those obtained by direct injection of the vitamin E standard (22.8 µg/ml) onto the column. The average percentage recovery was 92.8% and values of α-tocopherol in muscle samples were adjusted to account for percentage recovery.

7.2.5. Determination of fatty acids in LT muscle

Lipid fraction in raw minced LT samples was extracted following the method described by Bligh and Dyer (1959). The lipid fraction was transesterified to fatty acid methyl esters (FAME) using BF_3 in methanol as a catalyst following the method described by Park and Goins (1994). FAMEs were dissolved with isooctane, dried over anhydrous sodium sulphate (0.3 g) for 15 min and stored at -20 °C prior to gas chromatography analysis. The separation of FAME was carried out using a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA) using a WCOT fused silica capillary column (Varian CP-SIL 88 Tailor Made FAME, 60 m x 0.25 mm i.d. x 0.20 µm film thickness) and a flame ionisation detector. The column oven temperature was held at 150 °C for 25 min and programmed to increase from 150 °C to 240 °C at 4 °C/min and held for 2 min. The injector and detector temperatures were 270 ºC and 260 °C respectively. Helium was used as the carrier gas at a pressure of 30 psi. The injection was carried out using a Combi PAL (CTC Analytics AG, Zwingen, Switzerland) auto-injector. The injection volumes and split ratios for FAMEs were 1 µl and 1:2 split, respectively. Individual fatty acids were identified by comparing relative retention times with pure FAME standards (a mixture of Supleco 37 component FAME mix, *trans*-11 vaccenic acid methyl ester and conjugated linoleic acid

methyl ester; Sigma-Aldrich Ireland Ltd., Vale Road, Arklow, Wicklow, Ireland). Results were reported as individual fatty acids expressed as a percentage of the total fatty acids ((peak area of individual FAME/total peak area of FAME examined) x 100). The atherogenic index (AI) and thrombogenic index (TI) were calculated according to Ulbricht and Southgate (1991).

7.2.6. Determination of total phenol content and *in vitro* antioxidant activity

7.2.6.1. Preparation of muscle homogenates

Beef homogenates (10% w/v) were prepared as described by Qwele et al. (2013) for the determination of *in vitro* antioxidant activities. In brief, raw minced LT (5 g) was homogenised in 0.05 M phosphate buffer (45 ml) using an Ultra-turrax T25 homogeniser at 24,000 rpm for 3 min. Muscle homogenates were centrifuged at 7800 g for 10 min at 4 °C using an Avanti® J-E Centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA). The supernatant was filtered through Whatman No. 1 paper and the filtrate was analysed for ferric reducing antioxidant power (FRAP) and ferric ion chelating activity (FICA). For the determination of TPC and 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity assays, 10% trichloroacetic acid (5 ml) was added to muscle homogenates and the mixture was centrifuged at 7800 g for 10 min at 4 °C. The supernatant was filtered through Whatman No. 1 paper and analysed for TPC and DPPH assays.

7.2.6.2. Measurement of the total phenol content

Muscle extracts were analysed for TPC using the Folin-Ciocalteu method (Singleton et al., 1999) with minor modifications. Briefly, extracts (0.5 ml) were mixed with Folin-Ciocalteu reagent (2.5 ml, 20% in distilled water) and sodium carbonate (2 ml, 7.5% in distilled water) was added after 5 min. The mixture was stored in the dark for 2 h at room temperature and

absorbance measurements were recorded at 750 nm on a UV-vis spectrophotometer (Cary 300 Bio, UV-vis spectrophotometer, Varian Instruments, CA, USA) against a blank containing all reagents and distilled water. A calibration curve using standard solutions of aqueous gallic acid (20 – 100 μg/ml) was constructed and results are expressed as mg of gallic acid equivalents (GAE)/g of muscle.

7.2.6.3. Measurement of in vitro antioxidant activities

Radical scavenging activity in muscle was measured using the DPPH assay following a minor modification of the method described by Yen and Wu (1999). Muscle extract (0.6 ml) and distilled water (2.4 ml) were mixed with 0.2 mM DPPH in methanol (3 ml) and incubated in the dark for 1 h at room temperature. Absorbance measurements were recorded at 517 nm on a UV-vis spectrophotometer (Cary 300 Bio) against a methanol blank. An assay blank containing distilled water (3 ml) and 0.2 mM DPPH in methanol (3 ml) was used for calculation purposes. A calibration curve using standard solutions of methanolic Trolox (10 – 50 μg/ml) was constructed and results were expressed as mg of Trolox equivalents (TE)/g of muscle.

Total antioxidant activity in muscle was determined using FRAP assay following a minor modification of the method described by Benzie and Strain (1999). Briefly, muscle extract (0.45 ml) was mixed with 8.55 ml FRAP reagent (a mixture of 30 mM acetate buffer (pH 3.6), 10 mM 2,4,6–Tris (2-pyridyl)-s-triazine in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in distilled water in the ratio 10:1:1, respectively incubated at 37 °C for 10 min prior to use). The mixture was incubated for 30 min in the dark and absorbance was recorded at 593 nm on a UV-vis spectrophotometer (Cary 300 Bio) against a blank containing all reagents. A calibration curve using solutions of methanolic Trolox (0.033 – 0.1 mg/ml) was constructed and results are expressed as mg TE/g of muscle.

The iron-chelating activity of muscle was measured using a minor modification of FICA assay described by Yen and Wu (1999). Muscle extract (0.5 ml) was mixed with $FeCl_2$ (2 mM in distilled water, 0.1 ml), ferrozine solution (5 mM in distilled water, 0.2 ml) and distilled water (4.2 ml). The assay control contained $FeCl_2$ (0.1 m), ferrozine solution (0.2 ml) and distilled water (4.7 ml). The mixture was incubated for 1 h in the dark at room temperature and absorbance measurements were recorded at 562 nm against a water blank on a UV-vis spectrophotometer (Cary 300 Bio). Chelating activity was calculated as follows:

Chelating activity (%) = [1 − (absorbance of sample)/(absorbance of control)] × 100.

7.2.7. Determination of the oxidative stability and sensory properties of beef

7.2.7.1. Measurement of lipid oxidation and oxymyoglobin in muscle homogenates

Muscle homogenates (25%) were prepared by homogenising 15 g of LT in buffer (0.12 M KCL 5 mM histidine, pH 5.5) surrounded by crushed ice using an Ultra Turrax T25 homogeniser. Lipid oxidation in muscle homogenates (39.2 g) was initiated by the addition of pro-oxidants (45 μM $FeCl_3$/sodium ascorbate, 1:1) (O'Grady et al., 2001). Lipid oxidation (2-thiobarbituric acid reactive substances, TBARS) and oxymyoglobin (OxyMb) content in muscle homogenate were measured at 1 and 4 h of storage at 4 °C as described by Hayes et al. (2009).

7.2.7.2. Meat processing and packaging

The LT muscles were thawed overnight at 4 °C, trimmed of visible fat and connective tissue, and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). The minced muscle was formed into beef patties (100 g portions) using a meat former (Ministeak burger maker, O.L Smith Co. Ltd., Italy). For the fresh beef study, patties

were individually placed in a low oxygen permeable (<1 cm^3/m^2/24 h at STP) polystyrene/ethyl vinyl alcohol/polyethylene (PE) trays and flushed with 80% O_2:20% CO_2 (modified atmosphere packs, MAP) using a vacuum-sealing unit (VS 100, Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat-sealable layer. Fresh beef patties in MAP were stored for up to 14 days under fluorescent lighting (660 lx) at 4 °C. The gas atmosphere (% O_2 and % CO_2) in MAP was measured using a CheckMate 9900 (PBI-DanSensor, Denmark). The average gas composition in MAP was 79.87 ± 0.98% O_2 and 20.71 ± 0.16% CO_2 on day 1 of storage and contained 74.22 ± 0.88% O_2 and 25.63 ± 0.71% CO_2 on day 14 of storage.

For the cooked beef study, minced patties were individually placed on an aluminium foil-lined trays and cooked at 180 °C for 20 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72 °C was reached. Cooked beef patties were placed in PE trays over-wrapped with oxygen permeable film and stored for up to 6 days at 4 °C.

7.2.7.3. Measurement of lipid oxidation and surface colour

Lipid oxidation was measured in fresh beef patties on days 1, 4, 7, 11, and 14 of storage and on days 1, 3, and 6 in cooked beef patties (one beef patties/muscle/day). Lipid oxidation measurements were carried out following the method described by Siu and Draper (1978). Results were expressed as TBARS in mg malondialdehyde (MDA)/kg meat.

The surface colour of fresh beef patties was measured on days 1, 4, 7, 11, and 14 of storage (one beef patties/muscle/day) using a Konica Minolta CR-400 Chroma-Meter (Minolta Camera Co., Osaka, Japan). The Chroma-Meter consisted of a measuring head (CR-400),

with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-400). The Chrom-Meter was calibrated on the CIE LAB color space system using a white tile (D_c: L = 97.79, a = -0.11, b = 2.69). The 'L^*', 'a^*' and 'b^*' value represents lightness, redness and yellowness, respectively. Colour measurements were averaged for readings taken from four different locations on the surface of beef patties. Chroma (C^*) and hue angle (H^*) were calculated as $[(a^{*2} + b^{*2})^{1/2}]$ and $[\tan^{-1}b^*/a^*]$, respectively.

7.2.7.4. Measurement of textural properties of beef patties

The texture profile analysis (TPA) of fresh beef patties stored in MAP was measured on days 2 and 7 of storage. The TPA parameters (hardness (N), springiness (mm), cohesiveness (dimensionless), gumminess (N), chewiness (N × mm), adhesiveness (N)) were measured using a 30 kg load cell texture analyser (TA.XT2i Texture Analyser, Stable Micro Systems, UK) as described by Moroney et al. (2013).

7.2.7.5. Sensory acceptance testing of beef patties

Sensory acceptance of fresh beef patties (n = 8/treatment) stored in MAP was carried out by 40 untrained panellists in 2 sessions (20 panellists/session) on days 2 and 7 of storage as described by O'Sullivan et al. (2003). Beef patties were cooked for sensory analysis in a Zanussi oven at 180 °C for 20 min until an internal temperature of 72 °C was reached. Following cooking, patties were cooled to room temperature and cut into 2 cm × 2 cm cubes, identified with random three-digit codes. On each day of sensory analysis, beef samples were served to panellists in two separate sessions (morning and afternoon sessions). Prior to serving to panellists, beef samples were re-heated in a microwave for 10 s to release the meat odour and flavour. Sensory evaluation was performed in the panel booths of the University's sensory laboratory according to international standard regulations (ISO, 2007). Sample presentation was randomised to prevent any flavour

carryover effects (MacFie et al., 1989). Panellists were asked to indicate their degree of liking for appearance, odour, texture, juiciness, flavour and overall acceptability on a 10 cm line scale ranging from 0 (extremely dislike) to 10 (extremely like). Panellists were provided with water to cleanse their palates between samples.

7.2.8. Statistical analysis

All analyses were performed in duplicate. Data on proximate composition, antioxidant capacity and fatty acid profiles analyses were analysed using a general linear model including dietary treatment as a fixed factor and block as a random factor. The orthogonal polynomial contrast was used to determine the linear and quadratic responses of dietary inclusion levels of DCP. Data relating to the oxidative stability and sensory attributes were analysed using a general linear model with a full-repeated measures ANOVA. Effects of dietary treatment represented the 'between-subjects' factor and the effect of storage/incubation time was measured using the 'within-subjects' factor and the interaction between diet and storage/incubation time was tested. The effects of panellist and session were included as random terms in the model used for the analysis of sensory eating attributes. Tukey's HSD test was used for multiple comparisons of treatment means when significance was detected at $P \leq 0.05$ and a tendency for treatment effect was observed when $0.05 < P \leq 0.10$. All data analysis was performed using SPSS statistical software (IBM Statistics version 22).

7.3. RESULTS AND DISCUSSION

Animal dietary strategies provide feasible opportunities to enhance the nutritional quality of beef by increasing the PUFA and CLA content, linked to positive health effects such as decreased incidence of cardiovascular diseases (CVD) and cancer (Vahmani et al., 2015). Feeding DCP, compared to cereals, may improve the fatty acid profiles of ruminant meat or milk by inhibiting ruminal biohydrogenation due to its high concentration of phenolic compounds and lower starch content (Lanza et al., 2015; Santos et al., 2014). The phenolic concentration in the DCP ingredient fed in the present study is approximately 2-fold (18.34 *vs.* 10.87 g gallic acid equivalent/kg DM) greater than reported in a study by Luciano et al. (2017). This highlights the considerable variation in chemical composition of citrus pulp, which can be influenced by factors such as the fruit source and type of processing (Bampidis and Robinson, 2006). As expected, the substitution of barley with increasing levels (40% and 80%) of DCP substantially decreased the starch content and increased the concentration of phenolic compounds in concentrate supplements (Table 7.1). The inclusion of 40% and 80% DCP in concentrate supplement represents 17.7% and 35.3% of the total dietary DM intake (grass silage + concentrate), respectively. Replacing barley with DCP in a concentrate supplement offered to grass silage-fed steers may increase the proportion of beneficial unsaturated fatty acids in beef. In addition, DCP may increase the supply of dietary phenolic antioxidants which could enhance the antioxidant capacity and oxidative stability of beef without negatively influencing consumer acceptability.

7.3.1. Proximate composition of muscle

The proximate composition of LT beef is presented in Table 7.2. There was a quadratic response (P = 0.015) in muscle pH due to DCP inclusion levels. However, muscle pH measured in all dietary treatments is within the normal pH range (5.4 – 5.8) associated with

post-mortem muscle (Faustman and Cassens, 1990). Intramuscular fat (IMF) is an important meat quality trait considering its influence on the sensory characteristics and fatty acid composition of meat (Wood et al., 2008). It is known that high-starch diets promote the deposition of IMF because of increased availability of glucose used for lipogenesis in intramuscular adipocytes (Pethick et al., 2004). However, the present study demonstrated that IMF was unaffected ($P > 0.05$) by the reduction in dietary starch content when rolled barley was replaced with DCP in concentrate supplements (Table 7.1). This observation may be attributed to the positive effect of concentrate DCP, compared to rolled barley, on the ruminal production of propionate in grass silage-fed growing cattle (Lenehan et al., 2017). This is in line with the assertion that propionate serve as the main glucogenic precursor utilized for IMF synthesis in ruminants (Pethick et al., 2004). Previous studies have also shown that replacing barley with DCP in concentrate diets did not affect the IMF content of lamb meat (Costa et al., 2017; Lanza et al., 2015). Beef produced from the present study can be considered as lean meat according to the labelling criteria (<5% fat) stipulated by the Food Advisory Committee (1990).

Furthermore, moisture contents were unaffected ($P > 0.05$) by dietary treatment. However, protein and ash contents responded quadratically ($P < 0.01$) to increasing levels of DCP, with 80% DCP displaying the highest values for both parameters. The higher ash content present in the 80% DCP diet (Table 7.1) is reflective of the presence of many vitamins (mainly carotenoids, vitamin B complex, vitamin C) and minerals (potassium and calcium) present in DCP (Bampidis and Robinson, 2006). Presumably, DCP vitamins and minerals were subsequently deposited in LT muscle from steers fed 80% DCP (Table 7.2).

Table 7.2. Effect of increasing levels of dried citrus pulp (DCP) in supplementary concentrates on the pH, proximate composition, antioxidant status and antioxidant activity of *longissimus thoracis* muscle of steers

Parameter	Dietary treatment (% DCP)			SEM	*P*-value	Contrast effect	
	0	40	80			Linear	Quadratic
Muscle pH	5.47[a]	5.52[b]	5.49[a]	0.009	0.019	0.133	0.015
Proximate composition (g/100 g wet weight)							
Protein	23.67[a]	22.91[b]	23.79[a]	0.118	0.003	0.612	0.001
Intramuscular fat	2.57	2.54	2.31	0.098	0.511	0.287	0.660
Moisture	73.12	73.16	73.21	0.090	0.914	0.674	0.987
Ash	1.11[a]	0.94[b]	1.37[c]	0.031	<0.001	<0.001	<0.001
Antioxidant status							
α-tocopherol (μg/g muscle)	2.38	2.51	2.69	0.107	0.510	0.251	0.914
TPC (mg GAE/g muscle)	0.94	0.90	0.92	0.020	0.767	0.729	0.525
Antioxidant activity							
DPPH (mg TE/g muscle)	0.25	0.25	0.24	0.004	0.367	0.162	0.898
FRAP (mg TE/g muscle)	0.38	0.39	0.38	0.004	0.355	0.416	0.236
FICA (%)	55.22	56.29	53.81	1.294	0.749	0.662	0.537

[a,b]Means within the same row bearing different superscripts are significantly different ($P < 0.05$)
SEM: Standard error of mean
TPC: Total phenol content DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; FICA: Ferric ion chelating activity.
GAE: gallic acid equivalent; TE: trolox equivalent

7.3.2. Antioxidant capacity of muscle

The biological antioxidant system comprises of enzymatic (such as glutathione peroxidase, superoxide dismutase, catalase) and non-enzymatic (such as vitamins A, E and C, and phenolic compounds) factors that are important to delay the onset of lipid peroxidation in *post-mortem* muscle (Salami et al., 2016). The concentration of α-tocopherol (vitamin E) and phenolic compounds in meat primarily depend on their dietary intakes by animals (Castillo et al., 2013). In the present study, the α-tocopherol concentration and TPC in LT were unaffected ($P > 0.05$) by the inclusion of DCP in concentrate supplements (Table 7.2). This was consistent with the lack of differences ($P > 0.05$) in muscle antioxidant activities (DPPH-radical scavenging, FRAP and FICA) between treatments (Table 7.2).

The current result is contrary to the expectation that high concentration of phenolic compounds in dietary DCP would enhance the antioxidant capacity of beef. It is noteworthy that the inclusion of 40% and 80% DCP in concentrate supplements increased dietary TPC by 2-fold and 3-fold, respectively. Despite the high phenolic content in DCP diets, the lack of differences in muscle TPC suggests that absorption of citrus polyphenols into the muscle was restricted which could explain the lack of antioxidant effect in the muscle. In agreement with this theory, Bodas et al. (2012) showed that dietary supplementation of a citrus flavonoid, naringin, resulted in the accumulation of naringenin (aglycone fraction of naringin) and subsequent antioxidant activity in the liver but not in the muscle. Furthermore, the inclusion of 35% DCP in a concentrate diet of lambs resulted in a 2-fold increase in dietary TPC without affecting the TPC or antioxidant activities of plasma, liver and muscle, measured by radical scavenging and reducing power assays (Luciano et al., 2017). Similarly, supplementation of PUFA-rich concentrate diets with 0.5 or 5 g/kg of citrus extract did not affect the radical scavenging activity in plasma, muscle and liver of rats (Gladine et al., 2007a; Gladine et al., 2007b). However, other studies have shown that

feeding 18% pelleted CP increased the TPC and reducing power in milk (Santos et al., 2014) while the inclusion of up to 21% DCP in the concentrate diet of rabbits increased the total antioxidant capacity of the liver (Lu et al., 2018). Thus, it is possible that differences in the absorption and *in vivo* antioxidant effect of citrus polyphenols is influenced by factors such as animal species (monogastric *vs.* ruminants), interaction with other dietary ingredients and the tissue of measurement.

Luciano et al. (2017) indicated that dietary DCP enhanced the antioxidant status of lamb meat by increasing the deposition of α-tocopherol in the muscle. This observation contradicts results presented in the current study where no effect of dietary DCP on α-tocopherol levels was observed. A similar finding was reported by Mourão et al. (2008) who showed that inclusion of up to 10% DCP in concentrate diet of broilers did not influence α-tocopherol concentration in the breast meat. It should be emphasised that the TPC, DPPH, FRAP and FICA assays used in the present study are not specific for assessing the antioxidant activity related to only polyphenols but rather provide different information about the mechanism of total antioxidant activity in a particular substance or biological tissue (Niki, 2010). Thus, the combination of these assays is robust to detect any direct or indirect changes in the antioxidant capacity of muscle that may arise from the intake of dietary citrus polyphenols or other bioactive molecules and their *in vivo* metabolites. In this regard, the current results suggest that inclusion of DCP in concentrate supplements fed with grass silage did not enhance the antioxidant capacity of beef. Further studies are required to investigate other factors that might play a role in the *in vivo* antioxidant efficacy of DCP including potential effects on antioxidant enzyme activities.

7.3.3. Fatty acid profiles of muscle

The fatty acid composition of ruminant meat, including beef, is influenced by the fatty acid composition of the feed, metabolism (lipolysis and subsequent biohydrogenation) of dietary lipids in the rumen and endogenous fatty acid metabolism and synthesis in the muscle (Bessa et al., 2015). The replacement of barley with DCP in a concentrate supplement did not result in substantial differences in feed fatty acid composition (Table 7.1). Total SFA was similar ($P > 0.05$) between dietary treatments and this was consistent with the lack of dietary differences ($P > 0.05$) in C14:0 and the two most abundant individual SFA (C16:0 and C18:0) found in beef (Table 7.3). Costa et al. (2017) also reported that substituting barley with 42% DCP in concentrate diet did not affect the concentration of these SFA in the *longissimus* muscle of lambs and this effect was consistent with the lack of changes in the concentration of these fatty acid in the rumen of lambs (Lanza et al., 2015).

The percentages of total MUFA and TFA were unaffected ($P > 0.05$) by dietary treatment. However, dietary DCP levels linearly decreased the percentage of *c*-10 17:1 ($P < 0.01$). Dietary treatment tended ($P = 0.065$) to increase the percentage of the predominant MUFA, *c*-9 C18:1 (oleic acid), with a quadratic response as the dietary level of DCP increases. A similar tendency for a higher percentage of oleic acid in lamb meat was exclusively associated with the lower starch content in concentrate diets in which cereals (barley, corn and wheat) were replaced with a combination of DCP, hominy feed and palm kernel meal (Oliveira et al., 2017). The ability of DCP to increase the proportion of oleic acid in beef could contribute to a healthy human diet because this fatty acid potentially exerts an anti-inflammatory effect and reduces the risk of cardiovascular diseases (Calder, 2015a). Furthermore, diet tended ($P = 0.096$) to influence the proportion of *t*-11 18:1, with a linear increase as the dietary level of DCP increases.

Dietary *t*-11 18:1 potentially exhibits health-promoting properties in humans coupled with its role as the primary precursor for the *de novo* synthesis of bioactive *c*-9, *t*-11 CLA (Bessa et al., 2015; Vahmani et al., 2015). The presence of *t*-11 18:1 in ruminant meat or milk is associated with its synthesis from dietary linoleic and linolenic acid during ruminal biohydrogenation before being absorbed into the muscle or mammary tissues (Bessa et al., 2015). Therefore, a change in the proportion of *t*-11 18:1 in the muscle is indicative of a modulatory effect of DCP on the ruminal biohydrogenation. Previous studies have also demonstrated that feeding oil supplements plus concentrate diets containing DCP as a substitute for cereals (barley and corn) increased *t*-11 18:1 concentration in lamb meat (Costa et al., 2017) and ewe milk (Santos-Silva et al., 2016).

Table 7.3. Effect of increasing levels of dried citrus pulp (DCP) in supplementary concentrates to grass silage-fed steers on the fatty acid composition of *longissimus thoracis* muscle of steers

Fatty acids (% of total fatty acids)	Dietary treatment (% DCP)			SEM	*P*-value	Contrast effect	
	0	40	80			Linear	Quadratic
C12:0	0.07	0.08	0.07	0.009	0.753	0.903	0.461
C14:0	2.51	2.75	2.45	0.084	0.314	0.765	0.138
c-9 C14:1	0.44	0.35	0.37	0.041	0.676	0.529	0.538
C15:0	0.39	0.42	0.41	0.019	0.831	0.696	0.644
C16:0	22.99	20.43	21.82	1.610	0.822	0.770	0.583
C16:1	1.85	1.58	1.68	0.135	0.741	0.630	0.549
C17:0	0.86	0.85	0.85	0.024	0.980	0.848	0.957
c-9 C17:1	0.50[a]	0.27[b]	0.24[b]	0.032	<0.001	<0.001	0.073
C18:0	13.32	12.22	12.31	0.331	0.325	0.212	0.408
t-9 18:1	2.07	2.42	1.97	0.128	0.350	0.747	0.160
t-11 C18:1	0.95	1.00	1.15	0.041	0.096	0.039	0.529
c-9 C18:1	29.11	34.66	31.55	0.973	0.065	0.281	0.037
t-9,12 C18:2	0.28	0.40	0.40	0.052	0.559	0.349	0.601
c-9,12 C18:2	2.29	2.72	3.18	0.160	0.067	0.021	0.970
C20:0	0.02	0.01	0.10	0.032	0.414	0.291	0.423
c-9,12,15 C18:3	0.35	0.40	0.37	0.013	0.193	0.480	0.095
[1]CLA	0.14[a]	0.34[b]	0.41[b]	0.033	0.001	<0.001	0.292
C22:0	0.52	0.52	0.47	0.026	0.742	0.504	0.704
C20:4 *n*-6	1.10	1.19	1.08	0.028	0.116	0.432	0.639
C20:5 *n*-3	0.18[a]	0.38[b]	0.25[ab]	0.031	0.021	0.277	0.010
C22:5 *n*-3	0.44	0.46	0.47	0.043	0.216	0.771	0.086
Summary							
∑SFA	41.15	37.63	38.52	1.687	0.688	0.531	0.555
∑MUFA	36.98	40.69	37.65	0.912	0.221	0.653	0.125
∑PUFA	4.97[a]	6.04[b]	6.52[b]	0.185	0.002	0.001	0.357
Total trans	3.02	3.42	3.13	0.106	0.309	0.681	0.142
∑*n*-6 PUFA	3.87[a]	4.62[ab]	5.02[b]	0.159	0.020	0.006	0.825
∑*n*-3 PUFA	0.96[a]	1.24[b]	1.09[ab]	0.037	0.004	0.178	0.003
n-6:*n*-3	4.03	3.73	4.61	0.416	0.220	0.691	0.095
PUFA:SFA	0.12	0.16	0.17	0.011	0.458	0.321	0.443
[2]Atherogenicity index	0.82	0.70	0.76	0.045	0.562	0.568	0.365
[3]Thrombogenicity index	1.78	1.43	1.62	0.080	0.211	0.387	0.124

[a,b]Means in the same row bearing different superscripts are significantly different ($P \leq 0.05$).

SEM: Standard error of mean

[1]CLA: *c*-9 *t*-11 18:2 + *t*-9 *c*-11 18:2

[2]Atherogenic index: (C12:0 + [4 × C14:0] + C16:0)/(*n*-3 PUFA + *n*-6 PUFA + MUFA).

[3]Thrombogenic index: (C14:0 + C16:0 + C18:0)/([0.5 × MUFA] + [0.5 × *n*-6 PUFA] + [3 × *n*-3 PUFA] + [*n*-3/*n*-6 PUFA]).

CLA: conjugated linoleic acid; SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: poly-unsaturated fatty acids

The higher dietary fibre in DCP, compared to cereals, may have resulted in a stimulatory effect on fibrolytic bacteria such as the *Butyrivibrio* spp. linked to the synthesis of *t*-11 18:1, before the formation of stearic acid, in the last step of ruminal biohydrogenation (Enjalbert et al., 2017). However, Lanza et al. (2015) demonstrated that substituting barley with up to 35% DCP in concentrate diet of lambs increased the percentage of *t*-11 18:1 in the plasma but no difference was observed in the rumen and muscle tissue. Variation in the accumulation of *t*-11 18:1 in meat or milk from ruminants fed reduced dietary starch may be attributed to the formation of a competing intermediate (*t*-10 18:1) at the expense of *t*-11 18:1 in the ruminal biohydrogenation pathway (Costa et al., 2017). Further studies would prove useful to investigate strategies which could maximize the effect of DCP on the production of *t*-11 18:1 during ruminal biohydrogenation.

An increase in the proportion of PUFA, particularly *n*-3 PUFA and *c*-9,*t*-11 CLA, in beef fat would improve the nutritional quality of beef and contribute to a healthy human diet considering the physiological effects of these fatty acids in preventing or reducing the risk of human diseases such as CVD, cancer and obesity (Bessa et al., 2015; Vahmani et al., 2015). The inclusion of dietary DCP linearly increased ($P < 0.01$) the proportion of total PUFA driven by a linear increase in *c*-9,12 C18:2 (linoleic acid), CLA (*c*-9,*t*-11 C18:2 + *t*-9,*c*-11 C18:2), and a quadratic increase in C20:5 *n*-3 (Table 7.3). Quantitative analyses have shown that *c*-9,*t*-11 C18:2 is the major CLA isomer (60 – 85% of the total CLA) in beef fat (Fritsche et al., 2000), indicating that *c*-9,*t*-11 C18:2 is the predominant fatty acid in the co-eluted CLA peak identified in this study. In agreement with this observation, a similar increase in *c*-9,*t*-11 CLA has been reported in lamb meat (Lanza et al., 2015) and ewe milk (Santos-Silva et al., 2016) when cereals (barley and corn) were replaced with up to 35% DCP in concentrate diets. However, this effect was inconsistent as shown with a lack of

change in the concentration of *c*-9,*t*-11 CLA in lamb meat when barley was substituted with 42% DCP in a concentrate diet (Costa et al., 2017).

The availability of *c*-9,*t*-11 CLA in ruminant meat and milk is derived from its synthesis during ruminal biohydrogenation and endogenous desaturation of *t*-11 18:1 via Δ-9-desaturase activity in ruminant tissues (Bessa et al., 2015). However, endogenous synthesis is considered as the predominant source, accounting for up to 95% of *c*-9,*t*-11 CLA in ruminant meat and milk (Palmquist et al., 2005). In the present study, a linear increase in *c*-9,*t*-11 CLA is consistent with that of *t*-11 18:1. This data suggests that an observed increase in *c*-9,*t*-11 CLA could be attributed to the higher availability of endogenous substrate (*t*-11 18:1) originating from ruminal biohydrogenation. In addition to the possible inhibitory effect of DCP on ruminal biohydrogenation, a high phenolic content in DCP may enhance endogenous desaturation due to the effect of dietary phenolics, such as tannins, in stimulating the expression of Δ-9-desaturase in the muscle (Vasta et al., 2009). This speculation may partly explain the response effects of DCP diets in increasing the percentage of C20:5 *n*-3 which are endogenous derivatives of linoleic and linolenic acids, respectively (Simopoulos, 1991). This is consistent with the increased proportion of long-chain PUFA (C20:5 *n*-3 and C22:5 *n*-3) observed in the muscle of lambs fed concentrate diets containing DCP as a substitute for barley (Lanza et al., 2015). Consequently, the percentage of total *n*-6 PUFA linearly increased ($P < 0.05$) as the dietary level of DCP increases, with muscle from steers fed 80% DCP higher in total *n*-6 PUFA relative to 0% DCP. However, increasing levels of DCP resulted in a quadratic increase ($P < 0.05$) in total *n*-3 PUFA, with 40% DCP being higher compared to 0% DCP. Overall, the measurements of nutritional indices (*n*-6:*n*-3 PUFA, PUFA:SFA, atherogenicity index and thrombogenicity index) did not differ ($P > 0.05$) between dietary treatments.

7.3.4. Oxidative stability of beef

Lipid oxidation is a major cause of quality deterioration and reduced acceptability during the shelf life of meat. This is attributed to rancid flavour development and colour deterioration resulting from the conversion of oxymyoglobin (red) to metmyoglobin (brown) (Morrissey et al., 1998). Animal diets can influence the oxidative resistance of meat by altering the balance between pro-oxidant and antioxidant compounds in muscle especially by increasing the amount of oxidizable PUFA in the phospholipid membrane (Faustman et al., 2010). In the present study, the effect of dietary replacement of barley with DCP on beef oxidation was examined under three different oxidative conditions (MAP storage, iron/ascorbate-induced lipid oxidation and aerobic storage).

Lipid oxidation, measured as TBARS, increased ($P < 0.01$) in fresh beef patties stored in MAP (80% O_2:20% CO_2) over 14 days (Figure 5.1a). Despite the higher proportion of PUFA present in the muscle from steers fed DCP diets, dietary treatment did not affect ($P > 0.05$) the concentration of TBARS in fresh beef patties during the storage duration. As expected, surface discolouration progressively occurred in fresh beef patties stored in MAP over the 14-day storage period (Table 6.4). This is evident with a decrease ($P < 0.05$) in redness (a^*), yellowness (b^*) and chroma (C^*, colour vividness) and an increase in hue angle (H^*, trueness of red). However, the instrumental colour variables (L^*, a^*, b^*, C^*, H^*) were unaffected by dietary treatment and no diet × storage time interaction was observed over the duration of storage. The values of $a^* = 12$ and $C^* = 16$ are considered as thresholds for visual discolouration that limits consumer acceptability of beef (Van Rooyen et al., 2017). The current data indicated that values of a^* and C^* measured up to 10 days were greater than the threshold values, suggesting that visual acceptability of barley- and DCP-fed beef may extend up to 10 days of storage.

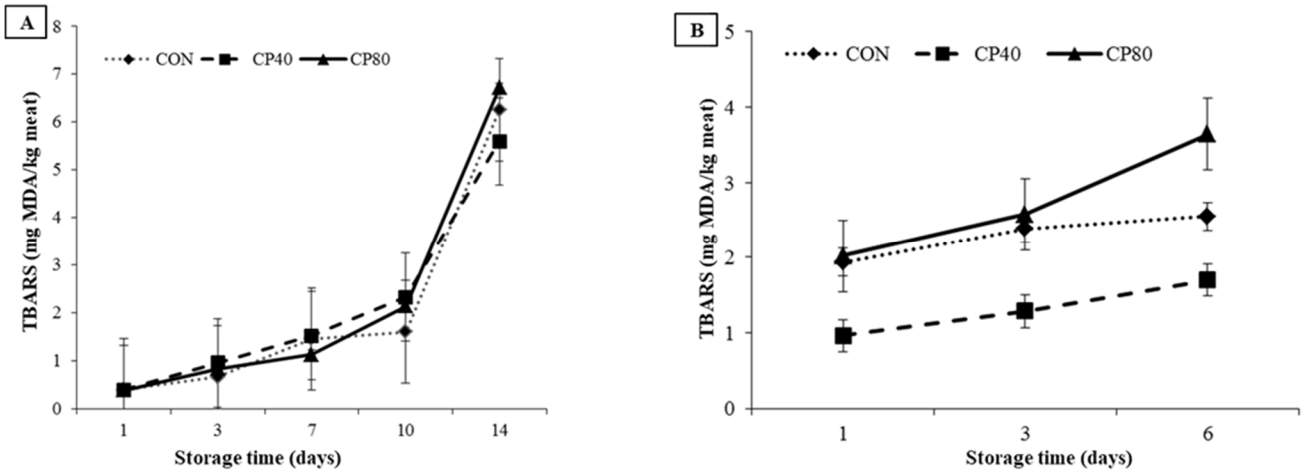


Figure 7.1. Effect of increasing levels of dried citrus pulp (DCP) in supplementary concentrates to grass silage-fed steers on the lipid oxidation (thiobarbituric acid reactive substances (TBARS)) of (A) fresh beef patties stored in modified atmosphere packs (80% O_2:20% CO_2) at 4 °C for up to 14 days (B) cooked beef patties stored in aerobic packs at 4 °C for up to 6 days.

There is a close interaction between processes involved in lipid oxidation and myoglobin oxidation due to the ability of free radicals generated in their biochemical reactions to influence these oxidative processes in a reciprocal manner (Faustman et al., 2010). This may partly explain why the lack of dietary effect on lipid oxidation was consistent with that of colour stability when fresh beef patties were stored in MAP. This is further supported by the lack of dietary effect ($P > 0.05$) on lipid and oxymyoglobin oxidation when muscle homogenates were subjected to a greater oxidative challenge by incubating with iron/ascorbate pro-oxidants (Table 7.5). To our knowledge, the present study is the first to report the effect of dietary DCP on the oxidative stability of beef. However, previous studies have shown that dietary supplementation of DCP or citrus flavonoids enhanced the oxidative stability of fresh meat from other animal species. The inclusion of up to 35% DCP

Table 7.4. Effect of increasing levels of dried citrus pulp (DCP) in supplementary concentrates to grass silage-fed steers on the colour stability of fresh beef patties stored in modified atmosphere packs for up to 14 days at 4 °C.

Dietary treatment (% DCP)	**Parameter**	**Storage time at 4 °C (days)**				
		1	**4**	**7**	**10**	**14**
0	Lightness L^*	48.02^{av}	48.86^{av}	48.46^{av}	49.24^{av}	51.65^{av}
40		48.19^{av}	47.89^{av}	47.75^{av}	48.44^{av}	52.33^{av}
80		48.72^{av}	48.67^{av}	48.47^{av}	48.89^{av}	51.78^{av}
SEM		0.65	0.52	0.54	0.76	0.63
0	Redness a^*	22.42^{av}	20.86^{aw}	18.98^{ax}	15.44^{ay}	5.90^{az}
40		22.79^{av}	20.67^{av}	18.58^{av}	13.80^{aw}	5.72^{ax}
80		23.04^{av}	21.31^{avw}	19.33^{aw}	13.75^{ax}	5.75^{ay}
SEM		0.265	0.340	0.254	0.577	0.209
0	Yellowness b^*	16.46^{av}	16.00^{av}	15.12^{avw}	14.15^{aw}	14.45^{aw}
40		16.76^{av}	15.61^{aw}	14.71^{awx}	13.40^{axy}	14.26^{ay}
80		16.86^{av}	16.10^{avw}	15.36^{awx}	13.62^{ay}	14.64^{axy}
SEM		0.170	0.168	0.131	0.186	0.107
0	Chroma[1] C^*	27.82^{av}	26.30^{av}	24.27^{aw}	20.96^{ax}	15.61^{ay}
40		28.29^{av}	25.91^{avw}	23.71^{aw}	19.30^{ax}	15.40^{ay}
80		28.55^{av}	26.71^{avw}	24.70^{aw}	19.36^{ax}	15.74^{ay}
SEM		0.269	0.342	0.237	0.486	0.079
0	Hue angle[2] H^*	36.28^{av}	37.50^{av}	38.53^{av}	42.53^{aw}	67.77^{ax}
40		36.37^{av}	37.07^{av}	38.43^{av}	44.61^{av}	68.17^{aw}
80		36.24^{av}	37.13^{av}	38.47^{av}	44.75^{aw}	68.57^{ax}
SEM		0.332	0.356	0.381	1.080	0.820

[a]Effect of dietary treatment: within each parameter, values in the same column are not significantly different ($P > 0.05$).
[v,w,x,y,z]Effect of storage time: values in the same row bearing different superscripts are significantly different ($P \leq 0.05$).
SEM: Standard error of mean.
[1]Chroma (colour vividness), higher values indicate greater saturation of red.
[2]Hue angle (trueness of red), lower values indicate a redder colour.

Table 7.5. Effect of increasing levels of dried citrus pulp (DCP) in supplementary concentrates to grass silage-fed steers on oxymyoglobin (oxyMb) and lipid oxidation (TBARS) in *longissimus thoracis* homogenates incubated with a ferric chloride/sodium ascorbate pro-oxidant system.

Dietary treatment (% DCP)	**Storage time (h)**			
	1		**4**	
	OxyMb[1] (%)	**TBARS[2]**	**OxyMb[1] (%)**	**TBARS[2]**
0	85.51^{ax}	2.23^{ax}	45.12^{ay}	6.19^{ay}
40	87.80^{ax}	2.15^{ax}	51.48^{ay}	6.18^{ay}
80	87.23^{ax}	2.48^{ax}	52.49^{ay}	6.39^{ay}
SEM	0.763	0.123	2.000	0.170

[a]Effect of dietary treatment: values in the same column are not significantly different ($P > 0.05$).
[x,y]Effect of storage time: values of OxyMb or TBARS in the same row bearing different superscripts are significantly different ($P \leq 0.05$).
SEM: Standard error of mean.
[1]TBARS: Thiobarbituric acid reactive substances expressed as mg malondialdehyde/kg meat.
[2]OxyMb: Oxymyoglobin, % of total myoglobin.

in concentrate diet reduced lipid and protein oxidation in lamb meat stored aerobically at 4 ºC for up to 6 days (Gravador et al., 2014; Inserra et al., 2014). Similarly, dietary supplementation of flavonoid compounds (naringin or hesperidin) reduced lipid oxidation and discolouration in aerobically-stored fresh broiler meat (Goliomytis et al., 2015) and lamb meat (Simitzis et al., 2018). In the present study, the inability of dietary DCP to enhance the oxidative stability of beef can be explained by the similarity in muscle antioxidant capacity as earlier discussed. Microbial metabolism of in the rumen is a major factor limiting the bioavailability and absorption of phenolics into the muscle tissue of ruminants (Vasta and Luciano, 2011). In contrast to large ruminants like cattle, positive effect of citrus phenolics on meat oxidative stability in lambs (small ruminants) or chickens (monogastric) may be attributed to a less-rigorous microbial activities in the gut, which allows for a sufficient amount of antioxidant compounds to be deposited in the muscle.

Heat processing induces the formation of free radicals which accelerate oxidative reactions and increase the susceptibility of meat to lipid peroxidation (Bekhit et al., 2013). In this regard, the effect of feeding DCP was further examined in cooked beef patties stored in aerobic packs at 4 ºC for up to 6 days (Figure 7.1b). Lipid oxidation increased ($P < 0.01$) as a function of storage time. In comparison to 0% and 80% DCP, feeding 40% DCP decreased ($P < 0.05$) lipid oxidation on day 1 and 3 of storage and a tendency ($P = 0.096$) for reduced levels of lipid oxidation was observed on day 6 of storage. The reason for the antioxidant effect of 40% DCP in cooked beef is unclear considering that dietary DCP did not enhance the antioxidant capacity or oxidative stability of LT muscle. However, heat treatment has been shown to enhance the liberation and activation of bound phenolic compounds from citrus resulting in increased antioxidant activity (Hayat et al., 2010; Jeong et al., 2004). Thus, it can be deduced that heating enhanced the antioxidant activity of DCP phenolics deposited in the muscle which could partly explain the discrepancy between the antioxidant effects observed in cooked beef patties from steers fed 40% DCP but not in fresh beef patties or muscle homogenates. Further studies are required to investigate the mechanism through which citrus phenolic compounds or their *in vivo* metabolites exert antioxidant effect in meat.

7.3.5. Texture profile and consumer acceptability of beef

Lipid and protein oxidation in *post-mortem* muscle potentially impact tenderness properties which influence the palatability and consumer acceptability of meat (Bekhit et al., 2013). Previous studies have demonstrated that feeding DCP did not affect the tenderness, measured by Warner–Bratzler shear force, of fresh beef (De Souza Duarte et al., 2011; Maia Filho et al., 2016). However, no information has been reported on the effect of dietary DCP on meat tenderness relative to the effect of oxidation during storage. Texture profile analysis provides a detailed characterization of textural attributes (hardness, springiness,

Table 7.6. Effect of increasing levels of dried citrus pulp (DCP) in supplementary concentrates to grass silage-fed steers on the texture profile parameters and eating attributes of beef patties stored in modified atmosphere packs at 4 °C for up to 7 days

	Storage time (day)/dietary treatment (DCP)							
	Day 2				**Day 7**			
Parameters	**0% DCP**	**40% DCP**	**80% DCP**	**SEM**	**0% DCP**	**40% DCP**	**80% DCP**	**SEM**
Textural characteristics								
Hardness	15.07^{ax}	18.66^{ax}	18.92^{ax}	0.714	25.45^{ay}	20.81^{ay}	22.72^{ay}	1.086
Springiness	0.83^{ax}	0.83^{ax}	0.86^{ax}	0.009	0.86^{ax}	0.84^{ax}	0.85^{ax}	0.005
Cohesiveness	0.59^{ax}	0.57^{ax}	0.60^{ax}	0.008	0.61^{ax}	0.57^{ax}	0.64^{ax}	0.017
Gumminess	8.88^{ax}	10.67^{ax}	11.45^{ax}	0.454	15.51^{ay}	11.83^{ax}	14.57^{ax}	0.956
Chewiness	7.32^{ax}	8.91^{ax}	9.89^{ax}	0.445	13.35^{ay}	9.96^{ax}	12.47^{ax}	0.849
Adhesiveness	-1.59^{ax}	-1.48^{ax}	-0.72^{ax}	0.194	-1.36^{ax}	-1.52^{ax}	-1.25^{ax}	0.081
Eating quality								
Appearance	5.38^{ax}	6.05^{ax}	6.06^{ax}	0.214	6.06^{ax}	6.22^{ax}	6.45^{ax}	0.194
Odour	5.87^{ax}	6.10^{ax}	6.10^{ax}	0.183	6.14^{ax}	6.00^{ax}	6.30^{ax}	0.170
Texture	4.58^{ax}	5.26^{ax}	4.55^{ax}	0.228	4.33^{ax}	5.14^{ax}	4.52^{ax}	0.200
Juiciness	3.47^{ax}	4.26^{ax}	3.22^{ax}	0.216	3.14^{ax}	4.16^{ax}	3.19^{ax}	0.188
Flavour	5.81^{ax}	6.07^{ax}	5.47^{ax}	0.200	5.73^{ax}	6.30^{ax}	5.94^{ax}	0.168
Overall acceptability	5.10^{ax}	5.63^{ax}	5.03^{ax}	0.200	5.04^{ax}	5.71^{ax}	5.13^{ax}	0.161

[a]Effect of dietary treatment: within each parameter and storage day, values in the same row are not significantly different ($P > 0.05$).

[x,y]Effect of storage time: within each dietary treatment and between storage time (day 2 *vs.* day 7), values in the same row bearing different superscripts are significantly different ($P \leq 0.05$). SEM: Standard error of mean.

cohesiveness, gumminess chewiness and adhesiveness) of meat. Hardness (force required to attain a given deformation at first bite) is considered as the most important textural attribute because of its direct relationship with the objective tenderness of meat (Caine et al., 2003). Hardness, gumminess and chewiness increased ($P < 0.05$) in CON beef patties over the 7 days of storage (Table 7.6), in agreement with the effect of the high-oxygen MAP (80% O_2:20% CO_2) whereby beef tenderness is reduced due to crosslinking/aggregation of myosin by protein oxidation (Kim et al., 2010). There was no significant effect of dietary treatment on all the texture profile parameters over the storage duration. The lack of dietary effect on the lipid oxidation of beef may partly explain why the texture profile parameters did not differ between dietary treatments.

The effect of diet on muscle composition such as fatty acids could influence oxidative development and consequently affect sensory perception and consumer acceptability of meat (Wood et al., 2008). There is limited information on the effect of dietary DCP on the eating quality of meat. Previous sensory evaluation studies have shown that feeding orange pulp did not affect the sensory characteristics and overall acceptance of meat from guinea pigs (Mínguez and Calvo, 2018) and lambs (Lanza et al., 2001). Similarly, the present study showed that naïve panellists did not detect differences in the eating quality sensory descriptors (appearance, odour, texture, juiciness, flavour and overall acceptability) of beef patties stored in MAP for up to 7 days. The lack of dietary impact on the oxidative stability and instrumental textural attributes of beef may partly explain the similarity between dietary treatments in the eating quality sensory scores.

7.4. CONCLUSIONS

Inclusion of up to 80% DCP in the concentrate supplement did not influence the antioxidant capacity of beef from steers offered grass silage. From a human nutrition perspective, dietary DCP improved the fatty acid composition of beef by increasing the percentage of CLA and PUFA. Feeding DCP did not compromise the oxidative stability of fresh beef stored in MAP while 40% DCP reduced the lipid oxidation in aerobically-stored cooked beef patties. Moreover, dietary DCP did not negatively influence the texture characteristics and consumer acceptability of beef.

CHAPTER 8 – General Discussion, Conclusions and Future Research

8.1. GENERAL DISCUSSION

The production of ruminant meat (beef, lamb meat and goat meat), expressed relative to their protein outputs, is associated with the largest environmental cost compared to other livestock products (milk, pork, poultry meat and egg). Feed production, transport, and utilisation play a major role in the environmental metrics of livestock production. Life cycle assessment (LCA) studies have shown that using human-inedible biomass (e.g. food wastes, crop residues and agro-industrial by-products) as animal feeds can mitigate the environmental impacts of livestock production. Large amounts of waste biomass are generated as agricultural and food industry by-products, accounting for approximately 30% of global agricultural production. The ruminant forestomach or rumen contains a diverse microbial population conferring an exclusive physiological adaptation for the greater utilisation of plant by-products (PBP) compared to monogastric livestock.

In this thesis (Chapter 1), a systematic review of the literature was conducted with the objective of highlighting the dual-impact of feeding PBP on environmental sustainability and meat quality attributes in ruminant meat production. A life cycle assessment (LCA)-based simulation study was conducted (using the US as the reference country) and results of different feeding scenarios indicated that replacement of human-inedible forages and fodder crops with up to 50% PBP reduced GHG emissions from ruminant meat production systems (beef cattle, sheep meat, and goat meat). In addition, PBP offers economical and resource-efficient benefits considering that the cultivation of conventional feed roughages (grass and legume pasture) occupies a significant proportion of land and requires an appreciable amount of inputs. It is noteworthy that most of the previous LCA studies focused on the comparison between environmental impacts of inedible feeds (e.g. food wastes, crop residues and agro-industrial by-product) and edible feeds (mainly grains)

biomass. Thus, the present results provide further insights suggesting that the choice of inedible feedstuffs requires careful consideration for strategies aimed at improving the sustainability of ruminant production. Furthermore, the simulation study identified feed transport and processing as significant hotspots for reducing GHG emissions if PBP are utilized as animal feeds. Therefore, harnessing the environmental benefit of feeding PBP demands that eco-friendly, and energy-efficient technologies are used in the processing of PBP. In addition, PBP should be used locally to reduce GHG emissions from feed transport.

In addition to the environmental benefits of feeding PBP, dietary PBP may offer an opportunity to improve the quality attributes of ruminant meat. Animal nutritional strategies that increase the ingestion of unsaturated fatty acids and polyphenols can inhibit ruminal biohydrogenation (RBH) of dietary lipids and therefore increase the accumulation of health-promoting unsaturated fatty acids in ruminant meat or milk. Moreover, the deposition of dietary phenolic antioxidants in the muscle can enhance the oxidative stability of meat. Thus, feeding strategies were undertaken with the hypothesis that by-products containing a considerable amount of residual unsaturated fat and/or phenolic compounds would enhance the nutritional quality and oxidative stability of ruminant meat. In this regard, 5 PBP (cardoon meal, dried corn gluten feed (CGF), dried corn (CDGS) and wheat (WDGS) distillers' grains with solubles, and dried citrus pulp (DCP)) were evaluated for their effect on the quality indices of lamb meat (cardoon meal) and beef (CGF, CDGS and WDGS, and DCP). Table 8.1 shows the total phenolic content (TPC) and antioxidant activity of the PBP and conventional feedstuffs substituted in the respective feeding trials. Cardoon meal, CGF and DCP contain considerably higher TPC compared to the conventional feedstuffs (dehydrated alfalfa, barley and soybean meal). In particular, cardoon meal contained a very high concentration of phenolic compounds with potent

Table 8.1. Total phenolic content (TPC), DPPH-free radical scavenging activity and ferric reducing antioxidant power (FRAP) of feedstuffs evaluated in the feeding trials of lambs and beef cattle

Feeding trial	Feedstuff	[1]TPC	[2]DPPH	[3]FRAP
LAMB	[3]Dehydrated alfalfa	15.51	3.73	19.35
	[4]Cardoon meal	60.39	22.99	103.94
BEEF CATTLE	[3]Barley	4.03	2.84	7.86
	[3]Soybean meal	4.31	1.25	3.99
	[4]Dried corn gluten feed	17.65	2.92	20.42
	[4]Dried maize distillers' grains with solubles	8.54	2.04	8.47
	[4]Dried wheat distillers' grains with solubles	8.39	2.00	8.32
	[4]Dried citrus pulp	18.35	3.14	23.45

[1]Expressed as g of gallic acid equivalent/kg dry matter of feedstuff
[2]Expressed as g of trolox equivalent/kg dry matter of feedstuff
[3]Conventional feedstuffs
[4]By-products
DPPH: 1,1-diphenyl-2- picrylhydrazyl

antioxidant activities (DPPH-free radical scavenging activity and ferric reducing antioxidant power).

Feeding diets containing a high concentration of phenolic compounds may exhibit negative effects on ruminal metabolism and the microbiome, which in turn impairs animal performance. Thus, as a prelude to this thesis, a feeding trial was conducted to characterize the rumen function response in lambs fed phenolic-rich diets where a concentrate-based diet was supplemented with two hydrolysable tannins (chestnut and tara) and condensed tannins (mimosa and gambier) for 75 days pre-slaughter (Chapter 2). Tannin extracts were selected as a source of phenolic compounds because of their wide application in animal nutrition and significant impacts on rumen function. Considering the structural complexity of phenolic compounds, different tannin sources (chestnut, tara, mimosa and gambier) were evaluated in order to cover a wide scope of possible effects on rumen function. An inclusion of 4% tannin extracts was supplemented in this experiment because the inclusion of 2.5 to 5% has been suggested as a moderate dosage threshold for the biological potency of tannins that would not impair nutrient intake and animal performance. It should be noted that these extracts are not 100% purified tannins and therefore contain other phenolic compounds in addition to tannins. The TPC in the control, chestnut, tara, mimosa and gambier-supplemented diets were 4.71, 24.32, 28.98, 25.27 and 11.67 g of tannic acid equivalents (TAE)/kg DM diet, respectively.

Dietary supplementation of tannin extracts did not negatively influence the ruminal metabolism and microbiome in lambs. Moreover, tara, mimosa and gambier extracts displayed specific antimicrobial activity against methanogens and protozoa linked to greenhouse methane production in ruminants. The result from this study provided a basis of establishing that dietary TPC of up to 29 g TAE/kg DM diet (equivalent to approximately 32 g of gallic acid equivalent (GAE)/kg DM diet) did not impair rumen function. Therefore,

by-products (cardoon meal, CGF, CDGS, WDGS and DCP) were included in the diets fed in subsequent experiments (Chapters 2 - 7) without exceeding the dietary TPC threshold (32 g GAE/kg DM diet).

Cardoon meal is a by-product retained after the extraction of oil from the seeds of cultivated cardoon (*Cynara cardunculus* var. *altilis*), a perennial herb native to the Mediterranean region and widespread in parts of Europe, Americas and Oceania. Approximately 75 - 80% of the cardoon seed biomass is retained as a by-product (cardoon meal) after oil extraction. Cardoon meal is a novel by-product that has not been evaluated for its feeding potential and effect on meat quality. In chapters 3 and 4, a feeding trial was conducted in which lambs were fed a concentrate diet containing either 15% dehydrated alfalfa or cardoon meal and subsequent effects on the rumen function and meat quality was examined. Dietary cardoon meal significantly increased the intake of unsaturated fatty acids (oleic acid and linoleic acid) and polyphenols but reduced the intake of α-linolenic acid, which is usually predominant in forages such as alfalfa (Chapter 3). Results indicated that dietary cardoon meal did not negatively influence ruminal fermentation, animal performance and carcass traits. However, the diet containing cardoon meal increased ruminal biohydrogenation through a modification of the rumen bacterial community. At the genus level, feeding cardoon meal mediated a specific shift from *Prevotella*, *Alloprevotella*, *Solobacterium* and *Fibrobacter* to *Ruminobacter*, suggesting that these bacterial genera may play an important role in ruminal biohydrogenation (RBH). This observation contradicts the hypothesis that high phenolic content in cardoon meal could reduce RBH compared to dehydrated alfalfa. However, it should be emphasized that inhibition of RBH by dietary phenolic compounds is inconsistent across studies due to the interaction of factors such as structural complexity, dosage, type of diets, animal species and physiological status, as well as characteristics inherent to the basal diet.

The main phenolic compounds in cultivated cardoon are flavonoids (luteolin, apigenin, quercetin etc.) and caffeoylquinic acid derivatives, of which chlorogenic acid and trans-cinnamic acid are abundant. Indeed, there is limited information on the efficacy of these compounds in inhibiting RBH. It has been shown that hydroxycinnamic acids including caffeic and chlorogenic acids can be metabolised by the gut microflora. This suggests that the phenolic compounds present in dietary cardoon meal might have been metabolised by the rumen microbiota, resulting in lack of efficacy of CMD in inhibiting RBH. Moreover, it was postulated that heat treatment involved in the production of dehydrated alfalfa, caused changes in the protein matrix surrounding the fat droplets resulting in a simultaneous reduction in ruminal proteolysis and RBH. Thus, the application of feed processing methods such as heat treatment may also prove useful to protect cardoon meal against ruminal protein degradation and biohydrogenation.

The increased ruminal biohydrogenation observed with dietary cardoon meal resulted in a lower concentration of potential health-promoting fatty acids (vaccenic and conjugated linoleic acid (CLA)) in lamb meat (Chapter 4). Despite the higher antioxidant activities exhibited by cardoon meal diet compared to the control, feeding cardoon meal did not enhance the oxidative stability of raw and cooked lamb meat stored aerobically at 4 ºC for up to 7 and 4 days, respectively. Again, this suggests that possible microbial metabolism of cardoon phenolics in the rumen may have prevented the deposition of phenolic antioxidants in the muscle, accounting for the lack of an effect on the oxidative stability of lamb meat. In order to evaluate the antioxidant effect of cardoon phenolics in a muscle model system, a phenolic-rich extract manufactured from cardoon meal was examined in ovine LTL homogenates subjected to $FeCl_3$/sodium ascorbate-induced lipid oxidation. Addition of 5% cardoon extract significantly increased (+114.3%) the TPC in LTL homogenates compared to the control. Similarly, the addition of 5% extract inhibited (-77.6%) lipid oxidation

relative to the control. Pearson's correlation analysis showed that TPC negatively correlated with TBARS values, suggesting that cardoon phenolics contributed to the inhibition of lipid oxidation in LTL homogenates. Results demonstrated the antioxidant potential of compounds present in cardoon meal and the potential use of cardoon extract as a natural additive for developing functional meat products with extended shelf-life characteristics.

In chapter 5, quality indices of beef from steers fed grass silage and concentrate supplements containing varying levels (0%, 25%, 50% or 75%) of dried CGF substituted for barley/soybean meal was examined. The inclusion of up to 75% CGF in the supplementary concentrate diet improved the fatty acid profile of beef by decreasing the proportion of C14:0 and increasing CLA, C18:3 *n*-3, C20:5 *n*-3 and C22:5 *n*-3 fatty acids. This study showed that increasing the inclusion levels of CGF resulted in corresponding increases in dietary unsaturated fatty acids (oleic acid and linoleic acid) which might have favourably altered the fatty acid composition of beef. In addition, the inclusions of CGF decreased dietary starch levels which could mitigate the impairment of ruminal digestion that may occur from supplementation of forage with cereals. Thus, it is possible that CGF exhibited a complementary effect in protecting dietary unsaturated fatty acids against ruminal biohydrogenation. Results from linear discriminant function analysis suggest that muscle fatty acid profiles may be a potential chemical marker for discriminating beef from cattle finished on a forage-based diet and concentrate supplements containing CGF. This approach may be relevant to authenticate meat produced from sustainable feeding systems that promote the substitution of by-products for cereal grains in animal diets.

CGF consists of bran and steep liquor that are rich sources of phenolic antioxidants such as protocatechuic acid, vanillic acid, *p*-coumaric acid, ferulic acid, sinapic acid and quercetin. Although CGF diets contained higher TPC than the control (barley/soybean meal), results from this study showed that feeding CGF did not enhance the antioxidant capacity and

oxidative stability of beef patties. Low bioavailability of these phenolic antioxidants may explain why phenolic compounds were not deposited in the muscle for antioxidant activity in this study. However, feeding CGF did not negatively influence the textural attributes and sensory eating quality of beef patties stored in modified atmosphere packs (MAP; 80% O_2:20% CO_2) for up to 7 days.

In Chapter 6, beef quality from steers fed grass silage and concentrate supplements containing either barley/soybean meal (control), 80% CDGS or 80% WDGS was examined. Dietary CDGS and WDGS increased the percentage of conjugated linoleic acids but tended to increase C18:3 *n*-3 and total polyunsaturated fatty acids (PUFA) relative to the control. Feeding CDGS or WDGS had a lesser impact in improving the fatty acid profile of beef compared to the effect observed with dietary CGF (Chapter 5). This suggests that even though distillers' grains with solubles (CDGS and WDGS) and CGF are by-products of grain milling (dry *vs*. wet), they may exhibit differential impact on beef quality traits. It is noteworthy that inclusion of both distillers' grains with solubles and CGF in concentrate diets resulted in corresponding increases in dietary unsaturated fatty acids and reduces dietary starch content. However, it may be hypothesised that higher phenolic content in dietary CGF, compared to CDGS or WDGS, might have contributed to the inhibition of RBH resulting in a greater accumulation of PUFA in beef.

Distillers' grains with solubles (DGS) is rich in phenolic compounds such as vallinic, caffeic, *p*-coumaric, ferulic and sinapic acids and this is evident with a slightly higher (+60%) TPC in CDGS and WDGS diets compared to the control diet. However, feeding DGS diets decreased the lipid and colour stability of beef patties stored in MAP, with CDGS exhibiting an intermediate effect between the control and WDGS. The retail shelf-life of beef patties from steers fed CDGS and WDGS may be limited to 7 days while that of the control diet may extend up to 10 days. Higher oxidation in beef from steers fed DGS may

be due to increased PUFA content which increases the amount of oxidizable substrate in the muscle. It is known that feeding DGS preferentially increases the PUFA content in the sarcoplasmic membrane which may enhance its instability and increases the susceptibility of muscle tissues to rapid oxidation. This is coupled with the effect of beef mincing employed in this study, which contributed to the disruption of muscle cell structure exposing labile lipid components to oxygen resulting in rapid lipid oxidation in beef. Results from this study also showed that dietary DGS did not enhance the antioxidant potential of muscle in order to prevent beef oxidation. Dietary supplementation of vitamin E could be an effective strategy to improve the lipid and colour stability of fresh beef from cattle fed DGS diets. In contrast to MAP, packaging methods such as vacuum packaging reduce the exposure of meat to oxygen and may be useful to improve the oxidative stability of fresh beef from cattle fed DGS diets. Nonetheless, feeding CDGS or WDGS did not negatively influence the sensory eating acceptance of beef patties stored in MAP for up to 7 days.

In Chapter 7, quality indices of beef from grass silage-fed steers offered concentrate supplements in which barley was replaced with varying levels (0%, 40% and 80%) of DCP was examined. The inclusion of up to 80% DCP improved the fatty acid profile of beef by increasing the percentage of CLA and PUFA in beef. The positive impact of dietary DCP on the beef fatty acid profile of ruminant meat or milk may be due to its effects on ruminal biohydrogenation attributed to a reduced dietary starch content and/or a high concentration of phenolic compounds.

The predominant phenolic compounds in citrus by-products are flavonoids such as naringin, hesperidin, quercetin, rutin and luteolin. Despite DCP diets containing up to 3-fold higher in TPC, dietary DCP did not enhance the TPC content of muscle which may explain the lack of differences between dietary treatments in terms of antioxidant capacity

and oxidative stability in beef. The lack of antioxidant effect of dietary DCP on muscle antioxidant capacity may be attributed to the lack of deposition of citrus polyphenols in the muscle tissues. However, it was observed that feeding 40% DCP reduced the lipid oxidation in aerobically-stored cooked beef patties. The reason for the antioxidant effect of 40% DCP in cooked beef is unclear considering that dietary DCP did not enhance the antioxidant capacity of muscle. However, heat treatment may enhance the liberation and activation of bound citrus phenolic compounds resulting in an increased antioxidant activity. Therefore, heating may have enhanced the antioxidant activity of DCP phenolics deposited in the muscle which could partly explain the discrepancy between the antioxidant effects observed in cooked beef patties from steers fed 40% DCP but not in fresh beef patties or muscle homogenates. Nonetheless, feeding up to 80% DCP did not negatively influence the texture characteristics and sensory eating acceptability of beef.

Altogether, these experiments showed that feeding PBP containing residual unsaturated fatty acids and phenolic compounds can improve the fatty acid profile of ruminant meat. However, this positive effect appears to depend on how the context of concentrate feeding strategies influences ruminal biohydrogenation. The increased ruminal biohydrogenation observed with the concentrate replacement of dehydrated alfalfa with cardoon meal highlights the constraints in replacing processed forages with PBP for improving the fatty acid composition of lamb meat. On the other hand, the replacement of cereal (barley) with PBP (CGF, CDGS, WDGS, DCP) in concentrate supplements appears to have a consistent effect in improving the fatty acid profile of beef from grass silage-fed steers. In addition to the unsaturated fat and/or phenolic compounds present in these PBP, their ability to decrease dietary starch level, and mitigate a decline in ruminal pH and impairment of forage digestion might have contributed in modulating the ruminal biohydrogenation. Furthermore, the experiments showed that feeding cardoon meal, CGF, CDGS, WDGS and

DCP did not enhance lamb or beef oxidative stability. These results highlight the inconsistency of phenolic-rich PBP to deposit antioxidant phenolics in muscle tissues, irrespective of the type of phenolic compounds present or concentrate feeding strategy employed.

8.2. CONCLUSIONS

The general conclusions from this thesis are as follows:

- Feeding strategies that increase the utilization of PBP in animal diets can improve the environmental impacts of ruminant meat production through a reduction of greenhouse gas emissions.
- Feed transport and processing represent significant hotspots for harnessing environmental benefits from the utilization of plant by-products as inedible feedstuffs.
- Cardoon meal can be included at 15% level in a concentrate-based diet of lambs without negatively influencing animal performance and carcass characteristics, and with minimal impact on meat quality traits.
- Phenolic-rich extract from cardoon meal extract exhibited a potent antioxidant activity against lipid oxidation in a muscle model system, demonstrating its potential as a natural additive for developing functional meat products with extended shelf-life characteristics.
- The replacement of barley/soybean meal with up to 75% CGF in a concentrate supplement increased the proportion of health-promoting PUFA and CLA in beef without negatively influencing the oxidative stability and sensory acceptance properties.
- Barley/soybean meal can be substituted with 80% CDGS or WDGS in a supplementary concentrate, with a positive impact on the fatty acid profile of beef but negative effects on the retail shelf-life of beef should be considered.
- The inclusion of 80% DCP substituted rolled barley in a supplementary concentrate with an increase in the beneficial PUFA and CLA in beef without negatively influencing the oxidative stability and eating quality of beef.

- Antioxidant phenolics present in cardoon meal, CGF, CDGS, WDGS and DCP seems not to have been deposited in the muscle and therefore, does not enhance the oxidative stability of lamb meat or beef.

8.3. FUTURE RESEARCH

This thesis provides a basis for identifying future research direction that would enhance the application of plant by-products (PBP) as eco-friendly inedible feedstuffs in concentrate feeding strategies for improving ruminant meat quality. Some of these future research work include:

- Comprehensive life-cycle assessments should be conducted to compare the environmental impacts of PBP and other human-inedible feed resources with reference to specific feeding conditions/production systems in different countries.
- Characterize the phenolic composition of cardoon meal, CGF, DGS and DCP using advanced analytical techniques such as high-performance liquid chromatography with diode-array detection and mass spectrometry.
- Investigate the microbial metabolism of PBP phenolic compounds in the rumen vis-à-vis the mechanism of absorption of phenolics and their metabolites into the muscle tissue to provide a better understanding on their bioavailability and antioxidant activity in the meat.
- Assess the application of feed processing methods such as heat treatment for protecting unsaturated fats in cardoon meal against ruminal biohydrogenation and the consequent effect on the fatty acid profile of ruminant meat.
- Culture-based microbiological methods can be coupled with transcriptomics techniques to study the functional role of *Prevotella, Alloprevotella*, *Solobacterium, Fibrobacter* and *Ruminobacter* in the synthesis of ruminal biohydrogenation intermediates in relation to feeding conditions such as dietary PBP.
- Evaluate the use of cardoon extract as a natural ingredient for enhancing the shelf-life and quality parameters of meat products.

- Explore the potential of biological and chemical markers (such as fatty acids and bioactive compounds) in meat as effective tools for discriminating meat from ruminants fed certain threshold of PBP, in order to facilitate the authentication of meat produced from sustainable feeding systems that promote the substitution of edible cereals with PBP.
- Assess the application of strategies such as dietary supplementation with vitamin E and vacuum packaging method for extending the shelf-life of meat from ruminants fed concentrate diet containing DGS.
- Investigate the use of nutritional strategies such as PUFA-enrich supplements (oil and marine algae) that could maximize the effect of DCP in concentrate diets on the ruminal synthesis of vaccenic acid and the consequent effect on endogenous desaturation into bioactive CLA.

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APPENDIX

Table S1.1. Feed formulation used for the evaluation of environmental impacts of feeding scenarios (FS) based on increased use of plant by-products (PBPs) in ruminant rations as simulated in the Global Livestock Environmental Assessment Model (GLEAM)

	Beef cattle herds								**Sheep meat and goat meat herds**			
	Adult females and, adult males and replacement animals				**Fattening animals**				**All animal categories[1]**			
Feed components (%)	**FS default**	**FS1**	**FS2**	**FS3**	**FS default**	**FS1**	**FS2**	**FS3**	**FS default**	**FS1**	**FS2**	**FS3**
Fresh grass	37.0	27.0	27.0	17.0	22.0	17.0	14.0	10.0	25.8	20.8	10.8	5.8
Hay/silage from grass	37.3	37.3	27.3	27.3	18.0	18.0	16.0	15.0	34.0	29.0	29.0	24.0
Hay/silage from alfalfa	2.8	2.8	2.8	2.8	4.0	4.0	4.0	4.0	1.5	1.5	1.5	1.5
Silage from whole maize plant	0.9	0.9	0.9	0.9	4.0	4.0	4.0	4.0	0.0	0.0	0.0	0.0
Silage from whole grain plant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	1.4	1.4	1.4
Total roughage	78.0	68.0	58.0	48.0	48.0	43.0	38.0	33.0	62.7	52.7	42.7	32.7
Maize	1.2	1.2	1.2	1.2	28.0	28.0	28.0	28.0	1.2	1.2	1.2	1.2
Grains	0.8	0.8	0.8	0.8	14.0	14.0	14.0	14.0	16.1	16.1	16.1	16.1
Total grains	2.0	2.0	2.0	2.0	42.0	42.0	42.0	42.0	17.3	17.3	17.3	17.3
By-products from soy	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.5	0.5	0.5	0.5
By-products from cottonseed	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Molasses	1.0	1.0	2.0	2.0	0.5	1.0	1.0	1.0	0.5	1.0	1.5	2.0
Maize gluten meal	2.0	3.0	4.0	4.0	1.0	1.5	2.0	2.0	2.0	3.0	3.0	4.5
Maize gluten feed	1.5	2.5	3.5	5.5	0.5	1.5	2.0	3.0	2.0	2.5	3.0	4.0
Dry-by products from grain industries	8.0	13.0	18.0	23.0	5.0	7.0	10.0	12.0	10.0	15.0	20.0	23.0
Wet-by products from grain industries	7.0	10.0	12.0	15.0	3.0	4.0	5.0	7.0	5.0	8.0	12.0	16.0
Total PBP	20.0	30.0	40.0	50.0	10.0	15.0	20.0	25.0	20.0	30.0	40.0	50.0
Total ration	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[1]Animal categories include adult females, adult males and replacement animals, and fattening animals.

Table S2.1. List of primers used for quantitative PCR and ion torrent next generation sequencing (NGS)

Target gene and group	References	Primer set	Sequence (5` to 3` direction)	T[a]	Amplicon (bp)
Quantitative PCR					
18S rDNA for total protozoa	Sylvester, Karnati, Yu, *et al.* (2004)	PSSU-316f PSSU-539R	F: GCTTTCGWTGGTAGTGTATT R: CTTGCCCTCYAATCGTWCT	55	223
16S rRNA for total bacteria	Maeda, Fujimoto, Haruki, *et al.* (2003)	Univ16S_1048-1067 Univ16S_1175_1194	F: GTGSTGCAYGGYTGTCGTCA R: ACGTCRTCCMCACCTTCCTC	61	150
[a]*mcrA* gene for methanogenic archaea	Denman, Tomkins and McSweeney (2007)	qmcrA-f qmcra-r	F: TTCGGTGGATCDCARAGRGC R: GBARGTCGWAWCCGTAGAATCC	56	140
18S rRNA and ITS1 for total anaerobic fungi	Denman and McSweeney (2006)	Denfun f Denfun r	F: GAGGAAGTAAAAGTCGTAACAAGGTTTC R: CAAATTCACAAAGGGTAGGATGATT	62	120
Ion torrent NGS					
Bacterial Adaptors			F: CCATCTCATCCCTGCGTGTCTCCGACTCAG R: CCTCTCTATGGGCAGTCGGTGAT		
Bacterial primers	Spear, Sikaroodi, Zariffard, *et al.* (2008)	27F 357R	F: AGAGTTTGATCMTGGCTCAG R: CTGCTGCCTYCCGTA	58	348
Methanogens adaptors			F: CCATCTCATCCCTGCGTGTCTCCGACTCAG R: CCTCTCTATGGGCAGTCGGTGAT		
Methanogens primers	Wright and Pimm (2003) Turner, Pryer, Miao, *et al.* (1999)	86F 519R	F: GCTCAGTAACACGTGG R: GWATTACCGCGGCKGCTG	58	433

[a]mcrA gene: methyl-coenzyme M reductase alpha subunit gene

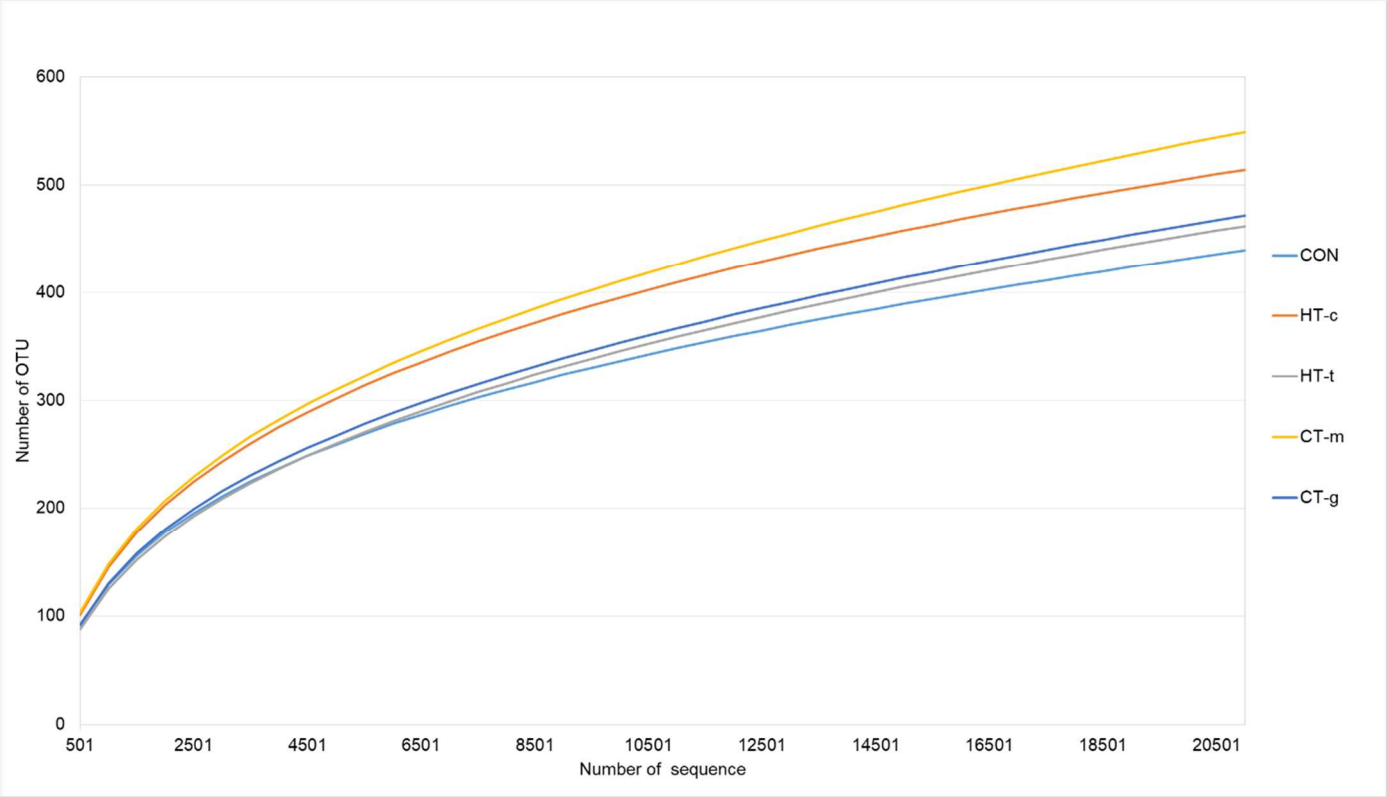


Figure S2.1. Rarefaction curves illustrating the sequencing depth of the rumen bacterial communities in lambs fed different tannin extracts. Dietary treatments are: CON, control; HT-c, chestnut hydrolysable tannin; HT-t, tara hydrolysable tannin; CT-m, mimosa condensed tannin; CT-g, gambier condensed tannin.

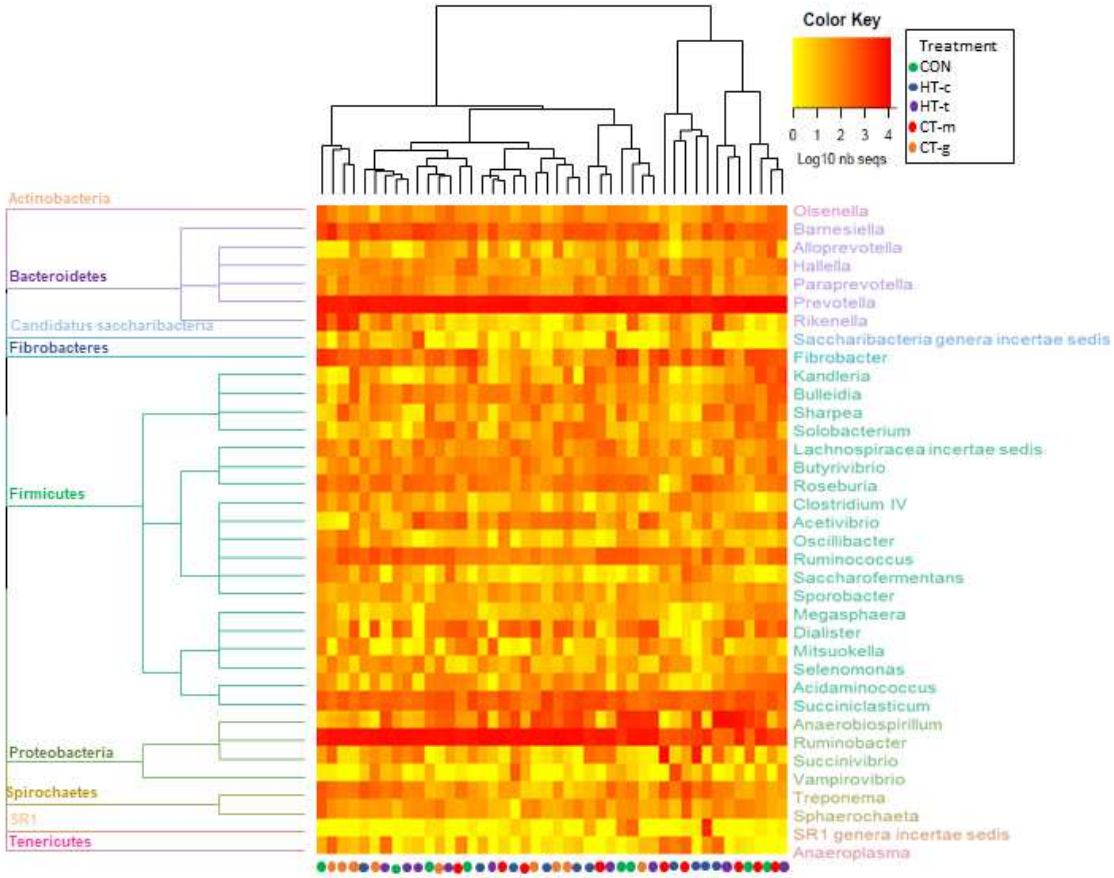


Figure S2.2. Heat map illustrating the abundance of bacterial genera in the rumen of lambs fed a control diet (CON) and four dietary treatments supplemented with different tannin extracts. Dietary treatments are: CON, control; HT-c, chestnut hydrolysable tannin; HT-t, tara hydrolysable tannin; CT-m, mimosa condensed tannin; CT-g, gambier condensed tannin. Bacterial genera with relative abundance less than 0.1% were discarded and a dendrogram was created based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering of the Bray-Curtis distances.

Table S2.2. Correlation coefficients of canonical correspondence analysis indicating relationship between rumen bacterial community structure, fermentation and microbial variables

	R^2	*P*-value
Rumen pH	0.085	0.265
Protein content	0.351	0.002
NH_3-N	0.007	0.897
Total volatile fatty acids	0.047	0.434
Acetate	0.156	0.058
Propionate	0.036	0.475
Iso-butyrate	0.038	0.528
Butyrate	0.579	0.001
Iso-valerate	0.033	0.599
Valerate	0.073	0.239
Acetate/propionate ratio	0.793	0.001
Total bacteria	0.033	0.453
Total archaea	0.105	0.152
Total fungi	0.060	0.344
Total protozoa	0.053	0.438
Archaea/bacteria ratio	0.113	0.109
Bacterial diversity	0.415	0.001
Methanogen diversity	0.047	0.444
Lipase activity	0.063	0.218
Carboxymethyl cellulase activity	0.020	0.745
Xylanase activity	0.064	0.286
Amylase activity	0.152	0.075

Table S2.3. Correlation coefficients of canonical correspondence analysis indicating relationship between rumen methanogen community structure, fermentation and microbial variables

	R^2	*P*-value
Rumen pH	0.017	0.744
Protein content	0.002	0.959
NH_3-N	0.036	0.469
Total volatile fatty acids	0.014	0.776
Acetate	0.033	0.532
Propionate	0.005	0.905
Iso-butyrate	0.006	0.905
Butyrate	0.028	0.550
Iso-valerate	0.035	0.461
Valerate	0.039	0.446
Acetate/propionate ratio	0.045	0.316
Total bacteria	0.149	0.062
Total archaea	0.090	0.147
Total fungi	0.078	0.196
Total protozoa	0.046	0.404
Archaea/bacteria ratio	0.033	0.470
Bacterial diversity	0.033	0.508
Methanogen diversity	0.015	0.729
Lipase activity	0.003	0.933
Carboxymethyl cellulase activity	0.075	0.206
Xylanase activity	0.044	0.399
Amylase activity	0.146	0.047

Table S3.1. Correlation coefficients of canonical correspondence analysis indicating relationship between rumen bacterial community structure, fermentation and microbial variables

Fatty acids	**R^2**	***P*-value**
4:0	0.034	0.847
5:0	0.144	0.398
6:0	0.039	0.649
8:0	0.015	0.924
9:0	0.351	0.085
10:0	0.226	0.313
11:0	0.107	0.567
12:0	0.150	0.480
cis-9 12:1	0.020	0.866
13:0	0.039	0.530
14:0	0.212	0.286
cis-9 14:1	0.097	0.675
trans-9 14:1	0.058	0.695
iso 14:0	0.060	0.689
15:0	0.015	0.913
iso 15:0	0.080	0.718
anteiso 15:0	0.049	0.762
16:0	0.029	0.769
cis-7 16:1	0.236	0.190
cis-9 16:1	0.090	0.728
trans-7 16:1	0.227	0.327
iso 16:0	0.114	0.639
17:0	0.092	0.301
cis-9 17:1	0.116	0.462
iso 17:0	0.046	0.745
anteiso 17:0	0.232	0.226
18:0 SA[1]	0.181	0.328
cis-6 18:1	0.168	0.397
18:1 *n*-9 OA[1]	0.233	0.338
cis-11 18:1	0.066	0.774
cis-12 18:1	0.101	0.632
cis-13 18:1	0.182	0.443
cis-14 18:1	0.333	0.127
cis-16 18:1	0.544	0.009
trans-5 18:1	0.124	0.634
trans-6+8 18:1	0.294	0.165
trans-9 18:1	0.0128	0.966
trans-10 18:1	0.366	0.038
trans-11 18:1 VA[1]	0.171	0.464
trans-12 18:1	0.133	0.416
18:2 *n*-6 LA[1]	0.317	0.083
cis-9 trans-11 18:2 CLA	0.003	0.943
cis-9 trans-12 18:2	0.201	0.267
trans-8 *cis*-10 18:2 CLA	0.356	0.081
trans-8 *cis*-13 18:2	0.001	0.990
trans-9 *cis*-12 18:2	0.009	0.968
trans-9 *cis*-13 18:2	0.128	0.611
trans-10 *trans*-12 18:2 CLA	0.200	0.229
trans-11 *cis*-15 18:2	0.111	0.510

18:3 *n*-6	0.067	0.761
18:3 *n*-3 ALA[1]	0.458	0.022
20:0	0.043	0.803
cis-11 20:1	0.334	0.075
trans-11 20:1	0.456	0.091
20:4 *n*-6	0.255	0.234
20:5 *n*-3 EPA[1]	0.060	0.642
21:0	0.084	0.386
22:0	0.270	0.195
cis-13 22:1	0.265	0.235
22:2 *n*-6	0.056	0.789
22:4 *n*-6	0.137	0.469
22:5 *n*-6	0.029	0.94
22:5 *n*-3 DPA[1]	0.007	0.971
22:6 *n*-3 DHA[1]	0.203	0.341
23:0	0.098	0.577
24:0	0.078	0.490
Σ SFA	0.195	0.258
Σ MUFA	0.291	0.146
Σ PUFA	0.302	0.107
Σ OBCFA[2]	0.086	0.751
Σ trans 18:1	0.272	0.191

[1]SA: stearic acid; OA: oleic acid; LA: linoleic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; OBCFA: Odd-and branched-chain fatty acids

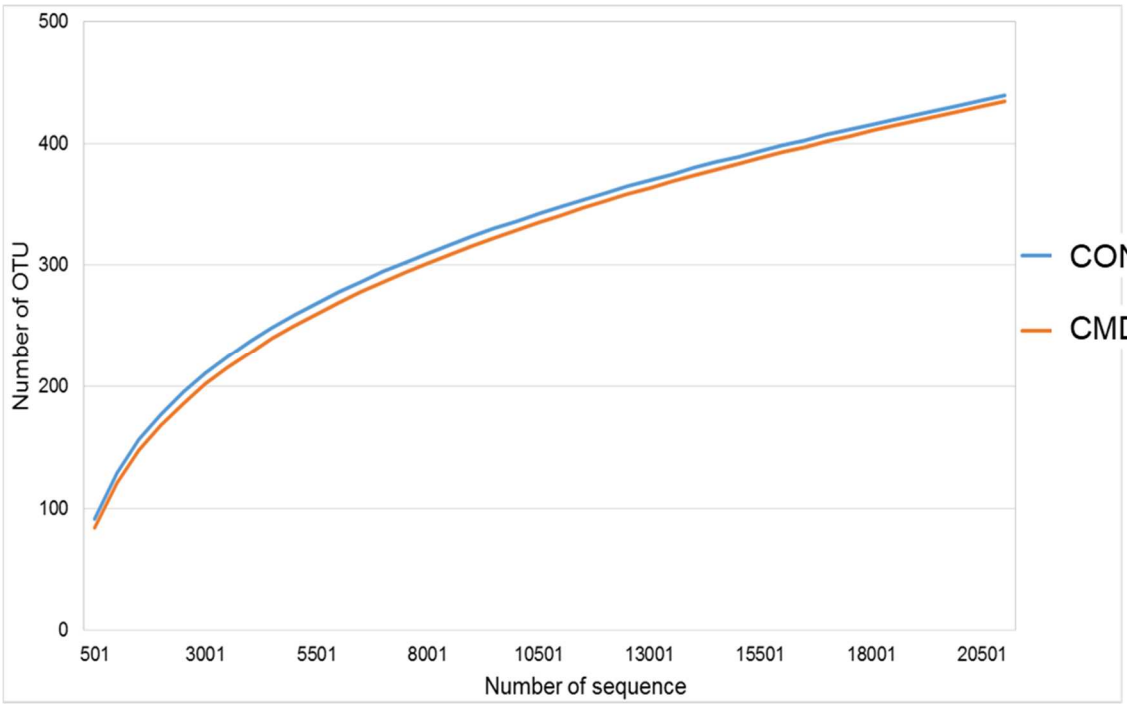


Figure S3.1. Rarefaction curves showing the sequencing depth of the rumen bacterial communities in lambs fed control diet (CON) and cardoon meal diet (CMD).

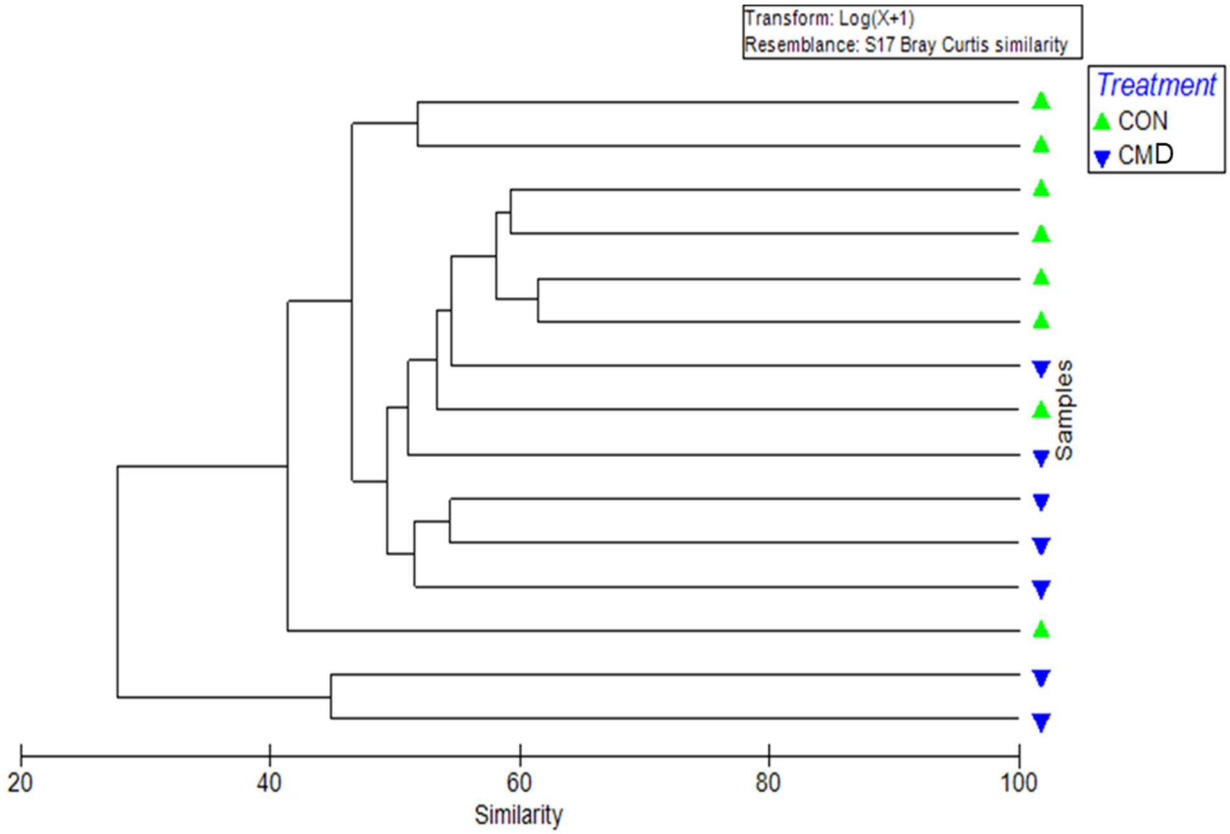


Figure S3.2. Dendrogram plot of hierarchical cluster analysis of rumen samples obtained from lambs fed a control diet (CON) and cardoon meal diet (CMD). Distance between clusters was calculated with group average of resemblance matrix created from log-transformed OTU data sets.

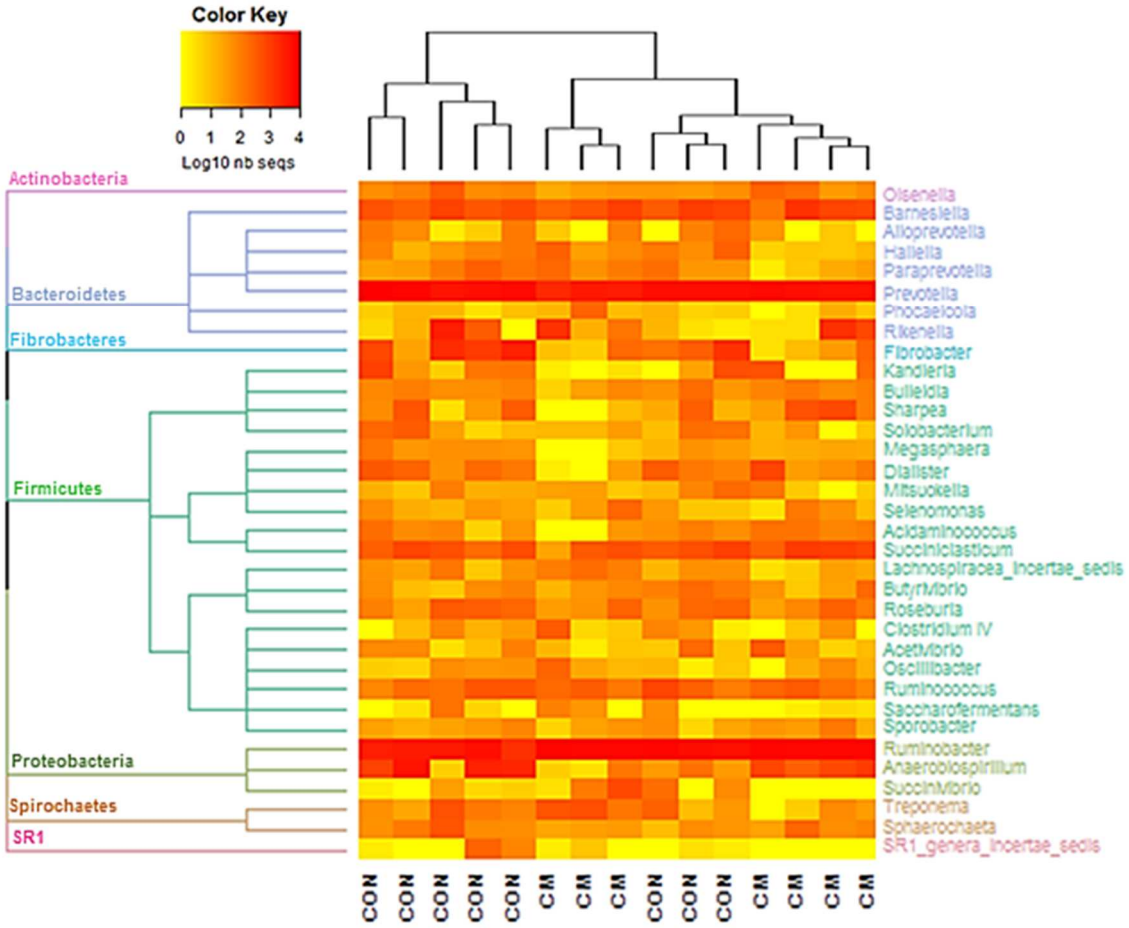


Figure S3.3. Heat map describing the abundance of bacterial genera in the rumen of lambs fed control diet (CON) and cardoon meal diet (CMD). Bacterial genera with relative abundance less than 0.1% were discarded and a dendrogram was created based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering of the Bray-Curtis distances.