The potential of quercetin to improve growth performance and meat quality in rabbits

by

Megan Kim North

Dissertation presented for the degree of

Doctor of Philosophy

(Agricultural Sciences)

at

Stellenbosch University

Department of Animal Sciences, Faculty of AgriSciences

Supervisor: Prof L. C. Hoffman (Department of Animal Sciences, Stellenbosch University, South Africa)

Co-supervisor: Prof A. Dalle Zotte (Department of Animal Medicine, Production and Health, University of Padova Agripolis, Italy)

April 2019

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 9 January 2019

ABSTRACT

Flavonoids are naturally-occurring bioactive compounds that may improve livestock production and product quality. Unfortunately, while their antioxidant, antimicrobial and other activities have been demonstrated, there has been limited research on their practical use as a livestock dietary supplement. This study investigated the effects of quercetin dihydrate (0 or 2 g/kg feed) on the growth performance and meat quality of growing New Zealand White rabbits.

Sixty-six rabbits (31 males, 35 females) were fed control (Ctrl, 34 rabbits) or quercetin-supplemented (Qrc, 32 rabbits) feeds from weaning (5 weeks) until slaughter. The growth, feed intake and feed conversion ratio (FCR) were measured, and serum hormone levels were determined at 11 weeks old. Sixteen males and 16 females were slaughtered at 12 weeks old; carcass and meat quality traits were measured and the fatty acid composition of the caecotrophes, dissectible fat, loin meat and hindleg meat were determined using GC-FID. Fifteen males and 19 females were slaughtered at 13 weeks old; caecal contents were collected from 12 rabbits for the 16S™ metagenome sequencing of the microbiome, and the loins were minced and stored for 1, 3 or 5 days at 3.2 °C under oxygen-permeable wrapping to test the shelf-life.

Quercetin-supplementation did not improve live performance, with Qrc rabbits only tending ($P \leq 0.10$) to have higher overall FCRs, smaller sex-differences in growth and FCR, and higher free triiodothyronine levels. Quercetin-supplemented rabbits also only had higher proportions of some *Firmicutes* families and *Anaerofustis* and lower proportions of *Roseburia*, *Oscillibacter* and *Ruminococcus albus* in the caecal microbiome. This limited effect may have been due to the aglycone being absorbed prior to the caecum. Strong correlations between the composition of the microbiome and live performance traits were found, supporting further research on this topic.

Quercetin-supplemented rabbits had higher hindleg meat:bone ratios due to lighter bones, which could increase meat yields but could also cause bone-breakage problems. This was contrary to previous findings that flavonoids decrease bone demineralisation, but may have been due to its effects on the connective tissue, which could have also caused the higher skin weight found. The largest effect of supplementation was on the loin fatty acid (FA) composition, increasing C18:3n-6, C20:3n-6, C20:3n-3 and C20:4n-6, and decreasing C20:2n-6, among others, thereby decreasing the n-6:n-3 ratio and improving the nutritional quality of the meat. This suggested an interaction between quercetin and endogenous lipid metabolism, which may have been influenced by the FA composition of the diet. The caecotrophes, dissectible fat and hindleg FAs were unchanged by quercetin. Quercetin-supplemented rabbits' meat samples had lower day one concentrations of hexanal (an oxidation indicator), but no difference in oxidation (TBARS and FRAP) or microbial status during the shelf-life study. Dietary quercetin therefore did not improve the shelf-life of rabbit meat.

Further research on the effects of quercetin on lipid metabolism should be done, particularly testing different inclusion levels and dietary FA compositions. The effects on bone-integrity, whole-carcass meat:bone ratio and

pelt/skin quality should also be looked into. While some sex differences were found in these studies, they did not meaningfully affect production or product quality.

OPSOMMING

Flavonoïde is natuurlike, bio-aktiewe komponente wat die produksie en die produkkwaliteit van vee mag verbeter. Die antioksidant en antimikrobiese aktiwiteit, asook ander voordelige eienskappe van flavonoïde, is reeds breedvoerig bewys in die literatuur, maar tog is daar baie min beskikbare inligting oor die praktiese implikasies van die insluiting daarvan in veevoeding. Daarom fokus hierdie studie daarop om die effek van kwersetien dehidraat (0 of 2 g/kg voeding) op die groei en vleiskwaliteit van Nieu-Seelandse Wit konyne te ondersoek.

Ses en sestig konyne in totaal (31 manlik en 35 vroulik) is gebruik, waarvan 34 van die konyne die kontrole voer en 32 konyne die kwersetien-aangevulde voer ontvang het, vanaf speen ouderdom (5 weke) tot slag. Die groei en voer-inname is gemeet, en die voer omset verhouding (VOV) is bereken, tesame met die bepaling van die serum hormoon vlakke op 11 weke ouderdom. Daar is 16 van elk manlike en vroulike diere geslag op 12 weke ouderdom, waarna die karkas- en vleiskwaliteitseienskappe bepaal is. Die vetsuursamestelling van die cecotropes, dissekteerbare vet en die lende en agterbeen vleis is ook bepaal op ‘n GC-FID toestel. Op 13 weke ouderdom is 15 manlike en 19 vroulike konyne geslag en die inhoud van 12 van die konyne se sekums is ingesamel om sodoende die 16S^{TM} metagenoom volgorde van die mikrobioom te bepaal. Die lendevleis is gemaal en toe gestoor vir 1, 3 of 5 dae by 3.2 °C onder suurstof-deurlaatbare, plastiek verpakking om sodoende die rakleeftyd te toets.

Die konyne wat die kwersetien aanvulling ontvang het, het nie ‘n verbeterde lewendige gewig getoon nie. Daar was slegs tendense ($P \leq 0.10$) van hoër algehele VOV, kleiner geslags verskille in groei en die VOV, asook hoër vrye triiodothyronine vlakke. Die mikrobiome het ook slegs hoër proporsies van sommige van die *Firmicutes* families en *Anaerofustis* en laer proporsies van *Roseburia*, *Oscillibacter* en *Ruminococcus albus* gehad. Die beperkte effek van kwersetien mag wees as gevolg van die absorbsie van die aglycone voor die cecum. Sterk korrelasies is gevind tussen die samestelling van die mikrobioom en die lewendige gewigseienskappe, wat die noodsaaklikheid vir verdere navorsing in terme van hierdie onderwerp versterk.

Die kwersetien aanvulling het gelei na ‘n hoër agterbeen vleis:been verhouding as gevolg van ‘n laer been gewig in die konyne wat dié voer ontvang het. Dit mag lei na ‘n verhoogte vleis opbrengs, maar kan ook die negatiewe gevolg hê van die breking van die agterbene wat makliker plaasvind. Hierdie bevinding is in teenstelling met vorige navorsing wat getoon het dat flavonoïde die de-miniraliserings proses verlaag. Dit mag wees dat die flavonoïde ‘n effek op die bindweefsel uitoefen wat ook kon gelei het na die hoër vel gewig van hierdie konyne. Die grootste effek van die aanvulling was egter op die samestelling van die rugstring spier se vetsuur inhoud. Die kwersetien in die voeding het gelei na toenemende vlakke van C18:3n-6, C20:3n-6, C20:3n-3 en C20:4n-6 vetsure, en ‘n laer C20:2n6 inhoud. Die n-6:n-3 verhouding is dus laer in die lende spier van hierdie konyne, wat ‘n positiewe effek op die voedingswaarde van die vleis het. Hierdie bevinding dui op ‘n interaksie tussen kwersetien en die endogene lipied metabolisme, en die feit dat dit moontlik beïnvloed is deur die vetsuursamestelling van die dieet. Die cecotropes, vet en vetsure van die agterbeen is onveranderd gelaat deur die kwersetien in die dieet. Die monsters van die konyne op die kwersetien aanvulling het laer vlakke van hexanal (‘n indikator van oksidasie) op

dag een getoon, maar geen verskil is opgemerk in die oksidasie (TBARS en FRAP) of die mikrobiese stand tydens die rakleeftyd studie. Die teenwoordigheid van kwersetien in die dieet het dus nie die rakleeftyd van die konynvleis verbeter nie.

Verdere navorsing op die invloed van kwersetien op lipied metabolisme is nodig om veral die effek van verskillende vetsure in die voer, asook verskillende insluitingskoerse, te ondersoek. Die invloed op been integriteit, heel karkas vleis:been verhouding, en vel kwaliteit is verdere faktore wat aangespreek moet word. Terwyl daar wel sommige geslagsverskille gevind is in die studie, het dit nie ‘n betekenisvolle invloed getoon op die produksie en produkkwaliteit nie.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following institutions for funding my research and supporting my studies:

- The National Research Foundation of South Africa, for awarding me the Scarce Skills Doctoral scholarship from 2016 to 2018.
- Stellenbosch University, for awarding me the Merit Bursary in 2016 and 2017.
- The South African Research Chairs Initiative of the Department of Science and Technology and the National Research Foundation of South Africa (UID: 84633), for funding my research. Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the National Research Foundation does not accept any liability in this regard.

I would like to thank the following people, in no particular order, for their support and assistance during the completion of my PhD:

- For his guidance and support of my project, as well as for allowing me the freedom to work independently, my supervisor Prof Louwrens Hoffman.
- For her insight during the writing of my thesis, my co-supervisor Prof Antonella Dalle Zotte.
- For her assistance with planning, ordering and lab analysis, and putting up with many last-minute panics about equipment, Ms Lisa Uys.
- For assistance with data and sample collection, the students of the Animal and Food Science Departments of Stellenbosch University. I mention no names for fear of unfairly omitting someone.
- For performing the statistical analysis of my data, Prof Martin Kidd from the Centre for Statistical Consultation at Stellenbosch University.
- For their assistance in the laboratory and constant good cheer, Ms Beverly Ellis, Mr Michael Mlambo and Ms Janine Booyse.
- For helping me with everything from building rabbit cages to working in the meat lab and helping close the -80 °C freezer, Mr Jonas Christiaan.
- For his assistance, patience and perseverance with collecting blood samples, Mr Noel Markgraaff.
- For not only performing the 16S™ metagenome sequencing of the caecal microbiome, but also going out of her way to explain the concepts and system to me, Ms Alvera Vorster, Senior Analyst of the DNA Sequencing Unit of the Central Analytical Facility of Stellenbosch University.
- For her assistance with the sample preparation and data analysis of the volatile compound profile, Dr Jeannine Marais.

- For their assistance with the analysis of the fatty acid composition and volatile compound profile, Mr Lucky Mokwena and Mr William Arries of the GC-MS Unit of the Central Analytical Facility of Stellenbosch University.
- For their assistance with determining the microbial status of the meat samples, Prof Pieter Gouws and Ms Nompumelelo Shange.
- For trusting me with their rabbits and helping me survive learning to be a rabbit farmer, as well as generously sharing their many years of experience and wisdom, Ms Sally Geibelmann and Mr Gavin Grgurin, as well as Ms Karoline Steenekamp.
- For allowing me the use of a bit of his farm, and occasionally his staff, as well as giving me pep talks when I thought I was failing as a 'farmer', Mr John Morris.
- For helping me keep my rabbits happy and alive, Mr Isaac Lukas.

I would also like to give a few people who not only helped this PhD happen (hopefully), but also kept me (somewhat) sane through the process, a special mention:

- Dr Tersia Needham, thank you for being a friend when I needed one, and for listening to many angry rants and periods of serious self-doubt. Doing this without you would have been a long, lonely process indeed, and I look forward to exchanging ideas and emotions with you for many years to come.
- Dr Greta Geldenhuys, thank you for mentoring me during my Master's and continuing to serve as a touchstone throughout my PhD.
- Lee, thank you for reminding me that there is a world outside, and that I am worth more than my work alone. Smithy and I look forward to sharing our lives with you for a long time yet.
- My parents, thank you for living with me through the anxiety and depression that were the main outcomes of this process. Hopefully in the end the nett outcome will be a positive one.

NOTES AND PUBLICATIONS

This thesis is presented in the format prescribed by the Department of Animal Sciences, Stellenbosch University. As each chapter has either been published or prepared for publication as a journal article, and was thus written as an individual entity, some repetition between chapters was unavoidable. Language, style and referencing are in accordance with the specifications of the journal *Meat Science*.

Results from this dissertation have been published in or submitted for publication in the following journals:

- North, M. K., Dalle Zotte, A. & Hoffman, L. C. (2018). Effect of quercetin supplementation on the growth, feed efficiency and serum hormone levels of New Zealand White rabbits. *South African Journal of Animal Science*, *48*(6), 1128 – 1139. (Chapter 3)
- North, M. K., Dalle Zotte, A. & Hoffman, L. C. (2018). The effects of quercetin supplementation on New Zealand White grower rabbit carcass and meat quality — A short communication. *Meat Science, 145*, 363 – 366. (Chapter 5)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

% Prob	Percentage probability of identification, based on mass spectra
ADF	Acid detergent fibre
ADG	Average daily gain
ADL	Acid detergent lignin
AI	Atherogenic index
AMSA	American Meat Science Association
AOAC	Association of Official Analytical Chemists
APC	Aerobic plate count
C*	Chroma
CC	Chilled carcass
CFU	Colony-forming units
CHD	Coronary heart disease
Ctrl/C	Control group rabbits
DM	Dry matter
EC	*E. coli*/Coliform plate count
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
F	Female
FA	Fatty acid
FAME	Fatty acid methyl ester
FCR	Feed conversion ratio
Fe^{2+} eq	Ferrous equivalent
FI	Daily feed intake
FRAP	Ferric reducing antioxidant power
fT3	Free triiodothyronine
fT4	Free thyroxine
GC	Gas-chromatograph(y)
GC-FID	Gas-chromatograph with flame-ionisation detector
GH	Somatotropin
GIT	Gastrointestinal tract
GM	M. *gluteus medius*
h/H	Ratio of hypocholesterolemic fatty acids to hypercholesterolemic fatty acids
H°	Hue angle

HC	Hot carcass
HL	Hindleg
HPA	Hypothalamo-pituitary-adrenal
hSHBG	Human plasma sex-hormone binding globulin
IMF	Intramuscular fat content
LAB	Lactic acid bacteria
LD	M. *longissimus dorsi*
LDPE	Low-density polyethylene
LSD	Least significance difference
LTL	Loin/M. *longissimus thoracis et lumborum*
LW	Live weight
M	Male
M:B	Meat to bone ratio
MDA	Malondialdehyde
MSM	Methylsulfonylmethane
MTBE	*Tert*-methyl butyl ether
MUFA	Monounsaturated fatty acid
NDF	Neutral detergent fibre
OTU	Operational taxonomic unit
PCA	Principle component analysis
pH_u	Ultimate pH
PUFA	Polyunsaturated fatty acid
Qrc eq	Quercetin equivalent
Qrc/Q	Quercetin-supplemented rabbits
RC	Reference carcasses
REML	Restricted maximum likelihood
RI	Retention index
RT	Retention time
SEM	Standard error of the mean
SFA	Saturated fatty acids
SM	M. *semimembranosus*
SPME	Solid-phase microextraction
SPME-GC-MS	Solid-phase microextraction with gas chromatography–mass spectrometry
SW	Slaughter weight
Σ	Sum

TAC	Total antioxidant capacity
TAM	Total aerobic mesophilic bacteria
TBARS	Thiobarbituric acid reactive substances
TI	Thrombogenic index
TMP	1,1,3,3-tetramethoxypropane
T3	Triiodothyronine
T4	Thyroxine
UPC^2-MS/MS	Ultra-performance convergence chromatography tandem mass spectrometry
USA	United States of America
VEPAC	Variance, estimation and precision
VFA	Volatile fatty acid
WBSF	Warner Bratzler shear force
WHC	Water-holding capacity

CHAPTER 1:

General introduction

The pressure on farmers to increase production and production efficiency is nothing new, and it has ever been the goal of agricultural researchers to help farmers achieve this. However, this goal has become somewhat more complex in recent years, largely as a result of the increasing role played by the consumer, as well as growing concerns about the consequences of a gain-at-all-costs approach to food production. Many modern consumers in the Western world are no longer only satisfied with a reasonable price for a reasonable product; they are also mindful of how their food is produced, and the welfare of the animals involved (Vanhonacker & Verbeke, 2014). In addition, they are concerned about the health implications of the food they eat, in terms of possible contamination with pathogens or residues (Verbeke & Viaene, 2000), and in many areas this has led to bans or restrictions on previously utilised methods of maximising production efficiency and herd health.

Two examples of this increase in regulation are the hormone-based and antibiotic growth promoters. The use of hormone-based growth promoters has been prohibited in the European Union (EU) since 1981, due to concerns about hormone residues in food products, and the effects these could have on consumers' health (Council of the European Union, 1981; Scientific Committee on Veterinary Measures relating to Public Health, 1999). While the use of these products is still allowed in many other countries, including the United States of America (USA), Canada and South Africa, the EU regulations apply not only to domestic production, but also to imported products, and therefore have an international impact (Scientific Committee on Veterinary Measures relating to Public Health, 1999; Serratosa *et al*., 2006). A similar situation exists for the use of antibiotic growth promoters. Antibiotics have long been used prophylactically in livestock to increase feed efficiency and reduce the prevalence of subclinical diseases. However, as the public health crisis of antibiotic resistance has become more apparent and wide-spread, the links between injudicious antibiotic use and the development of resistance have become impossible to ignore (Landers, Cohen, Wittum & Larson, 2012). This led to the EU banning the use of all growth-promoting antibiotics in member countries from 2006 (European Commission, 2005), and similar recommendations being made by the World Health Organisation (Ferber, 2003). Although the control of these products is not, as yet, as strict in other regions, official recommendations in the USA, Canada and Australia have also suggested that farmers limit their use of antibiotic growth promoters, and similar pressure has been applied by the private food industry (FDA Centre for Veterinary Medicine, 2013; Ferber, 2003; Landers *et al*., 2012; Serratosa *et al*., 2006).

These restrictions on conventional means to improve livestock production have made it necessary for researchers to investigate alternative feed additives, with natural bioactive compounds such as flavonoids being one possibility (Jouany & Morgavi, 2007). Flavonoids are secondary metabolites relatively ubiquitous in the plant kingdom (Havsteen, 2002), as well as being found in related products such as honey and propolis (Burdock, 1998). *In vitro* and physiological studies have described a diverse range of pharmacological properties for these

compounds, from antioxidant, antimicrobial and anti-inflammatory activities to effects on the gastrointestinal tract, cardiovascular system, and various hormone and enzyme systems, among others (Havsteen, 2002; Procházková, Boušová & Wilhelmová, 2011). Their roles as antioxidants and antimicrobials have attracted the most attention from the perspective of their potential as growth promoters, as they suggest that their addition to feed could reduce the effects of oxidative stress (Kamboh *et al*., 2015), and/or have similar growth promoting and disease-preventive effects to antibiotics. Unfortunately, the limited research that has investigated their practical application as growth promoters has had somewhat disappointing results (Goliomytis *et al*., 2015; Goliomytis *et al.*, 2014; Mason *et al*., 2005; Simitzis *et al.*, 2014; Simitzis *et al*., 2019; Simitzis, Symeon, Charismiadou, Ayoutanti & Deligeorgis, 2011), although results for the amelioration of the effects of applied stressors, such as heat or disease, have been more promising (Alhidary & Abdelrahman, 2014; Attia, Al-Hanoun & Bovera, 2011; Erener *et al.*, 2011; Greiner, Stahly & Stabel, 2001; Onderci *et al*., 2004; Tuzcu *et al*., 2008). However, despite the limited effects on live performance, studies have indicated possible effects on other aspects of meat production, particularly in terms of meat nutritional quality and shelf-life.

These effects on meat quality are important as changes in consumer demands have not been restricted to the pre-slaughter side of animal production. Modern consumers no longer consider meat, milk or eggs as simply a traditional part of the diet, and are no longer only worried about their appearance or flavour, although these sensory traits are still important components of quality. There is increasing interest in the healthfulness of food, with this including properties such as the fat and cholesterol content or fatty acid profile (Jiménez-Colmenero, Carballo & Cofrades, 2001; Verbeke & Viaene, 2000). This interest and awareness has promoted meat from certain species, while vilifying others (Verbeke & Viaene, 2000), and has motivated farmers and researchers to find ways of making their products of choice more attractive across the board (Jiménez-Colmenero *et al*., 2001). Previous research on flavonoids has found that dietary supplementation can alter the cholesterol content and fatty acid composition of meat from poultry (Kamboh & Zhuh, 2013; Sohaib, Butt, Shabbir & Shahid, 2015), ruminants (Andrés, Morán, *et al*., 2014; Tan *et al*., 2011) and rabbits (Simitzis *et al*., 2014); however, there is still a lack of consensus on the mechanism by which this occurs.

The potential protective effects of dietary flavonoids against oxidative changes in the meat during storage are also extremely interesting, as the transport and storage of meat products forms a vital part of their commercial production and sale, and many detrimental changes can take place during storage (Lambert, Smith & Dodds, 1991; Shahidi & Zhong, 2010). Moreover, concerns about the safety of some synthetic antioxidants has led to the restriction of their use in food products (Shahidi & Zhong, 2010), and consumers are becoming increasingly troubled by the incorporation of ‘unnatural’ additives in food, even in the absence of any evidence of negative health effects (Brewer, 2011). The risks of oxidation are also increased by an increase in the polyunsaturated fatty acid (PUFA) content (Wood *et al*., 2004), which is desired by health-conscious consumers. Flavonoids may thus provide an alternative, or additional, method of extending the shelf-life of meat, through both its antioxidant and antimicrobial activities. Indications of this effect have been reported for poultry (Goliomytis *et al.*, 2015;

Goliomytis *et al*., 2014; Jiang *et al*., 2007; Kamboh & Zhu, 2013; Simitzis *et al.*, 2011; Sohaib *et al*., 2015; Tang, Kerry, Buckley & Morrissey, 2001) and ruminants (Andrés, Huerga, *et al.*, 2014; Andrés, Morán, *et al*., 2014; Simitzis *et al*., 2019; Simitzis, Ilias-Dimopoulos, Charismiadou, Biniari & Deligeorgis, 2013; Zhong *et al.*, 2009).

It is therefore clear that dietary flavonoid supplementation presents a possible opportunity for a multi-pronged approach to improving production and product quality. However, their diverse array of pharmacological activities also makes researching their effects and mechanisms of action challenging, and the existing literature still lacks consensus on the effects of different flavonoids on different aspects of meat production. This dearth of information is particularly problematic for less widely utilised species, such as rabbits.

Rabbits are of interest for meat production for a number of reasons. From a live performance perspective, they have the advantage of being farmed on small areas of land, similar to pigs or poultry, while also utilising a high-fibre diet, which aligns with public concerns about the competition of livestock with humans for feed resources (Finzi, 2000). Rabbits are also highly prolific and grow rapidly, and have potential for not only large-scale commercial production, but also small, specialised or subsistence farming (Abu, Onifade, Abanikannda & Obiyan, 2008; Oseni, 2012). From the perspective of meat quality, rabbit meat aligns well to modern consumers' demands for healthy meat products, having a high protein, essential amino acid, PUFA and α-linolenic acid content, and a low cholesterol content (Dalle Zotte & Szendrő, 2011).

As lagomorphs that are hindgut fermenters and practice caecotrophy, rabbits are also highly specialised physiologically, which limits the value of extrapolating the results of previous flavonoid studies on poultry, pigs or ruminants. It is therefore necessary for comprehensive studies on the physiological, live performance and meat quality effects of dietary flavonoid supplementation to rabbits to be done.

The aim of this collection of studies was therefore to provide an initial description of the effects of dietary quercetin supplementation on the live performance and meat production of New Zealand White meat rabbits. Quercetin was chosen as it is one of the most widely distributed and well-studied flavonoids, for which purified extracts are readily available (Bischoff, 2008; Erlund, 2004). Furthermore, in an effort to provide some explanation for any observed effects on practical performance traits, the effects on serum hormone levels, the caecal microbiome, and the fatty acid content of the caecotrophes was also determined. It is hoped that the results of this research, although certainly not providing all the answers, will provide useful information for the formulation of further questions to be addressed by future research.

References

Abu, O. A., Onifade, A. A., Abanikannda, O. T. F. & Obiyan, R. I. (2008). Status and promotional strategies for rabbit production in Nigeria. *Proceedings of the 9th World Rabbit Congress — 10 – 13 June, 2008, Verona, Italy* (pp. 1499 – 1504).

Alhidary, I. A. & Abdelrahman, M. M. (2014). Effect of naringin supplementation on performance and physiological responses of heat stressed lambs. *Journal of Animal and Veterinary Advances*, *13*(19), 1126 – 1130.

Andrés, S., Huerga, L., Mateo, J., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Rotolo, L. & Giráldez, F. J. (2014). The effect of quercetin dietary supplementation on meat oxidation processes and texture of fattening lambs. *Meat Science, 96*, 806 – 811.

Andrés, S., Morán, L., Aldai, N., Tejido, M. L., Prieto, N., Bodas, R. & Giráldez, F. J. (2014). Effects of linseed and quercetin added to the diet of fattening lambs on the fatty acid profile and lipid antioxidant status of meat samples. *Meat Science, 97*, 156 – 163.

Attia, Y. A., Al-Hanoun, A. & Bovera, F. (2011). Effect of different levels of bee pollen on performance and blood profile of New Zealand White bucks and growth performance of their offspring during summer and winter months. *Journal of Animal Physiology and Animal Nutrition, 95*, 17 – 26.

Bischoff, S. C. (2008). Quercetin: potentials in the prevention and therapy of disease. *Current Opinion in Clinical Nutrition and Metabolic Care, 11*, 733 – 740.

Brewer, M. S. (2011). Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety, 10*, 221 – 247.

Burdock, G. A. (1998). Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology, 36*, 347 – 363.

Council of the European Union (1981). Council Directive 81/602/EEC of 31 July 1981 concerning the prohibition of certain substances having a hormonal action and of any substances having a thyrostatic action. *Official Journal of the European Communities, L222*, 32, 1 – 2.

Dalle Zotte, A. & Szendrő, Z. (2011). The role of rabbit meat as functional food. *Meat Science, 88*, 319 – 331.

Erener, G., Ocak, N., Altop, A., Cankaya, S., Aksoy, H. M. & Ozturk, E. (2011). Growth performance, meat quality and caecal coliform bacteria count of broiler chicks fed diet with green tea extract. *Asian-Australasian Journal of Animal Science, 24*(8), 1128 – 1135.

Erlund, I. (2004). Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research, 24*, 851 – 874.

European Commission (2005). *Ban on antibiotics as growth promoters in animal feed enters into effect*. Brussels, Belgium: European Commission, Available at: http://europa.eu/rapid/press-release_IP-05-1687_en.htm.

Ferber, D. (2003). WHO advises kicking the livestock antibiotic habit. *Science, 301*(5636), 1027.

Finzi, A. (2000). Raising rabbits for food security. *Proceedings of the 7th World Rabbit Congress — 4 – 7 July, 2000, Valencia, Spain, Volume B* (pp. 13 – 38).

Food and Drug Administration, Centre for Veterinary Medicine (2013). *Guidance for Industry #213: New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209.* Washington, D.C., USA: U.S. Department of Health and Human Services.

Goliomytis, M., Kartsonas, N., Charismiadou, M. A., Symeon, G. K., Simitzis, P. E. & Deligeorgis, S. G. (2015). The influence of naringin and hesperidin dietary supplementation on broiler meat quality and oxidative stability. *PLoS One, 10*(10), 1 – 11.

Goliomytis, M., Tsoureki, D., Simitzis, P. E., Charismiadou, M. A., Hager-Theodorides, A. L. & Deligeorgis, S. G. (2014). The effects of quercetin dietary supplementation on broiler growth performance, meat quality, and oxidative stability. *Poultry Science, 93*, 1957 – 1962.

Greiner, L. L., Stahly, T. S. & Stabel, T. J. (2001). The effect of dietary soy daidzein on pig growth and viral replication during a viral challenge. *Journal of Animal Science, 79*, 3113 – 3119.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67 – 202.

Jiang, Z. Y., Jiang, S. Q., Lin, Y. C., Xi, P. B., Yu, D. Q. & Wu, T. X. (2007). Effects of soybean isoflavone on growth performance, meat quality, and antioxidation in male broilers. *Poultry Science, 86*, 1356 – 1362.

Jiménez-Colmenero, F., Carballo, J. & Cofrades, S. (2001). Healthier meat and meat products: their role as functional foods. *Meat Science, 59*, 5 – 13.

Jouany, J.-P. & Morgavi, D. P. (2007). Use of "natural" products as alternatives to antibiotic feed additives in ruminant production. *Animal, 1*(10), 1443 – 1466.

Kamboh, A. A. & Zhu, W.-Y. (2013). Individual and combined effects of genistein and hesperidin supplementation on meat quality in meat-type broiler chickens. *Journal of the Science of Food and Agriculture, 93*, 3362 – 3367.

Kamboh, A. A., Arain, M. A., Mughal, M. J., Zaman, A., Arain, Z. M. & Soomro, A. H. (2015). Flavonoids: health promoting phytochemicals for animal production-a review. *Journal of Animal Health and Production, 3*(1), 6 – 13.

Lambert, A. D., Smith, J. P. & Dodds, K. L. (1991). Shelf life extension and microbiological safety of fresh meat — a review. *Food Microbiology, 8*, 267 – 297.

Landers, T. F., Cohen, B., Wittum, T. E. & Larson, E. L. (2012). A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Reports, 127*, 4 – 21.

Mason, L. M., Hogan, S. A., Lynch, A., O'Sullivan, K., Lawlor, P. G. & Kerry, J. P. (2005). Effects of restricted feeding and antioxidant supplementation on pig performance and quality characteristics of *longissimus dorsi* muscle from Landrace and Duroc pigs. *Meat Science, 70*, 307 – 317.

Onderci, M., Sahin, K., Sahin, N., Gursu, M. F., Doerge, D., Sarkar, F. H. & Kucuk, O. (2004). The effect of genistein supplementation on performance and antioxidant status of Japanese quail under heat stress. *Archives of Animal Nutrition, 58*(6), 463 – 471.

Oseni, S. O. (2012). Rabbit production in low-input systems in Africa — prospects, challenges and opportunities. *Proceedings of the 10th World Rabbit Congress — 3 – 6 September, 2012, Sharm El-Sheikh, Egypt* (pp. 719 – 731).

Procházková, D., Boušová, I. & Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia, 82*, 513 – 523.

Scientific Committee on Veterinary Measures relating to Public Health (1999). *Assessment of potential risks to human health from hormone resides in bovine meat and meat products*. Brussels, Belgium: European Commission.

Serratosa, J., Blass, A., Rigau, B., Mongrell, B., Rigau, T., Tortades, M., Tolosa, E., Aguilar, C., Ribo, O. & Balague, J. (2006). Residues from veterinary medicinal products, growth promoters and performance enhancers in food-producing animals: a European Union perspective. *Revue Scientifique et Technique (International Office of Epizootics), 25*(2), 637 – 653.

Shahidi, F. & Zhong, Y. (2010). Novel antioxidants in food quality preservation and health promotion. *European Journal of Lipid Science Technology, 112*, 930 – 940.

Simitzis, P. E., Babaliaris, C., Charismiadou, M. A., Papadomichelakis, G., Goliomytis, M., Symeon, G. K. & Deligeorgis, S. G. (2014). Effect of hesperidin dietary supplementation on growth performance, carcass traits and meat quality of rabbits. *World Rabbit Science, 22*, 113 – 121.

Simitzis, P. E., Charismiadou, M. A., Goliomytis, M., Charalambous, A., Ntetska, I., Giamouri, E. & Deligeorgis, S. G. (2019). Antioxidant status, meat oxidative stability and quality characteristics of lambs fed with hesperidin, naringin or α-tocopherol acetate supplemented diets. *Journal of the Science of Food and Agriculture, 99*(1), 343 – 349.

Simitzis, P. E., Ilias-Dimopoulos, V., Charismiadou, M. A., Biniari, E. E. & Deligeorgis, S. G. (2013). Effects of dietary hesperidin supplementation on lamb performance and meat characteristics. *Animal Science Journal, 84*, 136 – 143.

Simitzis, P. E., Symeon, G. K., Charismiadou, M. A., Ayoutanti, A. G. & Deligeorgis, S. G. (2011). The effects of dietary hesperidin supplementation on broiler performance and chicken meat characteristics. *Canadian Journal of Animal Science, 91*, 275 – 282.

Sohaib, M., Butt, M. S., Shabbir, M. A. & Shahid, M. (2015). Lipid stability, antioxidant potential and fatty acid composition of broilers breast meat as influenced by quercetin in combination with α-tocopherol enriched diets. *Lipids in Health and Disease, 14*(61), 1 – 15.

Tan, C. Y., Zhong, R. Z., Tan, Z. L., Han, X. F., Tang, S. X., Xiao, W. J., Sun, Z. H. & Wang, M. (2011). Dietary inclusion of tea catechins changes fatty acid composition of muscle in goats. *Lipids, 46*(3), 239 – 47.

Tang, S. Z., Kerry, J. P., Sheehan, D., Buckley, D. J. & Morrissey, P. A. (2001). Antioxidative effect of dietary tea catechins on lipid oxidation of long-term frozen stored chicken meat. *Meat Science, 57*, 331 – 336.

Tuzcu, M., Sahin, N., Karatepe, M., Cikim, G., Kilinc, U. & Sahin, K. (2008). Epigallocatechin-3-gallate supplementation can improve antioxidant status in stressed quail. *British Poultry Science, 49*(5), 643 – 648.

Vanhonacker, F. & Verbeke, W. (2014). Public and consumer policies for higher welfare food products: Challenges and opportunities. *Journal of Agricultural and Environmental Ethics, 27*, 153 – 171.

Verbeke, W. A. J. & Viaene, J. (2000). Ethical challenges for livestock production: Meeting consumer concerns about meat safety and animal welfare. *Journal of Agricultural and Environmental Ethics, 12*, 141 – 151.

Wood, J., Richardson, R., Nute, G., Fisher, A., Campo, M., Kasapidou, E., Sheard, P. & Enser, M. (2004). Effects of fatty acids on meat quality: a review. *Meat Science, 66*(1), 21 – 32.

Zhong, R. Z., Tan, C. Y., Han, X. F., Tang, S. X., Tan, Z. L. & Zeng, B. (2009). Effect of dietary tea catechins supplementation in goats on the quality of meat kept under refrigeration. *Small Ruminant Research, 87*, 122 – 125.

CHAPTER 2:

The use of dietary bioflavonoids in meat production: a review

Abstract

Flavonoids are ubiquitous plant polyphenols that have demonstrated an extensive range of pharmacological properties, many of which suggest that they may have the potential to improve livestock live performance and product quality. However, research on their effects on livestock is very limited and has not been reviewed. This paper therefore aimed to provide a summary of existing research on the practical applications of flavonoids, as well as recommend future avenues of research. An overview of the flavonoid content of existing livestock feed resources appeared to indicate that they may be relatively prevalent in standard diets. However, as the flavonoid content could vary widely depending on the formulation of the feed, it is recommended that future studies take the composition of the basal diet into account. Live performance studies suggest that flavonoids may have the potential to ameliorate the effects of stress-inducing conditions, such as heat or disease, and could thus have a role to play in mortality and morbidity prevention. Effects on growth performance under normal conditions have generally been limited. The effects on carcass and meat quality have been mixed and species-specific, but suggest that flavonoids may favourably modify the fatty acid composition and improve the oxidative stability of meat. However, more research is needed to confirm these findings, especially on pigs and rabbits. Further research on the dose-response curves of flavonoids, and their effects under stress-inducing conditions, also needs to be done.

2.1 Introduction

Two main drives in any livestock industry are to improve live performance and optimise product quality, and the pressure to achieve these is likely to increase in the future (Thornton, 2010). Live performance includes growth, feed conversion efficiency and health status, with the latter having a large influence on welfare. Product quality includes basic parameters such as flavour, tenderness and colour, as well as shelf-life and, more recently, the healthfulness of the product for the consumer. This last aspect includes factors such as the fat content and fatty acid composition, the cholesterol content, and the incorporation of potentially beneficial bioactive compounds, all of which contribute to the role of meat as a functional food (Dalle Zotte & Szendrő, 2011; Decker & Park, 2010).

Achieving some of these objectives has become more difficult in certain regions in recent years due to the increasing regulation of the use of antibiotics and hormone-based growth promoters in livestock farming (Council of the European Union, 1981; European Commission, 2005; FDA, 2013). These products not only increased yields and efficiency but also helped improve herd health status through their prophylactic use; however, their negative consequences — such as contributing to the development of antibiotic resistance and possibly having a negative impact on the health of consumers — ultimately outweighed their benefits (Landers, Cohen, Wittum & Larson, 2012; Scientific Committee on Veterinary Measures relating to Public Health, 1999). This has resulted in a need to find alternative products that can help farmers reach the required levels of livestock production and health, while avoiding undesirable consequences. Flavonoids are one of the many natural bioactive compounds that may be able to help achieve this.

Flavonoids are widely distributed plant polyphenols that have demonstrated numerous pharmacological properties in human, laboratory animal and *in vitro* studies. These have included antioxidant, antimicrobial, anti-inflammatory, antidiabetic, antimutagenic and hepatoprotective activities, as well as effects on the gastrointestinal tract and cardiovascular system (Havsteen, 2002; Narayana, Reddy, Chaluvadi & Krishna, 2001; Tapas, Sakarkar & Kakde, 2008). They also interact with numerous enzymes involved in a variety of metabolic pathways (Narayana *et al*., 2001; Tapas *et al*., 2008). Their antioxidant and antimicrobial effects are of particular interest in terms of their potential as growth promoters, as well as suggesting possible beneficial effects on meat shelf-life and microbial safety. In addition, considering their proposed health benefits to humans, the direct incorporation of flavonoids into meat is also of interest.

However, many of the flavonoids' bioactivities have primarily been demonstrated *in vitro* or *ex vivo*, or in human or rodent studies, and most of the numerous published reviews have focussed on their biochemical characterisation, in-depth physiological effects or potential for direct effects on human health (Havsteen, 2002). To the authors' knowledge, a review of the literature examining their potential for use in the livestock industry has not been done to date. This paper will therefore provide only a rudimentary summary of the chemical and pharmacological properties of flavonoids, and will focus more on their practical applications in the livestock industry.

2.2 The chemical structure of the flavonoids

Flavonoids are a group of polyphenolic pigments synthesized from phenylalanine and characterized by a triple-ring structure consisting of a benzene ring (A), condensed to an oxygen-containing, 6-member heterocyclic ring (C), with a third, phenyl ring (B), attached at the number 2- or 3-position of the C-ring (Beecher, 2002; Merken & Beecher, 2000; Narayana *et al*., 2001). They can be divided into six classes (Figure 2.1) based on the position of the attachment of the B-ring to the C-ring (2 for flavonoids, 3 for isoflavonoids), and the degree of saturation (no double bonds, position 2-3 double bond, or positions 1-2 and 2-3 double bonds) and functional groups found on the C-ring (Beecher, 2002).

The individual flavonoids are further differentiated based on the hydroxylation and conjugation patterns of the rings (particularly the B-ring), and the presence (glycosides) or absence (aglycones) of sugar moieties, which are usually attached to the A- or C-ring at positions 3 or 7 (Beecher, 2002; Narayana *et al*., 2001; Peterson & Dwyer, 1998). The majority of naturally-occurring flavonoids exist as glycosides, rather than aglycones. Polymerized flavonoids (tannins) also widely occur (Beecher, 2002), but will not be the focus of this review. Over 6000 individual flavonoid types had already been identified by the year 2000, and this number has likely grown since (Harborne & Williams, 2000).

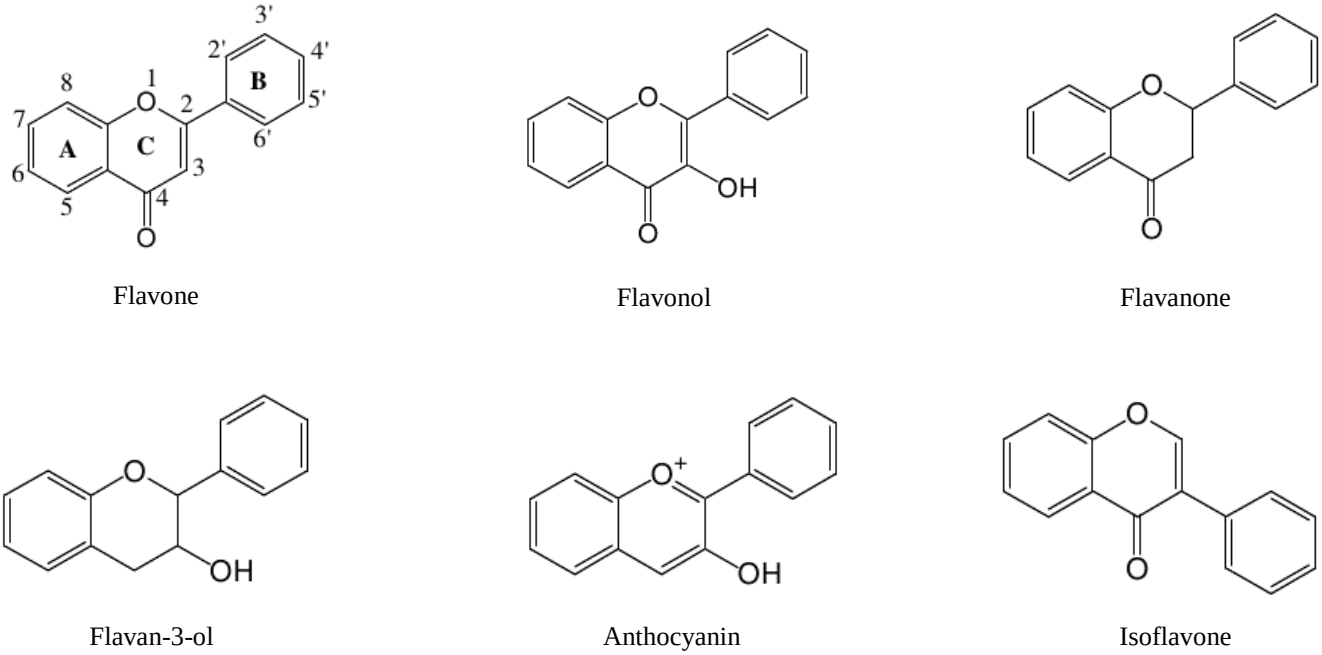

Figure 2.1 Generalised structures of the six main classes of dietary flavonoids (adapted from Tapas *et al*., 2008)

2.3 Flavonoids in livestock diets

Flavonoids are ubiquitous in the plant kingdom, and while they have not been found to participate directly in photosynthesis, they do appear to play an important role in gene regulation, growth metabolism, photosensitisation, energy transfer, control of respiration and photosynthesis, morphogenesis and sex determination (Cushnie &

Lamb, 2005; Havsteen, 2002). Despite this, their distribution is not uniform within or between species, and they tend to occur at particularly high concentrations in certain plants and products. Commonly considered sources of flavonoids in the human diet are vegetables and vegetable products, fruits and fruit products, tea and wine. Summaries of the most prevalent sources of dietary flavonoids for humans can be found in previous reviews (Beecher, 2002; Egert & Rimbach, 2011; Yao *et al*., 2004), and certain food composition databases have even begun to include flavonoids as listed nutrients (Bhagwat, Haytowitz & Holden, 2011).

However, research into the flavonoid content of current and potential livestock feed resources is more limited, and has often either been performed on the primary products (in the case of by-product livestock feeds) or with a different objective in mind, such as the study of allelopathy, plant physiology or plant taxonomy. The methods of extraction and determination also vary widely. Nevertheless, some discussion of the prevalence of flavonoids in livestock feeds is necessary, before the idea of supplementation can be considered.

2.3.1 Grass and forage crops

Ruminants, especially those farmed extensively, tend to have a large portion of their diets contributed by grazed forages. The species composition of these forages can vary from relative uniformity, in annually planted pastures, to highly diverse, in planted or naturally occurring permanent pastures. However, even annually planted pastures vary widely around the world, due to environmental differences. It is therefore challenging to provide a brief overview of the potential flavonoid intake from this feed resource, particularly in light of the limited research available. However, it does appear that one of the main components of pastures, the grasses (*Gramineae*), tend to have relatively low flavonoid contents, with ryegrass (*Lolium multiflorum* Lam.) varying from undetected to 1.54 mg/g, and orchard grass (*Dactylis glomerata* L.) containing around 0.2 mg/g (Hauck, Gallagher, Morris, Leemans & Winters, 2014; Ponce *et al*., 2009). There also seems to be consensus that the most widely distributed flavonoids present in grass forages are tricin and flavone C-glycosides (particularly apigenin and luteolin C-glycosides) (Harborne & Williams, 1976). The flavonols, quercetin and kaempferol, are conspicuous in their rarity in the grasses, as they are prolific in fruits and vegetables (Harborne & Williams, 1976; Peterson & Dwyer, 1998).

A second common and nutritionally important component of pastures, particularly planted pastures, are the legume forages, which fix nitrogen and help improve the protein and mineral content of grazing (McDonald, Edwards, Greenhalgh & Morgan, 2002). Some of the most popular legume species utilised in planted pasture are the clovers (*Trifolium* spp.). Like many members of the *Fabaceae* family, clovers are known for their content of isoflavones, with Oleszek, Stochmal and Janda (2007) reporting total contents of up to 97 mg/g dry matter (DM) for the 57 species tested. While isoflavones were also absent from some samples, flavonoids were present in all the species tested and varied from 0.61 to 32.4 mg/g DM, with quercetin glycosides being the most prevalent flavonoids in the flowers of white clover (Oleszek *et al*., 2007; Schittko, Burghardt, Fiedler, Wray & Proksch, 1999). The levels of flavonoids and isoflavones vary widely between species, and between the different components of the plants (leaves, stems and flowers), suggesting that considerable seasonal variation is also likely (Oleszek *et al*., 2007; Vetter, 1995). It is notable that breeding efforts during the 1970s largely aimed to reduce

the isoflavone content of clover, due to the potential negative effects of these compounds on animal fertility (Oleszek *et al*., 2007; Vetter, 1995).

Another key legume crop, although it is more often harvested for hay than grazed, is alfalfa or lucerne (*Medicago sativa* L.). Alfalfa is one of the oldest and most widely utilised legume forages internationally, and is an especially important component of feeds for ruminants and hindgut fermenters such as horses and rabbits (Radović, Sokolović & Marković, 2009). Like clover, it contains relatively high levels of flavonoids, with Stochmal and Oleszek (2007) reporting 27 – 37 mg/g DM, consisting primarily of tricin, luteolin and apigenin glycosides (Stochmal *et al*., 2001). The total flavonoid content also depended on when in the year the alfalfa was harvested, decreasing from the first to third cuts (Stochmal & Oleszek, 2007).

Other, less widely-utilised forage legume species include sainfoin (*Onobrychis viciifolia*), trefoil (*Lotus corniculatus*), sweet clovers (*Melilotus* species), vetches (*Vicia* species), burr medic (*Medicago polymorpha*) and *Stylosanthes* species (Graham & Vance, 2003). While data on the total flavonoid content of these species is limited, the stems and leaves of ten vetch species have been found to contain combined concentrations of myricetin, naringenin, kaempferol and isorhamnetin of 0.03 – 1.42 mg/g (Lee *et al*., 2017). Of these, naringenin was the most widely distributed across the species, and was present at the highest concentrations (Lee *et al*., 2017). The main flavonoid found in yellow sweet-clover (*Melilotus officinalis* (L.) Pall.) was rutin (Bubenchikova & Drozdova, 2004), while trefoil contained primarily flavonols (mainly aglycones of kaempferol and quercetin) and small quantities of isoflavonoids (Reynaud & Lussignol, 2005). The young leaves of sainfoin have similarly been found to contain flavonols as the main polyphenols, varying from 8 to 36 mg/g DM, with quercetin 3-rutinoside having the highest concentration (Thill *et al*., 2012). Apigenin 7-glucuronide and luteolin 7-glucuronide, both flavone glycosides, appeared to be the major flavonoids in burr medic, with no isoflavones and only one flavonol being detected (Saleh, Boulos, El-Negoumy & Abdalla, 1982). As species in the *Fabaceae* family tend to contain high levels of flavonoids, and appear to be relatively diverse in their contents and compositions, further research on both common and novel species would be of interest in order to allow the more accurate comparison between and within species and cultivars (Neugart, Rohn & Schreiner, 2015).

2.3.2 Formulated concentrates and total mixed rations

While pastures remain a vital feed resource for the livestock industry, an increasing proportion of ruminants are being supplemented with concentrate feeds, or are being fed total mixed rations, and for non-ruminants this is the norm. These formulated feeds have to be balanced for protein and energy, and raw materials are consequently often classified as either energy- or protein-sources.

Cereal grains are the most widely utilised sources of energy in the diets of most livestock species, and some whole grains can contain relatively high levels of phenolic compounds. While most of these are bound to cell wall materials and may be unavailable to monogastric animals, they can be released by microbial degradation and could therefore be absorbed by ruminants and rabbits (due to caecotrophy) (Liu, 2007). However, only a relatively small proportion of the total phenolic content of grains consists of flavonoids (1.68 µmol flavonoids/g, versus

15.55 µmol phenolics/g) (Adom & Liu, 2002), and relative to the total flavonoid content of some fruit and vegetable sources, the levels in grains are rather low. Žilić, Serpen, Akıllıoğlu, Gökmen and Vančetović (2012) reported total flavonoid contents of around 0.28 mg/g for some yellow maize varieties, and whole durum wheat grains contained 0.41 – 0.64 mg/g, depending on the cultivar (Dinelli *et al*., 2013). Nonetheless, considering the relatively large quantities of grains that are consumed by livestock, they may constitute a significant source of flavonoids in the diet, particularly in terms of effects on the gastrointestinal tract and rumen or caecal microbiome. In addition, the majority (79 %) of the flavonoid content is located in the bran/germ fraction (Liu, 2007), suggesting that grain by-products such as maize germ meal and wheat bran that are commonly utilised for livestock feed may further increase the contribution to the diet of grain-origin flavonoids.

Another source of energy used in livestock feeds are carbohydrate-rich roots and tubers such as potatoes (*Solanum tuberosum* L.), sweet potatoes (*Ipomoea batatas*), turnips (*Brassica rapa* var. *rapa* L.), swedes (*Brassica napobrassica*), mangels and fodder beets (*Beta vulgaris* cultivars) and cassava (*Manihot esculenta* Crantz). Potatoes and brassicas (family *Brassicaceae*) are generally utilised in temperate regions (Barry, 2013), while sweet potatoes and cassava are widely grown in tropical and subtropical areas. Members of the *Brassicaceae* family contain relatively high levels of flavonoids, particularly quercetin, kaempferol and isorhamnetin O-glycosides; however, more research has focussed on species utilised for their foliage, such as kale or cabbage, than those utilised for their roots (Cartea, Francisco, Soengas & Velasco, 2011). The flavonoid content of the roots tends to be lower than that of the foliage and flowers, as found for turnips by Fernandes *et al*. (2007) and Aires *et al*. (2011), with the leaves containing 3.5 – 8.7 mg total flavonoids/g, and the roots 0.7 – 7.6 mg/g, depending on the season and year. Data on the flavonoid contents of mangels and fodder beets is limited, but the leaves and stems of different varieties of the related Swiss chard (*Beta vulgaris* subspecies *cycla*) contained only 0.009 – 0.256 mg flavonoids/g fresh weight (Pyo, Lee, Logendra & Rosen, 2004), suggesting that the roots would also have a relatively low content. The total flavonoid content of cassava roots has been reported as 0.4 – 2.3 mg/g, depending on cultivar and storage time (Uarrota *et al*., 2014), and 2.2 – 2.7 mg/g, depending on the fertilizer treatment (Omar *et al*., 2012). Information on the total flavonoid content of sweet potato roots is limited, but the combined contents of quercetin, myricetin, kaempferol and luteolin have been reported at 0.05 – 0.6 mg/g, depending on the variety, with purple-fleshed varieties containing the highest concentrations and quercetin being the most prevalent flavonoid (Park *et al*., 2016). Potatoes had even lower levels of these flavonols and flavones, with a total of only 0.00013 mg/g (Chu, Chang & Hsu, 2000).

A number of by- or co-products of industries producing food for human consumption are also used as energy sources in livestock feeds, and some of these are notably rich sources of flavonoids. Citrus (genus *Citrus*) and grape (genus *Vitis*) products have particularly high levels, with citrus peel containing 31.1 – 49 mg/g extract powder, citrus tissues 1.2 – 17.1 mg/g and grape pomace up to 40 mg/g (Ghasemi, Ghasemi & Ebrahimzadeh, 2009; Lu & Foo, 1999; Wang, Chuang & Hsu, 2008). Hesperidin is the most prevalent flavonoid in citrus fruits, making up 5 mg/g of the total flavonoid content (49 mg/g) of citrus pomace from orange juice production

(Antongiovanni, Buccioni, Petacchi & Agnoletti, 2005). Whole apple (*Malus pumila*) pomace, consisting of varying proportions of peel, flesh, stem, core, seeds and juice, is also a good source of flavonoids, containing around 5.1 mg flavonoids/g DM, of which 4.46 mg/g were quercetin-glycosides (Lu & Foo, 1997). While citrus and apple by-products can be utilised at relatively high inclusion rates in livestock feeds, high tannin and fibre levels in grape pomace tend to restrict its utilisation, particularly for non-ruminants (Lu & Foo, 1999).

Unlike citrus, apple and grape pomaces, many widely-used and readily available by-products have low flavonoid contents. This is the case for both sugar beet (*Beta vulgaris*) residues (1.2 mg/g DM for a methanol extract) and sugarcane (genus *Saccharum*) bagasse (0.38 mg flavonoids/g), as well as their respective molasses by-products (Mohdaly, Hassanien, Mahmoud, Sarhan & Smetanska, 2013; Payet, Sing & Smadja, 2006; Valli *et al*., 2012). Subtropical fruits such as bananas (genus *Musa*) and mangoes (genus *Mangifera*) also have readily-available by-products in certain areas, but have low flavonoid contents (0.39 – 3.9 mg/g and 0.06 – 0.9 mg/g, respectively) (Fatemeh, Saifullah, Abbas & Azhar, 2012; Ma *et al*., 2011), as do tomato (*Lycopersicon esculentum* Mill.) wastes, with the skins having the highest flavonoid content, at around 0.2 mg/g (Toor & Savage, 2005).

By-products are also widely used as protein sources in livestock feeds, with soybean (*Glycine max*) oilcake or meal, which is produced during the extraction of soybean oil, making up two-thirds of the total world production of protein feed resources (Heuzé, Tran & Kaushik, 2017). Although soybean products are not necessarily high in total flavonoids, they are known to contain high levels of unique isoflavones, with the total isoflavone content of whole seeds varying from 1.16 – 3.09 mg/g, depending on season of harvest, location of growth and cultivar used (Eldridge & Kwolek, 1983; Hoeck, Fehr, Murphy & Welke, 2000). Genistin and diadzin (isoflavone glucosides) contribute the largest proportions of this (Eldridge & Kwolek, 1983; Kao & Chen, 2006). While the processing involved in extracting the oil and producing the soybean oilcake could influence these concentrations, it is notable that Eldridge and Kwolek (1983) found that defatted flakes had only slightly lower concentrations than full-fat flakes, on a fat-free basis.

Other widely used oilseed by-products include cottonseed (genus *Gossypium*) meal, groundnut or peanut (*Arachis hypogaea*) meal, palm kernel (*Elaeis guineensis*) meal, sunflower (*Helianthus annuus*) meal and canola or rapeseed (genus *Brassica*) meal. Data on the total flavonoid contents of cottonseed and canola meal are limited, but the major flavonoids present in the former are quercetin and kaempferol glycosides (Blouin, Zarins & Cherry, 1981), and in the latter, kaempferol glycosides (Shahidi & Ambigaipalan, 2015). Peanut skins contain predominantly proanthocyanidins, rather than monomeric flavonoids, with a total monomeric flavonoid content of 0.8 mg/g, and proanthocyanidin content of 5.0 mg/g (De Camargo *et al*., 2017). Dry-blanched peanut meal contains even lower levels, with no monomeric flavonoids detected and only 0.5 mg/g proanthocyanidins (De Camargo *et al*., 2017). Higher flavonoid contents have been found in palm kernel meal, with Wong *et al*. (2015) reporting values of 1.48 – 28.65 mg/g. Palm kernel meal is becoming increasingly available for utilization, particularly for use in ruminant and rabbit diets, as the demand for palm oil grows (Heuzé *et al*., 2016), and it can be included in diets for beef and dairy cattle at 50 – 80 % and 30 – 50 %, respectively (Zahari & Alimon, 2005),

suggesting that flavonoids from this source could make a considerable contribution to the diet. Sunflower seeds were similarly rich in flavonoids, with dehulled non-oil type kernels containing a total of 21.3 mg flavonoids/g, largely made up of quercetin glycosides (Karamać, Kosińska, Estrella, Hernández & Duenas, 2012).

Many forages and by-products are only readily available at certain times of the year, making storage necessary if year-round use is desired, and the most common methods of storage include drying and ensiling. As one would expect, the flavonoid content of any silage is largely determined by the flavonoid contents of the raw materials incorporated in it. Therefore, silages made using mixed foliage containing varying proportions of legumes and other non-grasses (4.9 mg/g DM) have higher flavonoid contents than those made from relatively pure grass crops (0.008 – 0.05 mg/g DM) (Njåstad *et al*., 2014). Red and white clover-based silages similarly reflect the variable isoflavone contents of the source material, with red clover silage containing higher levels of a number of compounds (Oleszek *et al*., 2007; Steinshamn, Purup, Thuen & Hansen-Moller, 2008). However, there does appear to be some evidence that the ensiling process may impact the flavonoid content, as it has been found to reduce the proanthocyanidin content of sainfoin (Ramsay *et al*., 2015), and the flavonoid content of sweet orange (*Citrus sinensis*) peels (Oluremi, Okafor, Adenkola & Orayaga, 2010). Drying appears to have a similar effect, with Toor and Savage (2006) reporting that semi-drying tomatoes at 42 °C decreased the flavonoid content, and air-dried birch (*Betula pendula* Roth) leaves containing lower levels of flavonoids than fresh or frozen samples (Keinänen & Julkunen-Tiitto, 1996). Nevertheless, the use of storage methods is unavoidable, and these potential sources of variation should therefore be taken into account when assessing the possible flavonoid content of a diet.

In conclusion, it appears that the largest contributions of flavonoids to livestock diets are made by the legume forages in grazing animals, and by by-products of the fruit and oilseed industries in concentrates and formulated complete rations, with alfalfa hay also making a large contribution to the latter. While there is a great deal of variation, it also appears that the flavonols quercetin and kaempferol are relatively prevalent, as are the isoflavones, particularly in legume crops, and flavones, in alfalfa. The proanthocyanidins are also widely distributed. Considering both the prevalence and variation in the flavonoid contents of different feed resources, it is important that future research on flavonoid supplementation to livestock takes the content of the basal control diet into account. In addition, it must be strongly emphasized that the flavonoid contents of the various feed resources are only provided in this review to give a rough indication of the relative contents of the different raw materials, and that due to the variation between papers in extraction and quantification methods no direct comparison of values is possible. Moreover, despite their wide usage for determining the total flavonoid content, the two aluminium complex-based spectrophotometric assays are limited in their selectivity, and have variable responses depending on the nature of the flavonoids present (Pękal & Pyrzynska, 2014). Further research specifically focussing on flavonoids in livestock feed resources and using standardised methods to provide relative values for different raw materials would therefore be of interest.

2.4 Relevant pharmacological properties

Flavonoids have been found to have numerous biological effects, and their complex pharmacology, as it is currently understood, has been extensively reviewed in previous literature (Havsteen, 2002; Williamson, Kay & Crozier, 2018), and will thus not be emphasized in this review. Certain properties, such as their effects on cancer, diabetes and hypercholesterolemia, are also less relevant in the scope of livestock production. Nonetheless, some understanding of their chemical and biological activities is necessary in order to provide insight into the mechanisms of the effects discussed in later sections, and they are thus summarised below.

2.4.1 Antioxidant activity

Possibly one of the most widely researched properties of the flavonoids is their behaviour as antioxidants, with this behaviour ultimately being responsible for many of their reported health benefits (Heim, Tagliaferro & Bobilya, 2002). Their modes of antioxidant activity include not only scavenging oxidizing species such as superoxide anions and hydroxyl and peroxy radicals, but also chelating metal ions and consequently neutralizing their catalytic activity, and reducing and thus regenerating α-tocopherol radicals (Harborne & Williams, 2000; Procházková, Boušová & Wilhelmová, 2011; Shahidi & Ambigaipalan, 2015). Flavonoids have also been found to inhibit the production of nitric oxide, a molecule which, while playing an important role in the cardiovascular system, can cause damage at high concentrations (Procházková *et al*., 2011).

The relative antioxidant abilities of the flavonoids vary depending on the specific assay used. The ranking of the five most active types according to the inhibition of malondialdehyde (MDA) formation has been reported as myricetin > myricitrin > quercetin > morin > fisetin, with all of these being considerably more effective than (+)-catechin (Ratty & Das, 1988). In contrast, epicatechin gallate, quercetin and cyanidin were reported to have the highest TEAC (Trolox equivalent antioxidant activity) values of the flavonoids tested by Heim *et al*., (2002), at 4.75 mM, 4.7 mM and 4.42 mM, while myricetin only had a TEAC value of 3.1 mM. Nonetheless, many flavonoids have been shown to have considerably higher *in vitro* antioxidant capacities than vitamins E and C (Procházková *et al*., 2011).

However, the *in vivo* functionality of an antioxidant depends not only on its chemical abilities, as assessed by *in vitro* assays, but also on its bioavailability. The bioavailability of flavonoids is impacted by a hugely complex array of interactions, including metabolism by gastrointestinal tract microorganisms, absorption and metabolism by gut epithelial cells, binding and transport in the circulatory system, further processing in organs such as the liver and final excretion (Williamson *et al*., 2018). Metabolic changes that take place include hydrolysis of glucuronides, sulphates and glycosides, dehydroxylation, demethylation and reduction of double bonds and ring cleavage by microbial enzymes in the gastrointestinal tract, and deglycosylation followed by glucuronidation, sulphation and O-methylation in the small intestine and liver (Procházková *et al*., 2011). Even further variation appears at the level of intracellular metabolism. Consequently, the structure of the flavonoid that ends up in circulation or in the tissues can be very different to that of the originally ingested molecule; for example, in a study using rats, mainly glucuronide and sulphate conjugates of quercetin and epicatechin were found in the blood after

the ingestion of the aglycones (Procházková *et al*., 2011). These changes can greatly impact the *in vivo* antioxidant potential of the ingested flavonoid. Nonetheless, *in vivo* antioxidant effects of procyanidins, quercetin, epicatechin aglycones and Cherokee rose flavonoids have been demonstrated (Frei & Higdon, 2003; Procházková *et al*., 2011). Some evidence also suggests that the consumption of tea polyphenols has a sparing effect on α-tocopherol, glutathione and endogenous antioxidant enzymes (Frei & Higdon, 2003).

The importance of antioxidants in the body is proportional to the influence that oxidising free radicals have on homeostasis. Free radicals play an important role in the body's immune response and in the functioning of enzymes such as the oxidoreductases (Havsteen, 2002), and are a normal by-product of cellular metabolism (Lykkesfeldt & Svendsen, 2007). However, when the body is under stress, such as through infectious disease, heat stress or simple metabolic overdrive due to very rapid growth, the balance between the levels of oxidants and reductants can be disturbed, resulting in a build-up of oxidants and oxidative stress (Belhadj Slimen, Najar, Ghram & Abdrrabba, 2016; Lykkesfeldt & Svendsen, 2007; Monaghan, Metcalfe & Torres, 2009). This build-up results in further oxidative damage at the macromolecule (DNA, proteins and lipids), cell and tissue levels, leaving the animal even more vulnerable to further disruptions and infections, and reducing productive performance (Lykkesfeldt & Svendsen, 2007). Unfortunately, the pressures of production and the environments under which many livestock are kept make them particularly likely to experience oxidative stress. Dietary antioxidants may thus play an important role in maintaining the health and production of livestock.

It is notable, though, that some flavonoids have also demonstrated pro-oxidant effects *in vitro* (Heim *et al*., 2002). Ratty and Das (1988), found that apigenin, flavone, flavanone, hesperidin, naringin and tangeritin increased MDA formation, and that this effect was dependent on not only the concentration of the flavonoid but also the presence and concentration of ascorbic acid. However, more research is necessary to clarify the extent and mechanisms of possible pro-oxidant behaviour *in vivo* (Heim *et al*., 2002). In addition, the pro-oxidant potential of flavonoid molecules has been linked to their ability to beneficially induce the production of important detoxifying enzymes, suggesting that 'pro-oxidant' may not be synonymous with 'detrimental' (Procházková *et al*., 2011).

2.4.2 Analgesia, inflammation and the immune response

Apart from possibly reducing the oxidative damage in injured tissue through their antioxidant activity, flavonoids may also reduce inflammation and aid the immune response through other means. Flavonoids have been found to interact with enzymes that form part of the body's response to invasion, particularly prostaglandin cyclooxygenase (Havsteen, 2002). They may also help activate the immune system by stimulating the secretion of interferons and the production of antibodies, and activating cytotoxic and natural killer T-lymphocytes (Havsteen, 2002). This suggests that they may be able to improve wound healing and help prevent inflammatory disease conditions such as lameness and foot lesions.

In addition, flavonoids interact with the nervous system, resulting in an effect on the pain response. This includes not only local, peripheral effects, when they are utilised as topical pain relievers, but also systemic

analgesic effects, presumably through interactions with the endogenous opiate system (Havsteen, 2002). While their analgesic properties may not suggest as widespread use in the livestock industry as their antioxidant effects, the use of painful procedures, such as physical castration, dehorning, tail docking, branding and debeaking, is still widespread, and many disease conditions and injuries also result in pain (Grandin, 2014). This suggests that flavonoids could play a role in improving the welfare of livestock.

2.4.3 Interaction with hormone systems

Flavonoids may also negatively impact livestock production through their interaction with hormone systems. Isoflavones have been found to act as phytoestrogens, which mimic endogenous oestrogens and consequently disrupt the normal functioning of the reproductive system. This was first reported for sheep eating fermented clover forage, which resulted in sexual stimulation (Havsteen, 2002). While this property of flavonoids may have potential for medicinal use in humans, such as in hormone therapy, it can be highly detrimental for livestock production. In Australia, clover disease describes the permanent infertility found in sheep consuming varieties of subterranean clover that are high in oestrogenic isoflavones, and similar effects have been seen in cattle and quail (Hughes, 1988). The isoflavonoid with the most potent oestrogenic activity is genistein, followed by diadzein, biochanin A and formononetin (Lone, 1997).

However, the oestrogenic activity of flavonoids may not necessarily always be negative, particularly in non-breeding stock. Apart from its vital role in fertility, oestrogen also influences both growth and bone integrity. Synthetic oestrogen analogues have long been used as growth promoters, in ruminant livestock in particular, as they are known to increase the growth rate and feed efficiency, as well as improve carcass composition (Preston, 1999). However, the use of hormone-based growth promoters has come under increasing scrutiny in recent years, and their use has been banned in the European Union (Serratosa *et al*., 2006). Oestrogenic flavonoids may therefore have similar beneficial effects on production, and may provide an alternative for synthetic oestrogens. There is also evidence suggesting that dietary supplementation with phytoestrogens may help prevent the loss of bone integrity (Nieves, 2013; Putnam, Scutt, Bicknell, Priestley & Williamson, 2007; Weaver, Alekel, Ward & Ronis, 2012). As bone breakage constitutes a serious economic and welfare problem in some livestock species, such as poultry, there may be potential for flavonoids to be used to reduce morbidity and mortality due to these types of injuries (Bradshaw, Kirkden & Broom, 2002).

Flavonoids have also been found to interfere with thyroid function, inhibiting the production of thyroid hormones and the uptake of iodine by the thyroid (Dos Santos, Gonçalves, Vaisman, Ferreira & de Carvalho, 2011). The effect on iodine uptake appears to be related to their inhibitory effect on the gene expression of the sodium/iodide symporter (NIS), while thyroxine production is reduced through the inhibition of thyroperoxidase activity. The mechanism of this inhibition varies among the different flavonoid types, but includes mechanism-based inactivation for structures containing a resorcinol moiety, non-competitive inhibition of tyrosine iodination and linear mixed-type inhibition (Dos Santos *et al*., 2011). In addition, hydrogen peroxide acts as a cofactor for thyroperoxidase, suggesting that the radical-scavenging behaviour of flavonoids may also contribute to its

inhibition. This results in lower levels of circulating levels of thyroxine (T4) and triiodothyronine (T3), thereby increasing the production of thyroid stimulating hormone and the risk of goitre (Dos Santos *et al*., 2011). Beyond their effects on thyroxine production, flavonoids also inhibit the conversion of the prohormone T4 to the biologically active T3 via the inhibition of the T4 5'-deiodinase enzyme (Dos Santos *et al*., 2011). They further impact the bioactivity of T3 and T4 by decreasing their binding to the serum transport protein transthyretin. This increases the proportion of free hormones, but could also potentially increase their rate of excretion, further negatively affecting their activity in the body (Dos Santos *et al*., 2011). As thyroid hormones are essential for normal post-natal skeletal and muscular growth and protein turnover, this activity of the flavonoids may negatively impact livestock health and production (Spencer, 1985).

The anti-thyroid nature of flavonoids varies among the different structural types, with the ranking of their inhibitory effect on thyroperoxidase as follows: myricetin > kaempferol > morin > quercetin > naringenin > biochanin A > fisetin > baicalein > naringin > catechin > rutin (Divi & Doerge, 1996). Their potency in inhibiting the type 1 iodothyronine deiodinase enzyme also varies, with baicalein > quercetin > catechin > morin > rutin > fisetin > kaempferol > biochanin A (Ferreira *et al*., 2002).

2.4.4 Interactions with enzymes

In addition to thyroperoxidase and deiodinase, flavonoids have been found to interact with enzymes including hydrolases, phosphatases, protein phosphokinases, DNA synthetases, RNA polymerases, oxygenases and amino acid oxidases (Havsteen, 2002). These interactions mostly involve allosteric inhibition, although some allosteric activation and competitive inhibition has also been reported (Havsteen, 2002). As one can imagine, these extensive effects on enzyme activity could potentially have a large impact on bodily function, and they have been found to be involved in flavonoids' antioxidant, anti-inflammatory and infection-fighting effects (Havsteen, 2002). Specifically, the activation of antioxidant enzymes and inhibition of oxidases helps support flavonoids' overall protective effect against oxidative damage (Procházková *et al*., 2011). Unfortunately, due to the complexity of effects, research on the links between specific enzyme interactions and implications for livestock production is limited.

2.4.5 Antimicrobial activity

Apart from the direct effects of flavonoids on animal physiology, they can also have a large effect on health through their impact on both pathogenic and beneficial commensal microbes. Flavonoids have demonstrated a wide range of antimicrobial properties, including antibacterial, antiviral and antifungal activities.

The antibacterial properties of natural products and extracts containing flavonoids have long been recognised and exploited in traditional medicine, and many of these beliefs have more recently been confirmed through scientific investigation (Cushnie & Lamb, 2005). Propolis, green tea and extracts of medicinal plants such as St. John's wort (*Hypericum* species) and shepherd's-purse (*Capsella* species) have shown *in vitro* antibacterial activity, as have a large number of pure flavonoid aglycones and glycosides (Cushnie & Lamb, 2005; Friedman,

2007). Beneficial interactions between different flavonoids and between flavonoids and other antibacterial agents have also been found, and various modifications of naturally-occurring flavonoids have been tested for efficacy. More recent research has also suggested that flavonoids interfere with bacterial virulence factors, thereby reducing their pathogenicity (Cushnie & Lamb, 2011).

In vivo studies have demonstrated effective protection by quercetin and quercitrin, and sophoraisoflavone A and diprenylgenistein, against *Shigella* and *Salmonella typhimurium* infections respectively in animal models (Cushnie & Lamb, 2005). Mixed extracts of green tea have also been shown to reduce symptoms and mortality in mice infected with *Escherichia coli* O157:H7 or *S. typhimurium*, and pigeon pea (*Cajanus cajan*) extracts reduced the effects of *Staphylococcus aureus* infections in mice (Friedman, 2007; Zu *et al*., 2010). Both *ex vivo* and *in vivo* studies have also reported effects of flavonoids on total gut microbial populations and on the composition of the gut microbiome, indicating that even in the complex environment of the gastrointestinal tract flavonoids exert selective pressure on microbes (Oskoueian, Abdullah & Oskoueian, 2013; Oteiza, Fraga, Mills & Taft, 2018). While the antibacterial activities of flavonoids tend to be less than that of more commonly utilised antibacterial agents such as antibiotics of bacterial origin, some have been found to have minimum inhibitory concentrations below 10 µg/ml, making them of great practical interest. These include panduratin A, isobavachalcone, bartericin A, scandenone, kaempferol 3-O-α-ι-(2”,4”-di-*E*-p- coumaroyl)-rhamnoside, sepicanin A, isolupalbigenin, flavone, 3’-O-methyldiplacol and licochalcone A (Cushnie & Lamb, 2011).

The possible uses of flavonoids as antibacterial agents in the livestock industry are extensive. Not only could they contribute to wound healing after topical application, they may also have potential for use as a prophylactic dietary supplement, or as part of the treatment of systemic diseases. Evidence of synergism with other antimicrobials also suggests possible roles as adjuncts. These roles will likely become increasingly important as restrictions on antibiotic use in livestock become more widespread, and as antibiotic resistance progressively limits the effectiveness of traditional antibiotics. Furthermore, species such as ruminants and hindgut fermenters rely heavily on their gut microbes for the efficient utilisation of dietary nutrients, and the alteration of this population by dietary flavonoids could have positive or negative effects on health and feed utilisation efficiency. The production of the greenhouse gas methane, which is becoming an increasingly important issue, could also possibly be impacted by the effects of flavonoids on the gut microbiome.

A substantial amount of research has demonstrated the antiviral properties of flavonoids. Viruses for which this activity has been reported include bovine herpes virus type 1, porcine reproductive and respiratory syndrome virus, bovine viral diarrhoea virus as well as numerous viruses related to human health (Martin, Crotty, Warren & Nelson, 2007). Flavonoids that have demonstrated antiviral activity include quercetin, biochanin A, genistein, prunetin, baicalin, luteolin, apiin, myricetin, chrysin, acacetin, morin, rutin, catechin and kaempferol, among others (Cushnie & Lamb, 2005; Liu *et al*., 2008; Martin *et al*., 2007). Synergistic antiviral effects have also been reported, both between different flavonoids, and flavonoids and other antiviral agents, suggesting that complex mixtures, such as those found in propolis, may be more effective than purified products (Cushnie & Lamb, 2005).

Considering the devastating effects that diseases such as foot and mouth, bluetongue, viral haemorrhagic disease, Newcastle disease, Rift Valley fever and many others have had and still have on livestock industries across the world, any possible method to ameliorate their spread or effects on production would be hugely valuable. However, considerably more research on the *in vivo* efficacy of flavonoid-based treatments is necessary.

Although fungal infections may not cause as widespread production losses in livestock as bacterial or viral diseases, some specific species, such as *Aspergillus fumigatus*, do cause problems, particularly in poultry (Arné *et al*., 2011). The antifungal properties of flavonoids are therefore worth a mention. Several flavonoids have shown activity against *Candida albicans* and other *Candida* species, as well as *Aspergillus flavus, Aspergillus tamarii, Cladosporium sphaerospermum, Penicillium digitatum* and *Penicillium italicum* (Cushnie & Lamb, 2005). However, research on the *in vivo* potential of flavonoids for treating fungal infections is limited, and the interpretation of results is often complicated by the anti-inflammatory and immune-stimulatory effects of the applied flavonoid. For example, both liquiritigenin and rutin have been found to aid recovery from *C. albicans* infections (disseminated and localised respectively) (Han, 2009; Lee *et al*., 2009). However, Lee *et al*. (2009) concluded that the effect of the former was due to its induction of Th1 cytokine production by CD4+ T cells, as *in vitro* activity against the fungus was not found, while Han (2009) suggested that the beneficial effect was due to both the anti-arthritic and anti-candidal properties of rutin.

2.5 Effects on live performance

Although most of the research on the pharmacological properties and biological effects of the flavonoids has looked at their value for human health management, there have been a number of studies considering their value to the livestock industry. This has included investigating the simple effects on growth, feed intake and feed efficiency, as well as more in-depth research into their potential roles as antioxidants or treatments or preventatives of disease.

Research into flavonoid use in livestock has included studies using pure flavonoid extracts, whole mixed extracts from naturally phenolic-rich plant materials and the crude materials themselves. While the latter two types are useful from a direct application perspective, they unfortunately have resulted in quite considerable variation in the results of these studies, possibly due to the conflicting effects of the various phenols and other compounds present in the unpurified sources. Additional variation as a result of the wide range of inclusion rates used and the specific measurements taken further muddy the water when trying to produce an overall assessment of the value of flavonoids for livestock production purposes.

In order to identify some effects of the different functional classes of flavonoids this review will focus on studies that utilized extracts containing one or only a few types of flavonoids, with limited discussion of more complex sources, with reference to their primary flavonoid components.

2.5.1 Poultry

From the perspective of pure commercial productive performance the results for flavonoid supplementation have thus far been somewhat disappointing. Goliomytis *et al*. (2014) found that supplementing quercetin at 0.5 or 1 g/kg feed to the diet of broiler chickens did not result in any significant change in growth, feed intake or feed conversion ratio (FCR), apart from a tendency for the FCR to detrimentally increase with increased supplementation. A similar lack of effect was found for *Gingko biloba* leaves (starter: 0.35 %, grower: 0.7 %) , which contain high levels of quercetin glycosides (Cao, Zhang, Yu, Zhao & Wang, 2012), and a citrus product (0.25 – 1 kg/ton feed) containing amino acids and equal quantities of quercetin and rutin (Peña *et al*., 2008). In contrast, Sohaib, Butt, Shabbir and Shahid (2015) reported a tendancy for quercetin supplementation (100 – 300 mg/kg feed) to increase broiler chicken growth and decrease the FCR. However, as the quercetin treatments in this study were all applied in conjunction with varying levels of α-tocopherol supplementation, the effect of quercetin alone could not easily be assessed.

A lack of effect on live performance was also found for the flavanones naringin (0.75 – 1.5 g/kg) and hesperidin (0.75 – 3.0 g/kg) by Goliomytis *et al*. (2015) and Simitzis, Symeon, Charismiadou, Ayoutanti and Deligeorgis (2011), and for citrus pulp (0 – 6 % of feed) containing these flavanones by Hajati, Hassanabadi and Yansari (2012). However, Kamboh and Zhu (2013) found that hesperidin at a much lower inclusion rate (20 mg/kg feed) increased the 42 day body weight and decreased the FCR of broiler chickens. This suggests that research investigating the effects of a wide range of inclusion rates *in vivo* would be of interest to determine optimal inclusion ranges for the different flavonoids. It is possible that flavonoids exhibit a U-shaped dose-response curve *in vivo* due to interactions between their many mechanisms of action, as has been found for oestrogenicity assays *in vitro* (Almstrup *et al*., 2002).

A study examining the effects of isoflavones supplemented to broiler diets at inclusion rates of 2- and 5-times the isoflavone content of the control diet (which contained 202 – 347 mg isoflavones/kg) found no effect on growth or feed intake, but a tendency for the gain:feed ratio to detrimentally decrease with supplementation (Payne, Bidner, Southern & McMillin, 2001). The addition of the isoflavone concentrate to a diet formulated using extracted soy protein (which is almost completely free of isoflavones) also failed to restore production levels to those of the control soybean meal diet, despite providing the same level of total isoflavones (Payne, Bidner, Southern & McMillin, 2001). In contrast, Jiang *et al*. (2007) found that supplementing broilers fed a low isoflavone diet with a synthetic soy isoflavone at 10 or 20 mg/kg increased weight gain, feed intake and feed efficiency (at 10 mg/kg) (Jiang *et al*., 2007). These conflicting results could be related to the older age of the broilers used in the latter study — 43 to 63 days, rather than 9 to 52 days, as used by Payne, Bidner, Southern and McMillin, (2001) — as they may have been experiencing metabolic stress at this stage. It could also be due to differences in the composition of the isoflavone supplements used, as Jiang *et al*. (2007) used one containing 98 % glycitein, whereas Payne, Bidner, Southern and McMillin (2001) did not provide the composition of the supplement used.

Kamboh and Zhu (2013) found no effect of genistein supplementation at 5 mg/kg on the live performance of broilers. In contrast, supplementation at 400 and 800 mg/kg to quail increased weight gain, decreased the FCR, and increased the serum levels of vitamins C, E and A, particularly under heat-stress conditions (Onderci *et al*., 2004). This suggests that the genistein helped alleviate the effects of heat stress, possibly through its role as an antioxidant (Onderci *et al*., 2004). In addition, Sahin *et al*. (2006) found that genistein supplemented at 200 – 800 mg/kg improved the apparent digestibility of dry matter, crude protein and ash. It also appeared to improve mineral absorption and deposition in the bones of quails under heat stress (Ca, P, Mg, Zn, Mn, Fe and Cu) and thermoneutral (Ca and P) conditions, possibly due to its oestrogenic activity. This suggests that genistein supplementation may reduce the risk of leg problems and bone-breakage (Sahin *et al*., 2006). A similar ameliorative effect on the consequences of heat stress in quails was found for epigallocatechin-3-gallate (200 – 400 mg/kg feed), with supplementation improving weight gain and feed efficiency, and preventing decreases in serum vitamins C, E and A and increases in serum MDA (Tuzcu *et al*., 2008). It therefore appears that flavonoid supplementation is more effective in heat stressed animals, which concurs with their role as antioxidants. Further research examining the potential of other flavonoids under heat-stress conditions would be valuable.

Several studies have investigated the effects of alfalfa flavonoids, which consist of predominantly tricin, luteolin and apigenin, on poultry performance. Ouyang, Xu, Jiang and Wang (2016) found that an alfalfa extract supplemented at 15 mg flavonoids/kg feed had a positive effect on the average daily gain (ADG) and FCR of broiler chickens and improved the birds' oxidative status. Geese provided with an alfalfa extract (81 % flavonoids) at 300 mg/kg also had improved growth, feed intake and nitrogen utilization, and heavier tibias, which could help reduce leg injuries (Chen *et al*., 2016). The higher weights of the bursa and spleen found in supplemented geese also suggested an improvement in the immune status of the birds. Dong *et al*. (2007) found that polysavone (alfalfa extract containing polysaccharides, saponins and flavonoids) supplementation had no effect on live performance, possibly due to the low inclusion rate used (0.06 %), but did improve the immune status of the chickens. It therefore seems that the combination of flavonoids present in alfalfa is particularly beneficial, and further research specifically examining the effects of these main flavonoid components individually and in combination would be of interest.

The results of research on the use of pure flavonoids to treat infectious disease conditions supported the suggested immune-stimulating effects of alfalfa flavonoids (Chen *et al*., 2016; Dong *et al*., 2007). Supplementation with a cranberry extract at 2 – 8 mg/bird/day enhanced humoral immunity through an increase in immunoglobulin B antibody levels (Islam, Oomah & Diarra, 2017). It also increased the antibody response to vaccinations for infectious bursal disease virus, infectious bronchitis virus and avian reovirus, suggesting a possible role as an adjuvant (Islam *et al*., 2017). However, no impact on live weight, feed intake or efficiency was found. The supplementation of a citrus by-product containing high levels of hesperidin and eriocitrin at 2 % (dry product) or 4 % (wet product) also prevented the occurrence of coccidiosis, and increased growth and the levels of immunoglobulin G in the blood (Antongiovanni *et al*., 2005). Catechins have also been found helpful for the

treatment of coccidiosis, with a study examining the potential of green tea leaf powder for treating or preventing *Eimeria maxima* finding that supplementation at 0.5 or 2 % reduced the shedding of faecal oocysts (Jang *et al*., 2007). However, the negative effects of infection on weight gain were not ameliorated. In contrast, Erener *et al*. (2011) found that green tea extract (mainly epigallocatechin gallate) at 0.1 or 0.2 g/kg reduced the caecal coliform count, as well as improving growth and feed efficiency. These differences may reflect the different concentrations of active components in dried tea leaves compared to a tea extract, despite the higher inclusion rate used by Jang *et al*. (2007).

Research on the use of flavonoids in the treatment of metabolic diseases has also had positive results, which concurs with their ameliorating effects on heat stress. Goliomytis *et al*. (2014) found that quercetin (0.5 – 1 g/kg feed) increased the proportion of the heart, which may help reduce the prevalence of metabolic disorders such as ascites and sudden death syndrome, and rutin supplementation (1 g/kg feed) reduced the incidence of exudative diathesis in selenium- and vitamin E-deficient broiler chicks (Jenkins, Hidiroglou & Collins, 1993). This may have been due to its activity as an antioxidant, or its strengthening effect on fibrous membranes, including the walls of the capillaries (Jenkins *et al*., 1993). Quercetin and morin (1 g/kg feed) also provided some protection against nutritional muscular dystrophy (Jenkins & Atwal, 1995). This further supports the idea that flavonoids may have a role to play in disease prevention.

A relatively large number of studies have investigated the effects of flavonoid-rich feed resources and crude extracts on poultry live performance. Propolis (0.5 – 5 g ethanol extract/kg diet) has exhibited beneficial effects on a range of variables, including the live weight, growth, feed intake and mortality of broilers under heat stress conditions (Seven & Seven, 2008; Seven, Seven, Yılmaz & Şimşek, 2008). This may have been due to its antioxidant effect, as indicated by decreases in MDA and aspartate transaminase and alkaline phosphatase (stress-indicators) levels (Seven *et al*., 2009). Kleczek, Majewska, Makowski and Michalik (2012) also found that combined propolis (0.025 %) and bee pollen (0.5 %) supplementation affected the geometric dimensions of broiler chicken tibia bones, although the shear strength of the bones was not impacted. While these benefits of bee products could be due to their chrysin, acacetin and naringenin contents, they also contain alcohols, terpenes and quinones, which exert a number of biological effects. It would therefore be of interest to investigate whether a purified flavonoid extract of propolis had the same benefits, as well as whether these effects were still observed in the absence of heat stress. Another natural source of flavonoids that has had promising results is the medicinal plant Baical skullcap root (*Scutellaria baicalensis radix*), which contains high levels of wogonin, baicalein, and baicalin (Kroliczewska, Zawadzki, Skiba, Kopec & Kroliczewska, 2008). Supplementation at 0.5 % of the diet significantly increased weight gain and decreased feed intake, without affecting the FCR. Further research on this medicinal plant, and its component flavonoids, should thus be done.

In contrast to the beneficial effects of propolis and Baical skullcap root, results for grape by-products, which are rich in catechins, have been less promising. Grape pomace (5 – 30 g/kg), grape pomace concentrate (15 – 60 g/kg) and grape seed extract (0.6 – 3.6 g/kg) have been found to have no effect on live performance parameters

(Brenes *et al*., 2008; Brenes *et al*., 2010; Goñi *et al*., 2007), while Lau & King (2003) found that supplementation with grape seed extract at 2.59 % and 5.18 % caused severe growth depression. These non-significant or negative effects may have been due to the relatively low flavonoid contents of some of these products (4.87 % total polyphenols for concentrated grape pomace) (Brenes *et al*., 2008), or, more likely, as a result of their high tannin contents (Lau & King, 2003). However, the presence of some physiological effects, such as the higher spleen weight in grape seed extract supplemented chickens (Brenes *et al*., 2010), does suggest that further research on specific flavonoid extracts of grape pomace may be valuable. Flavonoid extracts of fenugreek (*Trigonella foenum-graceum* L.) may also be worth researching, as although fenugreek seed powder supplementation (5 – 40 g/kg) decreased growth and increased the FCR, it also had an impact on blood glucose, triglyceride and cholesterol levels in a non-linear dose-dependent manner (Duru *et al*., 2013). The major flavonoids present in fenugreek are naringenin, quercetin, vitexin and tricin.

2.5.2 Pigs

Literature on the effects of flavonoid supplementation on the live performance of pigs is more limited than that for poultry. Only three studies testing the effects of pure flavonoids on pig live performance appear to have been done, all of which tested the efficacy of isoflavones.

Research by Cook (1998) on the effects of a mixed isoflavone extract containing genistein (14 %), diadzein (13.4 %) and glycitein (4.4 %) glycosides, supplemented at 1585 ppm, found that it tended to increase growth and estimated muscle gain from weaning to slaughter, without altering feed intake or efficiency. Cook (1998) also found that supplementation with a genistein-dominated (72 %) extract at 200 – 800 ppm had no effect on body or muscle growth, suggesting that most of the effect of the first mixed extract was due to the diadzein.

Greiner, Stahly and Stabel (2001a) found that diadzein supplementation at 200 or 400 ppm from weaning to 15 kg live weight improved growth, feed intake and feed efficiency, but only after the inoculation of the pigs with the porcine reproductive and respiratory syndrome (PRRS) virus. This effect was most significant during periods when the systemic virus concentrations were high, and supplementation also increased the serum levels of interferon, which signals the stimulation of macrophages, 4 days after inoculation. Similar results were found for genistin (genistein glycoside) at 200 – 800 ppm by Greiner, Stahly and Stabel (2001b), although genistin also reduced the serum levels of the virus, which was not found for diadzein. These results suggest that isoflavones may have potential for ameliorating the effects of viral infections in pigs, possibly through their interaction with the immune system. However, it is notable that during the first 17 days post-weaning, prior to inoculation, diadzein supplementation decreased the growth and the gain:feed ratio of the pigs (Greiner *et al*., 2001a).

In contrast with the results of Cook (1998) and Greiner *et al*. (2001a,b), Payne, Bidner, Southern and Geaghan (2001) found that the supplementation of a mixed isoflavone extract to a low flavonoid feed formulated using soy protein concentrate decreased growth and feed intake during the late finishing period, although there was no effect on overall live performance. The addition of the isoflavone concentrate to a standard corn-soybean meal also had no effect on growth or feed intake, despite the supplement being formulated to achieve isoflavone levels 2- and 5-

times that of the control diet (Payne, Bidner, Southern & Geaghan, 2001). Further research is clearly required to provide clarity on which isoflavones and at which inclusion rates, if any, may have beneficial effects. Considering the oestrogenic effects of isoflavones, research comparing their effects on different sex-types would also be of interest.

The only other flavonoid group that has been studied in a relatively purified form are the catechins, with Mason *et al*. (2005) using a mixture extracted from green tea containing 40 % epigallo-catechingallate, 24 % epigallocatechin, 12 % epicatechingallate and 10 % epicatechin. The supplementation of this extract at 200 mg/kg from 80 kg live weight to slaughter (105 kg), and in conjunction with rapeseed oil at 50 g/kg, did not improve feed intake or growth (Mason *et al*., 2005). Augustin *et al*. (2008) similarly found that green tea catechins at 10 or 100 mg/kg body weight had no effect on pig live performance, plasma antioxidant capacity or cholesterol content, or tissue tocopherol content. These results are in contrast with the general agreement in literature that green tea extracts do display *in vivo* antioxidant activity (Frei & Higdon, 2003). However, the lack of effect found may have been due to the relatively short supplementation period (*ca*. 33 days) and the lack of induced stress during supplementation. It may also have been due to the relatively high level of vitamin E in the control diet used by Augustin *et al*. (2008). A similar lack of effect on growth, feed intake or feed efficiency was found for the inclusion of black rice bran, which is high in cyanidin 3-glucoside, at up to 0.6 % of the diet of finisher pigs (Kil *et al*., 2006). However, this may have been due to the low inclusion rate and the very low number of replicates used in this study.

In contrast with the disappointing results for the green tea extract and rice bran, the inclusion (50 – 150 g/kg) of fermented *G. biloba* leaf residue in pig diets from 21 to 63 days of age increased growth, final body weight and the gain:feed ratio (at 100 g/kg), without changing feed intake (Zhou, Wang, Ye, Chen & Tao, 2015). It also increased the apparent total tract digestibility, possibly explaining the improved performance. Similarly, a combined extract of citrus fruit and chestnut tree (the active ingredients of which were flavonoids and tannins) supplemented at 0.2 %, increased weight gain to the same extent as a diet supplemented with apramycin and oxytetracycline, without changing feed intake or efficiency (Hong *et al*., 2004). The extract also appeared to reduce the prevalence of diarrhoea in the pigs, particularly in the first two days after weaning. However, in addition to its high flavonoid content (34 mg/g), fermented *G. biloba* residue contains substances such as terpenoids and phenolic acids, and tannins are known bioactive compounds. The results of these studies are consequently inconclusive in terms of establishing flavonoid efficacy, particularly in the absence of any research on the supplementation of pure flavanones and flavonols to pigs.

Apart from their systemic effects, ingested flavonoids may also impact gut health, as a large proportion of ingested flavonoids remain in the gut, rather than being absorbed. Sehm, Lindermayer, Dummer, Treutter and Pfaffl (2007) found that supplementing piglets with apple and grape pomace at 3.5 % dry matter caused significant changes in the histomorphometry of the gut. This included a decrease in villi growth in the jejunum (grape pomace), a decrease in Peyer's patch growth in the ilium (grape and apple pomace) and an increase in colon crypt

area (grape and apple pomace). These effects were generally considered positive, suggesting that dietary flavonoids could improve gut health, although supplementation had no effect on weight gain in this case (Sehm *et al*., 2007).

2.5.3 Rabbits

Few studies have investigated the effects on live performance of supplementing relatively pure flavonoids to rabbits. Simitzis *et al*. (2014) supplemented hesperidin at 1 or 2 g/kg feed to meat rabbits from weaning until slaughter at 80 days old and found that neither the final body weight nor feed efficiency were impacted by the supplementation. An extract of alfalfa (at 400 to 1200 mg/kg feed), which contained predominantly tricin and apigenin glycosides, and extracts of onion and cranberry (at 10 ppm total polyphenols), had similarly non-significant effects on growth, feed intake and feed efficiency when supplemented to growing meat rabbits (Dabbou *et al*., 2018; Koné e*t al*., 2016).

In contrast, supplementation with bee pollen at 100 – 300 mg/kg from 4 – 12 weeks of age improved the growth and survival of meat rabbits during both summer and winter, with 200 mg/kg showing the highest growth response (Attia, Al-Hanoun & Bovera, 2011). A similar effect was seen for adult bucks, with bee pollen supplementation increasing body weight gain and decreasing feed intake, resulting in a beneficial decrease in the FCR (Attia *et al*., 2011). Beneficial changes in biochemical blood parameters such as increased levels of glucose and protein and decreased urea and creatinine were also found. However, as bee pollen is a complex product also containing high levels of protein, essential amino acids, polyunsaturated fatty acids (PUFA), carbohydrates, vitamins, minerals and enzymes, these effects cannot be conclusively attributed to its flavonoid content.

Similarly, *Eucommia ulmoides* leaves contain, along with flavonoids (mostly quercetin and rutin), polyphenol acids, iridoid compounds, vitamins, minerals and amino acids. Nonetheless, it is interesting to note that supplementation with *E. ulmoides* leaf powder at 1 g/kg feed detrimentally decreased the feed intake of heat stressed rabbits from 42 to 63 days of age, but at 5 g/kg feed significantly increased growth, with both treatment levels beneficially reducing the feed conversion ratio (Li *et al*., 2018). Supplementation at 5 g/kg also increased the total antioxidant capacity (TAC) and glutathione peroxidase activity, and decreased the MDA levels in the serum, indicating an *in vivo* antioxidant effect. It thus seems likely that the active components in the *E. ulmoides* leaf powder helped ameliorate the heat-induced oxidative stress of the rabbits. However, further research including a negative control (not heat-stressed) would provide confirmation of the extent to which live performance could be improved by this product.

The replacement of berseem hay with dried strawberry by-products, which contain relatively high levels of flavonoids (fresh pomace *ca*. 3 mg/g flavonoids) also beneficially impacted rabbit performance, even in the absence of heat stress (Waly, Bassyony, Zedan & Galal, 2015). Inclusion at 25 % of the diet increased total body weight gain without changing feed intake, thus improving feed efficiency, and feed digestibility and nitrogen balance were also improved. While this confirms the value of strawberry pomace as a feed resource, once again, the presence of a multitude of other bioactive compounds precludes drawing conclusions on the role of flavonoids

in this effect, particularly as Koné *et al*. (2016) found no effect of supplementing a strawberry extract to rabbits at 10 ppm total polyphenol on their growth or FCR. However, the beneficial effects on the rabbits' oxidative status, including decreasing the MDA levels and increasing the TAC, do suggest that antioxidant components present in the strawberry product may have played a role (Waly *et al*., 2015).

In summary, while there is some suggestion that flavonoids may have value as growth promoters in rabbit diets, particularly under heat stress conditions, there is a serious lack of specific research on pure flavonoids to provide confirmation of this. Follow-up studies that supplement flavonoid extracts at similar inclusion levels as were provided by the whole products should be done.

2.5.4 Sheep, goats and cattle

In contrast to rabbits, a number of studies investigating the effects of pure flavonoids on the live performance of ruminants have been done, although they still only cover four of the thousands of flavonoid varieties, namely naringin, hesperidin, quercetin and catechins.

Alhidary and Abdelrahman (2014) found that 1 g naringin/day improved the ADG and feed efficiency of heat stressed lambs above that of the control, with 2 g/day having an intermediate effect. Supplementation also decreased serum creatinine and increased serum albumin, possibly through naringin's antioxidant activities. These effects on live performance under very similar heat stress conditions were confirmed by Alhidary and Abdelrahman (2016), with sheep on the lower inclusion rate (7 g/week) once again outperforming those receiving higher levels of naringin (14 g/week). In addition, the antioxidant effect was shown further as higher levels of superoxide dismutase and glutathione peroxidase activity in sheep receiving 14 g/week, and overall immune status was improved, based on higher antibody titres and cell mediated immunity (Alhidary & Abdelrahman, 2016). These significant effects concurred with the relatively high bioavailability found for naringin in ruminants, due to the ability of the rumen bacteria to deglycosylate naringin in the rumen, allowing the absorption of the naringenin aglycone in the small intestine (Gladine, Rock, Morand, Bauchart & Durand, 2007).

In contrast with these results, Simitzis *et al*. (2019) found that naringin supplemented at 2.5 g/kg feed had no effect on any live performance traits of lambs, despite decreasing the plasma MDA level. However, as the sheep used by Simitzis *et al*. (2019) were not experiencing heat stress, this discrepancy may suggest that under relatively stress-free conditions the antioxidant effects of the flavonoids were not as beneficial as the animals were not experiencing an oxidative imbalance. It is also interesting to note that naringin included at 4.5 % of the substrate had little to no impact on *in vitro* rumen microbial fermentation, as measured by the dry matter degradability, total volatile fatty acid (VFA) concentration, microbial enzyme activities and rumen microbial population, unlike some of the other flavonoids tested (Oskoueian *et al*., 2013).

Contrary to the findings of Oskoueian *et al*. (2013), Balcells *et al*. (2012) found that supplementation with a mixed flavonoid product consisting of predominantly naringin at 300 mg/kg DM helped protect dairy heifers against declines in rumen pH, including that induced through the administration of ground wheat. This may have been due to its effect on the rumen microbial population, including the lactate-consuming bacterium *Megasphaera*

elsdenii, or due to the stimulation of straw intake through an unknown mechanism. Supplementation also appeared to improve the efficiency of nitrogen usage in the rumen. However, these protective effects against induced acidosis did not translate into beneficial effects on growth or feed conversion efficiency in the absence of acidosis (Balcells *et al*., 2012). This suggests that in the absence of active digestive disturbances, the potential beneficial effects of flavonoids on rumen fermentation are less notable.

Simitzis *et al*. (2019) also tested the effect of hesperidin supplementation at 2.5 g/kg feed, and found that it decreased plasma MDA levels but did not improve growth or feed intake. A similar lack of any effect was found for hesperidin supplemented at 1.5 or 3 g/kg feed by Simitzis, Ilias-Dimopoulos, Charismiadou, Biniari and Deligeorgis (2013). It is interesting to note that in both of these studies vitamin E supplementation also had no effect on live performance, supporting the suggestion that under normal conditions antioxidant supplementation may have little effect.

Quercetin at 200 mg/kg feed has similarly been shown to have no effect on the feed intake of growing goats, although dry matter and crude protein digestibility were increased, as was the rumen total VFA concentration (Cho *et al*., 2010). This interaction of quercetin with the rumen microbial environment was confirmed by Oskoueian *et al*. (2013), who found that quercetin (4.5 % of substrate) increased *in vitro* gas production but reduced methane production; however, they found that it had no effect on VFA concentrations or dry matter degradability. The lack of effect of quercetin on live performance may be due to its very low bioavailability (Berger, Wein, Blank, Metges & Wolffram, 2012).

Green tea catechins, when supplemented at 2, 3 or 4 g/kg feed, improved the ADG of goats, with the 3 and 4 g/kg groups having the highest growth rate (Tan *et al*., 2011). It was speculated that this was due to their anti-microbial effects on the rumen, and/or antioxidant activity. The interaction with the rumen environment was somewhat supported by the findings of Oskoueian *et al*. (2013), although their results indicated a detrimental effect on dry matter degradability and rumen enzyme activity. Zhong *et al*. (2011) confirmed that ingested catechin (2 – 4 g/kg feed) interacted with the endogenous antioxidant glutathione system; however, the nature of the effect was dose dependant and suggested that the catechins actually increased the rate of reduced glutathione depletion in the plasma, especially at higher concentrations. Catechin supplementation also decreased plasma levels of cortisol, and increased insulin-like growth factor II at 2 g/kg (Zhong *et al*., 2011), which may suggest an alternative explanation for the effects on growth found by Tan *et al*. (2011).

The only pure flavonoid tested in cattle for its effect on live performance was an isoflavone, biochanin A, which is found in red clover. Harlow, Flythe, Kagan and Aiken (2017) found that the addition of biochanin A at 6.3 g/steer to a dried distillers grains concentrate resulted in improved ADGs in steers on grass pasture. They also established that at least one component of this effect was the interaction of the isoflavone with the rumen microbial population, with it having the specific effect of reducing the proportion of ingested protein degraded to ammonia, and thus increasing the bypass protein, by inhibiting hyper-ammonia-producing bacteria (Harlow *et al*., 2017). A similar effect on growth was found for sheep fed isoflavone-containing subterranean clover, which is high in

genistein, biochanin A, daidzein and formononetin (Pace *et al*., 2006), with the ADG of both ewes and male lambs being higher for those fed the diets containing subterranean clover.

A large number of studies have investigated the potential of high-flavonoid content by-product feed resources, such as grape (Economides & Georghiades, 1980; Malossini, Pinosa, Piasentier & Bovolenta, 1993; Voicu, Hăbeanu, Uță, Voicu & Gras, 2014), citrus (Fung, Sparkes, Van Ekris, Chaves & Bush, 2010; Lenehan, Moloney, O'Riordan, Kelly & McGee, 2017; Sharif *et al*., 2018) or apple (Taasoli & Kafilzadeh, 2008) pomace, in ruminants. Unfortunately, although these products are valuable sources of flavonoids such as naringin (citrus), quercetin (apple) and epicatechin (grape), the investigation of the effects of their inclusion in a crude form at high levels as replacements for conventional feedstuffs such as alfalfa, barley or lupin seeds, does not allow the assessment of the effects of the flavonoids. This is due to unavoidable confounding with other influential components such as sugars, pectin or lignin, as well as with the overall nutritional density of the product. Nonetheless, research assessing the effects of flavonoid extracts of these resources and other flavonoid-rich products may be of interest, as Gladine *et al*. (2007) found that aqueous extracts of both citrus pulp and grape pomace inhibited lipoperoxidation in blood plasma from sheep, after administration at 10 % DM through a rumen cannula. This suggests that these extracts do display *in vivo* antioxidant activity.

Furthermore, feed resources high in condensed tannins, such as grape pomace, pomegranate peels and some legume forages, may provide an additional source of epicatechins to ruminants. This is due to the ability of the rumen microbes to break down polymeric proanthocyanidins, releasing the component monomeric epicatechins and making them available for absorption in the small intestine (Gladine *et al*., 2007). Unfortunately, due to the direct nutritional (positive and negative) and anti-nematode effects of the tannins themselves, assessing their effects as sources of epicatechin would be challenging (Min, Barry, Attwood & McNabb, 2003).

2.6 Effects on carcass yields and meat quality

Carcass yields will always be economically important for producers, and organoleptic properties such as colour, flavour and texture will always play an important role in purchasing choices. However, modern Western consumers are becoming increasingly concerned about the healthfulness of the food they eat, as impacted by factors such as the total lipid content and the fatty acid composition (Jiménez-Colmenero, Carballo & Cofrades, 2001; Verbeke & Viaene, 2000). Moreover, the processing, transport and storage of meat products continues to play a vital role in commercial meat production, making a long shelf-life equally essential. It is therefore important that the possible implications, both positive and negative, of dietary flavonoid supplementation on these post-slaughter production parameters, is also determined.

2.6.1 Poultry

The effect of flavonoid supplementation on the slaughter yield of poultry has largely been non-significant (Goliomytis *et al*., 2015; Kamboh & Zhu, 2013; Payne, Bidner, Southern & McMillin, 2001; Peña *et al*., 2008), with the exception of the supplementation of propolis extract to heat stressed broilers, which increased both carcass

and breast yield, likely as a result of its positive effect on growth performance (Seven *et al*., 2008). The proportion (percentage of body weight) of the abdominal fat pad has also not generally been impacted by dietary flavonoid supplementation, with this being the case for quercetin at 0.5 or 1 g/kg feed (Goliomytis *et al*., 2014), naringin or hesperidin at 0.75 or 1.5 g/kg feed (Goliomytis *et al*., 2015; Simitzis *et al*., 2011), isoflavone concentrate at 2- or 5-times the control level (Payne, Bidner, Southern & McMillin, 2001), *G. biloba* leaf powder at 0.35 % (starter) and 0.7 % (grower) (Cao *et al*., 2012) or grape pomace concentrate at 15 – 60 g/kg (Brenes *et al*., 2008). It was similarly uneffected, even under heat stress conditions, by supplementation with a fermented citrus product containing quercetin and rutin at 0.25 – 1 kg/tonne feed (Peña *et al*., 2008) or propolis extract at 0.5 – 3 g/kg feed (Seven *et al*., 2008).

In contrast with the limited effects on carcass composition, there has been some evidence of interactions with basic physical meat quality parameters, particularly colour, pH and water-holding capacity (WHC), although consensus across studies is lacking. Goliomytis *et al*. (2014) found that breast meat lightness (L*) tended to decrease and redness (b*) tended to increase with increasing (0.5 to 1 g/kg feed) levels of quercetin supplementation, whereas a combination of genistein and hesperidin (1:4) at 20 mg/kg significantly increased lightness, 45 minute pH and WHC above that of the control (Kamboh & Zhu, 2013). The WHC was also increased by genistein at 5 mg/kg and hesperidin at 20 mg/kg. In contrast, Goliomytis *et al*. (2015) and Simitzis *et al*. (2011) both found no differences in colour or ultimate pH (pH_u) between hesperidin-supplemented broilers and controls, although supplementation at 1.5 g/kg caused higher L*, lower a* and lower pH_u values than supplementation at 3 g/kg (Simitzis *et al*., 2011). This suggests that dosage may play a large role in determining the observed effect, as was found for a mixed herb extract containing primarily luteolin and berberine (an isoquinoline derivative), which caused higher b* values when supplemented at 0.3 % than at 1 %, while neither treatment differed from the control (Jang *et al*., 2008). However, L* values were lower for both supplemented treatments than for the control samples (Jang *et al*., 2008). Isoflavone supplementation also tended to increase the WHC (40 mg/kg), L* (40 and 80 mg/kg) and pH_u (20 and 40 mg/kg), with the latter possibly being due to a decrease in lactic acid production *post-mortem* in supplemented birds (Jiang *et al*., 2007). It has also been suggested that the generally positive effects on the WHC were due to the supplemented flavonoids protecting the muscle tissue against stress-induced oxidative damage (Kamboh & Zhu, 2013). Nonetheless, many studies have not reported any effects on pH_u, WHC or colour (Cao *et al*., 2012; Goliomytis *et al*., 2015; Peña *et al*., 2008), and no effects on the cooking losses or toughness of poultry meat appear to have been reported.

In terms of the proximate chemical composition, Kroliczewska *et al*. (2008) found that the supplementation of Baical skullcap root increased the breast meat dry matter content and crude protein content, and decreased the crude fat content. This differed for the leg meat, for which supplementation tended to decrease the dry matter and did not impact the protein content, but decreased the fat and ash content. It is interesting to note that for both portions the effects on the fat content were greater for 0.5 and 1.0 % supplementation than 1.5 %. However, in

general the total intramuscular fat (IMF) content has not been impacted by flavonoid supplementation (Cao *et al*., 2012; Goliomytis *et al*., 2014; Jang *et al*., 2008; Kamboh & Zhuh, 2013; Simitzis *et al*., 2011).

In contrast, the muscle cholesterol content has been shown to be decreased by flavonoids (Kamboh & Zhuh, 2013), and the few studies that have determined the impact on the fatty acid composition have all reported significant effects. Kamboh and Zhuh (2013) found that genistein (5 mg/kg) and genistein+hesperidin (5, 10 or 20 mg/kg) supplementation tended to decrease the proportion of saturated fatty acids (SFA), while higher levels of the combined treatment also decreased the proportion of monounsaturated fatty acids (MUFA) and increased the proportion of PUFA. The ratios of n-6:n-3, and PUFA:SFA were also increased by genistein+hesperidin supplementation (Kamboh & Zhuh, 2013). Sohaib *et al*. (2015) similarly found that quercetin supplementation decreased the proportion of SFA; however, while quercetin at 100 mg/kg feed tended to decrease the MUFA as well, 200 and 300 mg/kg appeared to increase them, and the PUFA appeared to decrease with an increase in the quercetin inclusion level. There was also no effect on the PUFA:SFA ratio, which was generally much lower than that reported by Kamboh and Zhuh (2013). However, as the quercetin was supplemented in conjunction with varying amounts of α-tocopherol in this study, it was difficult to assess the effects of the flavonoid alone (Sohaib *et al*., 2015). Both quercetin and morin (1 g/kg) impacted the tissue fatty acid composition of vitamin E-deficient chicks (Jenkins & Atwal, 1995), decreasing several n-9 fatty acids, while increasing fatty acids of both the n-6 and n-3 series, including linoleic acid (C18:2n-6) and arachidonic acid (C20:4n-6).

Several mechanisms for the effects on the fatty acid composition have been suggested, including the antioxidant activity of the flavonoids helping protect PUFA from oxidation, and the inhibition of enzymes such as Δ9-desaturase, and other desaturases and elongases (Jenkins & Atwal, 1995; Kamboh & Zhuh, 2013). The specific increase in arachidonic acid found by both Jenkins and Atwal (1995) and Kamboh and Zhuh (2013) may also be linked to flavonoids' role as anti-inflammatory agents, as proinflammatory lipoxygenase metabolites (eicosanoids) are produced from this fatty acid. The inhibition of another enzyme, HMG-CoA reductase, could also possibly be responsible for the reduced cholesterol content, although flavonoids may also decrease the absorption of cholesterol and bile acids (Kamboh & Zhuh, 2013). Considerable further research is necessary to confirm the effects of flavonoids on meat fatty acid composition, as well as to investigate possible interactions with other dietary components.

Possibly the most distinctive effect of dietary flavonoids on poultry meat quality was on its oxidative stability, with the literature almost unanimously agreeing that flavonoid supplementation reduced the formation of MDA during storage. This was found for quercetin (Goliomytis *et al*., 2014: 0.5 – 1 g/kg; Sohaib *et al*., 2015: 100 – 300 mg/kg), naringin (Goliomytis *et al*., 2015: 0.75 – 1.5 g/kg), genistein and hesperidin (Kamboh & Zhu, 2013, G: 5 mg/kg, H: 20 mg/kg; Simitzis *et al*., 2011, H: 1.5 – 3 g/kg), isoflavones (Jiang *et al*., 2007: 10 – 80 mg/kg feed), tea catechins (Tang, Kerry, Buckley & Morrissey, 2001: 50 – 300 mg/kg feed), grape pomace (Brenes *et al*., 2008:15 – 60 g/kg feed; Goñi *et al*., 2007: 5 – 30 g/kg feed), *G. biloba* leaves (Cao *et al*., 2012: 0.35 % and 0.75 %) and a mixed herb extract (Jang *et al*., 2008: 0.3 – 1 %). In contrast, results for other measures of oxidation,

such as the TAC, as measured using the FRAP, DPPH-scavenging or $ABTS^{+}$-reducing activity, have not been as consistent. Rupasinghe, Ronalds, Rathgeber and Robinson (2010) reported no effect of supplementing quercetin (50 – 600 mg/kg body weight/day) or an apple product (50 – 150 mg total phenolics/kg body weight per day) to broiler chickens on the TAC of duodenum or liver tissue, nor did Jiang *et al*. (2007) for isoflavone supplementation on the TAC of the breast muscle tissue. However, Sohaib *et al*. (2015) reported that both FRAP and DPPH-scavenging activity tended to increase in response to increased quercetin supplementation, and Jang *et al*. (2008) found a similar response to a mixed herb extract, although there was less effect on DPPH and $ABTS^{+}$ activities than on the MDA content, as measured using the TBARS assay (thiobarbituric acid reactive substances).

While this effect on oxidative stability is clearly linked to the role of flavonoids as antioxidants, the exact mechanism is still uncertain. The most direct method would involve the incorporation of the supplemented flavonoid, or its metabolites, in the tissue, where it would protect PUFA and other molecules from oxidation. This is supported by the findings of Rupasinghe *et al*. (2010) and Sohaib *et al*. (2015), who detected quercetin glycosides in the tissue, and that of Sohaib *et al*. (2015) and Jang *et al*. (2008), who found an increase in the total phenol content of tissues in response to supplementation. An alternative, or possibly additional, mechanism could be through the sparing effects of supplemented flavonoids on other antioxidants, such as α-tocopherol. Evidence for this is the higher concentration of this vitamin found in the tissues of birds supplemented with various flavonoids or flavonoid-rich products (Goñi *et al*., 2007; Sohaib *et al*., 2015). Isoflavone supplementation has also been found to increase the activity of several antioxidant enzymes, including superoxide dismutase and catalase (Jiang *et al*., 2007). However, *G. biloba* supplementation did not affect superoxide dismutase or glutathione peroxidase activity, despite reducing MDA levels (Cao *et al*., 2012). More focussed research is needed to fully elucidate the mechanisms by which flavonoids exert their antioxidant effects in meat.

Only a single study appears to have investigated the effects of flavonoids on the sensory quality of poultry meat, and only for a mixed extract rather than a pure flavonoid. The rating of flavour, taste, texture and acceptability of cooked chicken breast meat on a 9-point hedonic scale appeared to be improved by the supplementation (0.3 % or 1 %), with flavour better after zero and three days chilled storage, but not 7, taste better after all three periods, texture only differing on day zero, and overall acceptability better for all the storage periods (Jang *et al*., 2008). Further sensory studies, particularly for the more complex mixed extracts and products, should be performed.

2.6.2 Pigs

Cook (1998) found that supplementing an isoflavone concentrate that provided 670 mg genistein, 705 mg daidzein and 210 mg glycitein/kg diet tended to increase the percentage of muscle in the carcass, and particularly favoured the growth of the red muscles. The specific effect on the growth of muscles consisting of mostly type-1 fibres was attributed to either the interleukin-3 stimulating effects of daidzein (and the further effect of interleukin on cellular glucose transporters), or to the oestrogenic effects of isoflavones (Cook, 1998). Cook (1998) also tested an extract containing predominantly genistein (47 %) at 200 – 800 ppm and found no effect on muscle growth, leading to the

conclusion that diadzein was largely responsible for the previous results. Similar effects were reported by Payne, Bidner, Southern and Geaghan (2001), who supplemented an isoflavone extract to a low-flavonoid diet formulated using soy protein extract, to reach a total isoflavone concentration equivalent to that of a standard corn-soy feed. Supplementation increased the dressing percentage, percentage lean and the lean:fat ratio, while decreasing the proportion of fat in the carcass, relative to that of the low-isoflavone diet. However, values for the supplemented pigs did not differ from those of the pigs on the standard corn-soy diet, apart from for the ham, which had the highest percentage lean and lowest total fat in the supplemented pigs (Payne, Bidner, Southern & Geaghan, 2001). The additional supplementation of isoflavones to the standard diet to achieve total isoflavone levels 2- and 5-times normal also did not have any effect on carcass quality, suggesting that the soybean meal normally utilised in pig diets provides sufficient levels of isoflavones for optimal performance (Payne, Bidner, Southern & Geaghan, 2001).

In contrast to the results for the isoflavones, supplementation with green tea extract (200 mg/kg feed) high in catechins had no effect on the dressing percentage, carcass lean, backfat depth or loin fat depth (Mason *et al*., 2005). The thickness of the shoulder and ham fat was higher in the supplemented pigs; however, this could have been due to the 50 g/kg rapeseed oil that was provided in conjunction with the tea extract (Mason *et al*., 2005).

In general, flavonoid supplementation had no impact on the proximate or physical quality of pork meat, with this being the case for an isoflavone concentrate (Cook, 1998; Payne, Bidner, Southern & Geaghan, 2001), green tea extract (Augustin *et al*., 2008: 10 or 100 mg/kg body weight), high cyanidin 3-glucoside black rice bran (Kil *et al*., 2006: 0.2 – 0.6 % of diet), grape seed extract (O'Grady, Carpenter, Lynch, O'Brien & Kerry, 2008) and a commercial flavonoid extract made from grapes, nuts and citrus fruit (González & Tejeda, 2007: 2 g/kg feed). O'Grady *et al*. (2008) found that grape seed extract at 100, 300 or 700 mg/kg feed had no effect on the pH_u or colour of raw pork loin, or the colour and sensory scores of cooked pork. This could have been due to the high proportion of condensed tannins in the grape seed extract (69 – 85 %), as they are known to be resistant to hydrolysis by mammalian digestive enzymes, and are not absorbed as is in the intestine. The amount of available catechin and epicatechin in the grape seed extract may therefore not have been sufficient to affect meat quality. However, Augustin *et al*. (2008) found a similar lack of effect for the supplementation of green tea extract at 10 or 100 mg/kg body weight on the pH_u, drip loss or colour, and the supplementation of the commercial flavonoid extract at 2 g/kg had no effect on the meat proximate composition and fatty acid profile (González & Tejeda, 2007).

Unlike for poultry, flavonoid supplementation has also not been found to improve the oxidative stability of pork meat. Neither green tea extract (Augustin *et al*., 2008) nor grape seed extract (O'Grady *et al*., 2008) decreased the degree of lipid oxidation (MDA content), even after 16 days of chilled storage or cooking (O'Grady *et al*., 2008). This lack of an antioxidant effect may reflect the very limited extent to which the flavonoids were deposited in the muscle tissue, as was found for quercetin after supplementation of the pure aglycone at 50 mg/kg body weight/day by Bieger *et al*. (2008), for catechins by Mason *et al*. (2005), and for total polyphenols by González

and Tejeda (2007). It may also be linked to their relatively short elimination half-life, as Augustin *et al*. (2008) suggested that the ingested tea catechins may have been purged from the tissue during the 24 hour *ante mortem* fasting period, thereby preventing any protective effect against oxidation *post-mortem*. This theory was supported by the finding that quercetin did not accumulate in the tissue, even after long-term supplementation, but that levels rather reflected the intake of the last meal (Bieger *et al*., 2008). Flavonoid supplementation to pigs also did not appear to have a sparing effect on other antioxidants, as the tocopherol content of the meat was not affected by supplementation (Augustin *et al*., 2008; González & Tejeda, 2007; Mason *et al*., 2005).

Nonetheless, while there were no significant effects, Mason *et al*. (2005) did note that MDA levels tended to be lower during storage in aerobically packaged meat from green tea extract-supplemented pigs, and there was some suggestion of an effect on the colour of meat under modified-atmosphere packaging, with green tea samples having higher L* and b* values after two days of storage (Mason *et al*., 2005). This may suggest that further research using a wider variety of inclusion rates may be valuable.

In general, there is a serious shortage of studies specifically testing the effects of pure flavonoids or flavonoid extracts on pork meat quality. Considering the increasing importance of the pork industry, as well as the relative sensitivity of pork, as meat from a monogastric animal, to dietary variations, further study is justified. Future research should preferably utilise well-characterised flavonoid supplements, and should particularly examine the effects on the fatty acid composition and oxidative deterioration during storage.

2.6.3 Rabbits

The supplementation of dietary flavonoids to rabbits has generally had no impact on carcass quality components; however, the available literature is unfortunately limited in research on pure flavonoids. While the results for hesperidin (Simitzis *et al*., 2014: 1 – 2 g/kg feed) and alfalfa flavonoids (Dabbou *et al*., 2018: 400 – 1200 mg/kg feed) can be considered as relatively reliable indications that flavonoids tend not to affect carcass components, the similarly non-significant results for chia (*Salvia hispanica* L.) seeds at 10 % or 15 % of the diet (Peiretti & Meineri, 2008), bilberry (*Vaccinium myrtillus* L.) pomace at 50 – 150 g/kg feed (Dabbou *et al*., 2017) and dry, ground purple loosestrife (*Lythrum salicaria*) leaves at 0.2 % or 0.4 % (Kovitvadhi *et al*., 2016) are not as conclusive. Although the flavonoids present in these supplements may have simply not had an impact on the carcass, their effects could also have been altered through interactions with other bioactive compounds, such as tannins. However, it is notable that dietary chestnut tannins have not previously been found to impact rabbit meat quality (Dalle Zotte *et al*., 2018). Similarly, while the positive effects of dried strawberry pomace at 25 % of the diet on the dressing percentage, edible giblet and total edible parts may have been due to its tiliroside (a glycosidic flavonoid) content, they could also have been related to other nutritional components (Waly *et al*., 2015).

As was found for the carcass composition, the effects on physical and chemical meat quality were limited, and those effects that were reported lacked consistency across studies. The only effect of hesperidin was a tendency for it to increase the cooking loss (Simitzis *et al*., 2014), while alfalfa flavonoids increased L* values for rabbit loin meat (Dabbou *et al*., 2018). Bilberry had no effect on the physical meat quality, but tended to increase the

meat protein content (Dabbou *et al*., 2017), and Baikal skullcap root at 9 % of the diet was similarly found to increase the crude protein and decrease the lipid and cholesterol contents of the hindleg. However, this effect was only seen for rabbits fed a high-cholesterol diet (Kroliczewska, Mista, Zawadzki, & Skiba, 2011). Waly *et al*. (2015) also found that strawberry pomace decreased the fat and ash content of the meat, but it had no effect on the protein content. Strawberry, cranberry and onion extracts had no effect on the protein or moisture content, or drip or cooking losses, but strawberry extract decreased the pH_u of the LD and BF, and the L* readings for the BF, and cranberry extract increased BF b* readings (Koné *et al*., 2016). In contrast, the supplementation of liquorice root extract (6 g/kg) increased the pH of minced rabbit hindleg meat (Dalle Zotte *et al*., 2017). Purple loosestrife had no effect on any aspect of meat quality (Kovitvadhi *et al*., 2016).

Determining the effects of dietary flavonoid-rich supplements on the fatty acid content of the meat lipids was complicated by interactions with the effects of the fatty acid content of the supplemented material. This was the case for chia seeds (Peiretti & Meineri, 2008), bilberry pomace (Dabbou *et al*., 2017) and fresh alfalfa (Dal Bosco *et al*., 2014), all of which also constitute rich sources of PUFA. Nonetheless, it has been suggested that the provision of flavonoids may increase the effects of PUFA-rich oil supplementation on the fatty acid composition of the meat, through the inhibition of PUFA oxidation (Dabbou *et al*., 2017). However, pure hesperidin decreased the PUFA content (particularly arachidonic acid), the PUFA n-6 series and the PUFA:SFA ratio (Simitzis *et al*., 2014). It is therefore clear that both the effects of pure flavonoids and their possible interactions with dietary fatty acids, need to be studied further.

The results for the effects on oxidative stability have also been mixed, with pure hesperidin, cranberry, onion and strawberry extracts, and purple loosestrife not having any effect on TBARS levels during storage, while alfalfa flavonoids effectively decreased the degree of oxidation in loin meat stored frozen for 30 days (Dabbou *et al*., 2018; Koné *et al*., 2016; Kovitvadhi *et al*., 2016; Simitzis *et al*., 2014). As the hesperidin was supplemented at a higher inclusion rate than the alfalfa flavonoids, this discrepancy may be related to the different flavonoids present, with alfalfa containing predominantly tricin and apigenin glycosides. The supplementation of bilberry and alfalfa sprouts also had no effect on TBARS levels in meat (Dabbou *et al*., 2017; Dal Bosco *et al*., 2015); however, the authors suggested that this may actually be an indication of effective antioxidant activity, as the high levels of PUFA also present in the supplements would likely have had a pro-oxidant effect in the absence of the flavonoids. This was supported by the increased diadzein content of loin meat from the alfalfa sprout-supplemented rabbits (Dal Bosco *et al*., 2015). Studies comparing the effects of flavonoid-free PUFA sources with or without coinciding natural flavonoid extracts could confirm whether this was the case.

In contrast with their lack of effect on the TBARS content, strawberry, cranberry and onion extracts did appear to improve the microbial status of the meat during storage (Koné *et al*., 2016). After 15 days of chilled storage under aerobic conditions rabbit thighs from supplemented rabbits had lower counts for total aerobic mesophilic (TAM) and presumptive lactic acid bacteria (LAB), with presumptive *Pseudomonas* counts being lower after 10 days of storage in samples from cranberry and onion supplemented rabbits (Koné *et al*., 2016). Fewer positive

effects were seen for samples stored under anaerobic conditions, but counts for TAM and presumptive LAB were lower in the fresh samples, and onion supplementation reduced both TAM and presumptive *Pseudomonas* counts after 15 days of storage. Similar beneficial effects on the *Pseudomonas* species count of minced rabbit hindleg meat stored aerobically for 0 – 6 days were found by Dalle Zotte *et al*. (2017), for rabbits supplemented with a liquorice root extract. However, there was no effect of the liquorice supplementation on the coliforms or total viable counts.

2.6.4 Sheep, goats and cattle

In general, flavonoid supplementation has not been found to affect carcass components or organ weights in ruminant livestock, with this being the case for naringin at 1.5 or 2.5 g/kg feed and hesperidin at 1.5, 2.5 or 3 g/kg feed (Bodas *et al*., 2012; Simitzis *et al*., 2013; Simitzis *et al*., 2019). The feeding of subterranean clover containing high concentrations of isoflavones similarly did not improve the slaughter yield or alter the carcass conformation or fatness scores, despite improving growth and therefore the cold carcass weight (Pace *et al*., 2006). However, the clover-fed sheep did have lower percentages of bone and higher percentages of intramuscular and total fat in the proximal pelvic limb than the control animals. Also, quercetin supplementation at 2 g/kg feed increased the weight of the kidney knob fat in lambs (Andrés *et al*., 2013). These effects on the percentages of fat and bone may have been due to the phytoestrogenic nature of some flavonoids, as oestradiol has been found to increase the deposition of subcutaneous fat (Andrés *et al*., 2013). This may also have been linked to a growth-promoting effect, which resulted in the earlier maturation of the supplemented animals, and thus the lower proportion of bone and increased fat deposition (Pace *et al*., 2006).

Despite the increase in depot fat in response to quercetin found by Andrés *et al*. (2013), there was no coinciding increase in the IMF content, or any change in the proximate composition of the meat. A similar lack of impact on the IMF content was reported for a product containing 10 % quercetin supplemented at 21 or 42 ppm by Kang *et al*. (2012), and for naringin at 1.5 g/kg (Bodas *et al*., 2011; Bodas *et al*., 2012), hesperidin (Simitzis *et al*., 2019: 2.5 g/kg feed) and tea catechins (2 – 4 g/kg feed), for the M. *longissimus dorsi* (LD) and the M. *gluteus medius* (GM) (Tan *et al*., 2011; Zhong *et al*., 2009). However, both Zhong *et al*. (2009) and Tan *et al*. (2011) found that the IMF content of M. *semimembranosus* (SM) was reduced by tea catechin supplementation at 3 g/kg feed. Tea catechins were also reported to decrease the moisture and ash content of the LD and increase the crude protein content of the SM, possibly through the improvement of nitrogen retention via the alteration of rumen fermentation (Tan *et al*., 2011). Further research is therefore required to provide some consensus on the effects on meat proximate composition.

Somewhat more impact on physical meat quality attributes has been found. Quercetin supplementation increased instrumentally measured cohesiveness in beef loins, and hardness in fresh lamb loins (Andrés, Huerga, *et al*., 2014: 2 g/kg feed; Kang *et al*., 2012). Moreover, sensory panellists rated meat from the supplemented lambs as being less tender than those from the controls (Andrés, Huerga, *et al*., 2014). However, lamb meat chewiness was not affected (Andrés, Huerga, *et al*., 2014), and neither hesperidin nor naringin supplementation, nor the

consumption of high-isoflavone clover, had any effect on lamb loin shear force (Pace *et al*., 2006; Simitzis *et al*., 2013; Simitzis *et al*., 2019). Meat from quercetin-supplemented (200 mg/kg feed) goats was also rated as being more desirable in colour, texture and overall acceptability than that from the controls (Cho *et al*., 2010).

Contradictory results have also been found for the effects of flavonoids on meat WHC and moisture retention. Tea catechin supplementation to goats decreased the drip loss in the GM and SM, without any apparent correlation with effects on the pH, leading to the suggestion that their antioxidant activity protected cell membranes from lipid oxidation, thereby enhancing their integrity and increasing WHC (Zhong *et al*., 2009). A clover-rich diet also decreased the drip loss of the LD muscle, without any impact on the drip loss of the *gluteus biceps* muscle (*sic*) or the pH_u (Pace *et al*., 2006). However, this effect on WHC and moisture loss was not found for the LD muscle of tea catechin-supplemented goats, or the LD of quercetin-supplemented goats, lambs or cattle (Andrés, Huerga, *et al*., 2014; Cho *et al*., 2010; Kang *et al*., 2012), or hesperidin or naringin-supplemented lambs (Simitzis *et al*., 2013; Simitzis *et al*., 2019). Considering the variation in the effects of supplementation on different muscles found by Tan *et al*. (2011) and Zhong *et al*. (2009), it would be advisable for future studies to repeat measurements on a number of muscle types.

Despite the limited effect on the muscle IMF content, the fatty acid profile of the meat lipids has proved labile. Quercetin supplementation (2 g/kg feed) tended to decrease the SFA content of lamb loins, resulting in a decrease in the atherogenic and saturation indices (Andrés, Morán, *et al*., 2014). Tan *et al*. (2011) similarly found that tea catechins decreased the SFA content, increased MUFA and increased the n-6:n-3 ratio, and at 3 or 4 g/kg feed increased the PUFA, resulting in an increase in the PUFA:SFA ratio. These changes were related to possible inhibitory effects that dietary flavonoids could have on the rumen microbial population, resulting in reduced biohydrogenation and thus increased absorption and deposition of unsaturated fatty acids. This proposition was supported by concurrent increases in the PUFA content and PUFA:SFA ratio of the rumen bacteria, although there was no coinciding change in the SFA content or n-6:n-3 ratio (Tan *et al*., 2011). Some indication of interactions between the dietary fatty acid content and supplemented flavonoids has also been found, with combined quercetin and linseed supplementation increasing the conjugated linoleic acid content of lamb meat more than either supplement on its own (Andrés, Morán, *et al*., 2014). This was particularly notable for rumenic and vaccenic acid, and considering the reported health benefits of these fatty acids (Decker & Park, 2010), more research on this type of interaction should be performed.

The effects of dietary flavonoids on ruminant meat oxidative stability have been measured using a wide variety of parameters. In general, the results for TBARS levels in meat during chilled storage have been promising, with quercetin (Andrés, Huerga, *et al*., 2014; Andrés, Morán, *et al*., 2014), hesperidin (Simitzis *et al*., 2013; Simitzis *et al*., 2019), naringin (Simitzis *et al*., 2019), tea catechins (Zhong *et al*., 2009) and a flavonoid-rich commercial product at 150 ppm (Muela, Alonso, Campo, Sañudo & Beltrán, 2014) all significantly decreasing the TBARS content. However, Bodas *et al*. (2012) found no effect of naringin supplementation on the TBARS content of meat during 9 days storage, despite the TBARS of the liver being significantly lower in the supplemented

animals. They also detected naringenin in the liver but not in the meat of the supplemented animals, and concluded that this lack of deposition in the meat explained the lack of any antioxidant effect. Simitzis *et al*. (2013) and Simitzis *et al*. (2019) also found that although MDA levels during chilled storage up to 9 days were reduced by hesperidin or naringin, the effect on stored frozen meat was limited, with hesperidin showing no antioxidant effect beyond 3 months of storage. It therefore appeared that the period of effectiveness of these flavonoids was relatively short. Dietary grape seed extract (50 mg/kg) and dried red grape pomace (5 %) had no effect on MDA levels during storage, suggesting that the rumen microbes were not able to release sufficient catechins from the condensed tannins in these supplements to produce physiological effects (Guerra-Rivas *et al*., 2016).

The results of other measures of oxidative stability have not always been as consistent as those provided by the TBARS assay. Studies using assays measuring antioxidant activity, such as the DPPH radical-scavenging assay and $ABTS^{+}$-reducing activity assay, are limited, and while Cho *et al*. (2010) found that quercetin increased the $ABTS^{+}$-reducing activity in fresh meat, it had no effect after 3 or 7 days of storage, and had no effect on the DPPH radical-scavenging activity in fresh or stored meat, as was also found by Kang *et al*. (2012). However, these studies did also both find no effect on meat TBARS values, or its total phenol content. Comparisons of the levels of oxidation products, particularly oxysterols and volatile aldehydes, have also had mixed results. Quercetin decreased the levels of oxysterols in lamb meat after 7 days chilled storage and cooking, but did not cause any difference in the volatile aldehyde content of the meat, although it is notable that two different muscles were used for these analyses (Andrés, Huerga, *et al*., 2014). Bodas *et al*. (2012) also found no effect of naringin supplementation on the oxysterol content, which concurred with the lack of effect on the meat TBARS content found in this study.

The effects of flavonoids on meat surface colour during storage have also varied. Quercetin at 2 g/kg feed had no effect on L* or a*, but decreased b* values, and thus the hue angle, of lamb, from 3 days storage onwards, with this being attributed to its inhibition of the oxidation of oxymyoglobin to metmyoglobin (Andrés *et al*., 2013). However, Muela *et al*. (2014) found no effect of a mixed flavonoid product on L*, hue angle or chroma, or the percentages of metmyoglobin, myoglobin or oxymyoglobin, despite lower levels of MDA in the supplemented lambs. They also had contradictory results for the visual assessment of colour intensity, with the flavonoid supplement increasing intensity after 6 and 12 days of storage, but apparently decreasing it after 7, 8 and 11 days. Bodas *et al*. (2012) also found no effect of naringin on meat colour during 9 days storage, concurring with the non-significant TBARS and oxysterol results. While grape seed extract- and grape pomace-supplemented lambs showed some variation in meat colour throughout the storage period, this did not result in any overall change in the discolouration of the meat (Guerra-Rivas *et al*., 2016). It also did not extend the shelf-life of the meat relative to that of the control, although grape pomace did seem to improve the overall rating and reduce the occurrence of off-odours after 11 days of storage. Pace *et al*. (2006) only measured the colour of the fresh meat, but found that the consumption of isoflavone-rich subterranean clover decreased both the meat L* values and the hue angle, without any effect on chroma. It would have been interesting to determine whether these effects persisted during

a chilled storage period, as Andrés *et al*. (2013) also found that a decreased hue angle in quercetin-supplemented lambs was present in fresh meat, but had no effect after one day of storage.

One of the well-known *in vitro* attributes of flavonoids is their antimicrobial activity; however, the possibility of dietary supplementation improving the microbial stability of red meat has only been directly investigated in two studies. While Kang *et al*. (2012) found no effect of quercetin supplementation on the levels of volatile basic nitrogen substances, which can be used as a measure of spoilage, Andrés *et al*. (2013) found that quercetin-supplemented samples had higher levels of extract release volume (ERV) after 7 days of storage. The ERV is the volume of extract released by a meat homogenate during a specified time period, and is reduced by microbial spoilage. Andrés *et al*. (2013) also found that quercetin tended to decrease the total viable bacteria count after 3 days storage, although it did not affect the counts for *Pseudomonas* species or moulds and yeasts. In contrast, the inclusion of grape seed extract and grape pomace in the diet increased the total viable count and *Pseudomonas* count of meat samples stored under modified-atmosphere packaging for 14 days (Guerra-Rivas *et al*., 2016), while counts for lactic acid bacteria and enterobacteria were unchanged.

2.7 Conclusion

Although specific research is limited, and the methods used in existing literature limit direct comparisons between studies, it appears that flavonoids are relatively prevalent in current livestock diets. Future research should therefore take into account both the total flavonoid content and flavonoid composition of the control feed used, and the testing of flavonoids that are generally not naturally-occurring in livestock feed resources could be of interest.

Despite the very brief overview of some of the more directly relevant pharmacological properties, it is nonetheless clear that flavonoids can be considered bioactive compounds that have wide-reaching and complex effects on both animal and microbial functioning. Unfortunately, reaching simple, concise conclusions on their effects on live performance and health were not as simple, due to the limited number of studies and the many contradictory results. However, it did appear that beneficial effects on growth and feed efficiency were more prevalent under stress-inducing conditions than not, with this most widely demonstrated for heat-stress. It also seemed that flavonoids may be able to reduce morbidity and mortality rates, having improved animal responses to coccidiosis and viral infections, and shown potential for improving bone health. This suggests that future research should focus on testing the effects of flavonoid supplementation under a variety of stress-inducing conditions, and should possibly include some sort of challenge-testing with common infectious diseases.

The effects on carcass and meat quality appeared to be more species-specific than those on live performance, and obtaining an overall idea of the effects on pig and rabbit meat was challenging due to the lack of research that has been performed for these species. However, in general it appeared that the effects on carcass and basic physical and proximate meat quality were limited, with dietary flavonoids having a greater impact on meat fatty acid composition and oxidative stability. Results for poultry and ruminants suggested that flavonoids tended to decrease the SFA content and increase the PUFA content, as well as reducing cholesterol levels, but considerable further

research is needed to confirm these results. In terms of the effects on shelf-life, research on poultry and ruminants appeared to indicate a clear antioxidant effect of dietary flavonoids, as shown by a reduction of the MDA content of the meat during storage. However, the results for other measures of oxidative status, such as the TAC, instrumental colour and levels of products of oxidation, were less conclusive, as were the results for pig and rabbit meat. There is also a lack of research correlating the muscle tissue content of flavonoids and their metabolites to changes in oxidative stability, which limits our understanding of the mechanisms involved. Results for a limited number of studies on rabbits and ruminants suggested that dietary flavonoids may improve the microbial status of meat, particularly during aerobic storage, but further research is required.

In general, there was some evidence that flavonoids may have non-linear dose-response curves, and more extensive inclusion rate studies are therefore required. Research investigating the effects of flavonoid extracts of various flavonoid-rich feed resources may also be of interest, as would comparisons between the effects of supplementing the main pure flavonoids in these extracts to supplementing the complex mixed extract. This would help distinguish the effects of flavonoids from those of other bioactive compounds, as well as identify which of the flavonoids present is the most effective, and whether interactions between flavonoids play a major role. In addition, it is clear that there is a serious lack of research on the effects of pure flavonoids on all aspects of production, with this being particularly evident for pigs and rabbits. The use of dietary flavonoids in livestock production is therefore a field of research that provides many opportunities for future study.

2.8 References

Adom, K. K. & Liu, R. H. (2002). Antioxidant activity of grains. Journal of Agricultural and Food Chemistry, 50, 6182 – 6187.

Aires, A., Fernandes, C., Carvalho, R., Bennett, R. N., Saavedra, M. J. & Rosa, E. A. S. (2011). Seasonal effects on bioactive compounds and antioxidant capacity of six economically important brassica vegetables. *Molecules, 16*, 6816 – 6832.

Alhidary, I. A. & Abdelrahman, M. M. (2014). Effect of naringin supplementation on performance and physiological responses of heat stressed lambs. *Journal of Animal and Veterinary Advances, 13*(19), 1126 – 1130.

Alhidary, I. A. & Abdelrahman, M. M. (2016). Effects of naringin supplementation on productive performance, antioxidant status and immune response in heat-stressed lambs. *Small Ruminant Research, 138*, 31 – 36.

Almstrup, K., Fernández, M. F., Petersen, J. H., Olea, N., Skakkebaek, N. E. & Leffers, H. (2002). Dual effects of phytoestrogens result in u-shaped dose-response curves. *Environmental Health Perspectives, 110*, 743 – 748.

Andrés, S., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Blanco, C. & Giráldez, F. J. (2013). Quercetin dietary supplementation of fattening lambs at 0.2% rate reduces discolouration and microbial growth in meat during refrigerated storage. *Meat Science, 93*, 207 – 212.

Andrés, S., Huerga, L., Mateo, J., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Rotolo, L. & Giráldez, F. J. (2014). The effect of quercetin dietary supplementation on meat oxidation processes and texture of fattening lambs. *Meat Science, 96*, 806 – 811.

Andrés, S., Morán, L., Aldai, N., Tejido, M. L., Prieto, N., Bodas, R. & Giráldez, F. J. (2014). Effects of linseed and quercetin added to the diet of fattening lambs on the fatty acid profile and lipid antioxidant status of meat samples. *Meat Science, 97*, 156 – 163.

Antongiovanni, M., Buccioni, A., Petacchi, F. & Agnoletti, A. (2005). Use of a citrus by-product in broilers diets. Italian *Journal of Animal Science, 4*(sup 2), 501 – 503.

Arné, P., Thierry, S., Wang, D., Deville, M., Le Loc'h, G., Desoutter, A., Féménia, F., Nieguitsila, A., Huang, W., Chermette, R. & Guillot, J. (2011). *Aspergillus fumigatus* in poultry. *International Journal of Microbiology*, article ID 746356, 1 – 14.

Attia, Y. A., Al-Hanoun, A. & Bovera, F. (2011). Effect of different levels of bee pollen on performance and blood profile of New Zealand White bucks and growth performance of their offspring during summer and winter months. *Journal of Animal Physiology and Animal Nutrition, 95*, 17 – 26.

Augustin, K., Blank, R., Boesch-Saadatmandi, C., Frank, J., Wolffram, S. & Rimbach, G. (2008). Dietary green tea polyphenols do not affect vitamin E status, antioxidant capacity and meat quality of growing pigs. *Journal of Animal Physiology and Animal Nutrition, 92*, 705 – 711.

Balcells, J., Aris, A., Serrano, A., Seradj, A. R., Crespo, J. & Devant, M. (2012). Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. *Journal of Animal Science, 90*, 4975 – 4984.

Barry, T. N. (2013). The feeding value of forage brassica plants for grazing ruminant livestock. *Animal Feed Science and Technology, 181*, 15 – 25.

Beecher, G. R. (2003). Overview of dietary flavonoids: Nomenclature, occurrence and intake. *The Journal of Nutrition, 133*, 3248S – 3254S.

Belhadj Slimen, I., Najar, T., Ghram, A. & Abdrrabba, M. (2016). Heat stress effects on livestock: molecular, cellular and metabolic aspects, a review. *Journal of Animal Physiology and Animal Nutrition, 100*, 401 – 412.

Berger, L. M., Wein, S., Blank, R., Metges, C. C. & Wolffram, S. (2012). Bioavailability of the flavonol quercetin in cows after intraruminal application of quercetin aglycone and rutin. *Journal of Dairy Science, 95*, 5047 – 5055.

Bhagwat, S., Haytowitz, D. B. & Holden, J. M. (2011). *USDA database for the flavonoid content of selected foods, release 3.1*. Beltsville, USA: US Department of Agriculture.

Bieger, J., Cermak, R., Blank, R., de Boer, V. C. J., Hollman, P. C. H., Kamphues, J. & Wolffram, S. (2008). Tissue distribution of quercetin in pigs after long-term dietary supplementation. *The Journal of Nutrition, 138*, 1417 – 1420.

Blouin, F. A., Zarins, Z. M. & Cherry, J. P. (1981). Role of flavonoids in the production of color in biscuits prepared with wheat and cottonseed flours. *Journal of Food Science, 46*, 266 – 271.

Bodas, R., Lopez-Campos, O., Prieto, N., Giraldez, F. J., Lopez, S. & Andres, S. (2011). The effect of naringin on plasma lipid profile, and liver and intramuscular fat contents of fattening lambs. In M. J. Ranilla, M. D. Carro, H. Ben Salem & P. Morand-Fehr (Eds.), *Challenging strategies to promote the sheep and goat sector in the current global context*. *Options Méditerranéennes A, no. 99* (pp. 223 – 226). Zaragoza, Spain: CIHEAM / CSIC / Universidad de León.

Bodas, R., Prieto, N., Jordán, M. J., López-Campos, O., Giráldez, F. J., Morán, L. & Andrés, S. (2012). The liver antioxidant status of fattening lambs is improved by naringin dietary supplementation at 0.15% rates but not meat quality. *Animal, 6*(5), 863 – 870.

Bradshaw, R. H., Kirkden, R. D. & Broom, D. M. (2002). A review of the aetiology and pathology of leg weakness in broilers in relation to welfare. *Avian and Poultry Biology Reviews, 13*(2), 45 – 103.

Brenes, A., Viveros, A., Goñi, I., Centeno, C., Saura-Calixto, F. & Arija, I. (2010). Effect of grape seed extract on growth performance, protein and polyphenol digestibilities, and antioxidant activity in chickens. *Spanish Journal of Agricultural Research, 8*(2), 326 – 333.

Brenes, A., Viveros, A., Goñi, I., Centeno, C., Sáyago-Ayerdy, S. G., Arija, I. & Saura-Calixto, F. (2008). Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. *Poultry Science, 87*, 307 – 316.

Bubenchikova, V. N. & Drozdova, I. L. (2004). HPLC analysis of phenolic compounds in yellow sweet-clover. *Pharmaceutical Chemistry Journal, 38*(4), 195 – 196.

Cao, F. L., Zhang, X. H., Yu, W. W., Zhao, L. G. & Wang, T. (2012). Effect of feeding fermented *Ginkgo biloba* leaves on growth performance, meat quality, and lipid metabolism in broilers. *Poultry Science, 91*, 1210 – 1221.

Cartea, M. E., Francisco, M., Soengas, P. & Velasco, P. (2011). Phenolic compounds in Brassica vegetables. *Molecules, 16*, 251 – 280.

Chen, Y., Gong, X., Li, G., Lin, M., Huo, Y., Li, S. & Zhao, G. (2016). Effects of dietary alfalfa flavonoids extraction on growth performance, organ development and blood biochemical indexes of Yangzhou geese aged from 28 to 70 days. *Animal Nutrition, 2*, 318 – 322.

Cho, S. K., Jo, C., Jung, S., Kim, M. K., Oh, H. M., Lee, B. D. & Lee, S. K. (2010). Effects of dietary quercetin on the feed utilization, blood parameters, and meat quality in Korean native goats. *Journal of Animal Science and Technology, 52*(4), 297 – 304.

Chu, Y.-H., Chang, C.-L. & Hsu, H.-F. (2000). Flavonoid content of several vegetables and their antioxidant activity. *Journal of the Science of Food and Agriculture, 80*, 561 – 566.

Cook, D. R. (1998). *The effect of dietary soybean isoflavones on the rate and efficiency of growth and carcass muscle content in pigs and rats* (Doctoral dissertation). Retrieved from Retrospective Theses and Dissertations, Iowa State University, https://lib.dr.iastate.edu/rtd/11915.

Council of the European Union (1981). Council Directive 81/602/EEC of 31 July 1981 concerning the prohibition of certain substances having a hormonal action and of any substances having a thyrostatic action. *Official Journal of the European Communities, L222*, 32, 1 – 2.

Cushnie, T. P. T. & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents, 26*, 343 – 356.

Cushnie, T. P. T. & Lamb, A. J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents, 38*, 99 – 107.

Dabbou, S., Gai, F., Renna, M., Rotolo, L., Dabbou, S., Lussiana, C., Kovitvadhi, A., Brugiapaglia, A., De Marco, M., Helal, A. N., Zoccarato, I. & Gasco, L. (2017). Inclusion of bilberry pomace in rabbit diets: Effects on carcass characteristics and meat quality. *Meat Science, 124*, 77 – 83.

Dabbou, S., Gasco, L., L., R., Pozzo, L., Tong, J. M., Dong, X. F., Rubiola, P., Schiavone, A. & Gai, F. (2018). Effects of dietary alfalfa flavonoids on the performance, meat quality and lipid oxidation of growing rabbits. *Asian-Australasian Journal of Animal Sciences, 31*(2), 270 – 277.

Dal Bosco, A., Mugnai, C., Roscini, V., Mattioli, S., Ruggeri, S. & Castellini, C. (2014). Effect of dietary alfalfa on the fatty acid composition and indexes of lipid metabolism of rabbit meat. *Meat Science, 96*, 606 – 609.

Dalle Zotte A., Cullere M., Giaccone V., Celia C., Szendrő Zs., Gerencsér Zs. & Matics Zs. (2017). Can liquorice extract administered as dietary and/or as food supplement improve rabbit meat shelf-life? *Proceedings of the 63rd International Congress of Meat Science and Technology — August 13 – 18, 2017, Cork, Ireland.* (pp. 498 – 499).

Dalle Zotte, A., Szendrő, Zs. (2011). The role of rabbit meat as functional food. A review. *Meat Science. 88*, 319 – 331.

Dalle Zotte A., Cullere M., Tasoniero G., Gerencsér Zs, Szendrő Zs., Novelli E., Matics Zs. (2018). Supplementing growing rabbit diets with chestnut hydrolyzable tannins: Effect on meat quality and oxidative status, nutrient digestibilities, and content of tannin metabolites. *Meat Science, 146*, 101 – 108.

De Camargo, A. C., Regitano-d'Arce, M. A. B., Rasera, G. B., Canniatti-Brazaca, S. G., do Prado-Silva, L., Alvarenga, V. O., Sant'Ana, A. S. & Shahidi, F. (2017). Phenolic acids and flavonoids of peanut by-products: Antioxidant capacity and antimicrobial effects. *Food Chemistry, 237*, 538 – 544.

Decker, E. A. & Park, Y. (2010). Healthier meat products as functional foods. *Meat Science, 86*, 49 – 55.

Dinelli, G., Marotti, I., Di Silvestro, R., Bosi, S., Bregola, V., Accorsi, M., Di Loreto, A., Benedettelli, S., Ghiselli, L. & Catizone, P. (2013). Agronomic, nutritional and nutraceutical aspects of durum wheat (*Triticum durum* Desf.) cultivars under low input agricultural management. *Italian Journal of Agronomy, 8*(e12), 85 – 93.

Divi, R. L. & Doerge, D. R. (1996). Inhibition of thyroid peroxidase by dietary flavonoids. *Chemical Research in Toxicology, 9*, 16 – 23.

Dong, X. F., Gao, W. W., Tong, J. M., Jia, H. Q., Sa, R. N. & Zhang, Q. (2007). Effect of polysavone (alfalfa extract) on abdominal fat deposition and immunity in broiler chickens. *Poultry Science, 86*, 1955 – 1959.

Dos Santos, M. C. S., Gonçalves, C. F. L., Vaisman, M., Ferreira, A. C. F. & de Carvalho, D. P. (2011). Impact of flavonoids on thyroid function. *Food and Chemical Toxicology, 49*, 2495 – 2502.

Duru, M., Erdoğan, Z., Duru, A., Küçükgül, A., Düzgüner, V., Kaya, D. A. & Şahin, A. (2013). Effect of seed powder of a herbal legume fenugreek (*Trigonella foenum-graceum* L.) on growth performance, body components, digestive parts, and blood parameters of broiler chicks. *Pakistan Journal of Zoology, 45*(4), 1007 – 1014.

Economides, S. & Georghiades, E. (1980). Grape marc as a substitute for barley grain in diets of fattening lambs. *Technical Bulletin, 29*, 3 – 8.

Egert, S. & Rimbach, G. (2011). Which sources of flavonoids: complex diets or dietary supplements. *American Society for Nutrition, 2*, 8 – 14.

Eldridge, A. C. & Kwolek, W. F. (1983). Soybean isoflavones: effect of environment and variety on composition. *Journal of Agricultural and Food Chemistry, 31*, 394 – 396.

Erener, G., Ocak, N., Altop, A., Cankaya, S., Aksoy, H. M. & Ozturk, E. (2011). Growth performance, meat quality and caecal coliform bacteria count of broiler chicks fed diet with green tea extract. *Asian-Australasian Journal of Animal Science, 24*(8), 1128 – 1135.

European Commission (2005). *Ban on antibiotics as growth promoters in animal feed enters into effect*. Brussels, Belgium: European Commission, Available at: http://europa.eu/rapid/press-release_IP-05-1687_en.htm.

Fatemeh, S. R., Saifullah, R., Abbas, F. M. A. & Azhar, M. E. (2012) Total phenolics, flavonoids and antioxidant activity of banana pulp and peel flours: influence of variety and stage of ripeness. *International Food Research Journal, 19*(3), 1041 – 1046.

Food and Drug Administration, Centre for Veterinary Medicine (2013). *Guidance for Industry #213: New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209.* Washington, D.C., USA: U.S. Department of Health and Human Services.

Fernandes, F., Valentão, P., Sousa, C., Pereira, J. A., Seabra, R. M. & Andrade, P. B. (2007). Chemical and antioxidative assessment of dietary turnip (*Brassica rapa* var. *rapa* L.). *Food Chemistry, 105*, 1003 – 1010.

Ferreira, A. C. F., Lisboa, P. C., Oliveira, K. J., Lima, L. P., Barros, I. A. & Carvalho, D. P. (2002). Inhibition of thyroid type 1 deiodinase activity by flavonoids. *Food and Chemical Toxicology, 40*(7), 913 – 917.

Frei, B. & Higdon, J. V. (2003). Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. The Journal of Nutrition, 133, 3275S – 3284S.

Friedman, M. (2007). Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Molecular Nutrition & Food Research, 51*, 116 – 134.

Fung, Y. T. E., Sparkes, J., Van Ekris, I., Chaves, A. V. & Bush, R. D. (2010). Effects of feeding fresh citrus pulp to Merino wethers on wool growth and animal performance. *Animal Production Science, 50*, 52 – 58.

Ghasemi, K., Ghasemi, Y. & Ebrahimzadeh, M. A. (2009). Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. *Pakistan Journal of Pharmaceutical Sciences, 22*(3), 277 – 281.

Gladine, C., Rock, E., Morand, C., Bauchart, D. & Durand, D. (2007). Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. *British Journal of Nutrition, 98*, 691 – 701.

Goliomytis, M., Kartsonas, N., Charismiadou, M. A., Symeon, G. K., Simitzis, P. E. & Deligeorgis, S. G. (2015). The influence of naringin and hesperidin dietary supplementation on broiler meat quality and oxidative stability. *PLoS One, 10*(10), 1 – 11.

Goliomytis, M., Tsoureki, D., Simitzis, P. E., Charismiadou, M. A., Hager-Theodorides, A. L. & Deligeorgis, S. G. (2014). The effects of quercetin dietary supplementation on broiler growth performance, meat quality, and oxidative stability. *Poultry Science, 93*, 1957 – 1962.

Goñi, I., Brenes, A., Centeno, C., Viveros, A., Saura-Calixto, F., Rebolé, A., Arija, I. & Estevez, R. (2007). Effect of dietary grape pomace and vitamin E on growth performance, nutrient digestibility, and susceptibility to meat lipid oxidation in chickens. *Poultry Science, 86*, 508 – 516.

González, E. & Tejeda, J. F. (2007). Effects of dietary incorporation of different antioxidant extracts and free-range rearing on fatty acid composition and lipid oxidation of Iberian pig meat. *Animal, 1*(7), 1060 – 1067.

Graham, P. H. & Vance, C. P. (2003). Legumes: importance and constraints to greater use. *Plant Physiology, 131*, 872 – 877.

Grandin, T. (2014). Animal welfare and society concerns finding the missing link. *Meat Science, 98*, 461 – 469.

Greiner, L. L., Stahly, T. S. & Stabel, T. J. (2001a). The effect of dietary soy daidzein on pig growth and viral replication during a viral challenge. *Journal of Animal Science, 79*, 3113 – 3119.

Greiner, L. L., Stahly, T. S. & Stabel, T. J. (2001b). The effect of dietary soy genistein on pig growth and viral replication during a viral challenge. *Journal of Animal Science, 79*, 1272 – 1279.

Guerra-Rivas, C., Vieira, C., Rubio, B., Martínez, B., Gallardo, B., Mantecón, A. R., Lavín, P. & Manso, T. (2016). Effects of grape pomace in growing lamb diets compared with vitamin E and grape seed extract on meat shelf life. *Meat Science, 116*, 221 – 229.

Hajati, H., Hassanabadi, A. & Yansari, A. T. (2012). Effect of citrus pulp on performance and some blood parameters of broiler chickens. *Proceedings of the 1st International and the 4th National Congress on Recycling of Organic Waste in Agriculture — April 26 – 27, 2012, Isfahan, Iran.*

Han, Y. (2009). Rutin has therapeutic effect on septic arthritis caused by *Candida albicans*. *International Immunopharmacology, 9*, 207 – 211.

Harborne, J. B. & Williams, C. A. (1976). Flavonoid patterns in leaves of the Gramineae. *Biochemical Systematics and Ecology, 4*, 267 – 280.

Harborne, J. B. & Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry, 55*, 481 – 504.

Harlow, B. E., Flythe, M. D., Kagan, I. A. & Aiken, G. E. (2017). Biochanin A (an isoflavone produced by red clover) promotes weight gain of steers grazed in mixed grass pastures and fed dried-distillers' grains. *Crop Science, 57,* 506 – 514.

Hauck, B., Gallagher, J. A., Morris, S. M., Leemans, D. & Winters, A. L. (2014). Soluble phenolic compounds in fresh and ensiled orchard grass (*Dactylis glomerata* L.), a common species in permanent pastures with potential as a biomass feedstock. *Journal of Agricultural and Food Chemistry, 62*, 468 – 475.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67 – 202.

Heim, K. E., Tagliaferro, A. R. & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry, 13*, 572 – 584.

Heuzé V., Tran G. & Kaushik S. (2017). Soybean meal. *Feedipedia, a programme by INRA, CIRAD, AFZ and FAO.* Available at https://feedipedia.org/node/674. Last updated on January 13, 2017, 15:45.

Heuzé V., Tran G., Sauvant D., Noblet J., Renaudeau D., Bastianelli D. & Lebas F. (2016). Palm kernel meal. *Feedipedia, a programme by INRA, CIRAD, AFZ and FAO.* Available at https://www.feedipedia.org/node/43. Last updated on March 23, 2016, 11:59.

Hoeck, J. A., Fehr, W. R., Murphy, P. A. & Welke, G. A. (2000). Influence of genotype and environment on isoflavone contents of soybean. *Crop Science, 40*, 48 – 51.

Hong, J. W., Kim, I. H., Kwon, O. S., Min, B. J., Lee, W. B. & Shon, K. S. (2004). Influences of plant extract supplementation on performance and blood characteristics in weaned pigs. *Asian-Australasian Journal of Animal Science, 17*(3), 374 – 378.

Hughes, C. L. J. (1988). Phytochemical mimicry of reproductive hormones and modulation of herbivore fertility by phytoestrogens. *Environmental Health Perspectives, 78*, 171 – 175.

Islam, M. R., Oomah, D. B. & Diarra, M. S. (2017). Potential immunomodulatory effects of non-dialyzable materials of cranberry extract in poultry production. *Poultry Science, 96*, 341 – 350.

Jang, S. I., Jun, M.-H., Lillehoj, H. S., Dalloul, R. A., Kong, I.-K., Kim, S. & Min, W. (2007). Anticoccidial effect of green tea-based diets against *Eimeria maxima*. *Veterinary Parasitology, 144*, 172 – 175.

Jang, A., Liu, X.-D., Shin, M.-H., Lee, B.-D., Lee, S.-K., Lee, J.-H. & Jo, C. (2008). Antioxidative potential of raw breast meat from broiler chicks fed a dietary medicinal herb extract mix. *Poultry Science, 87*, 2382 – 2389.

Jenkins, K. J. & Atwal, A. S. (1995). Flavonoids increase tissue essential fatty acids in vitamin E-deficient chicks. *Nutritional Biochemistry, 6*, 97 – 103.

Jenkins, K. J., Hidiroglou, M. & Collins, F. W. (1993). Influence of various flavonoids and simple phenolics on development of exuditive diathesis in the chick. *Journal of Agricultural and Food Chemistry, 41*, 441 – 445.

Jiang, Z. Y., Jiang, S. Q., Lin, Y. C., Xi, P. B., Yu, D. Q. & Wu, T. X. (2007). Effects of soybean isoflavone on growth performance, meat quality, and antioxidation in male broilers. *Poultry Science, 86*, 1356 – 1362.

Jiménez-Colmenero, F., Carballo, J. & Cofrades, S. (2001). Healthier meat and meat products: their role as functional foods. Meat Science, 59, 5 – 13.

Kamboh, A. A. & Zhu, W.-Y. (2013). Individual and combined effects of genistein and hesperidin supplementation on meat quality in meat-type broiler chickens. *Journal of the Science of Food and Agriculture, 93*, 3362 – 3367.

Kang, M., Kim, H. J., Jang, A., Gam, D. K., Yun, G. S. & Jo, C. (2012). Effect of dietary supplementation of quercetin on antioxidant activity and meat quality of beef cattle. *CNU Journal of Agricultural Science, 39*(1), 61 – 68.

Kao, T.-H. & Chen, B.-H. (2006). Functional components in soybean cake and their effects on antioxidant activity. *Journal of Agricultural and Food Chemistry, 54*, 7544 – 7555.

Karamać, M., Kosińska, A., Estrella, I., Hernández, T. & Duenas, M. (2012). Antioxidant activity of phenolic compounds identified in sunflower seeds. *European Food Research and Technology, 235*, 221 – 230.

Keinänen, M. & Julkunen-Tiitto, R. (1996). Effect of sample preparation method on birch (*Betula pendula* Roth) leaf phenolics. *Journal of Agricultural and Food Chemistry, 44*, 2724 – 2727.

Kil, D. Y., Ryu, S. N., Piao, L. G., Kong, C. S., Han, S. J. & Kim, Y. Y. (2006). Effect of feeding cyanidin 3-glucoside (C3G) high black rice bran on nutrient digestibility, blood measurements, growth performance and pork quality of pigs. *The Asian-Australasian Journal of Animal Science, 19*(12), 1790 – 1798.

Kleczek, K., Majewska, K., Makowski, W. & Michalik, D. (2012). The effect of diet supplementation with propolis and bee pollen on the physicochemical properties and strength of tibial bones in broiler chickens. *Archiv Tierzucht, 55*(1), 97 – 103.

Koné, A. P., Cinq-Mars, D., Desjardins, Y., Guay, F., Gosselin, A. & Saucier, L. (2016). Effects of plant extracts and essential oils as feed supplements on the quality and microbial traits of rabbit meat. *World Rabbit Science, 24*, 107 – 119.

Kovitvadhi, A., Gasco, L., Ferrocino, I., Rotolo, L., Dabbou, S., Malfatto, V., Gai, F., Peiretti, P. G., Falzone, M., Vignolini, C., Cocolin, L. & Zoccarato, I. (2016). Effect of purple loosestrife (*Lythrum salicaria*) diet supplementation in rabbit nutrition on performance, digestibility, health and meat quality. *Animal, 10*(1), 10 – 18.

Kroliczewska, B., Mista, D., Zawadzki, W. & Skiba, T. (2011). Effect of Baikal skullcap root (*Scutellaria baicalensis radix*) on cholesterol level and meat quality in rabbits fed a cholesterol rich diet. *Foliabiologica, 59*(3-4), 169 – 173.

Kroliczewska, B., Zawadzki, W., Skiba, T., Kopec, W. & Kroliczewska, J. (2008). The influence of Baical skullcap root (*Scutellaria baicalensis radix*) in the diet of broiler chickens on the chemical composition of the muscles, selected performance traits of the animals and the sensory characteristics of the meat. *Veterinarni Medicina, 7*, 373 – 380.

Landers, T. F., Cohen, B., Wittum, T. E. & Larson, E. L. (2012). A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Reports, 127*, 4 – 21.

Lau, D. W. & King, A. J. (2003). Pre- and post-mortem use of grape seed extract in dark poultry meat to inhibit development of thiobarbituric acid reactive substances. *Journal of Agricultural and Food Chemistry, 51*, 1602 – 1607.

Lee, J. Y., Lee, J.-H., Park, J. H., Kim, S.-Y., Choi, J. Y., Lee, S. H., Kim, Y. S., Kang, S. S., Jang, E-C. & Han, Y. (2009). Liquiritigenin, a licorice flavonoid, helps mice resist disseminated candidiasis due to *Candida albicans* by Th1 immune response, whereas liquiritin, its glycoside form, does not. *International Immunopharmacology, 9*, 632 – 638.

Lee, K. J., Lee, J.-R., Kim, H.-J., Raveendar, S., Lee, G.-A., Jeon, Y.-A., Park, E., Ma, K-H., Lee, S-Y. & Chung, J-W. (2017). Comparison of flavonoid contents and antioxidant activities of *Vicia* species. *Plant Genetic Resources: Characterization and Utilization, 15*(2), 119 – 126.

Lenehan, C., Moloney, A. P., O'Riordan, E. G., Kelly, A. & McGee, M. (2017). Comparison of rolled barley with citrus pulp as a supplement for growing cattle offered grass silage. *Advances in Animal Biosciences, 8*(s1), s33 – s37.

Li, S., Zhao, M., Jiang, T., Lv, W., Gao, S., Zhou, Y. & Miao, Z. (2018). Growth performance and antioxidant status of growing rabbits fed on diets supplemented with *Eucommia ulmoides* leaves. *World Rabbit Science, 26*, 35 – 41.

Liu, A.-L., Liu, B., Qin, H.-L., Lee, S. M., Wang, Y.-T. & Du, G.-H. (2008). Anti-influenza virus activities of flavonoids from the medicinal plant *Elsholtzia rugulosa*. *Planta medica, 74*(8), 847 – 851.

Liu, R. H. (2007). Whole grain phytochemicals and health. *Journal of Cereal Science, 46*, 207 – 219.

Lone, K. P. (1997). Natural sex steroids and their xenobiotic analogs in animal production: Growth, carcass quality, pharmacokinetics, metabolism, mode of action, residues, methods, and epidemiology. *Critical Reviews in Food Science and Nutrition, 37*(2), 93 – 209.

Lu, Y. & Foo, L. Y. (1997). Identification and quantification of major polyphenols in apple pomace. *Food Chemistry, 59*(2), 187 – 194.

Lu, Y. & Foo, L. Y. (1999). The polyphenol constituents of grape pomace. *Food Chemistry, 65*, 1 – 8.

Lykkesfeldt, J. & Svendsen, O. (2007). Oxidants and antioxidants in disease: Oxidative stress in farm animals. *The Veterinary Journal, 173*, 502 – 511.

Ma, X., Wu, H., Liu, L., Yao, Q., Wang, S., Zhan, R., Xing, S. & Zhou, Y. (2011). Polyphenolic compounds and antioxidant properties in mango fruits. *Scientia Horticulturae, 129*, 102 – 107.

Malossini, F., Pinosa, M., Piasentier, E. & Bovolenta, S. (1993). Grape marc and maize cobs in heavy lamb diets. *Annales de Zootechnie, 42*(3), 315 – 328.

Martin, J. H. J., Crotty, S., Warren, P. & Nelson, P. N. (2007). Does an apple a day keep the doctor away because a phytoestrogen a day keeps the virus at bay? A review of the anti-viral properties of phytoestrogens. *Phytochemistry, 68*, 266 – 274.

Mason, L. M., Hogan, S. A., Lynch, A., O'Sullivan, K., Lawlor, P. G. & Kerry, J. P. (2005). Effects of restricted feeding and antioxidant supplementation on pig performance and quality characteristics of *longissimus dorsi* muscle from Landrace and Duroc pigs. *Meat Science, 70*, 307 – 317.

McDonald, P., Edwards, R. A., Greenhalgh, J. F. D. & Morgan, C. A. (2002). *Animal nutrition* (6th ed.). Essex, UK: Pearson Education Limited.

Merken, H. M. & Beecher, G. R. (2000). Measurement of food flavonoids by high-performance liquid chromatography: A review. *Journal of Agricultural and Food Chemistry, 48*(3), 577 – 599.

Min, B. R., Barry, T. N., Attwood, G. T. & McNabb, W. C. (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Animal Feed Science and Technology, 106*, 3 – 19.

Mohdaly, A. A. A., Hassanien, M. F. R., Mahmoud, A., Sarhan, M. A. & Smetanska, I. (2013). Phenolics extracted from potato, sugar beet, and sesame processing by-products. *International Journal of Food Properties, 16*(5), 1148 – 1168.

Monaghan, P., Metcalfe, N. B. & Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology Letters, 12*, 75 – 92.

Muela, E., Alonso, V., Campo, M. M., Sañudo, C. & Beltrán, J. A. (2014). Antioxidant diet supplementation and lamb quality throughout preservation time. *Meat Science, 98*, 289 – 295.

Narayana, K. R., Reddy, M. S., Chaluvadi, M. R. & Krishna, D. R. (2001). Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Indian Journal of Pharmacology, 33*, 2 – 16.

Neugart, S., Rohn, S. & Schreiner, M. (2015). Identification of complex, naturally occurring flavonoid glycosides in *Vicia faba* and *Pisum sativum* leaves by HPLC-DAD-ESI-MS^n and the genotypic effect on their flavonoid profile. *Food Research International, 76*, 114 – 121.

Nieves, J. W. (2013). Skeletal effects of nutrients and nutraceuticals, beyond calcium and vitamin D. *Osteoporosis International, 24*, 771 – 786.

Njåstad, K. M., Adler, S. A., Hansen-Møller, J., Thuen, E., Gustavsson, A.-M. & Steinshamn, H. (2014). Gastrointestinal metabolism of phytoestrogens in lactating dairy cows fed silages with different botanical composition. *Journal of Dairy Science, 97*(12), 7735 – 7750.

O'Grady, M. N., Carpenter, R., Lynch, P. B., O'Brien, N. M. & Kerry, J. P. (2008). Addition of grape seed extract and bearberry to porcine diets: Influence on quality attributes of raw and cooked pork. *Meat Science, 78*, 438 – 446.

Oleszek, W., Stochmal, A. & Janda, B. (2007). Concentration of isoflavones and other phenolics in the aerial parts of *Trifolium* species. *Journal of Agricultural and Food Chemistry, 55*, 8095 – 8100.

Oluremi, O. I. A., Okafor, F. N., Adenkola, A. Y. & Orayaga, K. T. (2010). Effect of fermentation of sweet orange (*Citrus sinensis*) fruit peel on its phytonutrients and the performance of broiler starter. *International Journal of Poultry Science, 9*(6), 546 – 549.

Omar, N. F., Hassan, S. A., Yusoff, U. K., Abdullah, N. A. P., Wahab, P. E. M. & Sinniah, U. R. (2012). Phenolics, flavonoids, antioxidant activity and cyanogenic glycosides of organic and mineral-based fertilized cassava tubers. *Molecules, 17*, 2378 – 2387.

Onderci, M., Sahin, K., Sahin, N., Gursu, M. F., Doerge, D., Sarkar, F. H. & Kucuk, O. (2004). The effect of genistein supplementation on performance and antioxidant status of Japanese quail under heat stress. *Archives of Animal Nutrition, 58*(6), 463 – 471.

Oskoueian, E., Abdullah, N. & Oskoueian, A. (2013). Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *BioMed Research International*, article ID 349129, 1 – 8.

Oteiza, P. I., Fraga, C. G., Mills, D. A. & Taft, D. H. (2018). Flavonoids and the gastrointestinal tract: Local and systemic effects. *Molecular Aspects of Medicine, 61*, 41 – 49.

Ouyang, K., Xu, M., Jiang, Y. & Wang, W. (2016). Effects of alfalfa flavonoids on broiler performance, meat quality, and gene expression. *Canadian Journal of Animal Science, 96*, 332 – 341.

Pace, V., Carbone, K., Spirito, F., Iacurto, M., Terzano, M. G., Verna, M., Vincenti, F. & Settineri, D. (2006). The effects of subterranean clover phytoestrogens on sheep growth, reproduction and carcass characteristics. *Meat Science, 74*(4), 616 – 622.

Park, S.-Y., Lee, S. Y., Yang, J. W., Lee, J.-S., Oh, S.-D., Oh, S., Lee, S. M., Lim, M-H., Park, S. K., Jang, J-S., Cho, H. S. & Yeo, Y. (2016). Comparative analysis of phytochemicals and polar metabolites from colored sweet potato (*Ipomoea batatas* L.) tubers. *Food Science and Biotechnology, 25*(1), 283 – 291.

Payet, B., Sing, A. S. C. & Smadja, J. (2006). Comparison of the concentrations of phenolic constituents in cane sugar manufacturing products with their antioxidant activities. *Journal of Agricultural and Food Chemistry, 54*, 7270 – 7276.

Payne, R. L., Bidner, T. D., Southern, L. L. & Geaghan, J. P. (2001). Effects of dietary soy isoflavones on growth, carcass traits, and meat quality in growing-finishing pigs. *Journal of Animal Science, 79*, 1230 – 1239.

Payne, R. L., Bidner, T. D., Southern, L. L. & McMillin, K. W. (2001). Dietary effects of soy isoflavones on growth and carcass traits of commercial broilers. *Poultry Science, 80*, 1201 – 1207.

Peiretti, P. & Meineri, G. (2008). Effects on growth performance, carcass characteristics, and the fat and meat fatty acid profile of rabbits fed diets with chia (*Salvia hispanica* L.) seed supplements. *Meat Science, 80*, 1116 – 1121.

Pękal, A. & Pyrzynska, K. (2014). Evaluation of aluminium complexation reaction for flavonoid content assay. *Food Analytical Methods, 7*, 1776 – 1782.

Peña, J. E. M., Vieira, S. L., López, J., Reis, R. N., Barros, R., Furtado, F. V. F. & Silva, P. X. (2008). Ascorbic acid and citric flavonoids for broilers under heat stress: Effects on performance and meat quality. *Brazilian Journal of Poultry Science, 10*(2), 125 – 130.

Peterson, J. & Dwyer, J. (1998). Flavonoids: Dietary occurrence and biochemical activity. *Nutritional Research, 18*(12), 1995 – 2018.

Ponce, M. A., Bompadre, M. J., Scervino, J. M., Ocampo, J. A., Chaneton, E. J. & Godeas, A. M. (2009). Flavonoids, benzoic acids and cinnamic acids isolated from shoots and roots of Italian rye grass (*Lolium multiflorum* Lam.) with and without endophyte association and arbuscular mycorrhizal fungus. *Biochemical Systematics and Ecology, 37*, 245 – 253.

Preston, R. L. (1999). Hormone containing growth promoting implants in farmed livestock. *Advanced Drug Delivery Reviews, 38*, 123 – 138.

Procházková, D., Boušová, I. & Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia, 82*, 513 – 523.

Putnam, S. E., Scutt, A. M., Bicknell, K., Priestley, C. M. & Williamson, E. M. (2007). Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytotherapy research, 21*(2), 99 – 112.

Pyo, Y.-H., Lee, T.-C., Logendra, L. & Rosen, R. T. (2004). Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cycla*) extracts. *Food Chemistry, 85*(1), 19 – 26.

Radović, J., Sokolović, D. & Marković, J. (2009). Alfalfa — most important perennial forage legume in animal husbandry. *Biotechnology in Animal Husbandry, 25*(5-6), 465 – 475.

Ramsay, A., Drake, C., Brinkhaus, A. G., Girard, M., Copani, G., Dohme-Meier, F., Bee, G., Niderkorn, V. & Mueller-Harvey, I. (2015). Sodium hydroxide enhances extractability and analysis of proanthocyanidins in ensiled sainfoin (*Onobrychis viciifolia*). *Journal of Agricultural and Food Chemistry, 63*, 9471 – 9479.

Ratty, A. K. & Das, N. P. (1988). Effects of flavonoids on non-enzymatic lipid peroxidation: structure-activity relationship. *Biochemical Medicine and Metabolic Biology, 39*, 69 – 79.

Reynaud, J. & Lussignol, M. (2005). The flavonoids of *Lotus corniculatus*. *Lotus Newsletter, 35*(1), 75 – 82.

Rupasinghe, H. P. V., Ronalds, C. M., Rathgeber, B. & Robinson, R. A. (2010). Absorption and tissue distribution of dietary quercetin and quercetin glycosides of apple skin in broiler chickens. *Journal of the Science of Food and Agriculture, 90*, 1172 – 1178.

Sahin, N., Sahin, K., Onderci, M., Sarkar, F. H., Doerge, D., Prasad, A. & Kucuk, O. (2006). Effects of dietary genistein on nutrient use and mineral status in heat-stressed quails. *Experimental Animals, 55*(2), 75 – 82.

Saleh, N. A. M., Boulos, L., El-Negoumy, S. & Abdalla, M. F. (1982). A comparative study of the flavonoids of *Medicago radiata* with other *Medicago* and related *Trigonella* species. *Biochemical Systematics and Ecology, 10*(1), 33 – 36.

Schittko, U., Burghardt, F., Fiedler, K., Wray, V. & Proksch, P. (1999). Sequestration and distribution of flavonoids in the common blue butterfly *Polyommatus icarus* reared on *Trifolium repens*. *Phytochemistry, 51*, 609 – 614.

Scientific Committee on Veterinary Measures relating to Public Health (1999). *Assessment of potential risks to human health from hormone resides in bovine meat and meat products.* Brussels, Belgium: European Commission.

Sehm, J., Lindermayer, H., Dummer, C., Treutter, D. & Pfaffl, M. W. (2007). The influence of polyphenol rich apple pomace or red-wine pomace diet on the gut morphology in weaning piglets. *Journal of Animal Physiology and Animal Nutrition, 91*, 289 – 296.

Serratosa, J., Blass, A., Rigau, B., Mongrell, B., Rigau, T., Tortadès, M., Tolosa, E., Aguilar, C., Ribo, O. & Balague, J. (2006). Residues from veterinary medicinal products, growth promoters and performance enhancers in food-producing animals: a European Union perspective. *Revue Scientifique et Technique (International Office of Epizootics), 25*(2), 637 – 653.

Seven, P. T. & Seven, I. (2008). Effect of dietary Turkish propolis as alternative to antibiotic on performance and digestibility in broilers exposed to heat stress. *Journal of Applied Animal Research, 34*, 193 – 196.

Seven, P. T., Seven, I., Yılmaz, M. & Şimşek, U. G. (2008). The effects of Turkish propolis on growth and carcass characteristics in broilers under heat stress. *Animal Feed Science and Technology, 146*, 137 – 148.

Seven, P. T., Yılmaz, S., Seven, I., Cercı, I. H., Azman, M. A. & Yılmaz, M. (2009). Effects of propolis on selected blood indicators and antioxidant enzyme activities in broilers under heat stress. *Acta Veterinaria Brno, 78*, 75 – 83.

Shahidi, F. & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects — A review. *Journal of Functional Foods, 18*, 820 – 897.

Sharif, M., Ashraf, M. S., Mushtaq, N., Nawaz, H., Mustafa, M. I., Ahmad, F., Younas, M. & Javaid, A. (2018). Influence of varying levels of dried citrus pulp on nutrient intake, growth performance and economic efficiency in lambs. *Journal of Applied Animal Research, 46*(1), 264 – 268.

Simitzis, P. E., Babaliaris, C., Charismiadou, M. A., Papadomichelakis, G., Goliomytis, M., Symeon, G. K. & Deligeorgis, S. G. (2014). Effect of hesperidin dietary supplementation on growth performance, carcass traits and meat quality of rabbits. *World Rabbit Science, 22*, 113 – 121.

Simitzis, P. E., Charismiadou, M. A., Goliomytis, M., Charalambous, A., Ntetska, I., Giamouri, E. & Deligeorgis, S. G. (2019). Antioxidant status, meat oxidative stability and quality characteristics of lambs fed with hesperidin, naringin or α-tocopherol acetate supplemented diets. *Journal of the Science of Food and Agriculture, 99*(1), 343 – 349.

Simitzis, P. E., Ilias-Dimopoulos, V., Charismiadou, M. A., Biniari, E. E. & Deligeorgis, S. G. (2013). Effects of dietary hesperidin supplementation on lamb performance and meat characteristics. *Animal Science Journal, 84*, 136 – 143.

Simitzis, P. E., Symeon, G. K., Charismiadou, M. A., Ayoutanti, A. G. & Deligeorgis, S. G. (2011). The effects of dietary hesperidin supplementation on broiler performance and chicken meat characteristics. *Canadian Journal of Animal Science, 91*, 275 – 282.

Sohaib, M., Butt, M. S., Shabbir, M. A. & Shahid, M. (2015). Lipid stability, antioxidant potential and fatty acid composition of broilers breast meat as influenced by quercetin in combination with α-tocopherol enriched diets. *Lipids in Health and Disease, 14*(61), 1 – 15.

Spencer, G. S. G. (1985). Hormonal systems regulating growth. A review. *Livestock Production Science, 12*, 31 – 46.

Steinshamn, H., Purup, S., Thuen, E. & Hansen-Møller, J. (2008). Effects of clover-grass silages and concentrate supplementation on the content of phytoestrogens in dairy cow milk. *Journal of Dairy Science, 91*(7), 2715 – 2725.

Stochmal, A. & Oleszek, W. (2007). Seasonal and structural changes of flavones in alfalfa (*Medicago sativa*) aerial parts. *Journal of Food, Agriculture & Environment, 5*(2), 84 – 88.

Stochmal, A., Piacente, S., Pizza, C., De Riccardis, F., Leitz, R. & Oleszek, W. (2001). Alfalfa (*Medicago sativa* L.) flavonoids. 1. Apigenin and luteolin glycosides from aerial parts. *Journal of Agricultural and Food Chemistry, 49*, 753 – 758.

Taasoli, G. & Kafilzadeh, F. (2008). Effects of dried and ensiled apple pomace from puree making on performance of finishing lambs. *Pakistan Journal of Biological Sciences, 11*(2), 294 – 297.

Tan, C. Y., Zhong, R. Z., Tan, Z. L., Han, X. F., Tang, S. X., Xiao, W. J., Sun, Z. H. & Wang, M. (2011). Dietary inclusion of tea catechins changes fatty acid composition of muscle in goats. *Lipids, 46*(3), 239 – 47.

Tang, S. Z., Kerry, J. P. ad S. D., Buckley, D. J. & Morrissey, P. A. (2001). Antioxidative effect of dietary tea catechins on lipid oxidation of long-term frozen stored chicken meat. *Meat Science, 57*, 331 – 336.

Tapas, A. R., Sakarkar, D. M. & Kakde, R. B. (2008). Flavonoids as neutraceuticals: A review. *Tropical Journal of Pharmaceutical Research, 7*(3), 1089 – 1099.

Thill, J., Regos, I., Farag, M. A., Ahmad, A. F., Kusek, J., Castro, A., Schlangen, K., Carbonero, C. H., Gadjev, I. Z., Smith, L. M. J., Halbwirth, H., Treutter, D. & Stich, K. (2012). Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin). *Phytochemistry, 82*, 67 – 80.

Thornton, P. K. (2010). Livestock production: recent trends, future prospects. *Philosophical Transactions of the Royal Society, 365*, 2853 – 2867.

Toor, R. K. & Savage, G. P. (2005). Antioxidant activity in different fractions of tomatoes. *Food Research International, 38*, 487 – 494.

Toor, R. K. & Savage, G. P. (2006). Effect of semi-drying on the antioxidant components of tomatoes. *Food Chemistry, 94*, 90 – 97.

Tuzcu, M., Sahin, N., Karatepe, M., Cikim, G., Kilinc, U. & Sahin, K. (2008). Epigallocatechin-3-gallate supplementation can improve antioxidant status in stressed quail. *British Poultry Science, 49*(5), 643 – 648.

Uarrota, V. G., Moresco, R., Coelho, B., da Costa Nunes, E., Peruch, L. A. M., de Oliveira Neubert, E., Rocha, M. & Maraschin, M. (2014). Metabolomics combined with chemometric tools (PCA, HCA, PLS-DA and SVM) for screening cassava (*Manihot esculenta* Crantz) roots during postharvest physiological deterioration. *Food Chemistry, 161*, 67 – 78.

Valli, V., Gómez-Caravaca, A. M., Di Nunzio, M., Danesi, F., Caboni, M. F. & Bordoni, A. (2012). Sugar cane and sugar beet molasses, antioxidant-rich alternatives to refined sugar. *Journal of Agricultural and Food Chemistry, 60*, 12508 – 12515.

Verbeke, W. A. J. & Viaene, J. (2000). Ethical challenges for livestock production: Meeting consumer concerns about meat safety and animal welfare. *Journal of Agricultural and Environmental Ethics, 12*, 141 – 151.

Vetter, J. (1995). Isoflavones in different parts of common *Trifolium* species. *Journal of Agricultural and Food Chemistry, 43*, 106 – 108.

Voicu, D., Hăbeanu, M., Uţă, R. A., Voicu, I. & Gras, M. A. (2014). Effect of the dietary dry grape pomace on the performance and health state of fattening steers. *Scientific Papers. Series D. Animal Science, 57*, 118 – 124.

Waly, A. H., Bassyony, M. M., Zedan, A. H. & Galal, H. M. F. (2015). Inclusion of dried agro-industrial strawberry by-products in growing rabbit diets. *Egyptian Journal of Nutrition and Feeds, 18*(2), 371 – 381.

Wang, Y.-C., Chuang, Y.-C. & Hsu, H.-W. (2008). The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. *Food Chemistry*, *106*(1), 277 – 284.

Weaver, C. M., Alekel, D. L., Ward, W. E. & Ronis, M. J. (2012). Flavonoid intake and bone health. *Journal of Nutrition in Gerontology and Geriatrics*, *31*(3), 239 – 253.

Williamson, G., Kay, C. D. & Crozier, A. (2018). The bioavailability, transport, and bioactivity of dietary flavonoids: A review from a historical perspective. *Comprehensive Reviews in Food Science and Food Safety*, *0*, 1 – 59.

Wong, W. H., Lee, W. X., Ramanan, R. N., Tee, L. H., Kong, K. W., Galanakis, C. M., Sun, J. & Prasad, K. N. (2015). Two level half factorial design for the extraction of phenolics, flavonoids and antioxidants recovery from palm kernel by-product. *Industrial Crops and Products*, *63*, 238 – 248.

Yao, L. H., Jiang, Y. M., Shi, J., Tomas-Barberan, F. A., Datta, N., Singanusong, R. & Chen, S. S. (2004). Flavonoids in food and their health benefits. *Plant Foods for Human Nutrition*, *59*, 113 – 122.

Zahari, M. W. & Alimon, A. R. (2005). Use of palm kernel cake and oil palm by-products in compound feed. *Palm Oil Developments*, *40*, 5 – 9.

Zhong, R. Z., Tan, C. Y., Han, X. F., Tang, S. X., Tan, Z. L. & Zeng, B. (2009). Effect of dietary tea catechins supplementation in goats on the quality of meat kept under refrigeration. *Small Ruminant Research*, *87*, 122 – 125.

Zhong, R., Xiao, W., Ren, G., Zhou, D., Tan, C., Tan, Z., Han, X., Tang, S., Zhou, C. & Wang, M. (2011). Dietary tea catechin inclusion changes plasma biochemical parameters, hormone concentrations and glutathione redox status in goats. *Asian-Australian Journal of Animal Science*, *24*(12), 1681 – 1689.

Zhou, H., Wang, C., Ye, J., Chen, H. & Tao, R. (2015). Effects of dietary supplementation of fermented *Ginkgo biloba* L. residues on growth performance, nutrient digestibility, serum biochemical parameters and immune function in weaned piglets. *Animal Science Journal*, *86*, 790 – 799.

Žilić, S., Serpen, A., Akıllıoğlu, G., Gökmen, V. & Vančetović, J. (2012). Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (*Zea mays* L.) kernels. *Journal of Agricultural and Food Chemistry*, *60*, 1224 – 1231.

Zu, Y.-G., Liu, X.-L., Fu, Y.-J., Wu, N., Kong, Y. & Wink, M. (2010). Chemical composition of the SFE-CO extracts from *Cajanus cajan* (L.) Huth and their antimicrobial activity *in vitro* and *in vivo*. *Phytomedicine*, *17*, 1095 – 1101.

CHAPTER 3:

The effects of quercetin supplementation on the growth, feed conversion ratio and serum hormone levels of New Zealand White rabbits[#]

Abstract

This study investigated the effects of quercetin dihydrate (0 or 2 g/kg feed) on the live performance and serum free triiodothyronine (fT3), free thyroxine, somatotropin and cortisol levels of growing rabbits. It also provided the first growth and feed conversion ratio (FCR) data for South African New Zealand Whites. Control (Ctrl) and quercetin-supplemented (Qrc) diets were fed to 34 (16 male, 18 female) and 32 (15 male, 17 female) rabbits, respectively, from 5 to 12 weeks old. Live performance was measured weekly, and serum hormone levels were determined at 11 weeks old. The rabbits grew well, increasing from 1052 ± 13.4 g to 3192 ± 45.3 g. Females had higher cortisol levels than males, as found for other species (female: 10.9 ± 1.55 ng/ml, male: 3.89 ± 0.53 ng/ml). Qrc rabbits tended to have higher FCRs (Ctrl: 3.83 ± 0.08, Qrc: 4.01 ± 0.09), possibly due to lower feed digestibility, and to have smaller sex-differences in growth and FCR, possibly due to xenoestrogenic activity. They also tended to have higher fT3 levels (Ctrl: 3.62 ± 0.420 ng/ml, Qrc: 4.98 ± 0.599 ng/ml), possibly due to the inhibition of binding to transthyretin. However, quercetin-supplementation did not improve the live performance of the rabbits.

[#]Published with minor changes as: North, M. K., Dalle Zotte, A. & Hoffman, L. C. (2018). Effect of quercetin supplementation on the growth, feed efficiency and serum hormone levels of New Zealand White rabbits. *South African Journal of Animal Science*, *48*(6), 1128 – 1139.

3.1 Introduction

Rabbits show great potential in South Africa, not only for large-scale commercial meat production but also for smaller, rural development projects (Oseni, 2012). They can be farmed intensively on small areas of land, are highly prolific, and reach slaughter weight in a short period of time, resulting in rapid returns on capital investment (Abu, Onifade, Abanikannda & Obiyan, 2008; Oseni, 2012). Rabbits also grow efficiently on diets with a high-fibre, low grain content, reducing the direct competition with humans for feed resources, and allowing the supplementation of their diet with locally-available forages (Abu *et al*., 2008; Finzi, 2000). Their small size allows easy handling by women and children and reduces the need for cold-storage at slaughter, and relatively simple farming systems can be used (Abu *et al*., 2008; Finzi, 2000).

However, like all livestock industries, rabbit farming faces challenges in terms of shrinking profit margins (which necessitate increasing the efficiency of production) and demands by consumers to not only produce high quality, healthy products, but also ones that are environmentally and socially responsible (Dalle Zotte & Szendrő, 2011; Verbeke & Viaene, 2000). This has resulted in bans on the use of antibiotics as growth promotors in animal feeds in the European Union (EU), as well recommendations from the World Health Organisation that the non-therapeutic use of antibiotics in animals is limited worldwide. Similar guidelines have been released by authorities in the United States of America, Canada and Australia, and the EU regulations also apply to imported animal products, and thus impact production in exporting nations as well (European Commission, 2005; FDA Centre for Veterinary Medicine, 2013; Johnson, 2010; Landers, Cohen, Wittum & Larson, 2012; Serratosa *et al*., 2006). It is therefore necessary for agricultural researchers world-wide to investigate alternative, natural options, such as flavonoids.

Flavonoids are naturally occurring polyphenolic compounds that are produced by plants as secondary metabolites, and have long been recognised as having extensive pharmacological actions (Erlund, 2004; Havsteen, 2002; Tapas, Sakarkar & Kakde, 2008). Some, having prominent antimicrobial and antioxidant activities, have been brought to the attention of the livestock industry as potential growth promotors (Erlund, 2004; Tapas *et al*., 2008). However, due to the wide range of bioactivities possessed by these compounds, including mutagenic effects and interactions with hormone systems, the risks of negative effects of supplementation must also be considered (Dakora, 1995; Dos Santos, Gonçalves, Vaisman, Ferreira & de Carvalho, 2011; Havsteen, 2002).

Quercetin is one of the most well-studied and characterised flavonoids, and is the most widely occurring dietary flavonol (Erlund, 2004). Livestock feed resources containing quercetin include white clover flowers (Schittko, Burghardt, Fiedler, Wray & Proksch, 1999), trefoil (Reynaud & Lussignol, 2005), sainfoin (Thill *et al*., 2012), members of the *Brassicaceae* family (Cartea, Francisco, Soengas & Velasco, 2011), sweet potatoes (Park *et al*., 2016), apple pomace (Lu & Foo, 1997), cottonseed meal (Blouin, Zarins & Cherry, 1981) and sunflower meal (Karamać, Kosińska, Estrella, Hernández & Duenas, 2012). The biochemical activities of quercetin have been found to be extensive (Erlund, 2004); however, livestock studies are relatively limited, and studies on poultry have had varying results. Liu *et al*. (2014) found that intermediate levels (0.2 – 0.4 g/kg feed) of supplementation improved feed efficiency and laying rates in hens, but higher levels (0.6 g/kg feed) had a negative effect. Similarly,

Goliomytis *et al*. (2014) found that relatively high inclusion levels (0.5 – 1 g/kg feed) caused non-significant increases in the feed conversion ratio of broiler chickens. While no studies have been done looking at the effects of quercetin on meat rabbit growth parameters, there is evidence of effects on rabbit doe reproductive traits (Naseer *et al*., 2017), bone histology (Babosová *et al*., 2016) and thyrotropin secretion (Kováčik, Packová & Kolesárová, 2015).

The aim of this study was therefore to provide the first data on the effects of quercetin supplementation on the growth and feed efficiency of rabbits, as well as to investigate its impact on the serum levels of several key hormones. The choice of hormones was based on both their relative importance to growth and development and previous findings on the effects of flavonoids on their metabolism. Cortisol is well-established as an indicator of chronic stress levels (Möstl & Palme, 2002), and the antioxidant effects of flavonoids have been suggested to aid in ameliorating the effects of stress (Onderci *et al*., 2004; Tuzcu *et al*., 2008). Flavonoids have also been found to interact with the thyroid hormone system (Narayana, Reddy, Chaluvadi & Krishna, 2001), and both thyroid hormones and somatotropin are important for physiological development (Spencer, 1985).

In addition, this study provides the first reported growth data for South African New Zealand White rabbits, which have been genetically isolated from the rest of the world for *ca.* 33 years due to a ban on the importation of rabbits.

3.2 Materials and methods

Ethical clearance for this study was obtained from the Stellenbosch University Animal Care and Use Committee (protocol number SU-ACUD16-00094).

3.2.1 Dietary treatments

Two diets were tested, namely a control (Ctrl) and a quercetin dihydrate-supplemented (Qrc) treatment diet. The Ctrl diet was formulated to be a complete, nutritionally-balanced rabbit grower feed (Table 3.1), and the Qrc diet was produced from this Ctrl diet through the addition of 2 g/kg quercetin dihydrate (*Sophorae japonica* flower extract, from Chengdu Okay Plant and Chemical Co., Ltd, Qionglai, China). The quercetin dihydrate was added to the feed during initial mixing, prior to pelleting. Neither of the feeds contained coccidiostats, and both feeds were manufactured by Pennville (Pty) Ltd (Pretoria, South Africa).

The total flavonoid content of the two diets was determined colourimetrically. Flavonoid extracts were prepared by sonicating (Power Sonic 405, United Scientific, Cape Town, South Africa) 0.4 g of feed with 10 ml 1 % formic acid in 50 % methanol for two 15 minute periods, with a 10 minute interval. The extracts were then centrifuged (Sigma 2-16 K, Wirsam scientific, Cape Town, SA) at 2000 × g for 10 minutes, and the supernatant was filtered using a PES (Prefilter) 0.22 µm, 33 mm syringe filter (Agela Technologies, Wilmington, USA). The extract was stored at -20 °C until analysis. The colorimetric assay was performed as described by Herald, Gadgil and Tilley (2012), with adaptations. Briefly, 100 µl distilled water, 10 µl 5 % sodium nitrite and 10 µl extract or standard were combined in a 2 ml microcentrifuge tube and vortexed. After incubating at room temperature for 5 minutes, 15 µl 10 % aluminium chloride was added and the tube was vortexed and incubated at room temperature

for a further 6 minutes. Thereafter, 50 µl 1 M sodium hydroxide and 50 µl distilled water were added, and the tubes were vortexed, prior to centrifuging at 3220 × g for 5 minutes. A 200 µl aliquot of the supernatant was transferred to a clear 96 well microplate (Greiner Cellstar 96 well flatbottom plate, Sigma-Aldrich, St Louis, USA) and the absorbance at 510 nm was measured (Spectrostar Nano, BMG Labtech, Ortenberg, Germany). The total flavonoid content was determined relative to a quercetin (Sigma-Aldrich, Steinheim, Germany) standard (25 – 500 µg/ml; $y = 0.0007x + 0.0569$; $R^2 = 0.997$) and is expressed as milligrams quercetin equivalents per gram dry matter.

Table 3.1
Ingredients and chemical composition of the rabbit grower control (Ctrl) diet and the total flavonoid content of the Ctrl diet and the diet supplemented with 2 g quercetin dihydrate/kg feed (Qrc).

Ingredients:	**g/kg**
Alfalfa	362
Wheat bran	356
Sunflower meal	126
Wheat	50.0
Molasses	50.0
Soya hulls	31.0
Soybean oil	11.0
Vitamin and mineral premix*	5.00
Limestone	3.70
Mono dicalcium phosphate 21 %	2.10
L-Lysine	1.40
Salt	1.00
DL-Methionine	0.80
Chemical composition:	**g/kg**
Dry matter	883
Crude protein	185
Ether extract	37.1
Ash	83.1
Crude fibre	190
Neutral detergent fibre (NDF)	317
Acid detergent fibre (ADF)	177
Acid detergent lignin (ADL)	61.3
Total flavonoid content:	**(mg Qrc eq/g DM)**
Ctrl feed	5.03
Qrc feed	5.25

*Vitamins and minerals provided per kg of diet: 17500 IU vitamin A, 7500 IU vitamin D_3, 583 mg choline, 250 mg vitamin C, 208 mg antioxidant, 200 mg vitamin B_5, 183 mg manganese, 167 mg zinc, 100 mg vitamin E, 83 mg niacin, 67 mg iron, 17 mg copper, 8.3 mg vitamin B_2, 4.8 mg vitamin B_6, 3.3 mg vitamin K_3, 3.3 mg vitamin B_1, 3.3 mg folic acid, 3.3 mg iodine, 0.75 mg cobalt, 0.5 mg selenium, 0.15 mg biotin, 0.015 mg vitamin B_{12}.
DM: dry matter; Qrc eq: quercetin equivalents.

3.2.2 Rearing conditions

The growth trial was carried out on Mariendahl Experimental Farm outside Stellenbosch in the Western Cape of South Africa (33°51'02.9"S 18°49'35.2"E) from August to October 2017, using 66 purebred New Zealand White rabbits. The 31 male and 35 female rabbits were assigned to the two dietary treatment groups according to live weight and litter, such that the litters were split between the treatments and each treatment had a similar average starting weight (Ctrl males 1022 ± 32 g, Ctrl females 1074 ± 24 g, Qrc males 1069 ± 25 g, Qrc females 1042 ± 21 g). The sexes were distributed more-or-less equally among the dietary treatments (16 Ctrl males, 18 Ctrl females, 15 Qrc males, 17 Qrc females).

At weaning at five weeks of age the rabbits were transferred from doe-cages in the breeder house to individual grower cages in two grower rooms. In order to take into account any possible environmental differences between the two grower rooms, the dietary treatments and sexes were evenly distributed within and between the two rooms. The grower cages used were constructed out of welded wire mesh, and were 60 x 60 cm and 50 cm high, with the floors of the cages 1.5 m off the ground. Wood shavings were spread below the cages to absorb urine and were changed whenever necessary.

In order to reduce the risk of digestive disturbances the rabbits were provided with a commercial multi-strain powdered probiotic product containing *Lactobacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Streptococcus salvarius* subsp. *thermophilus* and *Enterococcus faecium*, with a minimum total viable count of 2×10^8 cfu/gram (Protexin Soluble, Kyron Laboratories Pty Ltd., Johannesburg, South Africa), sprinkled on their feed (3 g/kg) from one week prior to weaning until one week after weaning. The experimental feeds were provided *ad libitum* from the day of weaning until slaughter at 12 weeks of age, and fresh water was freely available at all times via automatic water lines supplemented with bottle-based nipple drinkers. Daily feed intake (FI) was determined weekly for each rabbit/cage by assigning a set weight of feed to the cage at the beginning of the week and weighing any remaining, refused or spilled feed at the end of the week. All rabbits were weighed individually at the same time each week to determine live weight (LW) and calculate average daily gain (ADG, calculated for each rabbit weekly by subtracting the previous week's LW and dividing by seven). Feed was not withheld prior to weighing, and LW values thus included the weight of the full gastrointestinal tract. The FI and ADG data were used for the calculation of the feed conversion ratio (FCR) on a weekly basis.

A 12L:12D light regime was used throughout the trial, and artificial ventilation was provided via fans in room A and an air-conditioning system in room B. Temperature and relative humidity were recorded in the two rooms throughout the trial using automatic temperature loggers (LogTag Humidity & Temperature Recorder, Model HAX0-8), with the average recorded temperature and humidity in rooms A and B during the growth period being 14.9 ± 0.21 °C, 72.3 ± 0.96 % and 16.4 ± 0.14 °C, 63.4 ± 0.73 %, respectively.

3.2.3 Blood sampling

At 11 weeks of age 18 rabbits from each dietary treatment (Ctrl and Qrc), consisting of both sexes and distributed throughout the two grower rooms, were selected for blood sampling (8 Ctrl males, 10 Ctrl females, 9 Qrc males, 9 Qrc females). Blood (*ca.* 5 ml per rabbit) was collected by a trained animal technician from the central ear artery, using Healcath™ I.V. Cannula 24 G needles with catheters (Harsoria Healthcare Pvt. Ltd., Haryana, India) and sterile 5 ml syringes (Avacare Health, Johannesburg, South Africa). After withdrawal, the blood was transferred to BD Vacutainer® SST™ II *Advance* Plus blood collection tubes (Becton Dickinson and Company, New Jersey, USA) for serum separation. In order to reduce stress and improve the ease of blood withdrawal *ca.* 200 µl Neurotranq (Virbac RSA (Pty) Ltd, Gauteng, South Africa) was injected into the caudal ear artery using an insulin syringe (Becton Dickinson and Company, New Jersey, USA) prior to blood withdrawal. A Techniplast rabbit restrainer (Labotec, Cape Town, South Africa) was used during the procedure.

The collected blood was allowed to clot at room temperature for at least 30 minutes, before centrifuging at 1000 × g for 15 minutes and transferring the separated serum into 2 ml graduated microtubes (Scientific Specialities Inc., California, USA)**.** The serum was stored at -20 °C until further analysis.

3.2.4 Serum hormone determination

The free thyroxine (fT4), free triiodothyronine (fT3) and somatotropin (GH) contents of the serum samples were determined in duplicate using commercially available ELISA (enzyme-linked immunosorbent assay) kits from Elabscience (fT4: E-EL-RB2049, fT3: E-EL-RB0919 and GH: E-EL-RB2030) purchased from Biocom Africa (Pty) Ltd (Centurion, South Africa). The standard curves used for quantification all had coefficients of determination above 99.7 %.

The cortisol content of the samples was determined using ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS), as described by Quanson *et al*. (2016) and Needham, Lambrechts and Hoffman (in press). Briefly, 500 µl serum was combined with 50 µl 0.3 ng/µl cortisol D-4 internal standard (Cambridge Isotope Laboratories, Andover, MA, USA) in glass tubes; 1.5 ml *tert*-methyl butyl ether (MTBE, Sigma-Aldrich, Steinheim, Germany) was added and the tubes were shaken at 1000 rpm for 10 minutes. After shaking, the tubes were frozen at -80 °C for 1 hour to remove the aqueous phase and the organic phase was transferred to clean glass tubes. The MTBE was evaporated at 55 °C under a flow of nitrogen gas before re-suspending in 150 µl 50 % methanol (ROMIL, Cambridge, England) and transferring into HPLC vials. The extract was frozen at -20 °C until analysis.

Chromatographic separation and identification of the extracted compounds was performed as described by Quanson *et al*. (2016), using an Acquity UPC^2 system with an Acquity UPC^2 BEH 2-EP (3 mm x 100 mm, 1.7 um) column (Waters Corporation, USA). A cortisol external standard curve ($y = 0.04x + 0.007$; $R^2 = 0.996$) with concentrations from 0.05 to 250 ng/ml was used for the quantification of the cortisol content.

3.2.5 Statistical analysis

The trial used a randomised block experimental design in order to take into account any environmental differences between the two grower rooms, and the main effects of interest — namely diet, sex and age — were combined in a three-factor factorial structure. As the rabbits were individually housed, each rabbit served as an experimental unit.

The data was analysed using Statistica version 13 software, with normality being tested using normal probability plots and the homoscedasticity assessed with Levene's test. The Variance, Estimation, Precision and Comparison (VEPAC) mixed-model function was used with the Restricted Maximum Likelihood (REML) estimation method to determine the significance of the blocks, main effects and second- and third-order interactions for the live performance data. Weaning weight was included as a covariate. In addition to the basic analysis, simple linear regression lines were fitted for each diet-sex treatment group for the live performance data, and the intercept and slope parameters were compared across the diet-sex treatment groups to determine whether any significant differences were present.

The R lm package was used to test the significance of the blocks, main effects and interactions for the hormone data, with LW at 11 weeks as a covariate. Pearson's and Spearman's correlation coefficients were calculated for the associations between the concentrations of the hormones and the FI, ADG and FCR at 11 weeks of age. The Fisher's least significant difference *post hoc* test was used to compare the individual values of the treatment groups if second- and/or third-order interactions were found to be significant, for both the live performance and hormone data.

Main effects and interactions with $P \leq 0.05$ were considered significant, whereas those with $P \leq 0.10$ were reported as trends. Values were reported as the LSMean ± standard error of the mean (SEM).

3.3 Results

The statistical analysis of the data found that the block effect was relatively limited, with only a single variable, the serum fT3 concentration ($P = 0.01$), differing between the blocks.

As can be seen in Figure 3.1, the rabbits increased ($P < 0.001$) in weight almost linearly during the growth period, from 1052 ± 13.4 g at weaning at 5 weeks to 3192 ± 45.3 g at 12 weeks old. There was no difference in the LW between the diet-sex treatment groups at any point, although the variation within and between the groups increased with age. The similarity of growth between the treatment groups is also demonstrated by the regression parameters in Table 3.2.

The ADG decreased ($P < 0.001$) over time, and showed far more variation both from week to week and between the treatment groups than the LW, as demonstrated by the diet-sex-week third-order interaction ($P = 0.001$). In order to simplify the interpretation of Figure 3.1, significance has only been indicated between the treatment groups within each time point, as seen at week 6 and 10. The ADG during the first week post-weaning was the most variable, with male Ctrl rabbits growing the fastest, female Ctrl rabbits the slowest and the Qrc rabbits having similar, intermediate gains. Thereafter, the ADG stabilised across the treatment groups and

remained relatively similar until 10 weeks of age, at which point Ctrl females were growing the fastest, Qrc females the slowest and the males were intermediate. In terms of the general pattern of ADG, it appeared that the Qrc groups showed less sex-related variation over the growth period, with the latter also being demonstrated by the similar slopes and intercepts of the regression lines for the male and female rabbits in the Qrc group (Table 3.2). This indicated that the sexes had similar patterns of growth in this group, which was not seen for the Ctrl group. While the very low R^2 values must be taken into account when interpreting these regression parameters, the poor fit is caused by generally high variation in the data rather than by a non-linear relationship, therefore it is felt that the parameters still have some merit.

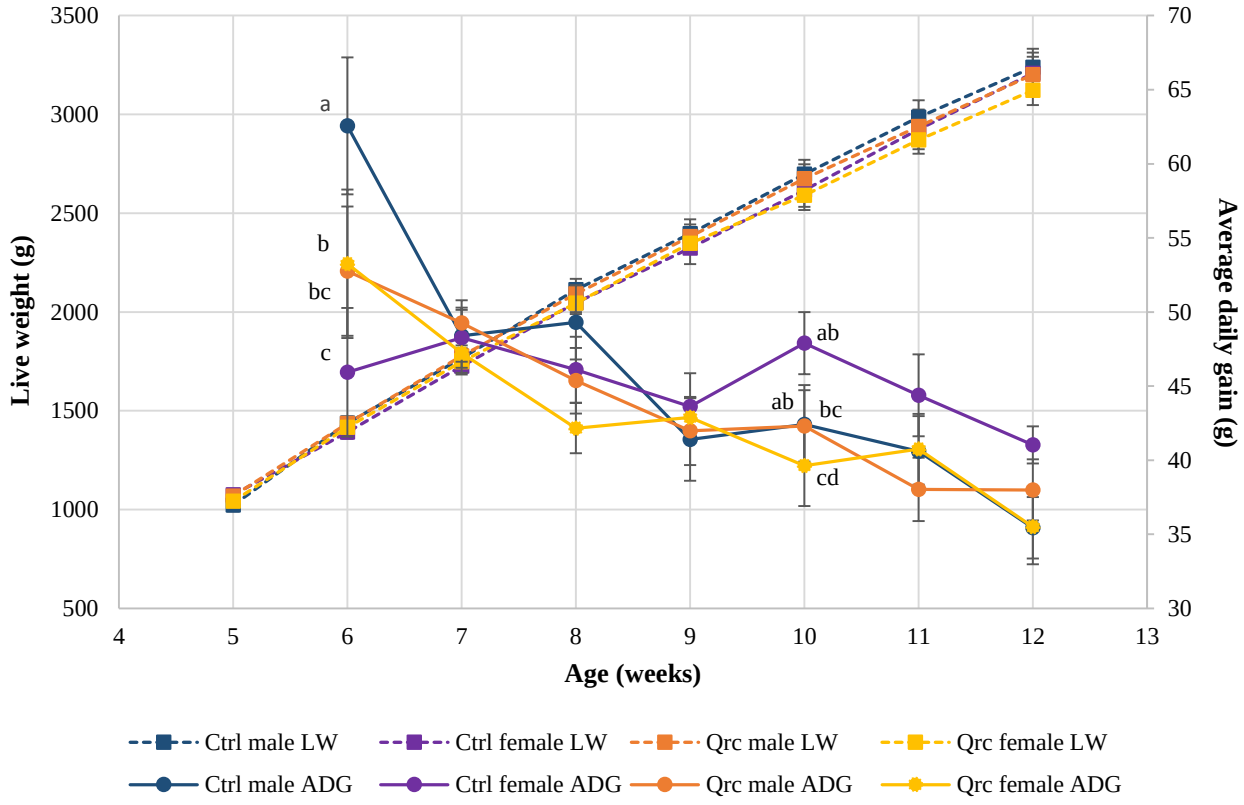

Figure 3.1 The live weight (LW) and average daily gain (ADG) from 5 to 12 weeks of age of male and female New Zealand White rabbits fed diets with (Qrc) or without (Ctrl) quercetin dihydrate supplemented at 2 g/kg. For ease of interpretation, differences ($P \leq 0.05$) between the diet-sex treatment groups are only shown on a weekly basis, as indicated by the significance letters adjacent to the data points. Error bars show the standard errors of the means.

The FI and FCR both increased ($P < 0.001$) with age (Figure 3.2). Similarly to the LW, there were no differences between the groups in the FI, or the changes in FI, over the growth period (Table 3.2). However, there was some tendency for a third-order interaction for the FCR ($P = 0.08$), and the LSD test found that the treatment groups differed at 6, 10 and 12 weeks of age. The variation at 6 weeks was most likely simply a reflection of the differences in ADG, as seen in Figure 3.1, with the high ADG of the Ctrl males resulting in a low FCR, and *vice versa* for the Ctrl females. This was also the case at 10 weeks of age. At 12 weeks, the continued high ADG of the

Ctrl females resulted in a lower FCR for this group, while the Ctrl males and Qrc groups retained similar, higher, FCR values. As was observed for the ADG, the sex-effect on the FCR over the growth period seemed less for the Qrc than for the Ctrl groups, with the slopes and intercepts for the FCR regressions not differing between the sexes in the Qrc group (Figure 3.2, Table 3.2).

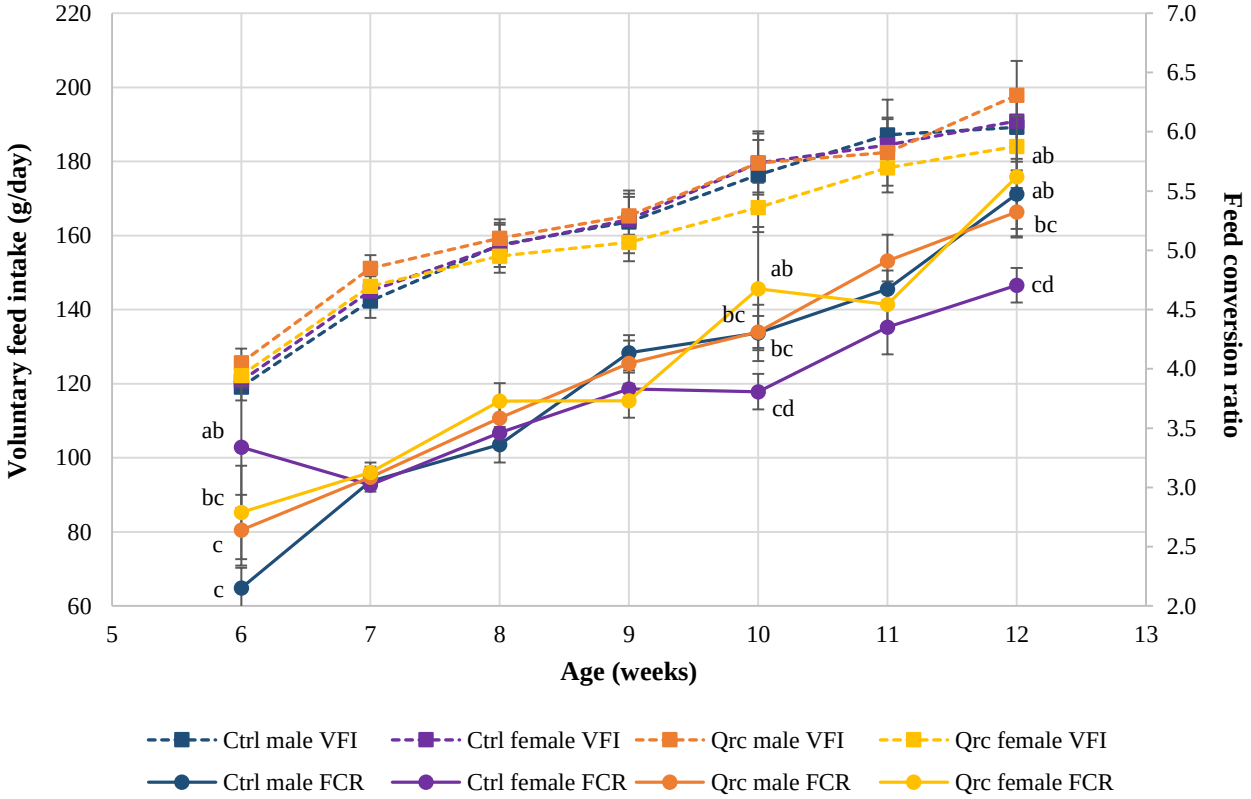

Figure 3.2 The daily feed intake (FI) and feed conversion ratio (FCR) from 6 to 12 weeks of age of male and female New Zealand White rabbits fed diets with (Qrc) or without (Ctrl) quercetin dihydrate supplemented at 2 g/kg. For ease of interpretation, differences ($P \leq 0.05$) between the diet-sex treatment groups are only shown on a weekly basis, as indicated by the significance letters adjacent to the data points. Error bars show the standard errors of the means.

There were no significant diet-sex interactions for the hormone data and the two main effects were consequently examined individually (Table 3.3). Only fT3 levels showed any sign of a dietary influence ($P = 0.06$), with the Qrc rabbits having higher fT3 concentrations in the serum than the Ctrl rabbits. The cortisol concentration showed a large sex-effect ($P < 0.01$), with female rabbits having much higher serum levels than males. None of the hormones correlated significantly with any of the 11 week live-performance data (Table 3.4); however, the Pearson's correlation coefficient between the FI and serum cortisol concentration tended towards significance ($P = 0.08$), suggesting a negative relationship between cortisol and feed intake.

3.4 Discussion

3.4.1 Live performance

The initial comparison of the growth data to values reported for New Zealand Whites in literature suggests that the rabbits in this study performed very well in terms of LW and ADG (Abdel-Samee, 1997; Anous, 1999; Marai, Ayyat, Gabr & Abdel-Monem, 1999; Nasr, Abd-Elhamid & Hussein, 2017; Ondruska *et al*., 2011). However, their FI was similarly high, resulting in FCR values that were comparable to some literature (Marai *et al*., 1999; Ondruska *et al*., 2011) but much poorer than those reported for commercial European farms by Maertens and Gidenne (2016). This may have been due to the wide-spread use of hybrid-line rabbits, rather than New Zealand Whites, in Europe. However, both the high growth rate and relatively high FCR may also have been due to the fairly low temperatures during the growth period, as winter conditions improve growth but also increase the FCR (Maertens & Gidenne, 2016). It may also have been linked to the individual housing conditions, as this has been found to increase growth and feed intake (Szendrő & Dalle Zotte, 2011).

Table 3.2
The intercepts, slopes and coefficients of determination (R^2) of linear regression lines fitted to live weight, average daily gain, daily feed intake and feed conversion ratio data for male and female New Zealand White rabbits fed a diet with (Qrc) or without (Ctrl) supplemented quercetin dihydrate (2 g/kg) from 5 to 12 weeks of age.

		Control (Ctrl)		Quercetin (Qrc)	
		Male	**Female**	**Male**	**Female**
Live weight	N	128	144	120	136
	Intercept	-473.5	-410.4	-367.5	-354.5
	Slope	313.5	304.2	302.8	293.7
	R^2	0.94	0.91	0.95	0.95
Average daily gain*	N	108	124	105	117
	Intercept	80.00^{a}	51.51^{c}	65.97^{ab}	65.75^{b}
	Slope	-3.73^{ab}	-0.74^{a}	-2.49^{b}	-2.51^{b}
	R^2	0.56	0.14	0.46	0.42
Voluntary feed intake	N	108	124	105	117
	Intercept	70.56	62.25	69.01	75.43
	Slope	10.53	11.15	10.69	9.26
	R^2	0.58	0.67	0.63	0.65
Feed conversion ratio	N	108	124	105	117
	Intercept	-0.72^{b}	1.53^{a}	0.01^{ab}	0.06^{ab}
	Slope	0.51^{a}	0.25^{b}	0.44^{a}	0.44^{a}
	R^2	0.81	0.46	0.77	0.55

^{abc} Means in the same row with different superscript letters differ significantly ($P \leq 0.05$)
*ADG calculated for each rabbit weekly by subtracting the previous week's live weight and dividing by seven

While the effects of feed composition on the FCR can certainly not be discounted, the comparison of the nutritional value of the feed used in this study (Table 3.1) to optimum acid detergent fibre (ADF: 16 – 17 %), neutral detergent fibre (NDF: 31 – 33 %) and acid detergent lignin (ADL: > 5 %) values, as well as normally-used

total lipid contents (2 – 4 %) seems to suggest that this was not a major contributing factor (Maertens & Gidenne, 2016). Although the ADF content (17.7 %) was slightly above the recommended optimum level, the NDF content was within range (31.7 %) and the lipid content was also towards the higher end of the normal range (3.71 %). It would, however, be of interest to determine the nutrient digestibility of the feeds used.

The patterns of change in both the ADG and FCR were as expected, with the growth rate declining (as indicated by the negative slope values for the ADG, Table 3.2) and the FCR increasing (positive slope values) as the rabbits matured, the effect on the latter being due to the increase in maintenance requirements and the shift towards fat deposition with age (Maertens & Gidenne, 2016; Pascual, Pla & Blasco, 2008).

As the 6 week ADG represented the growth rate during the first week post-weaning, the differences between the diet-sex treatment groups at this age (Figure 3.1), and thus the intercept values for the ADG linear regressions (Table 3.2), may have been due to variation between the groups in their response to the stress of the weaning process and the adaptation to the new housing conditions. It is uncertain why the Ctrl males and females responded so drastically differently to this. While a new relationship between the treatment groups had been established by 10 weeks of age, with Ctrl females outperforming the other groups, there were no further differences in the weekly ADG up until slaughter. However, the slope values for the ADG regressions did differ, particularly between the Ctrl females and the Qrc rabbits, with the latter showing a greater decline in growth rate with age (Table 3.2).

As one could expect, the FCR values followed a very similar pattern to those of the ADG (Figure 3.2), with Ctrl females having the highest values for week 6 (and thus the highest intercept values, Table 3.2) but the lowest for weeks 10 and 12, resulting in them also having the lowest slope value (Table 3.2). The FCR also showed a tendency of an effect of diet ($P = 0.08$), with Qrc rabbits having a higher overall FCR than the Ctrl rabbits. This is detrimental from a production perspective, and appeared to be more due to a slightly higher ADG in the Ctrl than Qrc rabbits, particularly towards the end of the growth period, as seen by the differences in the regression parameters for the ADG, than any difference in FI. Goliomytis *et al.* (2014) reported a similar negative effect of quercetin supplementation on the FCR of broiler chickens, and attributed this to nutrient dilution by the increasing quercetin content of the diet. Although this seems unlikely considering that their maximum inclusion level was only 1 g/kg feed, it is possible that it provides an explanation for the results of this study, particularly since a higher inclusion rate was used (2 g/kg). It is also possible that the effect on the FCR was as a result of a lower digestibility of the Qrc than Ctrl feed, due to alterations of the composition of the caecal microbial population by the flavonoid. Quercetin is known to exhibit antimicrobial activity, and flavonoids have been found to influence the gut microbiome in human studies (Cushnie & Lamb, 2005; Tzounis *et al*., 2008). Alternatively, flavonoids have been found to reduce the *in vitro* digestibility of proteins and starch in bread through the formation of indigestible complexes with the nutrients, and by inhibiting the activity of several digestive enzymes (Rohn, Rawel & Kroll, 2002; Świeca, Gawlik-Dziki, Dziki, Baraniak & Czyż, 2013).

The apparent reduction of the effect of sex on the ADG and FCR by the supplementation of quercetin was unusual and does not seem to have been reported for quercetin or any other flavonoid in previous livestock trials. However, there is evidence that some flavonoids act as xenoestrogens, interacting with the sex hormone systems

by binding with oestrogen receptors and human plasma sex-hormone binding globulin (hSHBG), which is involved in the transport of steroid hormones in the body (Déchaud, Ravard, Claustrat, de la Perrière & Pugeat, 1999; Erlund, 2004; Kuiper *et al*., 1998). Flavonoids have also been found to influence the activity of several enzymes that are involved in oestrogen, androgen and progestin metabolism (Narayana *et al*., 2001), and naringenin, in particular, has been reported to exhibit some antiestrogenic activity (Déchaud *et al*., 1999; Erlund, 2004; Kuiper *et al*., 1998; Ruh *et al*., 1995). This may provide some explanation for the interaction observed in this trial. In a study on heat-stressed rabbit does, Naseer *et al*. (2017) found that the supplementation of quercetin had a positive effect on the number of ovarian follicles, as well as the quality of oocytes and granulosa cell apoptosis; however, this was ascribed to the antioxidant effects of the quercetin rather than to a direct effect on the sex-hormone system. Considerable further research is necessary to not only confirm the smaller sex-differences in Qrc than Ctrl rabbits found in this study, but also to investigate the possible mechanism of action.

Table 3.3
Concentrations (ng/ml) of free triiodothyronine, free thyroxine, somatotropin and cortisol in serum from 11 week old male and female New Zealand White rabbits fed diets with (Qrc) or without (Ctrl) quercetin dihydrate supplemented at 2 g/kg (LSMean ± SEM).

	Overall	Diet		Sex	
		Ctrl	Qrc	Male	Female
N	36	18	18	17	19
Free triiodothyronine	4.31 ± 0.375	3.62^{β} ± 0.420	4.98^{α} ± 0.599	4.31 ± 0.482	4.29 ± 0.577
Free thyroxine	6.72 ± 0.542	7.29 ± 0.769	6.19 ± 0.765	6.59 ± 0.709	6.88 ± 0.824
Somatotropin	1.45 ± 0.107	1.35 ± 0.147	1.56 ± 0.155	1.57 ± 0.160	1.34 ± 0.143
Cortisol	7.55 ± 1.03	6.13 ± 1.03	8.66 ± 1.76	3.89^{b} ± 0.53	10.90^{a} ± 1.55

^{ab} Main effect means in the same row with different superscript letters differ significantly ($P \leq 0.05$)
$^{\alpha\beta}$ Main effect means in the same row with different superscript Greek letters tend to differ ($P \leq 0.10$)

3.4.2 Serum hormone levels

Somatotropin plays an important role in the endocrine regulation of growth, stimulating the production of somatomedins, which act as both hypertrophic and hyperplastic agents, thereby increasing tissue proliferation (Spencer, 1985). However, somatotropin also exhibits catabolic behaviour, which can complicate the relationship between its serum levels and growth rates. This may explain the lack of effect of dietary treatment or sex on somatotropin levels, as well as the lack of correlation with the ADG.

In contrast with somatotropin, cortisol plays a purely catabolic role in the body, decreasing overall DNA synthesis, suppressing muscle protein synthesis, increasing muscle degradation rates and generally inhibiting growth (Spencer, 1985). In addition, the corticotropin-releasing factor system, which forms part of the hormonal stress response and ultimately stimulates the synthesis of cortisol, inhibits feed intake (Richard, Lin & Timofeeva, 2002; Shibasaki *et al*., 1988). This concurs with the negative Pearson's correlation coefficient found for the relationship between cortisol levels and FI (Table 3.4), which suggested that intake tended to be lower when the serum cortisol level was higher.

The interpretation of serum cortisol levels is always complicated by the natural circadian variation in the secretion of this hormone (Möstl & Palme, 2002); nonetheless, Gunn, Middleton, Davies, Revell and Skene (2016) found that women had consistently higher cortisol levels than men, throughout the circadian cycle, which supports our findings that female rabbits had higher cortisol levels than males (Table 3.3). While this difference does not appear to have been previously reported in rabbits, female rats have been found to have higher baseline corticosterone levels and a number of studies have demonstrated differences in the stress response linked to sex in other species (Green & McCormick, 2016; Kirschbaum, Wüst & Hellhammer, 1992; Tilbrook, Turner & Clarke, 2000). The sex-effect on the hypothalamo-pituitary-adrenal (HPA) axis has been linked to circulating androgen and oestrogen levels, with the former decreasing and the latter increasing the HPA axis function (Green & McCormick, 2016; Handa, Burgess, Kerr & O'Keefe, 1994). It is interesting to note that this sex effect was already present at 11 weeks of age in the rabbits in this study.

Table 3.4
The relationships between serum hormone concentrations and live performance parameters of 11 week old male and female New Zealand White rabbits fed diets with (Qrc) or without (Ctrl) quercetin dihydrate supplemented at 2 g/kg, as indicated by Pearson's and Spearman's correlation coefficients.

		Free triiodothyronine		**Free thyroxine**		**Somatotropin**		**Cortisol**	
		r	***P*-value**	**r**	***P*-value**	**r**	***P*-value**	**r**	***P*-value**
ADG	Pearson	-0.19	0.26	-0.10	0.56	0.04	0.81	-0.25	0.14
	Spearman	-0.19	0.26	-0.15	0.38	0.04	0.80	-0.06	0.72
FI	Pearson	-0.12	0.48	-0.08	0.63	-0.11	0.54	-0.29	0.08
	Spearman	-0.05	0.76	-0.11	0.52	-0.16	0.36	-0.16	0.36
FCR	Pearson	0.14	0.43	0.03	0.86	-0.15	0.38	0.02	0.91
	Spearman	0.20	0.23	0.02	0.90	-0.16	0.35	-0.11	0.53

ADG: average daily gain, FI: daily feed intake, FCR: feed conversion ratio, r: correlation coefficient.

The only effect of diet on the hormone levels was a tendency for Qrc rabbits to have higher concentrations of fT3 than Ctrl rabbits (Table 3.3). While this exact effect does not seem to have been previously found in rabbits, the effects of flavonoids, including quercetin, on thyroid hormone metabolism are widely reported (Narayana et al., 2001). This effect is generally considered to be anti-thyroid, with flavonoids inhibiting iodothyronine deiodinase isozymes, iodide organification and thyroid peroxidase enzyme activity, as well as down-regulating the expression of several genes involved in thyroid function, which makes the higher fT3 in Qrc than Ctrl rabbits seem counterintuitive (Ferreira et al., 2002; Formica & Regelson, 1995; Giuliani et al., 2014). However, flavonoids have also been found to inhibit the binding of T3 and T4 to serum transthyretin, resulting in higher proportions of fT3 and fT4, which could provide an explanation for the findings of this study (Kohrle et al., 1989; Lueprasitsakul et al., 1990; Radović, Mentrup & Köhrle, 2006). While the difference in serum fT3 levels did not appear to cause significant differences in growth, it is possible that it may have been linked in some way to the higher FCR and smaller sex-differences found for Qrc than Ctrl rabbits. Considering the important role that thyroid hormones play

in both growth and feed intake, as well as the complex and dose-dependant catabolic and anabolic effects of these hormones (Spencer, 1985), further investigation of the total and bound levels of T3 and T4 may be warranted.

3.5 Conclusion

From a production perspective, the conclusions of this study are extremely limited. While it is heartening to see the South African New Zealand Whites performing well in terms of growth, improved selection methods and/or the development of hybrid-lines will be necessary in order to reach international standards of feed efficiency. In addition, the quercetin-supplementation did not appear to have any immediately beneficial effects on production performance, and in fact had a detrimental effect on the FCR, possibly by lowering the digestibility of the feed. It therefore does not seem that the provision of quercetin is justified from a commercial perspective, at the live performance level at least. Further research is necessary to identify possible causes for the effects found in this study, and to determine whether quercetin supplementation has any effect on nutrient digestibility *in vivo*.

However, there were some results that may suggest interesting avenues for further investigation. Considering the possible diet effect on sex-differences in growth and feed efficiency, it could be interesting to look at the impact of quercetin on the sex-hormone levels in breeding stock. Further work on the interaction of quercetin with thyroid hormone metabolism in rabbits may also be of interest. In addition, this study has provided some evidence that sex-differences in the hypothalamo-pituitary-adrenal axis previously reported for other species also exist in growing rabbits.

3.6 References

Abdel-Samee, A. M. (1997). Response of New Zealand White rabbits to thermal stress and its amelioration during winter and summer of North Sinai, *Egyption Journal of Arid Environments, 36*, 333 – 342.

Abu, O. A., Onifade, A. A., Abanikannda, O. T. F. & Obiyan, R. I. (2008). Status and promotional strategies for rabbit production in Nigeria. *Proceedings of the 9th World Rabbit Congress — June 10 – 13, 2008, Verona, Italy* (pp. 1499 – 1504).

Anous, M. R. (1999). Growth performance, slaughter and carcass compositional traits in rabbits of a local strain and New Zealand White breed raised in Burundi. *World Rabbit Science, 7*, 139 – 143.

Babosová, R., Omelka, R., Ďúranová, H., Kováčová, V., Lukáčová, M., Capcarová, M. & Martiniaková, M. (2016). Quercetin-induced changes in femoral bone microstructure of adult male rabbits. *Potravinarstvo Scientific Journal for Food Industry, 10*, 300 – 307.

Blouin, F. A., Zarins, Z. M. & Cherry, J. P. (1981). Role of flavonoids in the production of color in biscuits prepared with wheat and cottonseed flours. *Journal of Food Science, 46*, 266 – 271.

Cartea, M. E., Francisco, M., Soengas, P. & Velasco, P. (2011). Phenolic compounds in Brassica vegetables. *Molecules, 16*, 251 – 280.

Cushnie, T. P. T. & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents, 26*, 343 – 356.

Dakora, F. D. (1995). Plant flavonoids: Biological molecules for useful exploitation. *Australian Journal of Plant Physiology, 22*, 87 – 99.

Dalle Zotte, A. & Szendrő, Zs. (2011). The role of rabbit meat as functional food. *Meat Science, 88*, 319 – 331.

Déchaud, H., Ravard, C., Claustrat, F., de la Perrière, A. B. & Pugeat, M. (1999). Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG). *Steroids, 64*, 328 – 334.

Dos Santos, M. C. S., Gonçalves, C. F. L., Vaisman, M., Ferreira, A. C. F. & de Carvalho, D. P. (2011). Impact of flavonoids on thyroid function. *Food and Chemical Toxicology, 49*, 2495 – 2502.

Erlund, I. (2004). Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research, 24*, 851 – 874.

European Commission (2005). *Ban on antibiotics as growth promoters in animal feed enters into effect.* Brussels, Belgium: European Commission, Available at: http://europa.eu/rapid/press-release_IP-05-1687_en.htm.

Ferreira, A. C. F., Lisboa, P. C., Oliveira, K. J., Lima, L. P., Barros, I. A. & Carvalho, D. P. (2002). Inhibition of thyroid type 1 deiodinase activity by flavonoids. *Food and Chemical Toxicology, 40*, 913 – 917.

Finzi, A. (2000). Raising rabbits for food security. *Proceedings of the 7th World Rabbit Congress — July 4 – 7, 2000, Valencia, Spain, Volume B.* (pp. 13 – 38).

Food and Drug Administration, Centre for Veterinary Medicine (2013). Guidance for Industry #213: *New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209.* Washington, D.C., USA: U.S. Department of Health and Human Services.

Formica, J. V. & Regelson, W. (1995). Review of the biology of quercetin and related bioflavonoids. *Food and Chemical Toxicology, 33*, 1061 – 1080.

Giuliani, C., Bucci, I., Di Santo, S., Rossi, C., Grassadonia, A., Piantelli, M., Monaco, F. & Napolitano, G. (2014). The flavonoid quercetin inhibits thyroid-restricted genes expression and thyroid function. *Food and Chemical Toxicology, 66*, 23 – 29.

Goliomytis, M., Tsoureki, D., Simitzis, P. E., Charismiadou, M. A., Hager-Theodorides, A. L. & Deligeorgis, S. G. (2014). The effects of quercetin dietary supplementation on broiler growth performance, meat quality, and oxidative stability. *Poultry Science, 93*, 1957 – 1962.

Green, M. R. & McCormick, C. M. (2016). Sex and stress steroids in adolescence: Gonadal regulation of the hypothalamic-pituitary-adrenal axis in the rat. *General and Comparative Endocrinology, 234*, 110 – 116.

Gunn, P.J., Middleton, B., Davies, S.K., Revell, V.L. & Skene, D.J. (2016). Sex differences in the circadian profiles of melatonin and cortisol in plasma and urine matrices under constant routine conditions. *Chronobiology International, 33*, 39 – 50.

Handa, R. J., Burgess, L. H., Kerr, J. E. & O'Keefe, J. A. (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Hormones and Behavior, 28*, 464 – 476.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67 – 202.

Herald, T. J., Gadgil, P. & Tilley, M. (2012). High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. *Journal of the Science of Food and Agriculture, 92*, 2326 – 2331.

Johnson, R. (2010). *Potential trade implications of restrictions on antimicrobial use in animal production*. Washington D.C., USA: Congressional Research Service.

Karamać, M., Kosińska, A., Estrella, I., Hernández, T. & Duenas, M. (2012). Antioxidant activity of phenolic compounds identified in sunflower seeds. *European Food Research and Technology, 235*, 221 – 230.

Kirschbaum, C., Wüst, S. & Hellhammer, D. (1992). Consistent sex differences in cortisol responses to psychological stress. *Psychosomatic Medicine, 54*, 648 – 657.

Kohrle, J., Fang, S. L., Yang, Y., Irmscher, K., Hesch, R. D., Pino, S., Alex, S. & Braverman, L. E. (1989). Rapid effects of the flavonoid EMD 21388 on serum thyroid hormone binding and thyrotropin regulation in the rat. *Endocrinology, 125*(1), 532 – 537.

Kováčik, A., Packová, D. & Kolesárová, A. (2015). Impact of the T-2 toxin and quercetin on rabbit plasma levels of thyrotropin and thyroxine in vivo. *Journal of Microbiology, Biotechnology and Food Sciences, 4*, 57 – 59.

Kuiper, G. G. J. M., Lemmen, J. G., Carlsson, B. O., Corton, J. C., Safe, S.H., van der Saag, P.T., van der Burg, B. & Gustafsson, J-A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology, 139*, 4252 – 4263.

Landers, T. F., Cohen, B., Wittum, T. E. & Larson, E. L. (2012). A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Reports, 127*, 4 – 21.

Liu, H. N., Liu, Y., Hu, L. L., Suo, Y. L., Zhang, L., Jin, F., Feng, X. A., Teng, N. & Li, Y. (2014). Effects of dietary supplementation of quercetin on performance, egg quality, cecal microflora populations, and antioxidant status in laying hens. *Poultry Science, 93*, 347 – 353.

Lu, Y. & Foo, L. Y. (1997). Identification and quantification of major polyphenols in apple pomace. *Food Chemistry, 59*(2), 187 – 194.

Lueprasitsakul, W., Alex, S., Fang, S. L., Pino, S., Irmscher, K., Kohrle, J., & Braverman, L. E. (1990). Flavonoid administration immediately displaces thyroxine (T4) from serum transthyretin, increases serum free T4, and decreases serum thyrotropin in the rat. *Endocrinology, 126*(6), 2890 – 2895.

Maertens, L. & Gidenne, T. (2016). Feed efficiency in rabbit production: Nutritional, techno-economical and environmental aspects. *Proceedings of the 11th World Rabbit Congress — June 15 – 18, 2016, Qingdao, China*, (pp. 337 – 351).

Marai, I. F. M., Ayyat, M. S., Gabr, H. A. & Abdel-Monem, U. M. (1999). Growth performance, some metabolites and carcass traits of New Zealand white broiler male rabbits as affected by heat stress and its alleviation, under Egyptian conditions. In A. Testik and M. Baselga (Ed.), *2nd International Conference on Rabbit Production in Hot Climates — Zaragoza, Spain*: CIHEAM, (pp. 35 – 42).

Möstl, E. & Palme, R. (2002). Hormones as indicators of stress. *Domestic Animal Endocrinology, 23*, 67 – 74.

Narayana, K. R., Reddy, M. S., Chaluvadi, M. R. & Krishna, D. R. (2001). Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Indian Journal of Pharmacology, 33*, 2 – 16.

Naseer, Z., Ahmad, E., Epikmen, E. T., Uçan, U., Boyacioğlu, M. & Akosy, M. (2017). Quercetin supplemented diet improves follicular development, oocyte quality, and reduces ovarian apoptosis in rabbits during summer heat stress. *Theriogenology, 96*, 136 – 141.

Nasr, M. A. F., Abd-Elhamid, T. & Hussein, M. A. (2017). Growth performance, carcass characteristics, meat quality and muscle amino-acid profile of different rabbits breeds and their crosses. *Meat Science, 134*, 150 – 157.

Needham, T., Lambrechts, H. & Hoffman, L. (in press). Influence of Immunocastration vaccine administration interval on serum androgen concentrations and spermatogenic activity in ram lambs. *Small Ruminant Research*. Retrieved from https://doi.org/10.1016/j.smallrumres.2018.11.016.

Onderci, M., Sahin, K., Sahin, N., Gursu, M. F., Doerge, D., Sarkar, F. H. & Kucuk, O. (2004). The effect of genistein supplementation on performance and antioxidant status of Japanese quail under heat stress. *Archives of Animal Nutrition, 58*(6), 463 – 471.

Ondruska, L., Rafay, J., Okab, A. B., Ayoub, M. A., Al-Haidary, A. A., Samara, E. M., Parkanyi, V., Chrastinova, L., Jurcik, R., Massanyi, P., Lukac, N. & Supuka, P. (2011). Influence of elevated ambient temperature upon some physiological measurements of New Zealand White rabbits. *Veterinarni Medicina, 56*, 180 – 186.

Oseni, S. O. (2012). Rabbit production in low-input systems in Africa — prospects, challenges and opportunities. *Proceedings of the 10th World Rabbit Congress — September 3 – 6, 2012, Sharm El-Sheikh, Egypt*, (pp. 719 – 731).

Park, S.-Y., Lee, S. Y., Yang, J. W., Lee, J.-S., Oh, S.-D., Oh, S., Lee, S. M., Lim, M-H., Park, S. K., Jang, J-S., Cho, H. S. & Yeo, Y. (2016). Comparative analysis of phytochemicals and polar metabolites from colored sweet potato (*Ipomoea batatas* L.) tubers. *Food Science and Biotechnology, 25*(1), 283 – 291.

Pascual, M., Pla, M. & Blasco, A. (2008). Relative growth of organs, tissues and retail cuts in rabbits selected for growth rate. *Proceedings of the 9th World Rabbit Congress — June 10 – 13, 2008, Verona, Italy*, (pp. 211 – 216).

Quanson, J. L., Stander, M. A., Pretorius, E., Jenkinson, C., Taylor, A. E. & Storbeck, K-H. (2016). High-throughput analysis of 19 endogenous androgenic steroids by ultra-performance convergence chromatography tandem mass spectrometry. *Journal of Chromatography B, 1031*, 131 – 138.

Radović, B., Mentrup, B. & Köhrle, J. (2006). Genistein and other soya isoflavones are potent ligands for transthyretin in serum and cerebrospinal fluid. *British Journal of Nutrition, 95*, 1171 – 1176.

Reynaud, J. & Lussignol, M. (2005). The flavonoids of *Lotus corniculatus*. *Lotus Newsletter, 35*(1), 75 – 82.

Richard, D., Lin, Q. & Timofeeva, E. (2002). The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. *European Journal of Pharmacology, 440*, 189 – 197.

Rohn, S., Rawel, H. M. & Kroll, J. (2002). Inhibitory effects of plant phenols on the activity of selected enzymes. *Journal of Agricultural and Food Chemistry, 50*, 3566 – 3571.

Ruh, M. F., Zacharewski, T., Connor, K., Howell, J., Chen, I. & Safe, S. (1995). Naringenin: a weakly estrogenic bioflavonoid that exhibits antiestrogenic activity. *Biochemical Pharmacology, 50*, 1485 – 1493.

Schittko, U., Burghardt, F., Fiedler, K., Wray, V. & Proksch, P. (1999). Sequestration and distribution of flavonoids in the common blue butterfly *Polyommatus icarus* reared on *Trifolium repens*. *Phytochemistry, 51*, 609 – 614.

Serratosa, J., Blass, A., Rigau, B., Mongrell, B., Rigau, T., Tortadès, M., Tolosa, E., Aguilar, C., Ribó, O. & Balagué, J. (2006). Residues from veterinary medicinal products, growth promoters and performance enhancers in food-producing animals: a European Union perspective. *Revue Scientifique et Technique (International Office of Epizootics), 25*(2), 637 – 653.

Shibasaki, T., Yamauchi, N., Kato, Y., Masuda, A., Imaki, T., Hotta, M., Demura, H., Oono, H., Ling, N. & Shizume, K. (1988). Involvement of corticotropin-releasing factor in restraint stress-induced anorexia and reversion of the anorexia by somatostatin in the rat. *Life Science, 43*, 1103 – 1110.

Spencer, G. S. G. (1985). Hormonal systems regulating growth. A review. *Livestock Production Science, 12*, 31 – 46.

Świeca, M., Gawlik-Dziki, U., Dziki, D., Baraniak, B. & Czyż, J. (2013). The influence of protein-flavonoid interactions on protein digestibility in vitro and the antioxidant quality of breads enriched with onion skin. *Food Chemistry, 141*, 451 – 458.

Szendrő, Zs. & Dalle Zotte, A. (2011). Effect of housing conditions on production and behaviour of growing meat rabbits: A review. *Livestock Science, 137*, 296 – 303.

Tapas, A. R., Sakarkar, D. M. & Kakde, R. B. (2008). Flavonoids as neutraceuticals: A review. *Tropical Journal of Pharmaceutical Research, 7*, 1089 – 1099.

Thill, J., Regos, I., Farag, M. A., Ahmad, A. F., Kusek, J., Castro, A., Schlangen, K., Carbonero, C. H., Gadjev, I. Z., Smith, L. M. J., Halbwirth, H., Treutter, D. & Stich, K. (2012). Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin). *Phytochemistry, 82*, 67 – 80.

Tilbrook, A. J., Turner, A. I. & Clarke, I. J. (2000). Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of Reproduction, 5*, 105 – 113.

Tuzcu, M., Sahin, N., Karatepe, M., Cikim, G., Kilinc, U. & Sahin, K. (2008). Epigallocatechin-3-gallate supplementation can improve antioxidant status in stressed quail. *British Poultry Science, 49*(5), 643 – 648.

Tzounis, X., Vulevic, J., Kuhnle, G. G. C., George, T., Leonczak, J., Gibson, G. R., Kwik-Uribe, C. & Spencer, J. P. E. (2008). Flavanol monomer-induced changes to the human faecal microflora. *British Journal of Nutrition, 99*, 782 – 92.

Verbeke, W. A. J. & Viaene, J. (2000). Ethical challenges for livestock production: Meeting consumer concerns about meat safety and animal welfare. *Journal of Agricultural and Environmental Ethics, 12*, 141 – 151.

CHAPTER 4:

The rabbit caecal microbiome: composition, correlations and the effects of dietary quercetin supplementation and sex thereupon

Abstract

The purpose of this study was to add to the current understanding of the rabbit caecal microbiome. This involved describing its microbial composition and linking this to live performance parameters, as well as determining the effects of dietary quercetin supplementation (2 g/kg feed) and sex on the microbial population. The weight gain and feed conversion ratio of twelve New Zealand White rabbits was measured from 5 to 12 weeks old, blood was sampled at 11 weeks old for the determination of serum hormone levels, and the rabbits were slaughtered and caecal samples collected at 13 weeks old. 16S^{TM} metagenome sequencing was used to determine the microbiome profile. The dominance of the phyla *Firmicutes*, *Lachnospiraceae* and *Ruminococcaceae* concurred with previous reports, but variation both between studies and individual rabbits was apparent beyond this. Extensive correlations between microbial families and live performance parameters were found, suggesting that further research into the mechanisms of these associations would be useful. Negative correlations with the caecal flavonoid content were found, but the latter was not effected by diet, and the effects of quercetin-supplementation on the microbiome were very limited, possibly due to the absorption of the quercetin aglycone from the gastrointestinal tract prior to the caecum. Nonetheless, a number of families from the phylum *Firmicutes* were more abundant in the quercetin-supplemented rabbits, as was the genus *Anaerofustis*. Several microbial families differed between the sexes, with most being more abundant in female rabbits.

4.1 Introduction

Rabbits, like most herbivores, rely heavily on their commensal gut microbiota for assistance in the digestion of fibrous plant material and thus the supply of important nutrients (Combes, Fortun-Lamothe, Cauquil & Gidenne, 2013). The majority of microbial fermentation in the rabbit takes place in the caecum, with short-chain fatty acid production through caecal fermentation being postulated to contribute 30 – 50 % of maintenance energy requirements (Combes *et al*., 2013). In addition, rabbits practice caecotrophy, which is the consumption of specifically produced soft faeces, allowing them to also take advantage of bacterial proteins, which can contribute 15 % or more of total ingested nitrogen in adults (Combes *et al*., 2013). The caecal bacterial population is also an essential source of vitamins, particularly biotin, thiamine, cobalamin, riboflavin, folic acid and niacin; in some cases producing sufficient quantities to meet all the needs of the host (McBee, 1971).

The complexity and stability of the gut microbial ecosystem is also an important factor in the occurrence of disease, as it serves as a protective barrier (colonization resistance) and plays a role in the development of the immune system and intestinal mucosa (Combes *et al*., 2013). This aspect of host-bacteria interaction is of particular interest in rabbits, for which post-weaning mortalities due to digestive disturbances are very high (Michelland *et al*., 2010). The mechanism of colonization resistance is uncertain; however, it has been speculated to involve direct competition for mucosal attachment sites or nutrients, or the production of antimicrobial agents by the commensal bacteria. Studies have also shown that the gut microbial population is important for cell proliferation, the development of the gut-associated lymphoid tissue, the vascularization of the intestinal villi, the development of antigen tolerance mechanisms and the expansion of the antibody repertoire (Combes *et al*., 2013).

From the perspective of meat quality, the health and nature of the gut microbial community may have an effect on the fatty acid composition of rabbit meat, although this would likely be to a lesser extent than is found in ruminants. Leiber *et al*. (2008) found that the fatty acid profile of the caecotrophes showed distinctive signs of biohydrogenation, although this had little to no effect on the nature of the lipids deposited in the tissue.

Nevertheless, it is important that the nature of the caecal microbiome is understood, and that the effects of any dietary manipulation on the gut microbiome are elucidated, particularly in the case of substances such as flavonoids. Flavonoids have been found to demonstrate antimicrobial activity in a number of studies (Cushnie & Lamb, 2005; Lee, Jenner, Low & Lee, 2006), as well as being reported to alter the gut microbial profile (Oteiza, Fraga, Mills & Taft, 2018). Quercetin, specifically, has been found to inhibit the growth of *Ruminococcus gauvreauii*, *Bacteroides galacturonicus* and *Lactobacillus* species (Duda-Chodak, 2012), while apple flavonoids altered the gut microbiota of mice, increasing the proportions of *Bifidobacterium* and decreasing the prevalence of *Lactobacillus* (Espley *et al*., 2014). Furthermore, this interaction is bidirectional, as the gut microbiota can play a large role in the action of the flavonoid in the body, by metabolising flavonoids and thereby altering their absorption and even their biological effects (Lin, Hsui, Hou, Chen & Chao, 2003; Oteiza *et al*., 2018).

The purpose of this study was therefore to describe the composition of the rabbit caecal microbiome and link this composition to various performance parameters, as well as to investigate the possible effects of dietary quercetin supplementation and sex on the microbial population.

4.2 Materials and methods

Ethical clearance for this study was obtained from the Stellenbosch University Animal Care and Use Committee (protocol number SU-ACUD16-00094).

4.2.1 Rearing and sampling

The rabbits were reared on Mariendahl Experimental Farm outside Stellenbosch in the Western Cape of South Africa (33°51'02.9"S 18°49'35.2"E). Twelve New Zealand White rabbits were used, with three males and three females per dietary treatment group. At 5 weeks old they were weaned and transferred into individual cages, and were assigned to treatment groups according to sex and weaning weight, such that the average weights of the rabbits in each treatment group were comparable. The dietary treatments were applied from weaning, and consisted of two complete, pelleted feeds, one control (Ctrl) and one supplemented with 2 g quercetin dihydrate/kg feed (Qrc). The control diet contained predominantly alfalfa meal (36.2 %), wheat bran (35.6 %) and sunflower meal (12.6 %), with 88.3 % dry matter (DM), 19.0 % crude fibre, 18.5 % crude protein, 8.3 % ash and 3.7 % ether extract. The quercetin dihydrate was extracted from *Sophorae japonica* flowers (Chengdu Okay Plant and Chemical Co., Ltd, Chengdu, China), and was added to the feed during mixing and prior to pelleting.

During the growth period, the live weights (LW) and daily feed intakes (FI) were determined weekly, and the feed conversion ratios (FCR) were calculated. At 11 weeks old blood samples were collected from the rabbits via the central ear artery, and the free thyroxine (fT4), free triiodothyronine (fT3), somatotropin (GH) and cortisol contents of the serum were determined, as described in Chapter 3.

At 13 weeks old the rabbits were slaughtered, without prior fasting, at an abattoir on the experimental farm. They were electrically stunned and then exsanguinated via the carotid arteries and jugular veins, and the gastrointestinal tracts were removed and collected. Samples of the caecal content for microbial DNA extraction were collected in a sterile manner from an area approximately 3 cm from the ileocaecal junction. After collecting the samples for microbiome analysis, additional samples were collected for the determination of the total flavonoid content, and the pH of the caecal content was measured using a calibrated Crison PH25 portable pH meter with a 50 54 electrode (Crison Instruments S.A., Barcelona, Spain).

4.2.2 Total flavonoid content determination

Caecal content samples were lyophilised (CHRIST, model ALPHA 1-4/LDC-1M) for a minimum of 72 hours, until a constant pressure of 0.037 – 0.04 mbar was attained, indicating complete drying. They were then ground using a mortar and pestle prior to flavonoid extraction.

Extraction was performed according to the method of Vasantha Rupasinghe, Wang, Huber and Pitts (2008), with modifications. Briefly, 0.4 g dry sample was combined with 10 ml 1 % formic acid in 50 % methanol, and

was sonicated (Power Sonic 405, United Scientific, Cape Town, South Africa) for two 15 minute periods with a 10 minute interval. The suspension was subsequently centrifuged (Sigma 2-16 K, Wirsam scientific, Cape Town, SA) at 2000 × g for 10 minutes, and the supernatant was filtered using a PES 0.22 µm syringe filter. Extracts were stored at -20 °C until analysis.

The colorimetric assay was performed as described by Herald, Gadgil and Tilley (2012), with adaptations. Briefly, 25 µl of extract was combined with 100 µl distilled water and 10 µl 5 % sodium nitrite in a 2 ml microcentrifuge tube and vortexed. After incubating at room temperature for 5 minutes, 15 µl 10 % aluminium chloride was added, and the microtubes were vortexed and incubated for a further 6 minutes at room temperature. Thereafter, 50 µl distilled water and 50 µl 1 M sodium hydroxide were added, and the tubes were vortexed and centrifuged at 3220 × g for 5 minutes. A 200 µl aliquot of the supernatant was transferred to a clear 96 well microplate (Greiner Cellstar 96 well flatbottom plate, Sigma-Aldrich, St Louis, USA) and the absorbance at 510 nm was measured (Spectrostar Nano, BMG Labtech, Ortenberg, Germany). The total flavonoid content was quantified using a quercetin (Sigma-Aldrich, Steinheim, Germany) standard (25 – 500 µg/ml, $R^2 = 0.997$), and is expressed as mg quercetin equivalents/g DM.

4.2.3 16S™ metagenome sequencing

Microbial DNA was extracted from fresh caecal content samples using the PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific) according to the manufacturer's protocol (MAN0014266 Rev. A.0). Briefly, *ca*. 100 mg of each sample was homogenized with 700 µl S1 Lysis buffer in a bead tube. The DNA was bound to the column and washed, and subsequently eluted in 100 µl buffer using two sequential 50 µl elution steps.

The total DNA contents of the extracts were quantified with the Qubit 2.0 Fluorometer, using the Qubit dsDNA HS assay kit according to the protocol (MAN0002326 REVA.0). To confirm the presence of bacterial gDNA in the sample material, the Femto™ Bacterial DNA Quantification Kit was used according to the manufacturer's protocol (Ver. 1.0.0). The bacterial DNA quantity and quality was considered sufficient for library construction.

The Ion 16S™ Metagenomics Kit was used to amplify hypervariable regions from the polybacterial samples according to the protocol (MAN0010799 REV C.0). Two primer sets were used, one which amplified hypervariable regions V2, V4 and V8, and the other V3, V6, V7 and V9. Target regions were amplified from 2 µl gDNA across 25 cycles on the SimplyAmp Thermal Cycler (ThermoFisher Scientific). The presence of amplified products was verified on the PerkinElmer LabChip GXII Touch using the DNA NGS 3K LabChip and Reagent Kit according to the protocol (CLS145099 Rev. D). Following verification, PCR products were pooled and purified with Agencourt™ AMPure™ XP reagent and eluted in 15 µl nuclease-free water. Purified amplicons were quantified with the Qubit 2.0 Fluorometer using the Qubit dsDNA HS assay kit.

Library preparation was performed from 50 ng pooled amplicons for each sample using the Ion Plus Fragment Library Kit according to the protocol (MAN0006846, REV B.0). Briefly, amplified fragments were end-repaired in preparation for blunt-end ligation to IonCode™ Barcode Adapters. The adapter-ligated, barcoded library was

purified with Agencourt™ AMPure™ XP reagent and subsequently quantified using the Ion Universal Library Quantitation Kit. The StepOnePlus™ Real-time PCR system was used to perform the qPCR amplification.

Libraries were diluted to a target concentration of 10 pM, and the 16S libraries were combined in equimolar amounts for template preparation, which was performed using the Ion 520™ and Ion 530™ Chef Kit. In brief, 25 µl of the pooled library was loaded on the Ion Chef liquid handler using reagents, solutions and supplies according to the protocol (MAN0010846, REVD.0). Enriched, template positive ion sphere particles were loaded onto an Ion 530™ Chip.

Massively parallel sequencing was performed on the Ion S5™ System using the Ion S5™ sequencing solutions and sequencing reagent kits according to the protocol (MAN0010846, REVD.0). Flow space calibration and basecaller analyses were performed using default analysis parameters in the Torrent Suite Version 5.6.0 Software. Run data was uploaded to an IonReporter™ cloud account for mapping and annotation of the identified sequences, with the Basic Local Alignment Search Tool (BLAST) being used to align sequences to the curated MicroSEQ® 16S v2013.1 and Greengenes v13.5 reference libraries. Reads with fewer than 10 copies were ignored. Alpha and beta diversity analysis was also performed using the QIIME 1.9.1 open-source bioinformatics pipeline, through IonReporter™. The bacterial composition (per operational taxonomic unit, OTU) is reported as the percentage of the total mapped reads (for all primers) per sample.

4.2.4 Statistical analysis

Data was analysed using Statistica version 13 software, with normality being tested using normal probability plots, and homoscedasticity using Levene's test. Univariate tests of significance for the main effects (diet and sex) and their interaction, were performed using the general linear model procedure, with Fisher's least significant difference (LSD) test being performed to compare the individual diet-sex treatment groups. In the event that the Levene's test indicated that the variances of the treatment groups were not homogenous ($P \leq 0.05$), the Games-Howell *post hoc* test was used to compare the means. Pearson's correlation coefficients were calculated to measure the strength and direction of any relationships between the bacterial groups at each phylogenetic level, as well as between the bacterial families and the total LW gain from 5 to 12 weeks old, the average FCR from 5 to 12 weeks, the serum hormone levels and the caecal conditions (flavonoid content and pH).

Main effects and interactions with $P \leq 0.05$ were considered significant, whereas those with $P \leq 0.10$ are reported as trends. Values are reported as LSMeans ± standard error of the mean (SEM).

4.3 Results

The live performance data and serum hormone levels are discussed fully in Chapter 3; in summary, the LW gain did not differ between the sexes or dietary treatment groups, with an average of 2039 ± 103 g being gained from 5 to 12 weeks of age. The FCR similarly did not differ between the sexes, but tended to be higher ($P = 0.082$) for the Qrc rabbits. The serum fT3 content also tended to be higher in Qrc rabbits ($P = 0.063$), and the cortisol content was higher in females than males ($P < 0.001$), but no differences were found for the free fT4 or GH content. The

average hormone levels for fT3, fT4, GH and cortisol were 4.3 ± 0.37 ng/ml, 6.7 ± 0.54 ng/ml, 1.5 ± 0.10 ng/ml and 7.6 ± 1.03 ng/ml, respectively.

Neither the caecal pH nor total flavonoid content differed between the diets or sexes, with an overall average pH of 5.59 and an average flavonoid content of 3.90 mg quercetin equivalent/g DM being found.

For the metagenome sequencing, there was an average of 625783 ± 119957 total reads for the caecal microbial DNA samples, with 399460 ± 76621 valid reads and 162266 ± 36149 mapped reads per sample. The alpha diversity plots (Figure 4.1) provide a measure of the level of microbial diversity within each sample at the family level, as well as an indication of whether the samples were sequenced to a sufficient depth to be saturated. It is clear from Figure 4.1A and B that the sequencing depth used was more than sufficient, as indicated by the plateau in the rarefaction measures. This shows that the sequencing was appropriate for the complexity of the genetic library in the samples tested.

Figure 4.1 also demonstrates a lack of clear differences in diversity at the family level between the treatment groups; however, there was some tendency for females to have higher numbers of unique OTUs than males. This was also indicated by the statistical comparison of the rarefaction measures for males and females at 103962 sequences per sample, which tended to show higher values for female rabbits ($P = 0.057$). However, this sex effect was not apparent in the alpha diversity plots produced at the genus and species levels (not shown). The Chao1 rarefaction measure (Figure 4.1B), which attempts to estimate the true population diversity from the diversity of the sample sequenced, showed the same trends as were seen in the observed OTUs plot. Apart from the somewhat higher diversity of samples from females, Figure 4.1 also indicated that one sample, from a female rabbit in the Qrc group (Qrc female 16), had considerably higher levels of microbial diversity than the other samples, with a larger number of OTUs being identified. However, the percentage of mapped reads contributed by the dominant phyla were consequently relatively low for this sample (Figure 4.3).

The Bray-Curtis beta diversity at the family and species level, as visualised using principle coordinate plots (Figure 4.2), demonstrated the differences in the composition of the microbiome between the different samples, in other words the diversity across the samples. In accordance with the alpha diversity results, no clear clustering and separation of the treatment groups was seen at the species level, suggesting that the overall diversity was more due to differences between individual samples than differences between treatment groups. However, some separation between the dietary treatment groups according to PC1 at the family level could be seen. In addition, two samples from Qrc rabbits, one male (QM37) and one female (QF16), appeared to have very different microbiome compositions at the family-level to the other samples, with the latter concurring with the higher diversity seen in Figure 4.1.

The phylum-level composition of the caecal microbiome is shown in Figure 4.3. As can be seen, the dominant group in all the samples was *Firmicutes*, making up an average of 72 % of the total mapped reads, followed by *Proteobacteria* (11 %), *Bacteroidetes* (9.8 %) and *Actinobacteria* (3.7 %). A strong negative correlation ($r = -0.77$, $P < 0.01$) also indicated that samples with a greater abundance of *Bacteroidetes* tended to have fewer *Firmicutes*.

A higher abundance of *Bacteroidetes* also tended to associate with a lower abundance of *Tenericutes* (r = -0.59, *P* = 0.043) and *Verrucomicrobia* (r = -0.64, *P* = 0.024), whereas the latter was more abundant with higher *Firmicutes* abundance (r = 0.80, *P* < 0.01). While some of the phyla were only present at very low amounts, but were detected in all the samples, such as *Spirochaetes* (0.08 %) and *Synergistetes* (0.13 %), *Deinococcus-Thermus* (0.21 %) was only present in a few of the samples. *Nitrospinae* was in fact only detected in a single sample (0.01 % in a Qrc doe, QF60), and was consequently not included in Figure 4.3.

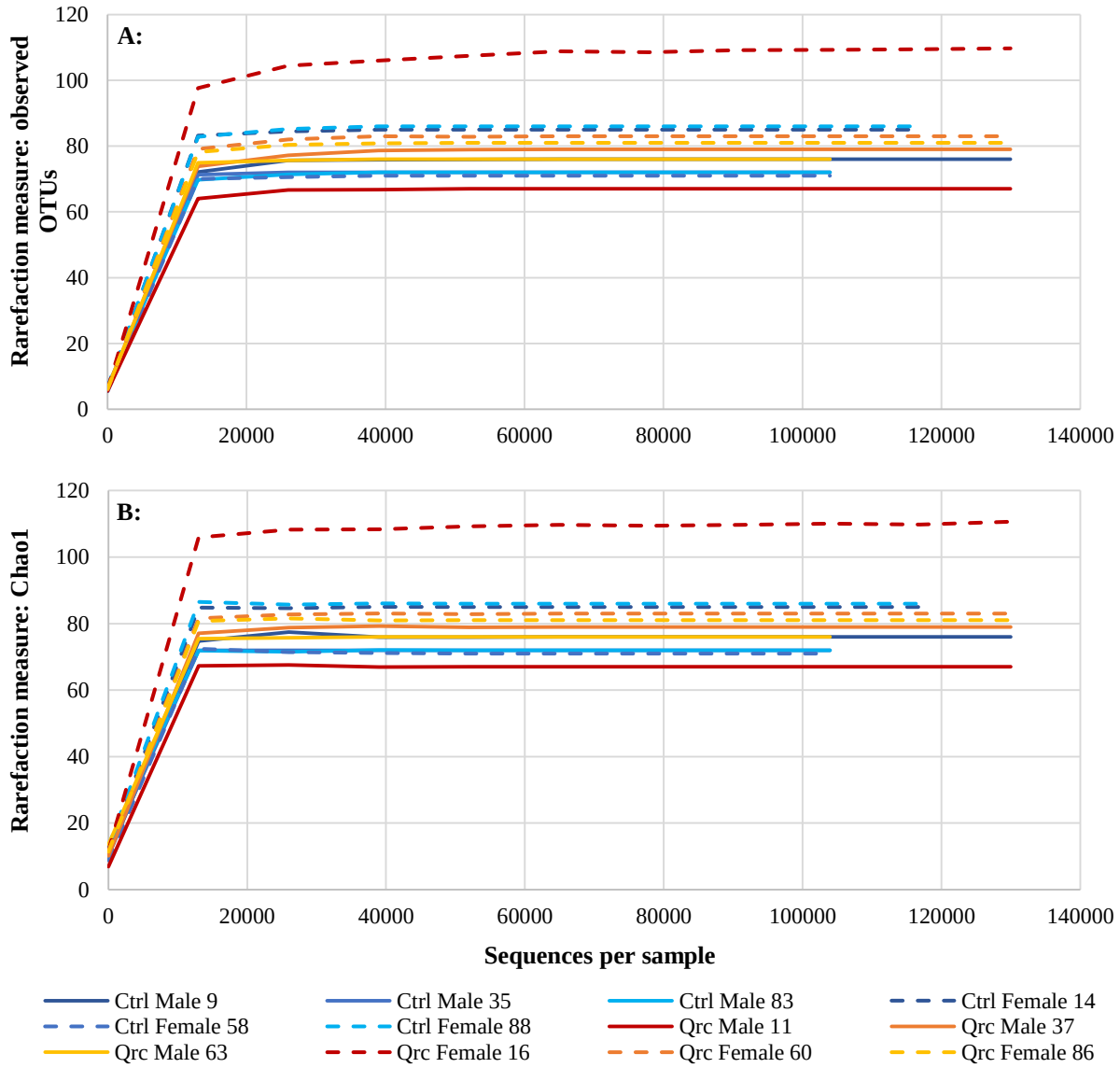

Figure 4.1 Alpha diversity at the family level of the caecal microbiome of male and female New Zealand White grower rabbits, fed either a control (Ctrl) or quercetin-supplemented (Qrc, 2 g/kg feed) diet: Rarefaction curves of average (A) observed operational taxonomic units (OTUs) at 97 % similarity, and (B) Chao1 values for each treatment group.

A similar pattern was observed at the family level (Figure 4.4), with families falling within the *Firmicutes* phylum (indicated in shades of green) dominating, particularly *Lachnospiraceae* (24 %), *Ruminococcaceae* (20 %) and *Clostridiaceae* (10 %), with these also all falling within the order *Clostridiales* (indicated in dotted shades of

green), which was the most prevalent order (62 %). The remaining prominent families were *Desulfovibrionaceae* (7.6 %, phylum *Proteobacteria*), *Eubacteriaceae* (3.8 %), and *Bacteroidaceae* (4.2 %) and *Porphyromonadaceae* (4.1 %, both phylum *Bacteroidetes*, shades of blue). The latter two both also belong to the order *Bacteroidales* (9.1 %, indicated in striped shades of blue), which was the second most prevalent order. The family *Coriobacteriaceae* (2.9 %) was also prevalent, and was the only member of the order *Coriobacteriales* (phylum *Actinobacteria*) detected. The majority of the families detected (67 of 83) were present at very low levels (less than 1 % of total mapped reads), and many were only present in a few of the samples, and were consequently not statistically analysed.

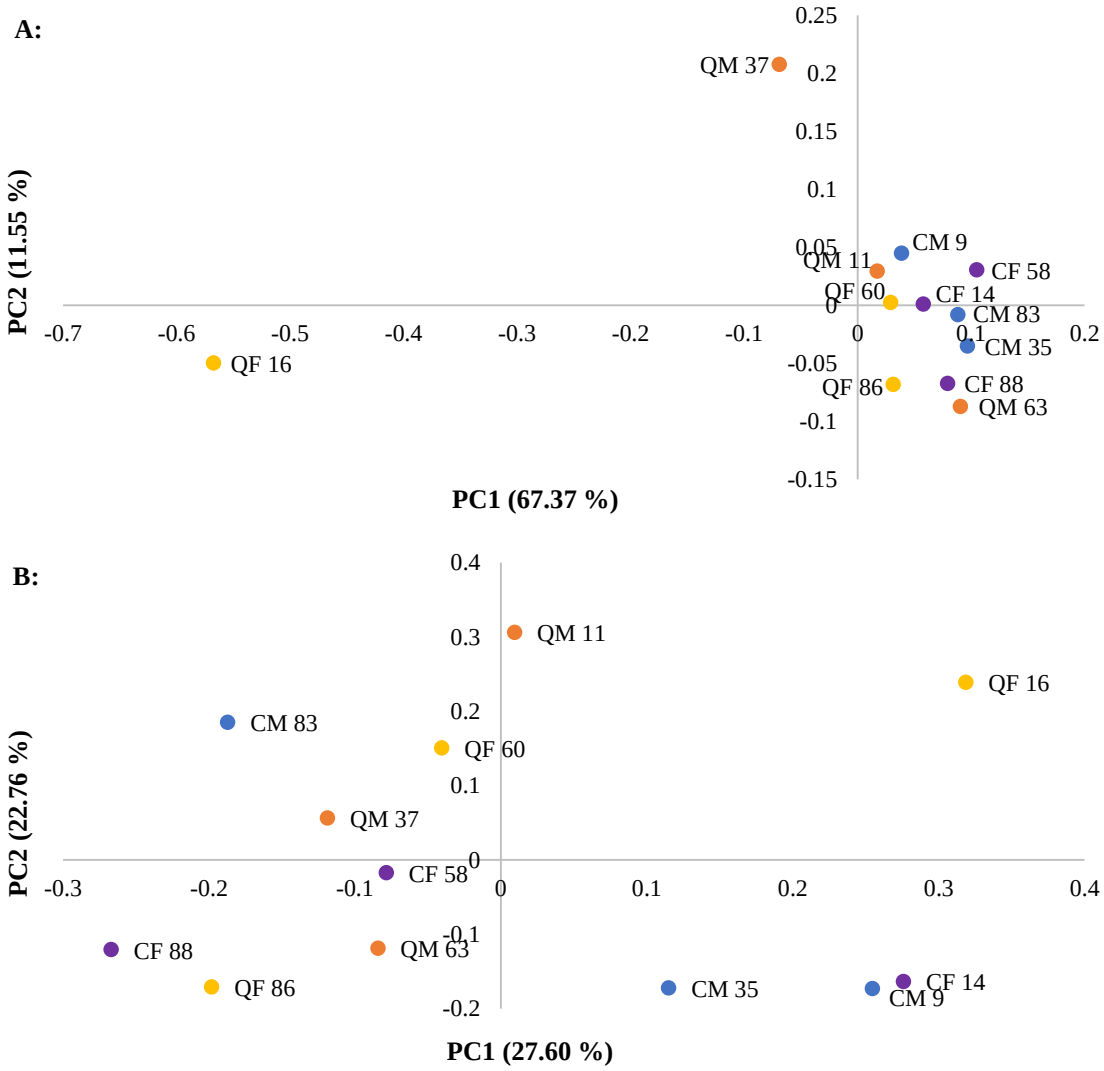

Figure 4.2 Bray-Curtis plots indicating the beta diversity at the (A) family and (B) species level of the caecal microbiome of male and female New Zealand White grower rabbits fed either a control (Ctrl) or quercetin-supplemented (Qrc, 2 g/kg) diet. Points indicate individual samples, with blue representing Ctrl males (CM), purple Ctrl females (CF), orange Qrc males (QM) and yellow Qrc females (QF).

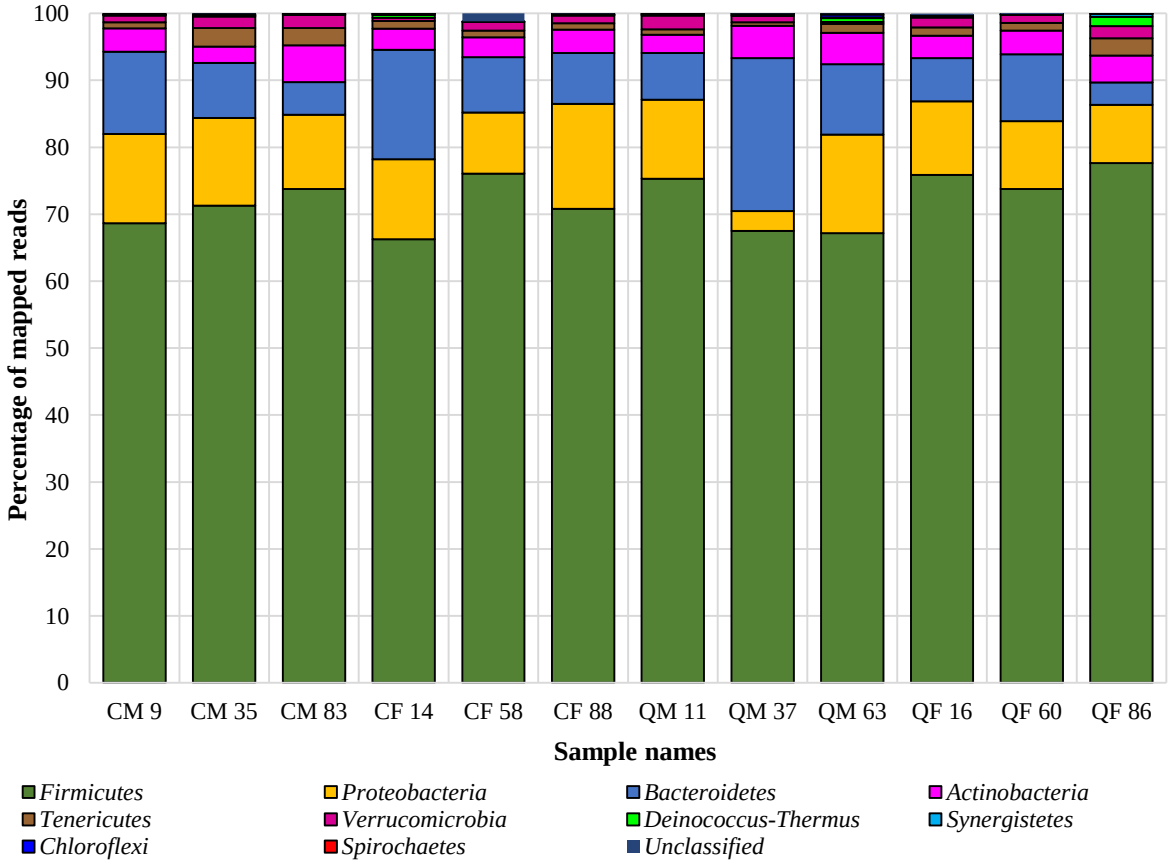

Figure 4.3 Relative abundance of the major bacterial groups in the rabbit caecum at the phylum level. Phyla are organised vertically from most to least prevalent (average across treatment groups), and samples are grouped horizontally by treatment (C: control; Q: dietary quercetin supplemented at 2 g/kg feed; M: male; F: female).

Only 3.8 % of the total mapped reads were identified down to the species level, with 85 % only having family level identification and 10.9 % only having genus level identification. The proportion of the total mapped reads identified as species also varied widely between individual samples, from 2.49 % to 6.76 %. The composition at the species level (Figure 4.4) was far more varied than was seen at the phylum or family levels. Some species, such as *Gemmiger formicilis* (0.72 %), *Stomatobaculum longum* (0.50 %) and *Ruminococcus gnavus* (0.40 %) were relatively consistently present in all the samples, whereas others, such as *Bacteroides dorei* (0.50 %), *Bacteroides uniformis* (0.17 %) and *Bacteroides rodentium* (0.13 %) were present at high levels in some samples, but barely detected in others. The latter two species were also most often present in the same samples, with their abundance having a strong, positive correlation ($r = 0.92$, $P < 0.01$). Higher proportions of these species also coincided with higher abundances of *Moryella indoligenes* ($r_{uniformis} = 0.92$, $r_{rodentium} = 0.85$, $P < 0.01$) and *Ruminococcus albus* ($r_{uniformis} = 0.60$, $P = 0.04$; $r_{rodentium} = 0.74$, $P < 0.01$). *Ruminococcus* (confirmed taxonomy, family *Ruminococcaceae*) was the most prevalent genus by percentage of mapped reads (2.17 %), with four identified species, of which *R. albus* (0.44 %) was the 4th most prevalent overall, whereas *Ruminococcus* (proposed taxonomy, family *Lachnospiraceae*, 2.03 %), was the 2nd most abundant genus, but only had only a single identified species, *Ruminococcus gnavus* (0.40 %). In contrast, eight of the identified species belonged to the genus

Bacteroides, despite it being only the 5th most prevalent genus identified (1.32 %). *M. indoligenes* was the only identified species in the genus *Moryella*, and the 6th most abundant species overall (0.19 %). A number of species (18 of 38) were only identified in a few of the samples, generally at very low levels, although some, such as *Bacteroides fragilis* and *Akkermansia muciniphila*, were quite abundant in the few samples in which they were detected.

Despite the relatively small sample size, a number of the bacterial families were found to correlate with the live performance data (Table 4.1). *Eubacteriaceae, Natranaerobiaceae, Peptococcaceae* and *Syntrophomonadaceae* (all in the phylum *Firmicutes*) all had moderate to strong positive Pearson's correlations with the total weight gain, suggesting that rabbits for which these families were more abundant, tended to gain more weight from weaning to 12 weeks old. In contrast, rabbits with higher proportions of *Hyphomicrobiaceae* tended to grow less ($r = -0.62$). Only three families correlated with the FCR, *Clostridiaceae, Erysipelotrichaceae* and *Haloplasmataceae*, with all three having moderate to strong negative correlations, suggesting that a higher abundance of these families coincided with a lower FCR, and thus an improvement in the efficiency of feed utilization.

There were also several correlations between bacterial families and serum hormone levels (Table 4.1). *Carnobacteriaceae, Coriobacteriaceae* (class *Coriobacteriia*), *Streptosporangiaceae, Thermoanaerobacterales Family III. Incertae Sedis*, *Thermoanaerobacterales Family IV. Incertae Sedis* and *Thermosporotrichaceae* all correlated positively with serum fT3 levels, whereas *Entomoplasmataceae* and *Hyphomicrobiaceae* had moderate to strong negative correlations. *Carnobacteriaceae*, although correlating positively with fT3 levels, had a negative correlation with fT4 levels, as did *Campylobacteraceae* and *Clostridiales Family XVI. Incertae Sedis*. *Acidaminococcaceae, Peptostreptococcaceae* and *Sutterellaceae*, on the other hand, tended to be more abundant when serum fT4 levels were higher. Only a single family correlated with the serum GH levels, *Clostridiales Family XII. Incertae Sedis*, which did not correlate with any of the other hormones. *Alicyclobacillaceae* had the only negative correlation with the serum cortisol level, while *Flavobacteriaceae, Rhizobiaceae* and *Rhodospirillaceae* correlated positively with this hormone.

Despite the lack of an effect of sex or diet on the caecal flavonoid content or pH, there were several bacterial families that did correlate with these variables. *Acetobacteraceae, Erythrobacteraceae, Microbacteriaceae, Mycoplasmataceae, Synergistaceae* and *Veillonellaceae* (order *Selenomonadales*) all tended to be less abundant in samples with higher flavonoid contents, while proportions of *Coriobacteriaceae* (class *Actinobacteria*) and *Veillonellaceae* (order *Clostridiales*) were higher. Three families correlated with the pH of the caecal content, *Clostridiales Family XI. Incertae Sedis*, *Desulfuromonadaceae* and *Spiroplasmataceae*, with all three being more abundant at higher pH levels. There was no apparent correlation between the pH and caecal flavonoid content.

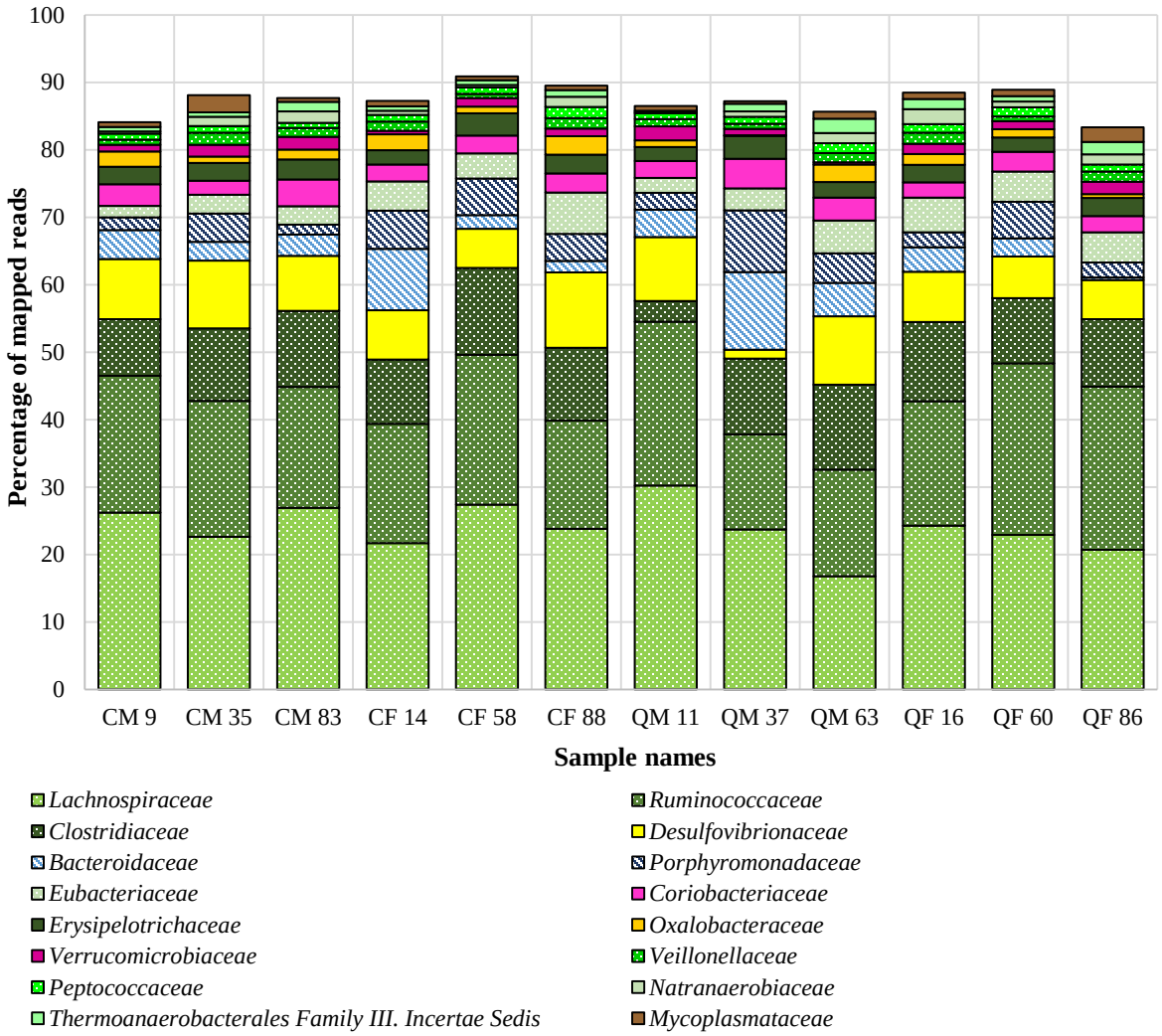

Figure 4.4 Relative abundance of the major bacterial groups (those contributing more than 1 % of total mapped reads) in the rabbit caecum at the family level. Families are organised vertically from most to least prevalent (average across treatment groups), and samples are grouped horizontally by treatment (C: control; Q: dietary quercetin supplemented at 2 g/kg feed; M: male; F: female).

The large degree of variation between the individual samples and the relatively small sample number made the identification of any effects of diet or sex difficult; however, some differences between the sexes and dietary treatments were detected (Table 4.2), as well as some tendencies towards significant interactions (Table 4.3).

Few dietary effects were observed (Table 4.2), although *Clostridiales Family XIII. Incertae Sedis* ($P = 0.005$) and *Anaerofustis* ($P = 0.003$) were more abundant in the Qrc group. A number of OTUs also tended ($P \leq 0.10$) to be more abundant in either the Qrc (*Caldicoprobacteraceae*, *Clostridiales Family XVI Incertae Sedis*) or Ctrl (*Sutterellaceae*, *Roseburia*, *Oscillibacter*, *Parasutterella*) rabbits, with *Ruminococcus albus*, the only species impacted, tending to be more abundant in the Ctrl group ($P = 0.093$). In addition, the Qrc group tended to have a greater proportion of sequences only identified to the family level ($P = 0.097$), whereas the Ctrl group had more identified at the genus level ($P = 0.094$).

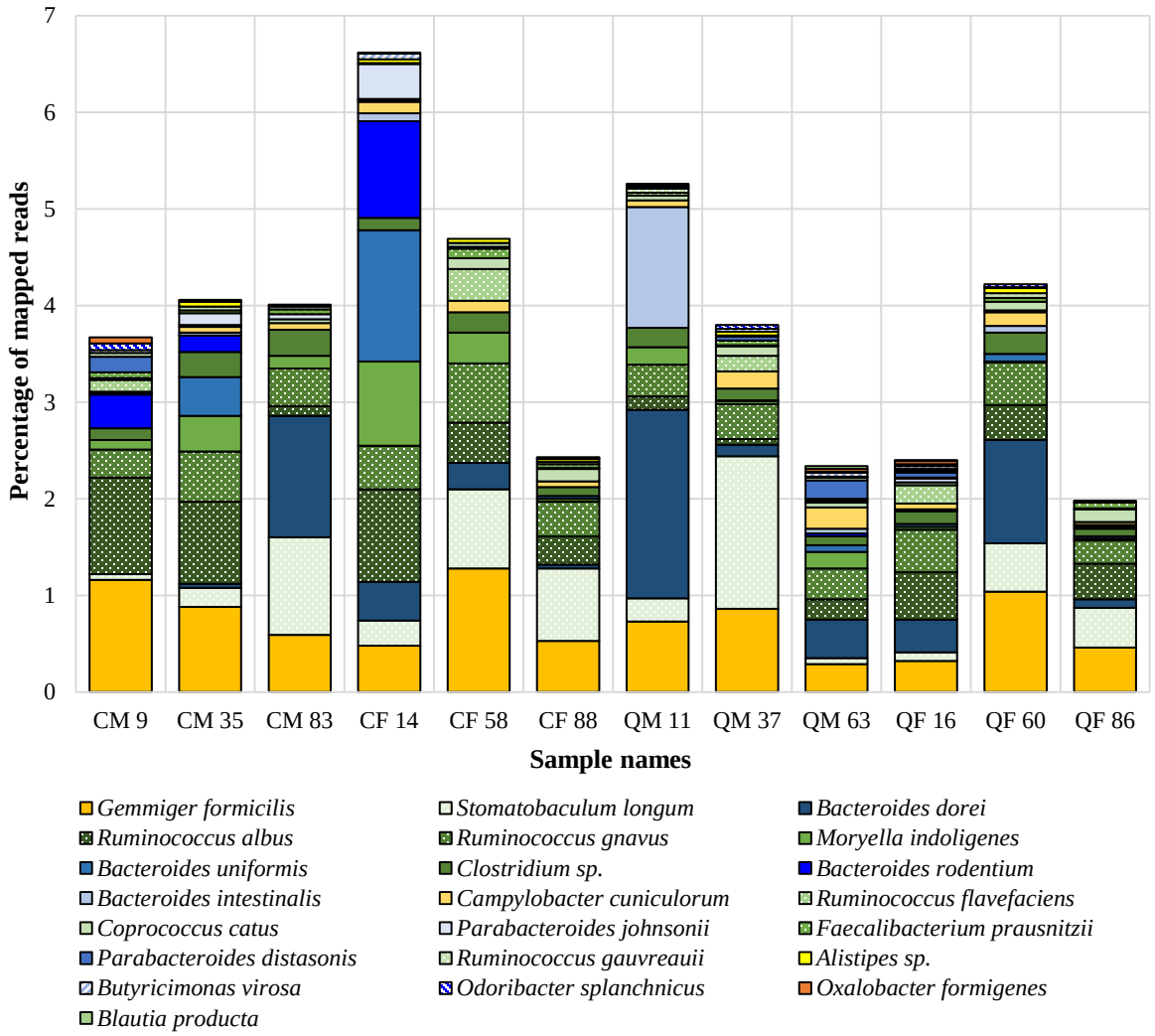

Figure 4.5 Relative abundance of the major bacterial species identified in the rabbit caecum. Species are organised vertically from most to least prevalent (average across treatment groups), and samples are grouped horizontally by treatment (C: control; Q: dietary quercetin supplemented at 2 g/kg feed; M: male; F: female).

The majority of the main effects found were differences between the sexes (Table 4.2), and in most cases this involved higher levels of the particular OTU being found in caecal samples from female rabbits. Females had higher levels of *Flavobacteriia* ($P = 0.032$), due to a higher abundance of *Flavobacteriales* and *Flavobacteriaceae*, as well as higher levels of *Desulfuromonadales* ($P = 0.038$), *Eubacteriaceae* ($P = 0.013$) and *Geobacteraceae* ($P = 0.023$). In addition, they also tended to have greater proportions of *Spiroplasmataceae* ($P = 0.087$), *Rhizobiaceae* ($P = 0.073$) and *Aurantimonadaceae* ($P = 0.081$), but lower proportions of *Lactobacillaceae* ($P = 0.098$), and thus *Lactobacillales* ($P = 0.060$), and *Alicyclobacillaceae* ($P = 0.094$). While the majority of those found to be higher in females fell within the phylum *Proteobacteria*, and both found to be higher in males fell under *Firmicutes*, this did not translate into any sex effect at the phylum level.

For a few bacterial groups the effect of sex tended to differ between the diets. At the class level (Table 4.3), male rabbits in the Ctrl group tended to have higher levels of *Mollicutes* than Ctrl females, whereas the sexes did

not differ for the Qrc rabbits (P = 0.051). The opposite was found for *Clostridiales Family XI. Incertae Sedis*, which was higher in Qrc females than males, but did not differ in the Ctrl group (P = 0.064). The genus *Campylobacter* was not effected by sex within either of the dietary treatment groups, but was more abundant in Qrc males than Ctrl males (P = 0.066), as was the species, *Campylobacter cuniculorum* (P = 0.066). *Marvinbryantia*, which made a very small contribution at the genus level, was higher in Ctrl males than Ctrl females, but did not differ in Qrc rabbits (P = 0.055).

4.4 Discussion

4.4.1 The microbiome profile

The overall dominance of *Firmicutes* found in this study (Figure 4.3) was in agreement with previous studies of the rabbit caecal microbiome, and the relative abundance (72 %) fits within the previously reported range, which, admittedly, is rather wide (Bäuerl, Collado, Zúñiga, Blas & Martínez, 2014; Monteils, Cauquil, Combes, Godon & Gidenne, 2008; Zhu, Wang & Li, 2015). The occurrence of *Proteobacteria* and *Bacteroidetes* in the top three most abundant phyla also concurs with the existing literature (Monteils *et al*., 2008; Zhu *et al*., 2015), although some studies report considerably lower proportions of *Proteobacteria* (Bäuerl *et al*., 2014; Massip, Combes, Cauquil, Zemb & Gidenne, 2012). It is notable that the abundance of *Proteobacteria* was reportedly influenced by the occurrence of Epizootic Rabbit Enteropathy (ERE), tending to be higher in rabbits with the disease (Bäuerl *et al*., 2014). However, ERE did not occur in this study.

The abundance of *Actinobacteria* and *Tenericutes* was high relative to the results of Massip *et al*. (2012) and Zhu *et al*. (2015); however, Bäuerl *et al*. (2014) and Badiola, De Rozas, Gonzalez, Aloy and Carabaño (2016) found a higher proportion of *Tenericutes* but minor amounts of *Actinobacteria*. Reported results for *Verrucomicrobia* abundance also vary, with it forming part of the top four or five phyla (2.4 – 3.9 %) in some studies (Bäuerl *et al*., 2014; Monteils *et al*., 2008; Zhu *et al*., 2015); but not being detected by Massip *et al*. (2012), and only contributing 1.28 % (6th most prevalent) in this study. Both *Tenericutes* and *Verrucomicrobia* have also been found to be influenced by ERE, with *Verrucomicrobia* increasing and *Tenericutes* decreasing in diseased rabbits (Bäuerl *et al*., 2014). *Verrucomicrobia* has also been found to be influenced by both the age of the rabbit at sampling and the starch to fibre ratio of the diet used, which may explain some of the variation between studies (Zhu *et al*., 2015).

Even less consistency was seen in the less abundant phyla, with *Chloroflexi* not being detected by either Massip *et al*. (2012) or Zhu *et al*. (2015), who also detected a number of phyla not found in this study, particularly *Euryarchaeota*, *Fibrobacteres*, *Fusobacteria* and *TM7*. However, this may also be linked to the number of samples used, the processing and sequencing methods used and the depth of sequencing.

At the family level (Figure 4.4), the predominance of *Lachnospiraceae* and *Ruminococcaceae* concurred with the findings of Zhu *et al*. (2015), Massip *et al*. (2012) and Bäuerl *et al*. (2014), although the proportions were much lower than reported by the latter two. Members of these families are thought to play an important role in fibre-digestion, particularly of peptose and cellulose (Gosalbes *et al*., 2011), and are significant producers of short-

chain fatty acids (Zhu *et al*., 2015). Unfortunately, Massip *et al*. (2012) and Bäuerl *et al*. (2014) did not report the fibre-contents of the feeds used, and a comparison with the composition of the feed used in this study can therefore not be done. In addition, *Ruminococcaceae* has been associated with secondary metabolite, specifically antibiotic, biosynthesis in a metatranscriptomic study on the human gut microbiome, suggesting a role in gut health (Gosalbes *et al*., 2011).

Clostridiaceae, the 3rd most abundant family in this study, was not reported as a main bacterial family in rabbits by Massip *et al*. (2012); however, it was present in rabbit caeca (Bäuerl *et al*., 2014), and was a dominant family in the capybara (García-Amado *et al*., 2012) and healthy horses (Steelman, Chowdhary, Dowd, Suchodolski & Janečka, 2012). Members of the *Clostridiaceae* family have been found to prefer simpler carbohydrates, rather than the fibrous components utilized by the *Lachnospiraceae* and *Ruminococcaceaea*, and its prevalence in the caecum was thus somewhat surprising (Biddle, Stewart, Blanchard & Leschine, 2013).

The 4th most prevalent bacterial family, *Desulfovibrionaceae*, was also detected by Bäuerl *et al*. (2014) and Massip *et al*. (2012), and was linked to feed intake levels in the latter study. *Desulfovibrionaceae* forms part of the sulphate-reducing group of bacteria, which play an important role in the sulphur cycle by degrading relatively simple organic compounds — such as ethanol, formate and lactate — into acetate, while reducing sulphate to sulphide and competing with methanogens for hydrogen (Brenner, Staley & Krieg, 2005; Muyzer & Stams, 2008).

The 5th and 6th most abundant families found were *Bacteroidaceae* and *Porphyromonadaceae*, which were also the most prevalent families from the order *Bacteroidales*. This is in contrast with the results of Bäuerl *et al*. (2014), who found that *Rikenellaceae* was the most abundant *Bacteroidales*. However, it partly concurs with the results of Badiola *et al*. (2016), who reported a similar percentage of *Bacteroidaceae* but no *Porphyromonadaceae*. Gosalbes *et al*. (2011) found that *Bacteroidaceae* was the main family involved in all the categorised functions in the human gut, and that both these families were among the most active of the gut microbiome. *Bacteroidaceae* was also found to be more abundant in rabbits with ERE (Bäuerl *et al*., 2014).

Eubacteraceae, of the phylum *Firmicutes*, contributed 3.8 % of the total mapped reads and was the 7th most abundant family. It did not appear to be detected by Badiola *et al*. (2016) or Zhu *et al*. (2015), but was detected in the rabbit caecum by Bäuerl *et al*. (2014). Members of this family generally utilize both carbohydrates and peptones and produce a variety of organic acids, and appear to be functionally versatile (De Vos *et al*., 2009; Gosalbes *et al*., 2011).

The 8th most abundant family, *Coriobacteraceae*, contributed 2.9 % of the total mapped reads. It was not detected in rabbit caecal contents by Badiola *et al*. (2016) or Zhu *et al*. (2015), whereas Massip *et al*. (2012) found it to be the third most abundant family overall. It has also been previously detected in the rabbit caecum by Bäuerl *et al*. (2014), in the colon and faeces of horses by Dougal *et al*. (2013) and in the chicken caecum by Sergeant *et al*. (2014).

Erysipelotrichaceae, the 9th most prevalent family in this study at 2.63 %, was reported as the 4th most abundant family by Massip *et al*. (2012), and was found in rabbit caeca by Badiola *et al*. (2016) and Bäuerl *et al*.

(2014) but not by Zhu *et al*. (2015). Of the remaining families contributing more than 1 % of the total mapped reads, *Oxalobacteraceae*, *Verrucomicrobiaceae*, *Veillonellaceae* and *Peptococcaceae* were all also reported by Bäuerl *et al*. (2014), although the rank for *Verrucomicrobiaceae* was much higher (as was seen for the phylum *Verrucomicrobia*), and that for *Veillonellaceae* much lower, than was found in this study. *Verrucomicrobiaceae* was also found to be more abundant in ERE rabbits by Bäuerl *et al*. (2014). *Natranaerobiaceae*, *Thermoanaerobacterales Family III. Incertae Sedis* and *Mycoplasmataceae* do not appear to have been previously reported, although the class under which *Mycoplasmataceae* falls, *Mollicutes*, was detected by Badiola *et al*. (2016).

The greatest extent of between-individual variation was seen at the species level (Figure 4.5), with not only the abundance of the different species varying, but also the proportion of the sequences that were identified to this level. Between 80 % and 91 % of the sequences were only identified to the family level, and a further 5.5 % to 15.8 % only to the genus level, which supports the speculation that rabbit caeca contain a large proportion of undescribed bacterial species (Michelland *et al*., 2010), and suggests that the composition of these novel species may vary between individuals.

Five species contributed 67 % of the total identified at the species level, and the two most prevalent of these were *Gemmiger formicilis* and *Stomatobaculum longum*. Neither species appear to have been identified in rabbit caeca before, although *G. formicilis* has been found in chicken ceca (Clench & Mathias, 1995) and human faeces (Salanitro, Muirhead & Goodman, 1976). *G. formicilis* uses carbohydrates as the only or major energy source, and produces mostly butyrate, lactate and formate, but does not produce any gas (Brenner *et al*., 2005). *S. longum* was only described and named in 2013, which may explain the lack of identification, but it has been previously isolated from the human oral cavity (Sizova *et al*., 2013).

The proportions of both *S. longum* and *Bacteroides dorei*, which both contributed 0.50 % of the total mapped reads, varied widely between individuals. The presence of the latter is of interest as it has been identified in rabbit gut samples previously, and members of the genus *Bacteroides* have been suggested to be largely responsible for pectinolytic activity in the rabbit caecum (Sirotek, Marounek, Rada & Benda, 2001). Furthermore, *B. dorei* has been proposed to be a beneficial species for the prevention of ERE. Badiola *et al*. (2016) observed that the inoculation of rabbit kits with *B. dorei* or *Bacteroides fragilis* reduced the expression of proinflammatory cytokines and increased the expression of MHC II, a surface antigen, as well as increasing immunoglobulin diversity. In contrast to *B. dorei*, *B. fragilis* was only identified in one of the 12 rabbit samples tested in this study.

Ruminococcus (family *Ruminococcaceae*) was the most prevalent genus, contributing 2.17 % of the total mapped reads, and the most abundant species identified in this genus was *Ruminococcus albus*. The prevalence of this genus concurs with Bäuerl *et al*. (2014), whereas Zhu *et al*. (2015) found larger proportions of the genus *Sporobacter*. *R. albus* is generally recognised as being one of the main cellulolytic bacteria in the rumen environment and has been previously reported in the rabbit caecum, so its abundance was expected (Abecia *et al*., 2005; Monteils *et al*., 2008; Rodríguez-Romero, Abecia & Fondevila, 2013). In contrast, *Ruminococcus gnavus*,

which has been tentatively assigned to the family *Lachnospiraceae*, does not appear to have been previously detected in rabbits, although it is reported to have been isolated in the hindgut of non-ruminants (Krause, Dalrymple, Smith, Mackie & McSweeney, 1999) and in the chicken caecum (Zhu, Zhong, Pandya & Joerger, 2002). *R. gnavus* has also been identified in the human gut, and has been found to be more abundant in people with Crohn's disease or ulcerative colitis (Png *et al*., 2010). *R. gnavus* is a mucus-associated, mucolytic species (Png *et al*., 2010).

A number of the genera and species previously identified in rabbit caeca were not identified in the caeca samples in this study, or were only present at very low levels. At the genus level, *Alistipes*, *Akkermansia* and *Subdoligranulum* were identified by Bäuerl *et al*. (2014) as being some of the most abundant genera in the rabbit caecal microbiome, whereas in this study *Alistipes* only contributed 0.13 %, *Subdoligranulum* was not detected, and *Akkermansia muciniphila*, the only species found in this genus, was only identified in a few of the samples. However, *A. muciniphila*, *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, *Clostridium perfringens* and *Clostridium coccoides* were identified as prevalent species in ERE rabbits (Badiola *et al*., 2016; Bäuerl *et al*., 2014), so their absence or very low presence in this study may be linked to the good health status of the rabbits used. In contrast, the relative scarcity of *Alistipes* — which is considered important for short chain fatty acid production — and abundance of *Bacteroides* found in this study, is more in line with the composition of the ERE rabbits described by Bäuerl *et al*. (2014). These discrepancies highlight the difficulty of comparing microbiome data from different studies.

4.4.2 Correlations with live performance and caecal conditions

While the comparison of the caecal microbiome profile to that previously reported for rabbits provides useful information on the diversity of the microbiome, it is limited when it comes to determining the productive implications of this profile. Examining the correlations between the abundance of bacterial families and live performance parameters, while certainly not providing causative information, can serve as an initial indication of which families may play important roles in performance (Table 4.1).

Total weight gain and the average FCR are two measures of the productive value of a grower rabbit, with maximum weight gain and minimum FCR being desirable. In this case, more families were related to weight gain than FCR, with seven showing positive correlations, four of which, *Eubacteriaceae*, *Peptococcaceae*, *Natranaerobiaceae* and *Syntrophomonadaceae*, had correlation coefficients exceeding 0.60. Of these, the first three all contributed more than 1 % of the total mapped reads, with *Eubacteraceae* making the largest contribution. *Eubacterium*, a genus in *Eubacteraceae*, has previously been found to have a strong positive correlation with the average milk fat yield in dairy cows, and a moderate positive correlation with the residual feed intake (RFI) (Jami, White, & Mizrahi, 2014). Only two families tended to be more abundant in rabbits showing lower growth, namely *Hyphomicrobiaceae* and *Entomoplasmataceae*.

Four families had negative correlations with the FCR, indicating a beneficial association with feed efficiency, with these being *Clostridiaceae*, *Erysipelotrichaceae*, *Haloplasmataceae* and *Rhizobiaceae*. The genus

Clostridium, which was the only member of the *Clostridiaceae* family detected in this study, was not found to have any significant correlation with the FCR of dairy cattle by Jami *et al*. (2014). However, it did have a moderate positive correlation with the RFI, suggesting that higher proportions of *Clostridium* associated with poorer feed efficiency, which directly contradicts the findings of this study. This discrepancy could be due to the differing physiology of ruminants and rabbits, as well as the complexity of the relationship between the microbiome and live performance.

A number of families were found to correlate significantly with serum levels of fT3, fT4, GH and cortisol (Table 4.1). While these specific interactions do not seem to have been previously investigated or reported, research has previously found links between the gut microbiome and serum leptin levels (Sanz, Santacruz & Gauffin, 2010). Furthermore, the short-chain fatty acids produced by the microbial population of the gut have been found to interact extensively with receptors in a variety of tissue types; this has been proposed as one possible route for the extensive metabolic effects of the gut microbiome, and may suggest an interaction with hormone systems (Kasubuchi, Hasegawa, Hiramatsu, Ichimura & Kimura, 2015). In addition, the relationship between stress hormones and the immune system may suggest a mechanism behind the correlations found between bacterial families and serum cortisol levels (Sanz *et al*., 2010), most of which were positive. The extensive correlations between fT3 and gut bacteria found in this study may also be linked to the excretion of thyroid hormones in the bile, as intestinal bacteria are known to deconjugate some of the sulfo- and glucuronide-conjugated thyroid hormones that are excreted, thus allowing their reabsorption and possibly increasing circulating levels (Yen, 2001). However, the existence of correlations alone does not necessarily indicate a physiological link or causation, and considerable further research is required before any assumptions of a direct link between serum hormones and the gut microbiome can be made.

Based on the known antimicrobial effects of the flavonoids, the correlations between the total flavonoid content of the caecum and the composition of the microbiome were anticipated (Cushnie & Lamb, 2005). Also somewhat unsurprisingly, the majority of the correlations found were negative, indicating that the families tended to be less abundant in samples with a higher caecal flavonoid content. The most abundant families with negative correlations were *Veillonellaceae* (order *Selenomonadales*) and *Mycoplasmataceae*. This result for *Veillonellaceae* concurred with previous findings that this family decreased in response to dihydromyricetin (Fan *et al*., 2018), whereas *Mycoplasmataceae* was found to be lower after dietary supplementation with an essential oil high in flavonoids (De Nardi *et al*., 2016). *Coriobacteriaceae* (class *Actinobacteria*) was a notably abundant family that was more prevalent in samples with a higher total flavonoid content, in contrast with the results of Huang *et al*. (2016), who found that the flavonoids tested (quercetin, catechin and puerarin) had no effect on this family. Both *Veillonellaceae* (order *Selenomonadales*) and *Hyphomicrobiaceae* also correlated with total weight gain, positively and negatively respectively. This, in conjunction with their correlations with the total flavonoid content, may suggest a possible detrimental impact of the caecal flavonoid content on performance.

Table 4.1

Significant Pearson's correlation coefficients between the abundance of bacterial families and the live performance data, serum hormone levels and caecal content conditions of New Zealand White grower rabbits. *P*-values provided in parentheses.

	Total weight gain	**Average FCR**	**Serum fT3**	**Serum fT4**	**Serum GH**	**Serum cortisol**	**Caecal flavonoid content**	**Caecal pH**
Clostridiaceae	-	-0.67 (0.02)	-	-	-	-	-	-
Eubacteriaceae	0.62 (0.03)	-	-	-	-	-	-	-
Coriobacteriaceae (class Actinobacteria)	-	-	-	-	-	-	0.64 (0.02)	-
Erysipelotrichaceae	-	-0.61 (0.04)	-	-	-	-	-	-
Veillonellaceae (order Selenomonadales)	0.56 (0.06)	-	-	-	-	-	-0.66 (0.02)	-
Peptococcaceae	0.60 (0.04)	-	-	-	-	-	-	-
Natranaerobiaceae	0.70 (0.01)	-	-	-	-	-	-	-
Thermoanaerobacterales Family III. Incertae Sedis	0.53 (0.07)	-	0.58 (0.05)	-	-	-	-	-
Mycoplasmataceae	-	-	-	-	-	-	-0.69 (0.01)	-
Hyphomicrobiaceae	-0.62 (0.03)	-	-0.61 (0.03)	-	-	-	0.53 (0.08)	-
Flavobacteriaceae	-	-	-	-	-	0.62 (0.03)	-	-
Acidaminococcaceae	-	-	-	0.58 (0.05)	-	-	-	-
Clostridiales Family XI. Incertae Sedis	-	-	-	-	-	-	-	0.64 (0.03)
Acetobacteraceae	-	-	-	-	-	-	-0.74 (<0.01)	-
Microbacteriaceae	-	-	-	-	-	-	-0.59 (0.04)	-
Haloplasmataceae	-	-0.68 (0.02)	-0.56 (0.06)	-	0.52 (0.08)	-	-	-
Synergistaceae	-	-	-	-	-	-	-0.62 (0.03)	-
Campylobacteraceae	-	-	-	-0.58 (0.05)	-	-	-	-
Erythrobacteraceae	-	-	-	-	-	-	-0.60 (0.04)	-
Thermosporotrichaceae	-	-	0.67 (0.02)	-	-	-	-	-
Carnobacteriaceae	-	-	0.66 (0.02)	-0.64 (0.02)	-	-	-	-
Sutterellaceae	-	-	-	0.62 (0.03)	-	-	-	-
Peptostreptococcaceae	-	-	-	0.65 (0.02)	-	-	-	-
Desulfuromonadaceae	-	-	-	-	-	-	-	0.69 (0.01)
Syntrophomonadaceae	0.63 (0.03)	-	-	-	-	-		-

Table 4.1 *(continued)*

	Total weight gain	Average FCR	Serum fT3	Serum fT4	Serum GH	Serum cortisol	Caecal flavonoid content	Caecal pH
Clostridiales Family XII. Incertae Sedis	-	-	-	-	0.65 (0.02)	-	-	-
Spiroplasmataceae	-	-	-0.52 (0.08)	-	-	-	-	0.72 (<0.01)
Alicyclobacillaceae	-	-	-	-	-	-0.65 (0.02)	-	-
Coriobacteriaceae (class Coriobacteriia)	-	-	0.69 (0.01)	-	-	-	-	-
Veillonellaceae (order Clostridiales)	-	-	-	-	-	-	0.61 (0.03)	-
Rhodospirillaceae	-	-	-	-	-	0.70 (0.01)	-	-
Entomoplasmataceae	-0.51 (0.09)	-	-0.68 (0.01)	-	0.53 (0.08)	-	-	-
Rhizobiaceae	-	-0.55 (0.06)	-0.50 (0.09)	-	-	0.60 (0.04)	-	-
Thermoanaerobacterales Family IV. Incertae Sedis	0.53 (0.08)	-	0.62 (0.03)	-	-	-	-	-
Streptosporangiaceae	-	-	0.61 (0.04)	-	-	-	-	-
Clostridiales Family XVI. Incertae Sedis	-	-	0.54 (0.07)	-0.58 (0.05)	-	-	-	-

N = 12 for all correlations; Families listed from most to least abundant
Total weight gain: weight gain from 5 to 12 weeks of age; Average FCR: Average feed conversion ratio from 5 to 12 weeks of age; fT3: free triiodothyronine; fT4: free thyroxine; GH: somatotropin

Surprisingly few families associated with the caecal pH, with *Clostridiales Family XI. Incertae Sedis*, *Desulfuromonadaceae* and *Spiroplasmataceae* all associating with higher pH values. This general preference for a higher pH is in agreement with the known detrimental effects of acidic conditions on rumen microbial function (Russell & Wilson, 1996).

4.4.3 Effects of diet

Quercetin-supplementation affected few bacterial groups (Table 4.2), but the total proportions of mapped reads identified at the family and genus level did tend to differ, with Qrc rabbits having a greater proportion only identified to the family level, while the Ctrl rabbits had more identified down to the genus level. This may suggest that more novel species were present in the Qrc samples, and could explain the slight separation of the dietary treatment groups seen in the Bray-Curtis plot at the family level (Figure 4.2).

The families *Caldicoprobacteraceae*, *Clostridiales Family XVI. Incertae Sedis* and *Clostridiales Family XIII. Incertae Sedis* all tended to be more abundant in the Qrc rabbits. The effects of flavonoids on these groups specifically does not appear to have been previously studied; however, quercetin has been found to increase the proportion of *Firmicutes*, the phylum to which they belong (Huang *et al*., 2016). In addition, the abundance of another member of the order *Clostridiales* (*Ruminococcaceae*) has also been found to increase with quercetin supplementation (Huang *et al*., 2016). In contrast, the administration of quercetin and resveratrol to rats on a high-fat diet reduced the proportion of *Firmicutes*, and did not impact the abundance of *Clostridiales* (Zhao *et al*., 2017). However, interactions appear to be specific to both the flavonoid and the microbe in question, as well as most likely being context-sensitive, so further research would be necessary to confirm the results of this study.

The genus *Anaerofustis*, which belongs to the family *Eubacteraceae*, was also more abundant in the Qrc rabbits (Table 4.2). *Eubacterium ramulus*, another species within this family that is a known component of the human gut microbiome, has been found previously to degrade quercetin and is proposed to be key species responsible for the degradation of flavonoids (Braune, Gütschow, Engst & Blaut, 2001). The ingestion of flavonoids, including quercetin, has also been found to stimulate the growth of this bacterium, consequently increasing its relative abundance, most likely by acting as a substrate for its growth (Simmering, Pforte, Jacobasch & Blaut, 2002). Possibly a similar interaction was responsible for the higher levels of *Anaerofustis* in the supplemented rabbits in this case.

The abundance of the genera *Roseburia* and *Oscillibacter* (both order *Clostridiales*), and *Parasutterella* (and its family, *Sutterellaceae*) all tended to be lower in the Qrc rabbits. *Roseburia* and *Parasutterella* did not appear to be detected in the rabbit caecum by Bäuerl *et al*. (2014), while *Oscillobacter* was, at similarly low levels as found in this study. However, both *Oscillibacte*r and *Roseburia* were detected in horse faeces (O'Donnell *et al*., 2013). As *Roseburia* species typically utilize carbohydrates to produce large amounts of butyrate (De Vos *et al*., 2009), and butyrate is an important energy source for the intestinal epithelia and plays a large role in overall colon health in humans (Hamer *et al*., 2008), this lower abundance of *Roseburia* could be detrimental. However, as this

genus only contributed 0.13 % of the total mapped reads, it seems unlikely that this effect would be extensive. *Oscillibacter* and *Parasutterella* made even smaller contributions to the overall microbiome.

Only a single identified species, *R. albus*, tended to differ between the diets, being more abundant in the Ctrl rabbits. As *R. albus* was the 4th most prevalent species, and is an important primary fermenter of fibrous cell-wall components, this difference could be of interest (McAllister, Cheng, Okine & Mathison, 1996). However, this result contrasts with the findings of Oskoueian, Abdullah and Oskoueian (2013), who reported no effect of quercetin on this, or other fibrolytic species, despite other flavonoids tested having an effect. It must however be noted that Oskoueian *et al*. (2013) studied the effect on rumen fluid components, *in vitro*.

The relatively limited effects of quercetin supplementation on the caecal microbiome, and the lack of effect on the caecal total flavonoid content, may be related to the use of the quercetin aglycone. The absorption of flavonoid glycosides in the small intestine is thought to be limited by their sugar moiety, and this results in their accumulation in the caecum (Oteiza *et al*., 2018). It may therefore be possible that most of the ingested quercetin aglycone was absorbed prior to the caecum, preventing any increase in the caecal total flavonoid content and reducing its effect on the microbiome.

4.4.4 Effects of sex

The sex-differences found for the abundance of a number of OTUs may explain the somewhat higher alpha diversity of females seen in Figure 4.1, and may be linked to testosterone levels, as reported in mice by Markle *et al*. (2013). However, none of the families found to be impacted by sex in this study were identified as sex-dependent by Markle *et al*. (2013).

The most abundant family to show a sex effect was *Eubacteraceae*, which was more prevalent in females than males (Table 4.2). Considering the versatility of function of this group it is difficult to identify how or whether this would impact the overall health and efficiency of the microbiome (De Vos *et al*., 2009; Gosalbes *et al*., 2011). However, as this family did appear to correlate with improvements in weight gain, this sex difference may be linked to the tendency for a higher average daily gain in female rabbits towards the end of the growth period (Chapter 3).

A number of other families were also affected. *Flavobacteriaceae*, *Geobacteraceae*, *Spiroplasmataceae*, *Rhizobiaceae* and *Aurantimonadaceae* were all more abundant or tended to be more abundant in females, with this effect carrying over to the order (*Flavobacteriales*, *Desulfuromonadales*) and even class (*Flavobacteriia*) level. *Flavobacteraceae*, which was the most abundant of these families (0.54 %), has not generally been found to be a major family in the rabbit caecal microbiome, with Bäuerl *et al*. (2014) only detecting it at very low levels. Badiola *et al*. (2016) also only detected *Flavobacteraceae* in younger rabbits (25 or 39 days old), whereas by 70 days old it was no longer present. Unfortunately, sex was not reported by Badiola *et al*. (2016), as it would have been interesting, in light of the sex effect found in this study, to see whether the evolution in the abundance of *Flavobacteraceae* with age differed for males and females. The other families found to be more abundant in females were only present at very low levels in the samples, and, in the case of *Geobacteraceae*,

Spiroplasmataceae and *Aurantimonadaceae*, did not appear to be common species in the gut environment (Badiola *et al*., 2016; Bäuerl *et al*., 2014; Zhu *et al*., 2015).

The families *Lactobacillaceae* (and thus order *Lactobacillales*) and *Alicyclobacillaceae*, and the genus *Pseudoflavonifractor*, also tended to differ between the sexes, but were higher in males than females (Table 4.2). *Lactobacillaceae*, the most abundant of these, was detected by Bäuerl *et al*. (2014) and Badiola *et al*. (2016) in rabbit caeca, and was identified as being one of the core microbial communities in the horse ileum (Dougal *et al*., 2013). In contrast, Zhu *et al*. (2015) notably did not detect the genus *Lactobacillus*, which falls within the *Lactobacillaceae* family. Lactobacilli are generally considered beneficial bacteria, stimulating the immune system, helping prevent antibiotic-associated diarrhoea and reducing the actions of harmful enzymes, and are consequently utilised as a probiotic (Reid, 2006). However, a number of genera fall within this family, so it is not necessarily the case that the greater abundance of the family *Lactobacillaceae* in males was beneficial.

The presence of the family *Alicyclobacillaceae* was unexpected and seemed somewhat unlikely, as bacteria in this family are generally aerobic (De Vos *et al*., 2009), and it was not detected by Badiola *et al*. (2016), Bäuerl *et al*. (2014) or Zhu *et al*. (2015). *Pseudoflavonifractor* was similarly not found in previous studies.

4.4.5 Diet × sex interactions

For a number of bacterial groups the effects of diet and sex tended to interact, with the effect of one depending on the other (Table 4.3). The most prevalent group showing this effect was the class *Mollicutes*, which was lower in Qrc than Ctrl males, but did not differ in females. This class has been reported in rabbit caeca previously (Badiola *et al*., 2016), and is considered a normal part of the rumen microbiome, although it is usually represented by members of the family *Anaeroplasmataceae*, which are the only obligate anaerobe *Mollicutes* (Joblin & Naylor, 2002). Surprisingly, this family was not one of those identified in the rabbit caeca in this study, with the families that were identified being facultative rather than obligate anaerobes. Many, if not most, *Mollicutes* exist as parasites in their various habitats, and some display bacteriolytic characteristics, particularly against gram-negative bacteria. This class may thus play a regulatory role in the caecal microbiome (Joblin & Naylor, 2002). It is of interest to note that the abundance of *Mollicutes* was found to be lower in rabbits presenting with ERE (Badiola *et al*., 2016).

At the family level, *Clostridiales Family XI. Incertae Sedis* was more abundant in Qrc females than in Ctrl females, with males not differing. This effect in the female rabbits aligns to what was found for the other *Clostridiales* families (*XVI* and *XIII*), which were more abundant in Qrc rabbits, although why it was restricted to this sex is uncertain. The opposite trend was seen for the genus *Marvinbryantia*, which was higher in Ctrl males than in all the other treatment groups.

Table 4.2

Levels of bacteria, identified at the class, order, family, genus or species level, in the caecal microbiome of male and female New Zealand White grower rabbits fed either a control or quercetin-supplemented (2 g/kg) diet.

		Diet		Sex	
	Overall	**Control**	**Quercetin**	**Male**	**Female**
Class level					
Flavobacteriia	0.54 ± 0.136	0.56 ± 0.230	0.53 ± 0.169	0.25^{b} ± 0.128	0.84^{a} ± 0.173
Order level					
Lactobacillales	1.29 ± 0.286	1.24 ± 0.367	1.33 ± 0.475	1.87^{α} ± 0.473	0.71^{β} ± 0.061
Flavobacteriales	0.54 ± 0.136	0.56 ± 0.230	0.53 ± 0.169	0.25^{b} ± 0.128	0.84^{a} ± 0.173
Desulfuromonadales	0.25 ± 0.060	0.19 ± 0.042	0.32 ± 0.113	0.14^{b} ± 0.047	0.37^{a} ± 0.093
Family level					
Eubacteriaceae	3.83 ± 0.379	3.58 ± 0.633	4.07 ± 0.456	2.93^{b} ± 0.437	4.73^{a} ± 0.341
Caldicoprobacteraceae	0.89 ± 0.086	0.74^{β} ± 0.117	1.04^{α} ± 0.096	0.80 ± 0.154	0.98 ± 0.074
Lactobacillaceae	0.79 ± 0.285	0.76 ± 0.360	0.82 ± 0.478	1.30^{α} ± 0.503	0.28^{β} ± 0.040
Flavobacteriaceae	0.54 ± 0.136	0.56 ± 0.230	0.53 ± 0.169	0.25^{b} ± 0.128	0.84^{a} ± 0.173
Geobacteraceae	0.18 ± 0.042	0.13 ± 0.029	0.23 ± 0.077	0.09^{b} ± 0.027	0.26^{a} ± 0.064
Sutterellaceae	0.09 ± 0.013	0.11^{α} ± 0.019	0.06^{β} ± 0.008	0.09 ± 0.017	0.09 ± 0.020
Spiroplasmataceae	0.06 ± 0.010	0.06 ± 0.012	0.06 ± 0.017	0.04^{β} ± 0.013	0.08^{α} ± 0.011
Alicyclobacillaceae	0.05 ± 0.009	0.05 ± 0.015	0.05 ± 0.010	0.07^{α} ± 0.009	0.03^{β} ± 0.012
Rhizobiaceae	0.03 ± 0.009	0.03 ± 0.017	0.03 ± 0.010	0.01^{β} ± 0.004	0.04^{α} ± 0.015
Clostridiales Family XVI. Incertae Sedis	0.02 ± 0.006	0.01^{β} ± 0.003	0.03^{α} ± 0.010	0.02 ± 0.009	0.02 ± 0.009
Clostridiales Family XIII. Incertae Sedis	0.01 ± 0.003	0.00^{b} ± 0.002	0.02^{a} ± 0.004	0.01 ± 0.004	0.01 ± 0.006
Aurantimonadaceae	0.01 ± 0.005	0.01 ± 0.005	0.01 ± 0.010	0.00^{β} ± 0.002	0.02^{α} ± 0.009
Total family level ID	85.1 ± 1.021	83.3^{β} ± 1.234	87.0^{α} ± 1.307	85.0 ± 1.659	85.3 ± 1.352
Genus level					
Anaerofustis	0.01 ± 0.002	0.00^{b} ± 0.000	0.01^{a} ± 0.003	0.00 ± 0.002	0.01 ± 0.003
Roseburia	0.13 ± 0.033	0.19^{α} ± 0.055	0.07^{β} ± 0.020	0.11 ± 0.025	0.15 ± 0.062
Oscillibacter	0.03 ± 0.006	0.04^{α} ± 0.008	0.02^{β} ± 0.007	0.02 ± 0.009	0.03 ± 0.007
Pseudoflavonifractor	0.06 ± 0.011	0.04 ± 0.008	0.07 ± 0.019	0.07^{α} ± 0.019	0.04^{β} ± 0.004
Parasutterella	0.08 ± 0.012	0.11^{α} ± 0.019	0.06^{β} ± 0.008	0.08 ± 0.016	0.08 ± 0.020
Total genus level ID	10.9 ± 0.778	12.3^{α} ± 0.966	9.58^{β} ± 1.006	11.0 ± 1.475	10.8 ± 0.698
Species level					
Ruminococcus albus	0.44 ± 0.095	0.60^{α} ± 0.156	0.27^{β} ± 0.066	0.39 ± 0.170	0.48 ± 0.099

Means with different superscript letters in the same row (within main effect) differ significantly (^{ab}, $P \leq 0.05$) or tend to differ ($^{\alpha\beta}$, $P \leq 0.10$)

The species *Campylobacter cuniculorum*, and thus its genus (*Campylobacter*) also showed a tendency for a significant interaction, with Qrc males having higher levels than Ctrl males, but females not differing. This species was first isolated in rabbit caecal contents, and was named accordingly (Zanoni, Debruyne, Rossi, Revez & Vandamme, 2009), and while the pathogenicity of this specific species is unknown, many other members of this genus are known pathogens (Lastovica & Allos, 2008). The apparent favouring of this species by quercetin could consequently have implications for gut health. Quercetin has been previously found to have no effect on the viability of *Campylobacter jejuni in vitro* (Gañan, Martínez-Rodríguez & Carrascosa, 2009).

Table 4.3
Proportions of bacteria, identified at the class, family, genus or species level, in the caecal microbiome of New Zealand White grower rabbits, as influenced by the combined effects of sex and diet (control or quercetin-supplemented, 2 g/kg feed).

	Overall	Control		Quercetin	
		Male	Female	Male	Female
Class level					
Mollicutes	1.42 ± 0.222	2.10^{α} ± 0.586	1.04^{β} ± 0.034	0.90^{β} ± 0.248	$1.65^{\alpha\beta}$ ± 0.463
Family level					
Clostridiales Family XI. Incertae Sedis	0.25 ± 0.061	$0.23^{\beta\gamma}$ ± 0.167	0.12^{γ} ± 0.062	0.16^{γ} ± 0.063	$0.49^{\alpha\beta}$ ± 0.076
Genus level					
Campylobacter	0.10 ± 0.018	0.05^{γ} ± 0.015	$0.10^{\beta\gamma}$ ± 0.020	$0.16^{\alpha\beta}$ ± 0.045	$0.08^{\beta\gamma}$ ± 0.033
Marvinbryantia	0.02 ± 0.010	0.06^{α} ± 0.025	0.00^{γ} ± 0.003	$0.01^{\beta\gamma}$ ± 0.007	$0.03^{\alpha\beta\gamma}$ ± 0.022
Species level					
Campylobacter cuniculorum	0.10 ± 0.018	0.05^{γ} ± 0.015	$0.10^{\beta\gamma}$ ± 0.020	$0.16^{\alpha\beta}$ ± 0.045	$0.08^{\beta\gamma}$ ± 0.033

$^{\alpha\beta\gamma}$ Means with different superscript Greek letters in the same row tend to differ ($P \leq 0.10$)

4.5 Conclusion

The comparison of the composition of the microbiome found in this study to previous research suggested that certain bacterial groups, such as the *Firmicutes* at the phylum level and *Lachnospiraceae* and *Ruminococcaceae* at the family level, are relatively consistently dominant in the rabbit caecum. However, consensus beyond the phylum level was extremely limited, particularly for identified species, with the two dominant species found in this study having not been previously detected in rabbit caecal contents. A considerable amount of variation in the species composition was also found within this study, indicating that even within a single population, individual variation in the caecal microbiome is highly prevalent. This absence of a clear, consistent microbiome profile in the rabbit caecum will present a challenge for any future research attempting to understand or manipulate this population. Nonetheless, the extensive correlations found between live performance traits and bacterial families suggest that further research may be of value. Families of particular interest for weight gain and feed efficiency were *Eubacteraceae* and *Clostridiaceae*, both of which were abundant members of the bacterial population. While the correlations with serum hormone levels found may not have had any physiological basis, they should nonetheless be kept in mind during future research on the links between growth performance and the caecal microbiome.

Considering the antimicrobial nature of the flavonoids, the correlations between the caecal flavonoid content and the various bacterial families was unsurprising. However, any clear pattern in this relationship has yet to be elucidated, with studies varying in which bacterial groups were affected, and whether these effects were positive or negative. Once again, it seems that the context-specificity of these associations makes drawing general conclusions impossible. Despite the strong correlations between the caecal flavonoid content and the microbiome, the effects of sex were more extensive than the effects of the dietary quercetin supplementation. The minimal effects of diet may have been due to the use of the quercetin-aglycone, which could have been absorbed from the gastrointestinal tract prior to the caecum. Nonetheless, a number of families from the *Firmicutes* phylum were more abundant in the supplemented rabbits, as was the genus *Anaerofustis*, a member of the *Eubacteraceae* family. The only species impacted, *R. albus*, tended to be lower in Qrc rabbits, which may be of interest as it is an important fibre-fermenting bacterium and an abundant species.

The sex differences largely involved higher levels of bacterial groups in samples from female rabbits, supporting the somewhat higher abundance in females indicated by the alpha-diversity plot. *Eubacteraceae* was one of the most abundant families affected by sex, along with *Flavobacteriaceae*, but in the absence of genus or species-level identification relating these differences to functional changes in the caecum was not possible.

Overall, the practical implications of this study were very limited, as is typical of exploratory studies of this kind. However, the information presented does provide avenues for further research, and adds to the limited picture of the rabbit caecal microbiome currently available.

4.6 References

Abecia, L., Fondevila, M., Balcells, J., Edwards, J. E., Newbold, C. J. & McEwan, N. R. (2005). Molecular profiling of bacterial species in the rabbit caecum. *FEMS Microbiology Letters, 244*, 111 – 115.

Badiola, I., de Rozas, P., Gonzalez, J., Aloy, N. & Carabaño, R. (2016). Recent advances in ERE in growing rabbits. *Proceedings of the 11th World Rabbit Congress — June 15 – 18, 2016, Qingdao, China* (pp. 491 – 502).

Bäuerl, C., Collado, M. C., Zúñiga, M., Blas, E. & Martínez, G. P. (2014). Changes in cecal microbiota and mucosal gene expression revealed new aspects of epizootic rabbit enteropathy. *PLoS One, 9*(8), 1 – 12.

Biddle, A., Stewart, L., Blanchard, J. & Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity, 5*, 627 – 640.

Braune, A., Gütschow, M., Engst, W. & Blaut, M. (2001). Degradation of quercetin and luteolin by *Eubacterium ramulus*. *Applied and Environmental Microbiology, 67*(12), 5558 – 5567.

Brenner, D. J., Staley, J. T. & Krieg, N. R. (2015). Classification of procaryotic organisms and the concept of bacterial speciation. In W. B. Whitman, F. Rainey, P. Kämpfer, M. Trujillo, J. Chun, P. DeVos, B. Hedlund, & S. Dedysh, (Eds.), *Bergey's Manual of Systematics of Archaea and Bacteria* (pp. 27 – 32). New Jersey, USA: John Wiley and Sons.

Clench, M. H. & Mathias, J. R. (1995). The avian cecum: A review. *The Wilson Bulletin, 107*(1), 93 – 121.

Combes, S., Fortun-Lamothe, L., Cauquil, L. & Gidenne, T. (2013). Engineering the rabbit digestive ecosystem to improve digestive health and efficacy. *Animal, 7*(9), 1429 – 1439.

Cushnie, T. P. T. & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents, 26*, 343 – 356.

De Nardi, R., Marchesini, G., Li, S., Khafipour, E., Plaizier, K. J. C., Gianesella, M., Ricci, R., Andrighetto, I. & Segato, S. (2016). Metagenomic analysis of rumen microbial population in dairy heifers fed a high grain diet supplemented with dicarboxylic acids or polyphenols. *BMC Veterinary Research, 12*, 1 – 9.

De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K-H. & Whitman, W. B. (2009). *Bergey's Manual of Systematic Bacteriology — Vol. 3, The Firmicutes* (2nd edition). New York, USA: Springer.

Dougal, K., de la Fuente, G., Harris, P. A., Girdwood, S. E., Pinloche, E. & Newbold, C. J. (2013). Identification of a core bacterial community within the large intestine of the horse. *PloS One, 8*(10), e77660.

Duda-Chodak, A. (2012). The inhibitory effect of polyphenols on human gut microbiota. *Journal of Physiology and Pharmacology, 63*(5), 497 – 503.

Espley, R. V., Butts, C. A., Laing, W. A., Martell, S., Smith, H., McGhie, T. K., Zhang, J., Paturi, G., Hedderley, D., Bovy, A., Schouten, H. J., Putterill, J., Allan, A. C. & Hellens R. P. (2014). Dietary flavonoids from modified apple reduce inflammation markers and modulate gut microbiota in mice. *Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions, 144*, 146 – 154.

Fan, L., Zhao, X., Tong, Q., Zhou, X., Chen, J., Xiong, W., Fang, J., Wang, W. & Shi, C. (2018). Interactions of dihydromyricetin, a flavonoid from vine tea (*Ampelopsis grossedentata*) with gut microbiota. *Journal of Food Science, 83*(5), 1444 – 1453.

Gañan, M., Martínez-Rodríguez, A. J. & Carrascosa, A. V. (2009). Antimicrobial activity of phenolic compounds of wine against *Campylobacter jejuni*. *Food Control, 20*, 739 – 742.

García-Amado, M. A., Godoy-Vitorino, F., Piceno, Y. M., Tom, L. M., Andersen, G. L., Herrera, E. A. & Domínguez-Bello, M. G. (2012). Bacterial diversity in the cecum of the world's largest living rodent (*Hydrochoerus hydrochaeris*). *Microbial Ecology, 63*(4), 719 – 725.

Gosalbes, M. J., Durbán, A., Pignatelli, M., Abellan, J. J., Jiménez-Hernández, N., Pérez-Cobas, A. E., Latorre, A. & Moya, A. (2011). Metatranscriptomic approach to analyze the functional human gut microbiota. *PLoS One, 6*(3), 1 – 9.

Hamer, H. M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F. J. & Brummer, R.-J. (2008). Review article: the role of butyrate on colonic function. *Alimentary Pharmacology & Therapeutics, 27*, 104 – 119.

Herald, T. J., Gadgil, P. & Tilley, M. (2012). High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. *Journal of the Science of Food and Agriculture, 92*, 2326 – 2331.

Huang, J., Chen, L., Xue, B., Liu, Q., Ou, S., Wang, Y. & Peng, X. (2016). Different flavonoids can shape unique gut microbiota profile *in vitro*. *Journal of Food Science, 81*(9), H2273 – H2279.

Jami, E., White, B. A. & Mizrahi, I. (2014). Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS One, 9*(1), e85423.

Joblin, K. N. & Naylor, G. E. (2002). The ruminal mycoplasmas: A review. *Journal of Applied Animal Research, 21*(2), 161 – 179.

Kasubuchi, M., Hasegawa, S., Hiramatsu, T., Ichimura, A. & Kimura, I. (2015). Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients, 7*, 2839 – 2849.

Krause, D. O., Dalrymple, B. P., Smith, W. J., Mackie, R. I. & McSweeney, C. S. (1999). 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. *Microbiology, 145*, 1797 – 1807.

Lastovica, A. J. & Allos, B. M. (2008). Clinical significance of *Campylobacter* and related species other than *Campylobacter jejuni* and *Campylobacter coli*. In I. Nachamkin, C. M. Szymanski & M. J. Blaser (Eds.), *Campylobacter* (3rd edition). Washington DC, USA: ASM Press.

Lee, H. C., Jenner, A. M., Low, C. S. & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Research in Microbiology, 157*, 876 – 884.

Leiber, F., Meier, J. S., Burger, B., Wettstein, H. R., Kreuzer, M., Hatt, J. M. & Clauss, M. (2008). Significance of coprophagy for the fatty acid profile in body tissues of rabbits fed different diets. *Lipids, 43*, 853 – 865.

Lin, Y. T., Hsui, S. L., Hou, Y. C., Chen, H. Y. & Chao, P. D. L. (2003). Degradation of flavonoid aglycones by rabbit, rat and human fecal flora. *Biological and Pharmaceutical Bulletin, 26*(5), 747 – 751.

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., von Bergen, M., McCoy, K. D., Macpherson, A. J. & Danska, J. S. (2013). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science, 339*, 1084 – 1088.

Massip, K., Combes, S., Cauquil, L., Zemb, O. & Gidenne, T. (2012). High throughput 16S-DNA sequencing for phylogenetic affiliation of the caecal bacterial community in the rabbit : Impact of the hygiene of housing and of the intake level. *Proceedings of the VIIIth INRA-RRI Symposium on Gut Microbiology — June 18 – 21, 2012, Clermont-Ferrand, France* (pp. 57).

McAllister, T. A., Cheng, K. J., Okine, E. K. & Mathison, G. W. (1996). Dietary, environmental and microbiological aspects of methane production in ruminants. *Canadian Journal of Animal Science, 76*, 231 – 243.

McBee, R. H. (1971). Significance of intestinal microflora in herbivory. *Annual Review of Ecology and Systematics, 2*, 165 – 176.

Michelland, R. J., Combes, S., Monteils, V., Cauquil, L., Gidenne, T. & Fortun-Lamothe, L. (2010). Molecular analysis of the bacterial community in digestive tract of rabbit. *Anaerobe, 16*, 61 – 65.

Monteils, V., Cauquil, L., Combes, S., Godon, J.-J. & Gidenne, T. (2008). Potential core species and satellite species in the bacterial community within the rabbit caecum. *FEMS Microbiology Ecology, 66*, 620 – 629.

Muyzer, G. & Stams, A. J. M. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature Reviews Microbiology, 6*(6), 441 – 454.

O'Donnell, M. M., Harris, H. M. B., Jeffery, I. B., Claesson, M. J., Younge, B., O' Toole, P. W. & Ross, R. P. (2013). The core faecal bacterial microbiome of Irish Thoroughbred racehorses. *Letters in Applied Microbiology, 57*, 492 – 501.

Oskoueian, E., Abdullah, N. & Oskoueian, A. (2013). Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *BioMed Research International*, 1 – 8.

Oteiza, P. I., Fraga, C. G., Mills, D. A. & Taft, D. H. (2018). Flavonoids and the gastrointestinal tract: Local and systemic effects. *Molecular Aspects of Medicine, 61*, 41 – 49.

Png, C. W., Lindén, S. K., Gilshenan, K. S., Zoetendal, E. G., McSweeney, C. S., Sly, L. I., McGuckin, M. A. & Florin, T. H. J. (2010). Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *American Journal of Gastroenterology, 105*, 2420 – 2428.

Reid, G. (2006). Probiotics. In E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson (Eds.). *The Prokaryotes — Human Microbiology* (pp. 45 – 56). Berlin, Germany: Springer Reference.

Rodríguez-Romero, N., Abecia, L. & Fondevila, M. (2013). Microbial ecosystem and fermentation traits in the caecum of growing rabbits given diets varying in neutral detergent soluble and insoluble fibre levels. *Anaerobe, 20*, 50 – 57.

Russell, J. B. & Wilson, D. B. (1996). Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? *Journal of Dairy Science, 79*, 1503 – 1509.

Salanitro, J. P., Muirhead, P. A. & Goodman, J. R. (1976). Morphological and physiological characteristics of Gemmiger formicilis isolated from chicken ceca. *Applied and Environmental Microbiology, 32*(4), 623 – 632.

Sanz, Y., Santacruz, A. & Gauffin, P. (2010). Gut microbiota in obesity and metabolic disorders. *The Proceedings of the Nutrition Society, 69*, 434 – 441.

Sergeant, M. J., Constantinidou, C., Cogan, T. A., Bedford, M. R., Penn, C. W. & Pallen, M. J. (2014). Extensive microbial and functional diversity within the chicken cecal microbiome. *PLoS One, 9*(3), 1 – 13.

Simmering, R., Pforte, H., Jacobasch, G. & Blaut, M. (2002). The growth of the flavonoid-degrading intestinal bacterium, *Eubacterium ramulus*, is stimulated by dietary flavonoids in vivo. *FEMS Microbiology Ecology, 40*(3), 243 – 248.

Sirotek, K., Marounek, M., Rada, V. & Benda, V. (2001). Isolation and characterization of rabbit caecal pectinolytic bacteria. *Folia Microbiologica, 46*(1), 79 – 82.

Sizova, M. V., Muller, P., Panikov, N., Mandalakis, M., Hohmann, T., Hazen, A., Fowle, W., Prozorov, T., Bazylinski, D. A. & Epstein, S. S. (2013). *Stomatobaculum longum* gen. nov., sp. nov., an obligately anaerobic bacterium from the human oral cavity. *International Journal of Systematic and Evolutionary Microbiology, 63*, 1450 – 1456.

Steelman, S. M., Chowdhary, B. P., Dowd, S., Suchodolski, J. & Janečka, J. E. (2012). Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. *BMC Veterinary Research, 8*, 231.

Vasantha Rupasinghe, H. P., Wang, L., Huber, G. M. & Pitts, N. L. (2008). Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder. *Food Chemistry, 107*, 1217 – 1224.

Yen, P. M. (2001). Physiological and molecular basis of thyroid hormone action. *Physiological Reviews, 81*(3), 1097 – 1142.

Zanoni, R. G., Debruyne, L., Rossi, M., Revez, J. & Vandamme, P. (2009). *Campylobacter cuniculorum* sp. nov., from rabbits. *International Journal of Systematic and Evolutionary Microbiology, 59*, 1666 – 1671.

Zhao, L., Zhang, Q., Ma, W., Tian, F., Shen, H. & Zhou, M. (2017). A combination of quercetin and resveratrol reduces obesity in high-fat diet-fed rats by modulation of gut microbiota. *Food and Function, 8*, 4644 – 4656.

Zhu, Y., Wang, C. & Li, F. (2015). Impact of dietary fiber/starch ratio in shaping caecal microbiota in rabbits. *Canadian Journal of Microbiology, 61*, 771 – 784.

Zhu, X. Y., Zhong, T., Pandya, Y. & Joerger, R. D. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Applied and Environmental Microbiology, 68*(1), 124 – 137.

CHAPTER 5:

The effects of quercetin supplementation on the carcass and meat quality of New Zealand White grower rabbits[#]

Abstract

This study aimed to provide initial data on the effects of quercetin supplementation on rabbit carcass and meat quality. Two diets, a control (Ctrl) and the control supplemented with 2 g/kg quercetin dihydrate, were fed to eight males (M) and eight females (F) per treatment group (in total, 32 rabbits) from weaning (5 weeks old) until slaughter (12 weeks old). Slaughter weight, carcass and organ weights, meat yields and physical and proximate meat quality were determined. Quercetin-supplemented rabbits (Qrc) had higher hindleg meat to bone ratios (Ctrl: 3.8 ± 0.10; Qrc: 4.2 ± 0.07) due to lower hindleg bone weights, contrary to the current understanding of the effect of quercetin on the skeletal system. However, this and the higher skin weight (Ctrl: 18.1 ± 0.24 %; Qrc: 19.0 ± 0.32 %) may have been due to the effects of quercetin on the connective tissue. Sex only affected the reference carcass yield (F: 85.1 ± 0.32 %; M: 84.1 ± 0.22 %), spleen weight (F: 0.049 ± 0.003 %; M: 0.042 ± 0.004 %), head weight (F: 3.7 ± 0.06 %; M: 3.9 ± 0.08 %) and loin pH_u (F: 5.62 ± 0.015; M: 5.70 ± 0.025), without commercial implications.

[#]Partly published as: North, M. K., Dalle Zotte, A. & Hoffman, L. C. (2018). The effects of quercetin supplementation on New Zealand White grower rabbit carcass and meat quality — A short communication. *Meat Science, 145*, 363–366.

5.1 Introduction

Farmers are coming under increasing pressure to produce high quality products that are not only visually appealing and enjoyable to eat, but that can also be considered healthy and functional foods (Dalle Zotte & Szendrő, 2011). In addition, they are expected to conform to increasingly stringent animal welfare standards (Vanhonacker & Verbeke, 2014), and use methods of practice that are deemed socially responsible, such as the exclusion of antibiotics as growth promotors or prophylactics (European Commission, 2005; FDA Centre for Veterinary Medicine, 2013). This has made it necessary for researchers to investigate alternative solutions, such as flavonoids.

Flavonoids, a large group of ubiquitous polyphenolic plant secondary metabolites, are considered functional ingredients, with the potential to provide medical or health benefits to the consumer (Havsteen, 2002). They exhibit a wide range of biological and pharmacological actions, including antioxidant, antimicrobial, anti-inflammatory, antidiabetic, antimutagenic and hepatoprotective activities, as well as effects on the gastrointestinal tract and cardiovascular system (Havsteen, 2002). From the perspective of livestock research, their antioxidant and antimicrobial nature is of particular interest, as it suggests potential for their use as both growth promoters and as a method of improving the functional nature of the end-products (Ahmad, Gokulakrishnan, Giriprasad & Yatoo, 2015; Falowo, Fayemi & Muchenje, 2014; Tipu, Akhtar, Anjum & Raja, 2006).

Quercetin is one of the most well-studied and abundant flavonoids, and is particularly prevalent in onions, capers and lovage, with purified extracts also being readily available (Bischoff, 2008; Erlund, 2004). Research on its use in livestock is limited, but it has been found to influence meat quality in lambs, including colour, toughness, fatty acid profile, microbial spoilage and oxidation during storage (Andrés *et al*., 2013; Andrés *et al*., 2014a, Andrés *et al*., 2014b). Goliomytis *et al*. (2014), similarly found that quercetin supplementation improved the oxidative shelf-life of chicken meat, as well as effecting meat colour and heart weight. A study on beef cattle, although limited, similarly found a tendency for quercetin supplementation to increase toughness, as well as meat ultimate pH (Kang *et al*., 2012).

Although no research on the effects of pure quercetin supplementation on the meat quality of rabbits appears to have been done, Naseer *et al*. (2017) did find that the reproductive potential of heat stressed rabbit does was improved by the supplementation of quercetin. In contrast, Koné *et al*. (2016) found that the supplementation of onion, cranberry or strawberry extracts, which are high in flavonoids, to rabbits had very limited effects on meat quality, despite tending to decrease the ultimate pH of the loin meat, and increase redness and decrease lightness values of the M. *biceps femoris*.

The aim of this study was thus to provide initial information on the effects of supplementing a purified quercetin extract to the diet of growing meat rabbits, with a particular focus on the carcass composition, and the physical quality and proximate chemical composition of the meat. As the breakdown, absorption and metabolism of quercetin and its glycosides is complex and still incompletely understood, the quercetin aglycone was used in this study, rather than one of its glycosides, despite the much greater prevalence of the latter in natural sources.

5.2 Materials and methods

5.2.1 Dietary treatments

Two complete, pelleted diets were tested. The control diet contained predominantly alfalfa meal (36.2 %), wheat bran (35.6 %) and sunflower meal (12.6 %), with 88.3 % dry matter (DM), 19.0 % crude fibre, 18.5 % crude protein, 8.3 % ash and 3.7 % ether extract. The quercetin-supplemented diet was produced through the addition of 2 g/kg quercetin dihydrate (Chengdu Okay Plant and Chemical Co., Ltd, Qionglai, China), extracted from *Sophorae japonica* flowers, to the control diet during initial mixing, prior to pelleting. The diets were manufactured by Pennville (Pty) Ltd (Pretoria, South Africa), and neither diet contained coccidiostats.

5.2.2 Rearing conditions

The growth trial was carried out on Mariendahl Experimental Farm outside Stellenbosch in the Western Cape of South Africa (33°51'02.9"S 18°49'35.2"E), from August to October 2017. Ethical clearance was obtained from the Stellenbosch University Animal Care and Use Committee (protocol number SU-ACUD16-00094).

Thirty-two New Zealand White rabbits, 16 males and 16 females, were assigned to dietary treatment groups (control: Ctrl, or quercetin-supplemented: Qrc) according to weaning weight, litter and sex, such that each treatment group had equal numbers of each sex and approximately equal starting weights, and the kits in each litter were split between the treatment groups. At weaning at five weeks of age, the rabbits were transferred to individual cages in two rooms in a grower house, with the dietary treatments and sexes being evenly distributed within and between the two grower rooms. All rabbits were provided with a multistrain probiotic (Protexin Soluble, Kyron Laboratories Pty Ltd., Johannesburg, South Africa) from one week prior to one week after weaning to reduce the risk of weaning-associated digestive disturbances. The rabbits were fed the experimental diets *ad libitum* from the day of weaning, and fresh water was provided *ad libitum* via automatic water lines. A 12L:12D light regime was used throughout the trial, and artificial ventilation was provided via fans in room A and an air-conditioning system in room B. Temperature and relative humidity were recorded in the two rooms throughout the trial using automatic temperature loggers (LogTag Humidity & Temperature Recorder, Model HAX0-8), with average recorded temperatures and humidities in room A and B during the growth period being 14.9 ± 0.21 °C, 72.3 ± 0.96 % and 16.4 ± 0.14 °C, 63.4 ± 0.73 %, respectively.

5.2.3 Slaughter and carcass processing

The rabbits were slaughtered at 12 weeks old at an abattoir on the experimental farm. They were not fasted prior to slaughter and slaughter weight (ADAM Equipment CPW Plus 6, B&R Scale services, Durbanville, South Africa) was recorded no more than 2 h before death. The rabbits were electrically stunned before exsanguination via the carotid arteries and jugular veins. After skinning and removing the distal parts of the legs, all organs were removed and collected per rabbit and the whole hot carcass was weighed. The organs and carcasses were transported to Stellenbosch University Animal Science Department's meat laboratory for weighing and chilling, respectively.

The liver (excluding gall bladder) and kidneys were weighed individually, whereas the heart, lungs, thymus, oesophagus and trachea were weighed together (as recommended by Blasco and Ouhayoun, 1996) and the entire full gastrointestinal tract was weighed as a whole. After weighing, the spleen was removed from the gastrointestinal tract and weighed. Any scapular fat deposits present were removed from the skins prior to the skins being weighed, with the fat also being weighed as part of the dissectible fat content.

After 24 hours chilling at 2 – 4 °C the cold carcasses were weighed and the heads were removed and weighed. Reference carcasses (RC: carcasses excluding head and red offal) were weighed prior to removing the dissectible fat (perirenal, inguinal and scapular deposits), *longissimus thoracis et lumborum* (LTL) muscles, and hindlegs (HL), for which weights were also recorded. The left HL was further deboned, with the meat and bone being weighed for the determination of the meat to bone ratio. The excised right LTLs and deboned left HLs were vacuum-packed and frozen at -20 °C until proximate chemical analysis.

5.2.4 Physical meat quality

The left LTLs were used for the determination of physical quality parameters. Prior to excision, the ultimate pH (pH_u) was measured twice at the 5th lumbar vertebra using a Crison PH25 portable pH meter with a 50 54 electrode (Crison Instruments S.A., Barcelona, Spain, sourced from Lasec SA Pty Ltd, Cape Town, South Africa). The surface colour (L*, a* and b* values) was measured three times along the length of the excised muscle according to the American Meat Science Association (AMSA, 2012) guidelines, using a calibrated Spectro-guide 45/0 gloss CIELab colour meter (catalogue number 6801, BYK-Gardner, Geretsried, Germany), with the standard D65 illuminant, an aperture size of 11 mm, and an observer angle of 10 degrees. Chroma values were calculated as:

$$\text{Chroma } (C^*) = \sqrt{(a^*)^2 + (b^*)^2}$$

Hue angle values for samples with positive a* values were calculated as indicated by AMSA (2012):

$$\text{Hue angle } (H^\circ) = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

However, for those samples with negative a* values it was necessary to add 180° to the calculated value in order to accommodate a 360° representation and remove negative hue angle values (McLellan, Lind & Kime, 1995).

A portion of the cranial end of the LTL was removed, weighed (17.2 ± 0.69 g) and suspended inside an inflated plastic bag for 24 hours at 2 – 4 °C, where after it was weighed again to determine drip loss. The remaining left LTL was weighed (86.0 ± 2.20 g), vacuum-packed and cooked at 80 °C for 1 hour in a water bath, cooled overnight at 2 – 4 °C and then removed from the bag, patted dry and weighed to determine cooking loss (Honikel, 1998).

The cooked portions were cut to produce at least six, 2 cm long, 1 × 1 cm blocks, with the long axis of the block parallel to the fibre direction of the muscle. These were used for the determination of the Warner Bratzler shear force (WBSF; Honikel, 1998). The force required to shear each block perpendicularly to the direction of the muscle fibres was measured using an Instron 3343 (serial number J8415, from Advanced Lab Solutions, Randburg,

South Africa) with a 5000 N load cell and a crosshead speed of 200 mm/min. The maximum load in Newton was reported, with the average value for the six blocks being used as the WBSF for the sample.

5.2.5 Proximate chemical composition

The proximate chemical composition was determined for the right LTLs and deboned left HLs, with moisture, protein and ash being analysed according to the methodology of the Association of Official Analytical Chemists (AOAC, 2002).

For the determination of the moisture content, 2.5 g of the homogenised meat was dried at 100 – 105 °C for 24 hours and weighed (AOAC method 934.01). This dried sample was incinerated at 500 °C for at least 6 hours, cooled and weighed to provide an estimate of the ash content (AOAC method 942.05).

The total lipid content was determined using a rapid solvent extraction method, as described by Lee, Trevino and Chaiyawat (1996), with a 1:2 chloroform:methanol solution being used for the LTL samples due to their relatively low fat content (< 5 %, Dalle Zotte & Szendrő, 2011). An initial test determination of the lipid content of the HL found it to be above 5 %, and a 2:1 chloroform:methanol solution was consequently used for these samples (Lee *et al*., 1996).

The dried, defatted samples were ground and used for the determination of the total protein content using the LECO combustion or Dumas method (AOAC method 992.15). A 0.5 g aliquot of each sample was weighed into a LECO foil cup, which was incinerated and analysed for nitrogen content, using EDTA for calibration (LECO Corporation, St Joseph, MI, USA). The nitrogen content was converted to a protein content by multiplying by 6.25.

All proximate chemical components are reported as percentages of the wet meat weight.

5.2.6 Statistical analysis

In order to take into account the possible differences in temperature, humidity and ventilation between the two grower rooms, the trial was structured as a randomised block two-by-two factorial design. The two blocks were the two grower rooms used in the trial and the main effects were diet and sex. As the rabbits were individually housed each rabbit served as an experimental unit, with 8 replicates per diet-sex treatment combination.

The data was analysed using Statistica version 13 software, with normality being tested using normal probability plots and the homogeneity of variances tested with Levene's test. The R lm package was used to test the significance of the blocks, main effects and interactions, and Fisher's least significant differences *post hoc* test was used to compare the individual values of the diet-sex treatment groups. Slaughter weight was included as a covariate.

Main effects and interactions with $P \leq 0.05$ were considered significant, whereas those with $P \leq 0.10$ are reported as trends. Values are reported as LSMeans ± standard error of the mean (SEM).

5.3 Results

The statistical analysis of the data indicated that several parameters, namely the carcass chilling loss ($P = 0.01$), the proportion of the heart-lung organ cluster ($P = 0.01$), and the HL meat to bone ratio ($P = 0.01$) were impacted by the block effect. This supported the use of a randomised block design.

No significant diet-sex interactions were observed, although there were some suggestions of gender-specific responses to diet. For the skin weight ($P_{diet \times sex} = 0.10$), Qrc increased the proportion of the skins in males, but not in females, with Qrc males having the heaviest skins, Ctrl males the lightest and the females' being intermediate in weight. However, as these effects are not considered statistically significant as such, the main effects were considered.

Only four of the measured parameters were affected by the dietary treatment (Table 5.1). The HL meat to bone ratio was higher ($P < 0.001$) in the Qrc rabbits, apparently due to a lower bone weight ($P = 0.01$), with the proportion of the skin also being higher in the supplemented rabbits ($P = 0.03$), which likely contributed to the tendency for lower hot carcass weights for these rabbits ($P = 0.07$). However, diet had no effect on the chilled carcass weight or dressing percentage.

The effect of sex was more distinctive, with a number of carcass, organ (Table 5.1) and meat traits (Table 5.2) showing tendencies towards significance or significant effects. Females tended to have heavier ($P = 0.07$) LTL muscles and higher ($P = 0.08$) HL meat to bone ratios as a result of higher HL meat weights ($P = 0.06$). Females also had heavier spleens ($P = 0.04$), whereas males had larger heads ($P < 0.001$). Female rabbits also had higher ($P = 0.02$) RC yields.

Sex was the only main effect with an impact on physical or chemical meat quality, with males' LTLs having higher ($P = 0.02$) pH_u values, and females tending to have yellower (higher b* value, $P = 0.07$) and more intensely coloured (higher chroma, $P = 0.09$) LTL meat. The proximate composition of the meat varied only in terms of the lipid content of the deboned HL, with those from females tending to have higher ($P = 0.07$) total lipid contents.

5.4 Discussion

5.4.1 Effect of diet

In order to determine the cause of the greater HL relative meat yield in Qrc rabbits (Table 5.1), it was necessary to examine the components of this ratio, with this revealing that a lower HL bone weight in the Qrc group, rather than a heavier meat component, was responsible. This was in contrast with the results of Celia *et al*. (2016), who found that the supplementation of a herbal formulation had no effect on the HL bone weight. While flavonoid studies and reviews have clearly demonstrated an effect on bone structure (Babosová *et al*., 2016), they have generally suggested that their administration protected connective tissue from degradation, promoted its proliferation, and reduced bone demineralisation (Putnam, Scutt, Bicknell, Priestley & Williamson, 2007; Teixeira, 2002; Weaver, Alekel, Ward & Ronis, 2012). The supplementation of broiler chickens with propolis and bee pollen — which are known to be rich in flavonoids, including quercetin (Daleprane & Abdalla, 2013) — has

also been found to increase the dimensions of the tibia, although no change in weight or breaking strength was observed (Kleczek, Majewska, Makowski & Michalik, 2012). Considering this evidence, it appeared contradictory that the HL bone weight was decreased by quercetin supplementation. However, it may be that the inclusion of quercetin in the diet resulted in some change in the structure and development of the bone which resulted in the reduction of its weight. Further research investigating the breaking strength, geometric dimensions and cross-sectional structure of the bones would be of interest, particularly to determine whether the quercetin had any negative effect on skeletal structural integrity (Weaver *et al*., 2012). A decrease in bone strength could lead to an increase in bone-related morbidities and mortalities, as well as carcass-quality problems; however, if the higher meat:bone ratio extended to the rest of the carcass, this could be beneficial for farmers.

Table 5.1
The effects of sex and dietary quercetin supplementation at 2 g/kg on New Zealand White rabbit carcass yields and organ weights (LSMean ± SEM).

	Overall mean	Diet		Sex	
		Control	Quercetin	Male	Female
N	32	16	16	16	16
Slaughter weight (SW) (g)	3217 ± 65.9	3200 ± 101	3234 ± 87.9	3251 ± 106	3183 ± 81.3
Skin weight (% SW)	18.6 ± 0.21	18.1^{b} ± 0.24	19.0^{a} ± 0.32	18.5 ± 0.34	18.6 ± 0.27
Full GIT weight (% SW)	15.4 ± 0.19	15.5 ± 0.25	15.2 ± 0.29	15.3 ± 0.22	15.4 ± 0.31
Spleen weight (% SW)	0.050 ± 0.002	0.048 ± 0.004	0.043 ± 0.002	0.042^{b} ± 0.004	0.049^{a} ± 0.003
Liver weight (% SW)	3.5 ± 0.13	3.5 ± 0.21	3.5 ± 0.15	3.6 ± 0.17	3.3 ± 0.18
Kidney weight (% SW)	0.7 ± 0.03	0.7 ± 0.05	0.7 ± 0.03	0.7 ± 0.04	0.7 ± 0.04
Heart-lung weight (% SW)	0.8 ± 0.03	0.8 ± 0.04	0.8 ± 0.04	0.8 ± 0.03	0.8 ± 0.05
Head weight (% SW)	3.8 ± 0.05	3.8 ± 0.07	3.8 ± 0.07	3.9^{a} ± 0.08	3.7^{b} ± 0.06
Hot carcass (HC) weight (g)	1916 ± 39.0	1937^{α} ± 60.9	1896^{β} ± 50.6	1926 ± 66.2	1907 ± 42.4
Chilled carcass (CC) weight (g)	1843 ± 38.3	1862 ± 60.9	1824 ± 48.4	1852 ± 64.7	1835 ± 42.2
Carcass chilling loss (% HC)	3.9 ± 0.14	3.9 ± 0.23	3.8 ± 0.16	3.9 ± 0.21	3.8 ± 0.19
Dressing out percentage (% SW)	57.3 ± 0.37	57.9 ± 0.61	56.8 ± 0.40	57.5 ± 0.42	57.1 ± 0.63
Reference carcass (RC) weight (g)	1559 ± 31.5	1576 ± 50.7	1543 ± 39.1	1557 ± 53.8	1561 ± 34.6
RC yield (% CC)	84.6 ± 0.21	84.6 ± 0.31	84.6 ± 0.30	84.1^{b} ± 0.22	85.1^{a} ± 0.32
Dissectible fat weight (% RC)	6.4 ± 0.33	6.0 ± 0.47	6.7 ± 0.46	6.5 ± 0.55	6.2 ± 0.38
Average LTL weight (% RC)	6.7 ± 0.10	6.7 ± 0.15	6.7 ± 0.13	6.5^{β} ± 0.16	6.8^{α} ± 0.09
Left HL weight (% RC)	15.4 ± 0.17	15.3 ± 0.23	15.6 ± 0.24	15.3 ± 0.27	15.6 ± 0.20
Left HL meat weight (g)	191.7 ± 3.36	190.3 ± 4.97	193.0 ± 4.62	187.8^{β} ± 5.45	195.6^{α} ± 4.00
Left HL bone weight (g)	47.8 ± 0.94	49.9^{a} ± 1.32	45.8^{b} ± 1.18	48.0 ± 1.58	47.6 ± 1.05
HL M:B	4.0 ± 0.07	3.8^{b} ± 0.10	4.2^{a} ± 0.07	3.9^{β} ± 0.10	4.1^{α} ± 0.10

SEM: Standard error of the mean; HL: Hindleg; M:B: Meat to bone ratio; LTL: *Longissimus thoracis et lumborum* muscle
GIT: Gastrointestinal tract; Heart-Lung weight: Combined thymus, trachea, oesophagus, lung and heart weight
^{ab} Means with different superscript letters in the same row (within main effect) differ significantly ($P \leq 0.05$)
$^{\alpha\beta}$ Means with different superscript Greek letters in the same row (within main effect) show a tendency towards significant differences ($P \leq 0.10$)

The effect of flavonoids on connective tissue and the collagen matrix may also provide some explanation for the heavier skins from Qrc rabbits (Teixeira, 2002). Studies on the topical application of quercetin to cutaneous wounds have found that its application improved wound healing through the increased proliferation of fibroblasts, epithelium and collagen (Kant, Jangir, Nigam, Kumar & Sharma, 2017). In addition, the topical application of an extract of *Labisia pumila* (a medicinal herb rich in flavonoids), has been found to restore collagen synthesis to normal after UVB damage (Choi *et al*., 2010). It may thus be possible that the heavier skins were due to increased collagen production in this organ. However, considerable histological study would be necessary to confirm this speculation. The lack of any significant effect on the WBSF of the LTL muscle (Table 5.2), contrary to the results of Andrés *et al*. (2014a) and Kang *et al*. (2012), also casts doubt on this explanation. It must also be noted that while the increase in the skin weight of the Qrc rabbits did not impact the dressing out percentage in this case, despite decreasing the hot carcass weight, if any such effect did occur it would be an important point of consideration for commercial meat farmers.

The generally limited response to the quercetin supplementation found in this study may be linked to the composition of the basal control feed used, as many of the ingredients included, particularly alfalfa, contain high levels of flavonoids (Stochmal & Oleszek, 2007; Zhou, Su & Yu, 2004). This may have reduced the potential of the supplemented quercetin to modify carcass yields and meat quality. In addition, the relatively high slaughter weights recorded for the rabbits in this trial suggest that growing conditions were favourable (Nasr, Abd-Elhamid & Hussein, 2017; Ondruska *et al*., 2011). Further studies which include the induction of stress, such as high ambient temperatures, may produce different results, as the effects of the consequent cellular oxidative stress could be ameliorated by the antioxidant properties of the supplemented quercetin. While limitations in the bioavailability of the quercetin-aglycone may have also played a role in the limited impact on the carcass, it is notable that Koné *et al*. (2016) found a similar lack of effect on meat quality, despite using natural plant extracts.

5.4.2 Effect of sex

The somewhat higher proportion of the LTL muscle in female rabbits (Table 5.1) concurred with the results of Trocino, Xiccato, Queaque and Sartori (2003) for Grimaud Frères hybrid-line rabbits, but not with Yalçin, Onbaşilar and Onbaşilar (2006), who used New Zealand Whites. However, Yalçin *et al*. (2006) slaughtered at a much lower live weight and at only 11 weeks of age. This suggests that the results of this study may be related to differences between the sexes in the rate of maturation, with females maturing earlier and consequently favouring the development of the late-maturing loin region (Lawrie & Ledward, 2006; Pla, Guerrero, Guardia, Oliver & Blasco, 1998).

Females also tended to have a higher HL meat weights and thus meat to bone ratios than males (Table 5.1), which contradicts the results of Pla *et al*. (1998), who found males to have a higher percentage of hind part in the carcass. However, the higher deboned HL weight in females may have been as a result of greater fat deposition, as indicated by the tendency towards a higher total lipid content in the deboned HL (Table 5.2), as also reported by Pla *et al*. (1998). This further supports the suggestion that the sex-related differences found in this study are

linked to the later slaughter age used and consequently increased weight and maturity of the rabbits (Ouhayoun, 1984, in Ortiz Hernández & Rubio Lozano, 2001).

Table 5.2
The effects of sex and dietary quercetin supplementation at 2 g/kg on the physical quality and proximate composition of New Zealand White rabbit *longissimus thoracis et lumborum* (LTL) muscle, and on the proximate composition of deboned hindleg (HL) meat (LSMean ± SEM).

		Overall mean	Diet		Sex	
			Control	Quercetin	Male	Female
LTL	N	32	16	16	16	16
	pH_u	5.66 ± 0.016	5.67 ± 0.027	5.66 ± 0.019	5.70^{a} ± 0.025	5.62^{b} ± 0.015
	L*	54.1 ± 0.33	54.3 ± 0.44	53.8 ± 0.50	53.6 ± 0.44	54.5 ± 0.49
	a*	-1.00 ± 0.157	-0.93 ± 0.225	-1.07 ± 0.223	-1.18 ± 0.228	-0.82 ± 0.211
	b*	6.38 ± 0.197	6.67 ± 0.306	6.08 ± 0.234	6.03^{β} ± 0.242	6.72^{α} ± 0.289
	Hue angle (H°)	99.5 ± 1.55	98.8 ± 2.25	100.3 ± 2.17	101.5 ± 2.35	97.6 ± 1.94
	Chroma (C*)	6.53 ± 0.183	6.81 ± 0.279	6.24 ± 0.222	6.22^{β} ± 0.222	6.84^{α} ± 0.274
	Drip loss (%)	1.22 ± 0.042	1.18 ± 0.049	1.27 ± 0.068	1.21 ± 0.060	1.24 ± 0.060
	Cooking loss (%)	23.7 ± 0.36	23.3 ± 0.58	24.1 ± 0.41	23.4 ± 0.49	24.0 ± 0.53
	WBSF (N)	32.1 ± 1.06	30.4 ± 1.45	33.8 ± 1.46	33.8 ± 1.53	30.3 ± 1.37
	Moisture (%)	73.6 ± 0.20	73.8 ± 0.21	73.3 ± 0.32	73.8 ± 0.25	73.3 ± 0.30
	Protein (%)	22.7 ± 0.18	22.6 ± 0.31	22.9 ± 0.19	22.6 ± 0.31	22.9 ± 0.19
	Lipids (%)	2.90 ± 0.202	2.88 ± 0.271	2.92 ± 0.308	2.79 ± 0.270	3.01 ± 0.309
	Ash (%)	1.21 ± 0.010	1.21 ± 0.012	1.20 ± 0.017	1.21 ± 0.016	1.20 ± 0.014
HL	Moisture (%)	72.3 ± 0.24	72.6 ± 0.38	72.1 ± 0.29	72.5 ± 0.42	72.2 ± 0.24
	Protein (%)	20.4 ± 0.28	20.0 ± 0.39	20.7 ± 0.42	20.8 ± 0.4	20.0 ± 0.39
	Lipids (%)	6.44 ± 0.343	6.56 ± 0.407	6.32 ± 0.566	5.88^{β} ± 0.429	7.00^{α} ± 0.524
	Ash (%)	1.24 ± 0.019	1.25 ± 0.029	1.24 ± 0.025	1.25 ± 0.026	1.24 ± 0.028

SEM: Standard error of the mean; WBSF: Warner Bratzler shear force
Hue angle (H°) = $\tan^{-1}\left(\frac{b^*}{a^*}\right)$, (+ 180 for negative a* values); Chroma (C*) = $\sqrt{(a^*)^2 + (b^*)^2}$
^{ab} Means with different superscript letters in the same row (within main effect) differ significantly ($P \leq 0.05$)
$^{\alpha\beta}$ Means with different superscript Greek letters in the same row (within main effect) show a tendency towards significant differences ($P \leq 0.10$)

The higher proportions of the head found in males in this study (Table 5.1) are in contrast to the results of Ortiz Hernández and Rubio Lozano (2001), who found that 70 day old New Zealand White females had higher head yields than males. However, this finding concurred with the results of Bernardini Battaglini, Castellini and Lattaioli (1995), who included an older slaughter age in their study. It is also possible that the results of this study may be linked to the breed specifications for New Zealand Whites, which call for a heavier head structure in males than females (Steenekamp, 2014), and that this trait was expressed to a larger extent in this study due to the greater maturity of the rabbits at slaughter. The heavier heads in male rabbits most likely also contributed to the higher RC yields found for female rabbits; however, as commercial farmers normally slaughter at a lower live weight, this is unlikely to have economic or processing implications.

The cause of the larger spleens found in females (Table 5.1) is difficult to elucidate, as sex-differences in spleen weight do not appear to have been reported for rabbits previously. However, considering the important role

the spleen plays in both the immune response and blood storage and filtration (Guyton, 1981), this could be worth further investigation.

Only sex had any effect on meat quality (Table 5.2), with the higher LTL pH_u values found for males concurring with the findings of Yalçin *et al*. (2006) and Pla *et al*. (1998). This difference in the pH_u may be due to divergent differentiation in the fibre-type composition of the LTL between the sexes with increasing maturity. Previous studies have found that male animals had higher proportions of aerobic fibre-types (Dalle Zotte, Ouhayoun, Parigi Bini & Xiccato, 1996), and that the pH decline in aerobic muscles tended to be less due to their lower glycogen content (Lefaucheur, 2010). However, Carrilho, Campo, Olleta, Beltran and Lopez (2009), Dalle Zotte *et al*. (1996) and Trocino *et al*. (2003) did not find any sex effect on muscle pH_u.

While the effects of sex on the proximate meat composition were very limited, it is worth briefly comparing the values reported in Table 5.2 to those in literature. Particularly notable was the high lipid content of the meat, which well exceeded the normal values for rabbit loin and HL meat reported by Hernàndez and Dalle Zotte (2010). Surprisingly, this did not result in a proportionally lower protein content in the LTL; however, the HL protein content was below normal and, as one could expect, the moisture content was considerably reduced in both muscles. This atypical proximate composition supports the hypothesis that the rabbits in this study were more mature than normally used, with the deposition of fat being at a more advanced stage (Dalle Zotte, 2002). The relatively low LTL pH_u, though probably also influenced by the lack of transport and fasting prior to slaughter, also supports their greater maturity (Dalle Zotte, 2002; María *et al*., 2006). While it is possible that this maturity was simply a reflection of the 12 week rather than 11 week slaughter age (North, Nkhabutlane & Hoffman, 2017), it is also possible that it was a reflection of the nature of the New Zealand White rabbits present in South Africa, as this stock has been genetically isolated for approximately 33 years. It must also be noted that many more recent studies, from which the values reported by Hernàndez and Dalle Zotte (2010) may have been drawn, utilise hybrid-line rabbits rather than New Zealand White purebreds, limiting the value of a direct comparisons.

5.5 Conclusion

The effects of diet on carcass and meat traits were relatively limited; however, the changes that were observed were of considerable interest. While the increase in the meat to bone ratio would be beneficial from an edible meat yield perspective, particularly if it holds true for the carcass as a whole, the decrease in bone weight was concerning, as problems with skeletal integrity could increase morbidity and mortality, as well as potentially bone-breakage during slaughter. The increase in the skin weight could possibly be useful for the rabbit pelt industry if it translates into improved skin quality; however, if it leads to lower dressing out percentages this would be detrimental for meat rabbit farmers. Nevertheless, both of these aspects need extensive and in-depth further research before any recommendations can be made.

The observed differences between the sexes, while somewhat more than generally expected, were likely due to the greater maturity of the rabbits at slaughter than normally found in literature, as indicated by their greater

age, heavier slaughter weights, and high meat lipid contents. As most producers slaughter at a lighter weight than used in this study it seems unlikely that the effects found would have a commercial impact.

5.6 References

Ahmad, S. R., Gokulakrishnan, P., Giriprasad, R. & Yatoo, M. A. (2015). Fruit-based natural antioxidants in meat and meat products: A review. *Critical Reviews in Food Science and Nutrition, 55*(11), 1503 – 1513.

AMSA (2012). *Meat color measurement guidelines*. Champaign, Illinois: American Meat Science Association.

Andrés, A., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Blanco, C. & Giráldez, F. J. (2013). Quercetin dietary supplementation of fattening lambs at 0.2% rate reduces discolouration and microbial growth in meat during refrigerated storage. *Meat Science 93*, 207 – 212.

Andrés, S., Huerga, L., Mateo, J., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Rotolo, L. & Giráldez, F. J., (2014a). The effect of quercetin dietary supplementation on meat oxidation processes and texture of fattening lambs. *Meat Science, 96*, 806 – 811.

Andrés, S., Morán, L., Aldai, N., Tejido, M. L., Prieto, N., Bodas, R. & Giráldez, F. J. (2014b). Effects of linseed and quercetin added to the diet of fattening lambs on the fatty acid profile and lipid antioxidant status of meat samples. *Meat Science, 97*, 156 – 163.

AOAC International (2002). *Official methods of analysis* (17th ed.), Arlington, Virginia, USA: Association of Official Analytical Chemists Inc.

Babosová, R., Omelka, R., Ďúranová, H., Kováčová, V., Lukáčová, M., Capcarová, M. & Martiniaková, M. (2016). Quercetin-induced changes in femoral bone microstructure of adult male rabbits. *Potravinarstvo Scientific Journal for Food Industry, 10*, 300 – 307.

Bernardini Battaglini, M., Castellini, C. & Lattaioli, P. (1995). Effect of sire strain, feeding, age and sex on rabbit carcass. *World Rabbit Science, 3*, 9 – 14.

Bischoff, S. C. (2008). Quercetin: potentials in the prevention and therapy of disease. *Current Opinion in Clinical Nutrition and Metabolic Care, 11*, 733 – 740.

Blasco, A. & Ouhayoun, J. (1996). Harmonization of criteria and terminology in rabbit meat research. Revised proposal. *World Rabbit Science, 4*, 93 – 99.

Carrilho, M. C., Campo, M. M., Olleta, J. L., Beltran, J. A. & Lopez, M. (2009). Effect of diet, slaughter weight and sex on instrumental and sensory meat characteristics in rabbits. *Meat Science, 82*, 37 – 43.

Celia, C., Cullere, M., Gerencsér, Zs., Matics, Zs., Tasoniero, G., Dal Bosco, A., Giaccone, V., Szendrő, Zs. & Dalle Zotte, A. (2016). Effect of pre- and post-weaning dietary supplementation with Digesterom® herbal formulation on rabbit carcass traits and meat quality. *Meat Science, 118*, 89 – 95.

Choi, H., Kim, D., Kim, J. W., Ngadiran, S., Sarmidi, M. R. & Park, C. S. (2010). *Labisia pumila* extract protects skin cells from photoaging caused by UVB irradiation. *Journal of Bioscience and Engineering, 109*, 291 – 296.

Daleprane, J. B. & Abdalla, D. S. (2013). Emerging roles of propolis: Antioxidant, cardioprotective, and antiangiogenic actions. *Evidence-based Complementary and Alternative Medicine, article 175135*, 1 – 8.

Dalle Zotte, A. (2002). Perception of rabbit meat quality and major factors influencing the rabbit carcass and meat quality. *Livestock Production Science, 75*, 11 – 32.

Dalle Zotte, A., Ouhayoun, J., Parigi Bini, R. & Xiccato, G. (1996). Effect of age, diet and sex on muscle energy metabolism and on related physicochemical traits in the rabbit. *Meat Science, 43*, 15 – 24

Dalle Zotte, A. & Szendrő, Zs. (2011). The role of rabbit meat as functional food. *Meat Science 88*, 319 – 331.

Erlund, I. (2004). Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research, 24*, 851 – 874.

European Commission (2005). *Ban on antibiotics as growth promoters in animal feed enters into effect*. Brussels, Belgium: European Commission, available at: http://europa.eu/rapid/press-release_IP-05-1687_en.htm.

Falowo, A. B., Fayemi, P. O. & Muchenje, V. (2014). Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food Research International, 64*, 171 – 181.

Food and Drug Administration, Centre for Veterinary Medicine (2013). *Guidance for Industry #213: New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209.* Washington, D.C., USA: U.S. Department of Health and Human Services.

Goliomytis, M., Tsoureki, D., Simitzis, P. E., Charismiadou, M. A., Hager-Theodorides, A. L. & Deligeorgis, S. G. (2014). The effects of quercetin dietary supplementation on broiler growth performance, meat quality, and oxidative stability. *Poultry Science, 93*, 1957 – 1962.

Guyton, A. C. (1981). *Textbook of medical physiology* (6th ed.). Philadelphia, USA: W. B. Saunders Co Ltd.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67 – 202.

Hernàndez, P. & Dalle Zotte, A. (2010). Influence of diet on rabbit meat quality. In C. de Blas & J. Wiseman (Eds.), *Nutrition of the rabbit* (2nd ed., pp. 163 – 178). Oxfordshire, UK: CABI International.

Honikel, K. O. (1998). Reference methods for the assessment of physical characteristics of meat. *Meat Science, 49*(4), 447 – 457.

Kang, M., Kim, H. J., Jang, A., Gam, D. K., Yun, G. S. & Jo, C. (2012). Effect of dietary supplementation of quercetin on antioxidant activity and meat quality of beef cattle. *CNU Journal of Agricultural Science, 39*(1), 61 – 68.

Kant, V., Jangir, B. L., Nigam, A., Kumar, V. & Sharma, S. (2017). Dose regulated cutaneous wound healing potential of quercetin in male rats. *Wound Medicine, 19*, 82 – 87.

Kleczek, K., Majewska, K., Makowski, W. & Michalik, D. (2012). The effect of diet supplementation with propolis and bee pollen on the physicochemical properties and strength of tibial bones in broiler chickens. *Archiv Tierzucht, 55*, 97 – 103.

Koné, A. P., Cinq-Mars, D., Desjardins, Y., Guay, F., Gosselin, A. & Saucier, L. (2016). Effects of plant extracts and essential oils as feed supplements on the quality and microbial traits of rabbit meat. *World Rabbit Science, 24*, 107 – 119.

Lawrie, R. A. & Ledward, D. A. (2006). *Lawrie's Meat Science* (7th ed.). Cambridge: Woodhead Publishing Limited.

Lee, C. M., Trevino, B. & Chaiyawat, M. (1996). A simple and rapid solvent extraction method for determining total lipids in fish tissue. *Journal of AOAC International, 79*(2), 487 – 492.

Lefaucheur, L. (2010). A second look into fibre typing — Relation to meat quality. *Meat Science, 84*(2), 257 – 270.

María, G.A., Buil, T., Liste, G., Villarroel, M., Sañudo, C. & Olleta, J.L. (2006). Effects of transport time and season on aspects of rabbit meat quality. *Meat Science, 72*, 773 – 777.

McLellan, M. R., Lind, L. R. & Kime, R. W. (1995). Hue angle determinations and statistical analysis for multiquadrant Hunter L, a, b data. *Journal of Food Quality, 18,* 235 – 240.

Naseer, Z., Ahmad, E., Epikmen, E. T., Uçan, U., Boyacioğlu, M. & Akosy, M. (2017). Quercetin supplemented diet improves follicular development, oocyte quality, and reduces ovarian apoptosis in rabbits during summer heat stress. *Theriogenology, 96*, 136 – 141.

Nasr, M.A.F., Abd-Elhamid, T. & Hussein, M.A. (2017). Growth performance, carcass characteristics, meat quality and muscle amino-acid profile of different rabbits breeds and their crosses. *Meat Science, 134*, 150 – 157.

North, M. K., Nkhabutlane, P. & Hoffman, L. C. (2017). The effect of age on the carcass composition, portion yield and proximate composition of two rabbit genetic types in South Africa. *World Rabbit Science*, *25*(3), 281 – 288.

Ondruska, L., Rafay, J., Okab, A.B., Ayoub, M.A., Al-Haidary, A.A., Samara, E.M., Parkanyi, V., Chrastinova, L., Jurcik, R., Massanyi, P., Lukac, N. & Supuka, P. (2011). Influence of elevated ambient temperature upon some physiological measurements of New Zealand White rabbits. *Veterinarni Medicina, 56*, 180 – 186.

Ortiz Hernández, J. A. & Rubio Lozano, M. S. (2001). Effect of breed and sex on rabbit carcass yield and meat quality. *World Rabbit Science, 9*, 51 – 56.

Pla, M., Guerrero, L., Guardia, D., Oliver, M. A. & Blasco, A. (1998). Carcass characteristics and meat quality of rabbit lines selected for different objectives: I. Between lines comparison. *Livestock Production Science, 54*, 115 – 123.

Putnam, S. E., Scutt, A. M., Bicknell, K., Priestley, C. M. & Williamson, E. M. (2007). Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytotherapy Research, 21*, 99 – 112.

Steenekamp, K. (2014). *Rabbits: The South African all breeds standards of excellence.* Cape Town, South Africa: South African Rabbit Judges Council.

Stochmal, A. & Oleszek, W. (2007). Seasonal and structural changes of flavones in alfalfa (*Medicago sativa*) aerial parts. *Journal of Food, Agriculture & Environment, 5*(2), 84 – 88.

Teixeira, S. (2002). Bioflavonoids: Proanthocyanidins and quercetin and their potential roles in treating musculoskeletal conditions. *Journal of Orthopaedic and Sports Physical Therapy, 32*, 357 – 363.

Tipu, M. A., Akhtar, M. S., Anjum, M. I. & Raja, M. L. (2006). New dimension of medicinal plants as animal feed. *Pakistan Veterinary Journal, 26*(3), 144 – 148.

Trocino, A., Xiccato, G., Queaque, P. I. & Sartori, A. (2003). Effect of transport duration and gender on rabbit carcass and meat quality. *World Rabbit Science, 11*, 23 – 32.

Vanhonacker, F. & Verbeke, W. (2014). Public and consumer policies for higher welfare food products: Challenges and opportunities. *Journal of Agricultural and Environmental Ethics, 27*, 153 – 171.

Weaver, C. M., Alekel, D. L., Ward, W. E. & Ronis, M. J. (2012). Flavonoid intake and bone health. *Journal of Nutrition in Gerontology and Geriatrics, 31*, 239 – 253.

Yalçin, S., Onbaşilar, E. E. & Onbaşilar, İ. (2006). Effect of sex on carcass and meat characteristics of New Zealand White rabbits aged 11 weeks. *Asian-Australian Journal of Animal Science, 19*, 1212 – 1216.

Zhou, K., Su, L. & Yu, L. L. (2004). Phytochemicals and antioxidant properties in wheat bran. *Journal of Agricultural and Food Chemistry, 52*, 6108 – 14.

CHAPTER 6:

The effect of dietary quercetin supplementation and sex on the fatty acid profile of rabbit caecotrophe material, dissectible fat, hindleg meat and loin meat

Abstract

This study examined the effects of dietary quercetin on the fatty acid (FA) profile of rabbit caecotrophes, dissectible fat, loin (LTL) meat and hindleg (HL) meat. Sixteen male and sixteen female New Zealand White rabbits were fed either a control or quercetin-supplemented (2 g/kg quercetin dihydrate) diet from 5 to 12 weeks old (slaughter). Caecotrophes were collected from the gut, and the dissectible fat, LTL, and deboned HL were sampled. Lipids were extracted and transmethylated, whereafter they were identified and quantified using GC-FID. Quercetin-supplementation had the greatest effect on the LTL FAs, decreasing the n-6:n-3 ratio, and thus improving the nutritional quality of the meat. Sex mostly affected the caecotrophe FAs, and did not impact the meat's nutritional value, and while the FA profile of the LTL and HL differed, both aligned to nutritional recommendations. The caecotrophe FA profile was typical of the effects of microbial biohydrogenation, but the effect of this on the carcass FA seemed limited.

6.1 Introduction

Numerous studies have shown links between the consumption of saturated fatty acids (SFA) and the risk of developing coronary heart disease (CHD). Furthermore, many health benefits have been found to be associated with the consumption of polyunsaturated fatty acids (PUFA), including a reduction of the total:high-density lipoprotein cholesterol ratio, and thus the risk of CHD, and possible improvements in insulin resistance and systemic inflammation (Mozaffarian, Micha & Wallace, 2010). This has led to recommendations that the PUFA:SFA ratio of the human diet should be at least 0.4 (Wood *et al*., 2004).

While meat products from some species, such as rabbits and chickens, naturally have PUFA:SFA ratios well above this recommended value (0.84 and 0.83, respectively; Dalle Zotte & Szendrő, 2011), the nature of the PUFA present is also a concern. Studies have shown that a high n-6:n-3 PUFA ratio in the diet can increase the risk of a number of diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune conditions (Simopoulos, 2004). This is due to the role n-6 PUFA play as precursors of proinflammatory eicosanoids in the body (Patterson, Wall, Fitzgerald, Ross & Stanton, 2012). Furthermore, the intake of the essential fatty acids (FA) linoleic acid (C18:2-6) and α-linolenic acid (C18:3n-3), is also important, as both are involved in a wide range of biological functions. There is consequently considerable interest in the alteration of the FA composition of meat, in terms of increasing the total proportion of PUFA, decreasing the n-6:n-3 ratio (recommended range of 1:1 – 4:1), and adjusting the levels of the essential FA (Simopoulos, 2004).

The FA composition of meat from non-ruminants, such as poultry and rabbits, can relatively easily be manipulated through the manipulation of the FA profile of the feed provided; for example, through the supplementation of fish oils, vegetable oils or whole oilseeds, or the incorporation of fresh pasture in the diet (Dalle Zotte & Szendrő, 2011; Wood *et al*., 1999). However, an increase in the PUFA content of the meat also increases the risk of oxidation during storage, making it necessary to also supplement antioxidants in order to maintain meat quality.

Flavonoids, as bioactive compounds with demonstrated antioxidant activity, may have potential to ameliorate the detrimental effects of increased PUFA levels on meat quality. However, their many other properties, including antimicrobial activities and interactions with a number of enzyme and hormone systems, suggest that their effects may be more extensive than just that of an antioxidant (Havsteen, 2002). A number of animal studies have demonstrated that the dietary inclusion of various flavonoids directly impacts the FA composition of meat from poultry (Kamboh & Zhuh, 2013; Sohaib, Butt, Shabbir & Shahid, 2015), ruminants (Andrés *et al*., 2014; Tan *et al*., 2011) and rabbits (Simitzis *et al*., 2014), even in the absence of concomitant FA supplementation. Proposed mechanisms for this effect have included flavonoids protecting the PUFA from oxidation or biohydrogenation (through the manipulation of the rumen or caecal microbial population), or having a direct effect on endogenous lipid metabolism, through interactions with the enzymes involved in desaturation and elongation (Jenkins & Atwal, 1995; Kamboh & Zhuh, 2013; Tan *et al*., 2011). However, the results have been conflicting and species-specific in many cases, and research on rabbits, in particular, is very limited.

This study therefore aimed to determine the effects of quercetin supplementation on the FA profile of the loin and hindleg meat, as well as the dissectible fat, of growing New Zealand White rabbits. In addition, in order to provide some clarity on the specific activity of quercetin that caused any observed differences, the effects on the FA profile of the caecotrophe material was also determined.

6.2 Materials and methods

Ethical clearance for this study was obtained from the Stellenbosch University Animal Care and Use Committee (protocol number SU-ACUD16-00094).

6.2.1 Growth, slaughter and sample collection

Full descriptions of the dietary treatments, rearing conditions and slaughter protocols can be found in Chapter 3 and Chapter 5. Briefly, 32 individually-caged New Zealand White rabbits of both sexes (16 males, 16 females) were reared from 5 weeks (weaning) to 12 weeks (slaughter) of age. During this period they were fed either a control diet (Ctrl) or a diet supplemented with 2 g quercetin/kg feed (Qrc) *ad libitum*, with the sexes being evenly distributed between the two treatment groups. Representative feed samples for the determination of the FA profile were collected throughout the growth period and stored at -20 °C until analysis.

At slaughter, the gastrointestinal tracts were collected, and caecotrophe samples were taken from the stomach or colon, as described by Leiber *et al*. (2008). These samples were vacuum-packed and stored frozen (-20 °C) until analysis. As no apparent caecotrophes were present for two of the rabbits (one Ctrl male, one Qrc male), a total of 30 caecotrophe samples were collected.

The whole carcasses were chilled at 3.2 ± 0.4 °C for 24 hours, after which the dissectible fat content (perirenal, inguinal and interscapular depots) was removed, the right *longissimus thoracis et lumborum* (LTL) muscles were excised and the left hindlegs (HL) were removed and deboned. Samples of each (fat, LTL and HL, with the latter including all musculature) were homogenised, vacuum-packed and stored at -20 °C until analysis.

6.2.2 Lipid extraction and transmethylation

Meat (1 g) and fat (0.5 g) samples were extracted in 50 ml 2:1 (v/v) chloroform:methanol solution (Folch, Lees & Sloane-Stanley, 1957), which contained 0.01 % butylated hydroxytoluene as an antioxidant. The samples were homogenised for 30 seconds at 10 000 rpm using a polytron mixer (IKA® T18 digital ULTRA TURRAX®), after which they were passed through an extraction funnel fitted with a glass microfibre filter paper (Whatman, GF/A, diameter 47 mm, Cat no. 1820-047). A heptadecanoic acid internal standard (catalogue number H3500, Sigma-Aldrich, Gauteng, South Africa) was used to allow the quantification of the individual FA, with 0.5 ml of the 10 mg/ml solution being added to each sample prior to homogenisation.

A 250 µl aliquot of the lipid extract solution was evaporated in a water bath (45 °C, *ca.* 10 minutes) and subsequently transmethylated at 70 °C for 2 h using 2 ml of a 19:1 (v/v) methanol:sulphuric acid solution as the transmethylating agent. After allowing the resultant solution to cool to room temperature, the FA methyl esters (FAMEs) were extracted with water and hexane. This entailed adding 1 ml distilled water and 2 ml hexane to the

transmethylated solution, mixing thoroughly, allowing to settle and transferring the top phase (FAME-containing hexane) to a clean glass tube. The hexane extract was dried in a water bath at 45 °C under a flow of nitrogen, whereafter the dried FAME sample was resuspended in 100 µl hexane and transferred to a gas chromatography (GC) vial for analysis.

Feed and caecotrophe samples were lyophilised (CHRIST, model ALPHA 1-4/LDC-1M) for a minimum of 72 hours (until a constant pressure of 0.037 – 0.04 mbar was attained, indicating complete drying) and finely ground prior to extraction. These samples (0.5 g) were extracted and transmethylated by combining with hexane (2.5 ml for feed samples, 5 ml for caecotrophe samples) and 1 ml 2.5 % (v/v) sulphuric acid in methanol and incubating for 1 h at 80 °C. A 1 mg/ml (in hexane) heptadecanoic acid internal standard (catalogue number H3500, Sigma-Aldrich, Gauteng, South Africa) was added (100 µl for feed, 200 µl for caecotrophes) prior to incubation. After allowing the samples to cool to room temperature, 2 ml 20 % (w/v) sodium chloride solution was added, and the tubes were vortexed and then centrifuged to facilitate the separation of the solvent layers. An aliquot of the top phase (FAME-containing hexane) was transferred to a GC vial for analysis.

6.2.3 Fatty acid GC-FID analysis

The FAMEs were analysed using a Thermo TRACE 1300 series gas-chromatograph (Thermo Electron Corporation, Milan, Italy) equipped with a flame-ionisation detector (GC-FID) and coupled to a CTC Analytics PAL autosampler. Separation was performed using a polar ZB-WAX (30 m, 0.25 mm ID, 0.25 µm film thickness) Zebron 7HG-G007-11 capillary column. A sample volume of 1 µl was injected in a 5:1 split ratio and helium was used as the carrier gas at a flow rate of 1 ml/minute. The injector temperature was maintained at 250 °C. The oven temperature was programmed as follows: held at 50 °C for 2 minutes, then ramped up to 180 °C at a rate of 25 °C/minute for 5 minutes, followed by a ramping rate of 3 °C/minute for 2 minute until 260 °C.

The FAMEs in each sample were identified by comparing the retention times to those of a standard FAME mixture (Supelco™ 37 Component FAME mix, Cat no. CRM47885, Supelco, USA), and quantified by comparing the integrated areas to that of the internal standard. The quantitative results are expressed as a percentage of the total FAME content, and as the *cis* and *trans* isomers of C18:1n-9 and C18:2n-6 co-eluted in the majority of the samples, they are reported as single combined values.

6.2.4 Fatty acid index calculation

A number of indexes were calculated to provide measures of the nutritional value of the FA in each sample. These included simple parameters such as the total saturated FA (ΣSFA), total monounsaturated FA (ΣMUFA) and total polyunsaturated FA (ΣPUFA) content, as well as the total n-6 PUFA (Σn-6), total n-3 PUFA (Σn-3), PUFA:SFA ratio and n-6:n-3 ratio. In addition, the ratio of hypocholesterolemic FA to hypercholesterolemic FA (h/H) was calculated as recommended by Santos-Silva *et al*. (2002):

$$h/H = [(C18:1n-9 + C18:2n-6 + C18:3n-6 + C18:3n-3 + C20:3n-6 + C20:4n-6) / (C14:0 + C16:0)]$$

It should be noted that FA not indicated in the h/H formula above were not detected in the samples.

The atherogenic index (AI) and thrombogenic index (TI) were also calculated, as recommended by Ulbricht and Southgate (1991):

$$AI = [C12:0 + (4 \times C14:0) + C16:0] / [(\Sigma PUFA) + (\Sigma MUFA)]$$

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

6.2.5 Statistical analysis

As a result of analytical problems, several samples from each sample type were removed from the data set prior to statistical analysis. The replicates per treatment group are therefore indicated in each table of results (Tables 6.1 – 6.5).

Statistical analyses were performed using Statistica version 13 software. Normal probability plots of the raw residuals were used to assess the normality of the distribution and identify outliers, and when outliers were found to be present the data was winsorised, with the value of the outlier data point being reduced to the mean plus three-times the standard deviation. Variables for which the winsorised means are reported are indicated in the tabulated results. Statistical comparisons of the fixed effects (sex, diet and sample type, and their interactions) were performed using the restricted maximum likelihood (REML) estimation method in the variance, estimation and precision (VEPAC) software package of Statistica. Rabbit number was included as a random effect. Fisher's least significant difference (LSD) *post hoc* test was used to compare individual means for significant interactions. Sample type was included as a fixed effect in order to allow the comparison of treatment groups across sample types, as seen in Table 6.1; however, the results for the diet and sex comparisons are reported in tables per sample type (Tables 6.2 – 6.5), to ease interpretation.

In addition to the basic comparisons of means, principle component analysis (PCA) was performed using XLStat software (Version 2018, Addinsoft, New York, USA), in order to visualise the relationships between the treatments groups and variables, based on a Pearson's correlation matrix.

Main effects and interactions with $P \leq 0.05$ were considered significant, whereas those with $P \leq 0.10$ are reported as trends. Values are reported as LSMeans ± standard errors of the means (SEM).

6.3 Results

The PCA biplot generated for all the variables and sample types, including the feeds (Figure 6.1) clearly demonstrated a separation both between the different sample types and between them and the FAME profile of the feeds. In addition, it can be seen that the caecotrophe content associated most strongly with the ΣSFA, and thus the atherogenic and thrombogenic indexes. The caecotrophes also associated with a higher n-6:n-3 ratio. The position of the data points for the feed samples indicated a contrasting profile, associating with the ΣPUFA, PUFA:SFA ratio, Σn-6 and Σn-3 contents and thus the h/H FAME ratio. The fat and meat samples all seemed to demonstrate greater similarity to the FAME profile of the feed than that of the caecotrophes, with this being most obvious for the dissectible fat. While both the fat and caecotrophe samples clustered relatively tightly, suggesting little variation between the samples, the HL and LTL meat samples were more widely distributed, indicating less

intra-sample type consistency. The distributions of the two meat sample types overlapped to some extent, although the HL samples associated more closely with the fat samples than the LTL samples did. The ΣMUFA content associated most strongly with the HL samples, while a number of PUFA and MUFA associated with the LTL samples, despite the lack of a close association with the ΣPUFA content.

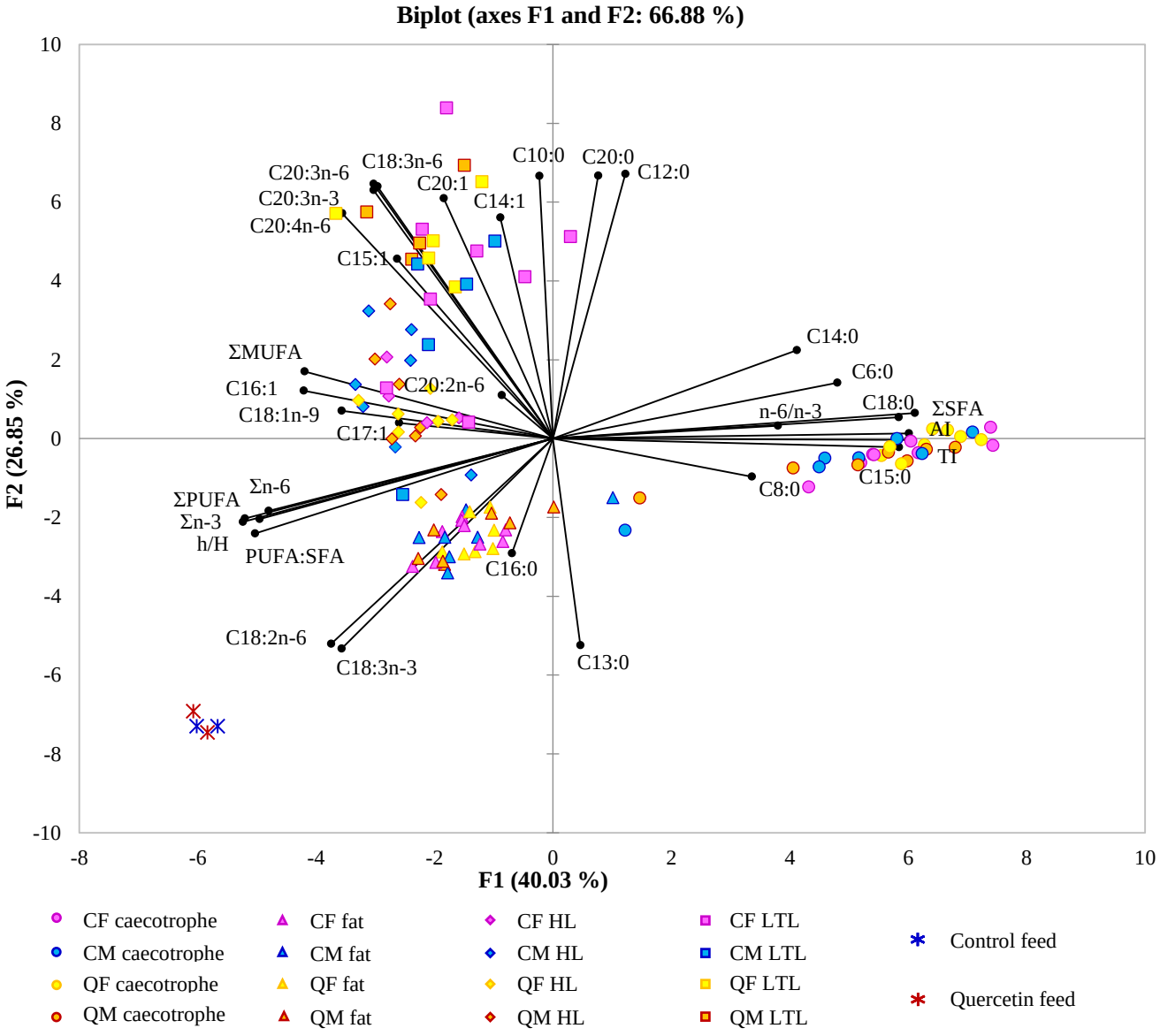

Figure 6.1 Principle component analysis (PCA) biplot of New Zealand White rabbit M. *longissimus thoracis et lumborum* (LTL) and hindleg (HL) meat, dissectible fat (fat) and caecotrophe fatty acid (FA) composition, classified according to diet (C: control, Q: quercetin supplemented at 2 g/kg feed) and sex (M: male, F: female). Black data points with labels indicate individual FA and calculated indexes: ΣPUFA: total polyunsaturated FA, ΣMUFA: total monounsaturated FA, ΣSFA: total saturated FA, Σn-6: total n-6 PUFA, Σn-3: total n-3 PUFA, h/H: hypocholesterolemic/hypercholesterolemic FA, AI: atherogenic index, TI: thrombogenic index.

Considering the distribution of the observations in the PCA biplot, the differences ($P < 0.001$) between the sample types (caecotrophes, fat, HL meat and LTL meat) in all the FA and calculated indexes were not surprising (Table 6.1).

Table 6.1
The fatty acid (FA) composition (% total FAME) of the control (Ctrl) and quercetin-supplemented (Qrc) feeds, as well as the caecotrophe material, dissectible fat, deboned hindleg (HL) meat and M. *longissimus thoracis et lumborum* (LTL) meat of New Zealand White rabbits (LSMeans ± SEM).

	Ctrl feed	Qrc feed	Caecotrophes	Fat	HL meat	LTL meat
N	2	2	30	30	25	22
C6:0	0.22	0.21	$4.20^{a} \pm 0.369$	$0.13^{c} \pm 0.010$	$0.69^{c} \pm 0.064$	$1.49^{b} \pm 0.090$
C8:0*	0.11	0.13	$0.36^{a} \pm 0.014$	$0.18^{b} \pm 0.017$	n.d	n.d
C10:0	0.35	0.33	$0.76^{b} \pm 0.043$	$0.24^{c} \pm 0.014$	$0.77^{b} \pm 0.065$	$1.83^{a} \pm 0.124$
C12:0	0.17	0.16	$1.18^{b} \pm 0.044$	$0.42^{d} \pm 0.027$	$0.86^{c} \pm 0.067$	$1.96^{a} \pm 0.121$
C13:0	1.80	1.45	$0.37^{a} \pm 0.012$	$0.36^{a} \pm 0.029$	n.d	n.d
C14:0	0.32	0.33	$2.78^{a} \pm 0.088$	$2.39^{b} \pm 0.062$	$1.52^{c} \pm 0.069$	$2.42^{b} \pm 0.106$
C15:0	0.21	0.22	$4.13^{a} \pm 0.159$	$0.55^{b} \pm 0.015$	$0.46^{b} \pm 0.019$	$0.64^{b} \pm 0.025$
C16:0	25.1	25.3	$27.2^{c} \pm 0.357$	$32.4^{a} \pm 0.331$	$28.6^{b} \pm 0.451$	$26.3^{c} \pm 0.701$
C18:0	3.07	3.13	$26.1^{a} \pm 0.777$	$7.44^{c} \pm 0.207$	$9.34^{b} \pm 0.209$	$9.45^{b} \pm 0.221$
C20:0	0.18	0.21	$0.47^{b} \pm 0.014$	$0.11^{c} \pm 0.005$	$0.40^{b} \pm 0.033$	$0.78^{a} \pm 0.055$
C14:1	n.d	n.d	$0.58^{c} \pm 0.028$	$0.46^{c} \pm 0.027$	$0.91^{b} \pm 0.040$	$1.09^{a} \pm 0.099$
C15:1	n.d	n.d	n.d	$0.11^{c} \pm 0.021$	$0.32^{b} \pm 0.035$	$0.50^{a} \pm 0.077$
C16:1	n.d	n.d	n.d	$5.76^{a} \pm 0.274$	$5.68^{a} \pm 0.318$	$4.97^{b} \pm 0.301$
C17:1	0.10	0.11	$0.12^{c} \pm 0.030$	$0.37^{b} \pm 0.007$	$0.59^{a} \pm 0.035$	$0.27^{b} \pm 0.087$
C18:1n-9	12.9	12.6	$14.0^{d} \pm 0.263$	$18.3^{b} \pm 0.250$	$20.5^{a} \pm 0.423$	$17.2^{c} \pm 0.507$
C20:1	0.54	0.51	$0.45^{c} \pm 0.014$	$0.40^{c} \pm 0.014$	$0.59^{b} \pm 0.027$	$0.85^{a} \pm 0.050$
C18:2n-6	35.7	35.1	$12.7^{c} \pm 0.436$	$19.8^{a} \pm 0.481$	$16.4^{b} \pm 0.257$	$13.5^{c} \pm 0.520$
C18:3n-6	n.d	n.d	n.d	$0.44^{c} \pm 0.080$	$1.57^{b} \pm 0.123$	$2.94^{a} \pm 0.244$
C18:3n-3	18.8	19.9	$4.28^{c} \pm 0.362$	$9.02^{a} \pm 0.193$	$6.21^{b} \pm 0.119$	$4.56^{c} \pm 0.245$
C20:2n-6*	0.36	0.40	$0.27^{d} \pm 0.017$	$0.37^{c} \pm 0.012$	$0.65^{b} \pm 0.046$	$0.80^{a} \pm 0.057$
C20:3n-6	n.d	n.d	n.d	$0.15^{c} \pm 0.006$	$0.70^{b} \pm 0.050$	$1.23^{a} \pm 0.088$
C20:3n-3	n.d	n.d	n.d	$0.30^{c} \pm 0.009$	$2.45^{b} \pm 0.163$	$4.20^{a} \pm 0.261$
C20:4n-6	n.d	n.d	n.d	$0.26^{c} \pm 0.009$	$0.49^{b} \pm 0.030$	$0.80^{a} \pm 0.068$
ΣSFA	31.6	31.4	$67.6^{a} \pm 0.813$	$44.2^{bc} \pm 0.441$	$42.8^{c} \pm 0.298$	$45.0^{b} \pm 0.862$
ΣMUFA	13.6	13.2	$15.2^{c} \pm 0.275$	$25.4^{b} \pm 0.448$	$28.5^{a} \pm 0.582$	$24.9^{b} \pm 0.734$
ΣPUFA	54.9	55.4	$17.2^{b} \pm 0.709$	$30.3^{a} \pm 0.681$	$28.7^{a} \pm 0.568$	$30.1^{a} \pm 1.263$
PUFA:SFA*	1.74	1.76	$0.26^{b} \pm 0.015$	$0.69^{a} \pm 0.020$	$0.67^{a} \pm 0.015$	$0.67^{a} \pm 0.038$
Σn-6*	36.1	35.5	$12.9^{b} \pm 0.443$	$21.0^{a} \pm 0.489$	$20.1^{a} \pm 0.405$	$20.8^{a} \pm 0.962$
Σn-3	18.8	19.9	$4.28^{b} \pm 0.362$	$9.32^{a} \pm 0.199$	$8.65^{a} \pm 0.185$	$8.82^{a} \pm 0.302$
n-6:n-3*	1.92	1.79	$3.23^{a} \pm 0.087$	$2.26^{b} \pm 0.015$	$2.30^{b} \pm 0.029$	$2.37^{b} \pm 0.132$
h/H	2.65	2.64	$1.04^{c} \pm 0.034$	$1.39^{b} \pm 0.026$	$1.53^{a} \pm 0.032$	$1.42^{b} \pm 0.046$
Atherogenic index	0.39	0.39	$1.24^{a} \pm 0.035$	$0.76^{b} \pm 0.016$	$0.62^{c} \pm 0.012$	$0.70^{bc} \pm 0.025$
Thrombogenic index	0.35	0.34	$2.17^{a} \pm 0.091$	$0.82^{b} \pm 0.021$	$0.78^{b} \pm 0.015$	$0.78^{b} \pm 0.024$

ΣSFA: total saturated FA content; ΣMUFA: total monounsaturated FA content; ΣPUFA: total polyunsaturated FA content; Σn-6: total n-6 PUFA; Σn-3: total n-3 PUFA; h/H: hypocholesterolemic/hypercholesterolemic FA ratio; n.d: not detected
abcd Means in the same row with different superscript letters differ ($P \leq 0.05$)
* Winsorised means and standard errors of the mean (SEM) reported

The ΣSFA content was the highest for the caecotrophes (67.6 ± 0.81 %), being nearly double that of the meat and fat samples ($P < 0.001$). The fat samples, in turn, did not differ from either meat sample, while the HL meat had a lower ΣSFA content than the LTL meat ($P = 0.024$). The opposite pattern was found for the ΣMUFA content, with the caecotrophes (15.2 ± 0.28 %) containing lower levels ($P < 0.001$) than the other samples, the LTL meat and fat not differing, and the HL meat containing the highest proportions (28.5 ± 0.58 %). In contrast, the ΣPUFA, while again lowest for the caecotrophes (17.2 ± 0.71 %), did not differ between the carcass samples. The FAME profile of the feed also concurred with the results of the PCA, containing predominantly ΣPUFA (54.9 – 55.4 %), and considerably lower levels of ΣMUFA and ΣSFA (13.2 – 13.6 %, 31.4 – 31.6 %, respectively).

The most prevalent FAMEs in the fat, HL meat and LTL meat were the same, namely C16:0, C18:1n-9 and C18:2n-6, with C16:0 making the largest contribution (26.3 – 32.4 %). The levels of these FAMEs differed between the sample types. For C16:0 and C18:2n-6 fat had the highest proportion and LTL meat the lowest, while for C18:1n-9 the order was HL > fat > LTL. In the caecotrophes, C16:0 and C18:0 were present at similar, high levels, followed by C18:1n-9. The feed samples contained relatively similar levels of C16:0 to those found in the meat and caecotrophe samples, but lower levels of C18:0 and C18:1n-9, and nearly double the proportion of C18:2n-6. The C18:3n-3 level was also much higher in the feed than in the other analysed samples.

Dietary treatment had no effect on the FAME profile of the caecotrophes or the HL meat samples. However, in the fat samples the proportion of C13:0 was higher in the Ctrl than Qrc rabbits ($P < 0.001$). The most extensive dietary effects were seen in the LTL meat samples. Qrc rabbits had higher levels of C18:3n-6 ($P = 0.001$), C20:3n-6 ($P < 0.001$), C20:3n-3 ($P = 0.001$) and C20:4n-6 ($P = 0.005$), but lower levels of C20:2n-6 ($P = 0.004$). As C20:3n-3 contributed the largest proportion of these PUFA, Qrc rabbits also had a lower n-6:n-3 ratio ($P < 0.001$) than Ctrl rabbits. Other FAMEs present at higher levels in the LTL meat of supplemented rabbits included C20:0 ($P = 0.003$), C15:1 ($P = 0.002$) and C20:1 ($P = 0.013$). These differences, along with tendencies for higher proportions of C10:0 ($P = 0.06$), C12:0 ($P = 0.07$) and C14:1 ($P = 0.09$) in Qrc rabbits, also resulted in tendencies for the Qrc rabbits to have higher ΣMUFA ($P = 0.09$) and lower ΣPUFA (0.08).

The greatest impact of sex was seen in the caecotrophes, with C15:0 ($P = 0.005$) and C18:0 ($P < 0.001$) having higher levels, and C18:2n-6 ($P = 0.05$) and C18:3n-3 lower levels ($P = 0.01$), in female than male rabbits. The TI ($P < 0.001$) and AI ($P = 0.05$) were also higher in females, due to the higher ΣSFA content ($P = 0.005$) and lower PUFA content ($P = 0.05$). Females also had a lower Σn-3 content ($P = 0.02$) and thus higher n-6:n-3 ratio ($P = 0.04$). In the dissectible fat samples, only a single FAME was effected by sex, with C16:1 being higher in females ($P = 0.008$). While this difference was only statistically significant for the fat samples, similar differences also tended to be present in both HL ($P = 0.095$) and LTL ($P = 0.090$) samples, resulting in a significant difference between the sexes across the samples ($P = 0.035$), and a lack of sex-sample type interaction ($P = 0.115$). A similar effect was seen for C18:1n-9. While a significant difference between the sexes was only seen in the LTL meat samples ($P = 0.044$), the higher levels in samples from male rabbits also tended towards significance in HL meat ($P = 0.051$), as well as being present, if not significant, in fat and caecotrophe samples, resulting in an effect of

sex across the sample types (P = 0.013). Male rabbits also had higher levels of C20:1 (P = 0.050) and C14:1 (P = 0.04) in HL and LTL meat samples, respectively.

6.4 Discussion

6.4.1 Effect of sample type

The results of both the PCA and standard statistical analyses clearly demonstrated that the sample types investigated had distinct FA profiles (Figure 6.1). This was most obvious for the comparison of the caecotrophes to the carcass components, but even within the fat and meat types there were notable, and unexpected, differences.

The two meat cuts differed in their ΣSFA and ΣMUFA contents, as well as in their h/H index, with this involving a higher ΣSFA and lower ΣMUFA content, and lower h/H ratio for the LTL than the HL (Table 6.1). This was in contrast with the findings of Papadomichelakis, Karagiannidou, Anastasopoulos and Fegeros (2010b), who reported that the *longissimus lumborum* muscle had a lower ΣSFA content and higher PUFA:SFA ratio than the *biceps femoris* muscle, and is opposite to what is normally reported in literature. Furthermore, it was unexpected considering the relative total lipid contents of these two portions, as discussed in Chapter 5 (LTL: 2.90 %, HL: 6.44 %). Previous research has found that the proportion of SFA tends to increase with an increase in fatness, as a result of the growing contribution of triacylglycerols (at the expense of phospholipids) to the total lipid content (De Smet, Raes & Demeyer, 2004), and this is supported by the high contents of both total lipids and ΣSFA of the meat samples in this study relative to previous literature (Hernández, Cesari & Blasco, 2008; Hernández & Dalle Zotte, 2010). The relatively high ΣSFA content of the LTL could have been related to the sampling and processing methods used, and confirmation of these differences is thus needed.

The relationship between the HL meat and the dissectible fat FAME profile (Table 6.1) also contrasts with literature, with the fat containing a greater proportion of ΣMUFA and lower proportion of ΣPUFA than the HL meat, the opposite to what was reported by Hernández *et al*. (2008). It is possible that the discrepancy in this case may be linked to the use of the combined dissectible fat, rather than the pure perirenal depot. Research on the FA composition of the fat depots of sheep, cattle and pigs has shown that the subcutaneous fat tends to be less saturated than the internal depots, with the degree of saturation of the internal depot sites also differing (Banskalieva, Sahlu & Goetsch, 2000). Unfortunately this does not seem to have been investigated in rabbits.

It is also interesting to note that the unusual relationship in the ΣSFA content between the fat, HL meat and LTL meat (Table 6.1) was not present for the most prevalent SFA, C16:0, but rather appeared to be driven by the relatively minor FAME, C14:0. The C16:0 content decreased from fat to HL meat to LTL meat, as would be expected, whereas C20:0, as well as the short-chain C6:0, C10:0 and C12:0, were highest in the LTL and lowest in the fat, with the HL meat having an intermediate content. The presence and abundance of these shorter-chain FA is highly unusual for meat samples. The lower ΣMUFA content in the LTL was due to lower levels of C16:1 and C18:1. Further research is needed to confirm these findings, and, if confirmed, provide a possible explanation. However, it should be emphasized that despite the differences between the two cuts of meat, both cuts still had PUFA:SFA ratios higher than the recommended minimum of 0.4 (Wood *et al*., 2004), and n-6:n-3 values well

within the recommended range of 1:1 – 4:1 (Simopoulos, 2004). The relatively high PUFA content agreed with values provided in previous literature, whereas the n-6:n-3 ratios were considerably lower than previously reported (Dalle Zotte & Szendrő, 2011). This may have been linked to the composition of the diet, which contained a large proportion of C18:3n-3, and/or to the high level of fatness of the rabbits used (De Smet *et al*., 2004). De Smet *et al*. (2004) suggested that meat with a higher fat content tended to have a lower C18:2n-6:C18:3n-3 ratio than leaner meat.

Table 6.2
The effects of dietary quercetin supplementation at 2 g/kg feed and sex (male and female) on the fatty acid (FA) composition (% total FAME) of rabbit caecotrophe material (LSMeans ± SEM).

	Diet			Sex		
	Ctrl	**Qrc**	***P*-value**	**Male**	**Female**	***P*-value**
N	15	15		14	16	
C6:0	4.10 ± 0.565	4.31 ± 0.494	0.62	4.25 ± 0.523	4.16 ± 0.536	0.83
C8:0*	0.37 ± 0.017	0.36 ± 0.022	0.64	0.35 ± 0.022	0.38 ± 0.017	0.31
C10:0	0.73 ± 0.052	0.79 ± 0.069	0.63	0.76 ± 0.053	0.77 ± 0.067	0.93
C12:0	1.15 ± 0.072	1.21 ± 0.051	0.68	1.18 ± 0.066	1.18 ± 0.060	0.97
C13:0	0.37 ± 0.022	0.37 ± 0.012	0.97	0.38 ± 0.021	0.36 ± 0.014	0.63
C14:0	2.74 ± 0.140	2.81 ± 0.112	0.67	2.76 ± 0.141	2.80 ± 0.115	0.80
C15:0	4.04 ± 0.219	4.23 ± 0.235	0.26	3.89 ± 0.274	4.38 ± 0.164	0.005
C16:0	27.2 ± 0.592	27.3 ± 0.421	0.97	27.5 ± 0.563	26.9 ± 0.459	0.50
C18:0	26.1 ± 0.936	26.2 ± 1.275	0.97	24.3 ± 1.155	27.9 ± 0.878	<0.001
C20:0	0.47 ± 0.019	0.47 ± 0.020	0.90	0.45 ± 0.020	0.49 ± 0.018	0.49
C14:1	0.56 ± 0.045	0.60 ± 0.033	0.66	0.61 ± 0.033	0.56 ± 0.043	0.56
C17:1	0.12 ± 0.046	0.11 ± 0.039	0.89	0.11 ± 0.045	0.12 ± 0.041	0.93
C18:1n-9	14.1 ± 0.326	13.9 ± 0.421	0.71	14.2 ± 0.424	13.8 ± 0.331	0.49
C20:1	0.46 ± 0.018	0.44 ± 0.022	0.81	0.45 ± 0.022	0.45 ± 0.018	0.92
C18:2n-6	12.5 ± 0.438	12.8 ± 0.769	0.77	13.5 ± 0.749	11.8 ± 0.409	0.05
C18:3n-3	4.56 ± 0.673	4.01 ± 0.281	0.27	4.94 ± 0.715	3.63 ± 0.183	0.01
C20:2n-6*	0.30 ± 0.022	0.25 ± 0.024	0.36	0.28 ± 0.021	0.27 ± 0.026	0.83
ΣSFA	67.4 ± 1.051	67.9 ± 1.273	0.66	65.9 ± 1.444	69.4 ± 0.654	0.005
ΣMUFA	15.3 ± 0.335	15.1 ± 0.445	0.83	15.4 ± 0.449	14.9 ± 0.340	0.61
ΣPUFA	17.4 ± 0.983	17.0 ± 1.056	0.80	18.7 ± 1.249	15.7 ± 0.586	0.05
PUFA:SFA*	0.26 ± 0.020	0.26 ± 0.024	0.84	0.29 ± 0.028	0.23 ± 0.010	0.14
Σn-6*	12.9 ± 0.447	13.0 ± 0.781	0.87	13.8 ± 0.759	12.1 ± 0.419	0.12
Σn-3	4.57 ± 0.673	3.98 ± 0.281	0.27	4.92 ± 0.715	3.63 ± 0.183	0.02
n-6:n-3*	3.14 ± 0.161	3.31 ± 0.068	0.22	3.09 ± 0.161	3.37 ± 0.075	0.04
h/H	1.05 ± 0.055	1.02 ± 0.040	0.65	1.09 ± 0.066	0.98 ± 0.022	0.10
Atherogenic index	1.23 ± 0.054	1.26 ± 0.045	0.58	1.20 ± 0.063	1.29 ± 0.032	0.05
Thrombogenic index	2.14 ± 0.136	2.21 ± 0.126	0.44	1.97 ± 0.139	2.38 ± 0.101	<0.001

Ctrl: control; Qrc: quercetin-supplemented; ΣSFA: total saturated FA content; ΣMUFA: total monounsaturated FA content; ΣPUFA: total polyunsaturated FA content; Σn-6: total n-6 PUFA; Σn-3: total n-3 PUFA; h/H: hypocholesterolemic/hypercholesterolemic FA ratio.
* Winsorised means and standard errors of the means (SEM) reported

Table 6.3
The effects of dietary quercetin supplementation at 2 g/kg feed and sex (male and female) on the fatty acid (FA) composition (% total FAME) of rabbit dissectible fat (LSMeans ± SEM).

	Diet			Sex		
	Ctrl	**Qrc**	***P*-value**	**Male**	**Female**	***P*-value**
N	15	15		14	16	
C6:0	0.14 ± 0.014	0.12 ± 0.014	0.97	0.14 ± 0.014	0.13 ± 0.014	0.98
C8:0*	0.18 ± 0.026	0.17 ± 0.024	0.74	0.18 ± 0.014	0.17 ± 0.031	0.82
C10:0	0.27 ± 0.025	0.21 ± 0.011	0.63	0.25 ± 0.019	0.24 ± 0.021	0.93
C12:0	0.42 ± 0.044	0.42 ± 0.033	0.99	0.40 ± 0.022	0.43 ± 0.047	0.79
C13:0	0.42 ± 0.049	0.31 ± 0.024	<0.001	0.38 ± 0.038	0.35 ± 0.043	0.42
C14:0	2.39 ± 0.103	2.39 ± 0.072	0.98	2.43 ± 0.090	2.34 ± 0.085	0.57
C15:0	0.57 ± 0.021	0.53 ± 0.021	0.82	0.56 ± 0.022	0.54 ± 0.020	0.89
C16:0	32.6 ± 0.586	32.2 ± 0.325	0.69	32.7 ± 0.662	32.0 ± 0.227	0.42
C18:0	7.06 ± 0.228	7.82 ± 0.321	0.38	7.22 ± 0.370	7.66 ± 0.211	0.61
C20:0	0.12 ± 0.007	0.11 ± 0.007	0.82	0.12 ± 0.008	0.11 ± 0.007	0.91
C14:1	0.47 ± 0.042	0.45 ± 0.034	0.85	0.42 ± 0.034	0.50 ± 0.038	0.40
C15:1	0.12 ± 0.031	0.11 ± 0.028	0.85	0.11 ± 0.029	0.12 ± 0.030	0.11
C16:1	5.69 ± 0.403	5.83 ± 0.384	0.76	5.14 ± 0.343	6.38 ± 0.370	0.008
C17:1	0.38 ± 0.010	0.36 ± 0.009	0.81	0.37 ± 0.009	0.36 ± 0.011	0.97
C18:1n-9	18.3 ± 0.368	18.4 ± 0.350	0.97	18.9 ± 0.350	17.8 ± 0.313	0.13
C20:1	0.38 ± 0.015	0.41 ± 0.023	0.48	0.44 ± 0.019	0.36 ± 0.016	0.10
C18:2n-6	19.8 ± 0.739	19.8 ± 0.641	0.95	19.8 ± 0.887	19.9 ± 0.490	0.88
C18:3n-6	0.52 ± 0.133	0.35 ± 0.093	0.44	0.43 ± 0.113	0.45 ± 0.113	0.91
C18:3n-3	9.06 ± 0.315	8.98 ± 0.233	0.87	8.97 ± 0.352	9.07 ± 0.201	0.84
C20:2n-6*	0.37 ± 0.016	0.37 ± 0.018	0.97	0.40 ± 0.018	0.35 ± 0.014	0.42
C20:3n-6	0.17 ± 0.009	0.14 ± 0.009	0.76	0.16 ± 0.009	0.15 ± 0.009	0.97
C20:3n-3	0.33 ± 0.013	0.27 ± 0.012	0.82	0.29 ± 0.013	0.31 ± 0.011	0.92
C20:4n-6	0.26 ± 0.014	0.26 ± 0.012	0.95	0.29 ± 0.012	0.23 ± 0.010	0.35
ΣSFA	44.1 ± 0.716	44.3 ± 0.539	0.90	44.4 ± 0.870	44.0 ± 0.358	0.75
ΣMUFA	25.4 ± 0.691	25.5 ± 0.594	0.89	25.3 ± 0.662	25.6 ± 0.628	0.80
ΣPUFA	30.5 ± 1.070	30.2 ± 0.881	0.88	30.2 ± 1.275	30.5 ± 0.670	0.88
PUFA:SFA*	0.70 ± 0.031	0.69 ± 0.031	0.82	0.69 ± 0.038	0.69 ± 0.018	0.93
Σn-6*	21.1 ± 0.757	21.0 ± 0.647	0.90	21.0 ± 0.918	21.1 ± 0.478	0.94
Σn-3	9.39 ± 0.324	9.25 ± 0.240	0.80	9.25 ± 0.362	9.38 ± 0.208	0.80
n-6:n-3*	2.25 ± 0.024	2.26 ± 0.020	0.93	2.27 ± 0.021	2.25 ± 0.023	0.87
h/H	1.39 ± 0.041	1.39 ± 0.032	0.98	1.39 ± 0.050	1.39 ± 0.021	0.95
Atherogenic index	0.77 ± 0.029	0.76 ± 0.017	0.87	0.78 ± 0.033	0.75 ± 0.012	0.55
Thrombogenic index	0.82 ± 0.036	0.83 ± 0.023	0.94	0.84 ± 0.043	0.81 ± 0.015	0.80

Ctrl: control; Qrc: quercetin-supplemented; ΣSFA: total saturated FA content; ΣMUFA: total monounsaturated FA content; ΣPUFA: total polyunsaturated FA content; Σn-6: total n-6 PUFA; Σn-3: total n-3 PUFA; h/H: hypocholesterolemic/hypercholesterolemic FA ratio.
* Winsorised means and standard errors of the mean (SEM) reported

The caecotrophes contained much higher proportions of ΣSFA, and lower proportions of ΣMUFA and ΣPUFA, and thus a lower PUFA:SFA ratio, than both the carcass components and the feed (Table 6.1). This

general prevalence of SFA in the caecotrophes agrees with the results of Leiber *et al*. (2008), and reflects the biohydrogenation activity of the caecal microbial population. This activity is further demonstrated by the much greater percentage of C15:0, a linear odd-chain FA which forms part of bacterial membranes, in the caecotrophe material than in the feed samples. C15:0 is thought to be produced predominantly by Gram-negative bacteria, which are largely responsible for xylanolytic and pectinolytic activity in the caecum (Papadomichelakis, Mountzouris, Paraskevakis & Fegeros, 2011). The high concentration of C18:0 and low proportions of C18:2n-6 and C18:3n-3 were also typical of the hydrogenation effects of the microbiome (Papadomichelakis *et al*., 2011).

Unfortunately, as the branched-chain FA were not analysed for in this study, and the *trans* FA co-eluted with their *cis* isomers in the meat and fat, clear conclusions regarding the transfer of lipids from the caecotrophes to the body tissues cannot be drawn, as these FA types are considered the main indicators of microbial lipid metabolism. However, the higher levels of C15:0 in the meat and fat than in the feed may suggest some effect of caecotrophy on the tissue lipid profile, as was concluded by Leiber *et al*. (2008). Furthermore, it seems likely that at least some of the shift in saturation away from PUFA and towards SFA as shown in Figure 6.1 was due to the effects of caecal fermentation and caecotrophy. Nonetheless, the comparison of the profiles of the different sample types does suggest that the FAME composition of the meat and fat is linked more to that of the feed than to that of the caecal microbial end-products, and that caecal biohydrogenation has a very limited effect on the meat FA profile.

6.4.2 Effect of diet

Somewhat surprisingly, the dietary supplementation of quercetin had no effect on the FAME profile of the caecotrophes (Table 6.2). This was unexpected considering the known antimicrobial effects of flavonoids, as well as the extensive effects that tea catechins were found to have on the FA composition of rumen bacteria (Tan *et al*., 2011). However, it concurs with the limited effect dietary quercetin had on the composition of the caecal microbiome. As discussed in Chapter 4, it is possible that the use of the quercetin aglycone allowed extensive absorption in the upper gastrointestinal tract, thereby limiting the amount of the flavonoid that actually reached the caecum.

There were similarly no differences between the Ctrl and Qrc rabbits in the HL meat (Table 6.4); however, differences were seen in the fat (Table 6.3) and LTL meat (Table 6.5) samples. In the dissectible fat, the Ctrl rabbits had a higher proportion of C13:0. This FAME was unfortunately not measured in either the only other study on the effects of a flavonoid (hesperidin) on rabbit meat FA composition (Simitzis *et al*., 2014), or in other studies on cattle, pigs or poultry. As C13:0, as an odd-chain FA, was most likely largely of dietary origin, this difference in meat content may suggest an effect of the quercetin on the absorption of this compound. However, as C13:0 made only a minor contribution to the FAME profile of the dissectible fat, and was not detected in either the HL or LTL meat, this differences is unlikely to have any major effect on meat quality.

The most extensive diet effects were seen in the LTL meat (Table 6.5), with samples from Qrc rabbits containing higher levels of C20:0, C15:1, C20:1, C18:3n-6, C20:3n-6, C20:3n-3 and C20:4n-6, and lower levels of C20:2n-6. Furthermore, largely due to their greater proportion of C20:3n-3, Qrc rabbits also had a lower n-6:n-3

ratio. They also tended to contain higher proportions of C10:0, C12:0 and C14:1, and lower ΣPUFA and higher ΣMUFA, although there was no effect on the PUFA:SFA ratio. The results for the ΣPUFA are similar to those of Simitzis *et al*. (2014), who found that dietary hesperidin supplementation decreased the ΣPUFA and PUFA:SFA ratio.

Table 6.4
The effects of dietary quercetin supplementation at 2 g/kg feed and sex (male and female) on the fatty acid (FA) composition (% total FAME) of deboned rabbit hindleg meat (LSMeans ± SEM).

	Diet			**Sex**		
	Ctrl	**Qrc**	***P*-value**	**Male**	**Female**	***P*-value**
N	11	14		14	11	
C6:0	0.76 ± 0.101	0.62 ± 0.083	0.77	0.70 ± 0.100	0.67 ± 0.076	0.94
C10:0	0.85 ± 0.094	0.70 ± 0.090	0.29	0.79 ± 0.100	0.76 ± 0.079	0.84
C12:0	0.92 ± 0.102	0.80 ± 0.090	0.41	0.88 ± 0.105	0.84 ± 0.077	0.77
C14:0	1.56 ± 0.080	1.49 ± 0.109	0.69	1.47 ± 0.078	1.58 ± 0.125	0.54
C15:0	0.48 ± 0.026	0.43 ± 0.026	0.83	0.43 ± 0.025	0.48 ± 0.028	0.83
C16:0	28.3 ± 0.701	28.9 ± 0.606	0.49	28.5 ± 0.639	28.7 ± 0.652	0.84
C18:0	9.50 ± 0.368	9.18 ± 0.249	0.74	9.15 ± 0.313	9.54 ± 0.272	0.69
C20:0	0.43 ± 0.050	0.37 ± 0.044	0.33	0.41 ± 0.052	0.39 ± 0.037	0.69
C14:1	0.95 ± 0.064	0.87 ± 0.052	0.41	0.91 ± 0.067	0.91 ± 0.038	0.94
C15:1	0.38 ± 0.044	0.26 ± 0.048	0.11	0.33 ± 0.052	0.32 ± 0.045	0.91
C16:1	5.56 ± 0.568	5.79 ± 0.369	0.65	5.26 ± 0.442	6.10 ± 0.428	0.09
C17:1	0.65 ± 0.032	0.53 ± 0.054	0.21	0.56 ± 0.057	0.61 ± 0.033	0.62
C18:1n-9	20.2 ± 0.626	20.7 ± 0.593	0.48	21.2 ± 0.577	19.7 ± 0.587	0.05
C20:1	0.61 ± 0.034	0.57 ± 0.040	0.40	0.64 ± 0.037	0.54 ± 0.030	0.05
C18:2n-6	16.1 ± 0.436	16.7 ± 0.315	0.52	16.4 ± 0.355	16.3 ± 0.383	0.90
C18:3n-6	1.67 ± 0.194	1.46 ± 0.160	0.38	1.60 ± 0.196	1.53 ± 0.135	0.79
C18:3n-3	6.09 ± 0.199	6.32 ± 0.145	0.67	6.12 ± 0.171	6.29 ± 0.163	0.76
C20:2n-6*	0.61 ± 0.064	0.70 ± 0.065	0.19	0.71 ± 0.055	0.59 ± 0.077	0.10
C20:3n-6	0.74 ± 0.080	0.65 ± 0.064	0.31	0.73 ± 0.080	0.67 ± 0.053	0.52
C20:3n-3	2.53 ± 0.271	2.37 ± 0.204	0.56	2.55 ± 0.264	2.35 ± 0.160	0.49
C20:4n-6	0.51 ± 0.043	0.47 ± 0.043	0.58	0.52 ± 0.046	0.45 ± 0.034	0.32
ΣSFA	43.0 ± 0.540	42.5 ± 0.341	0.72	42.4 ± 0.383	43.2 ± 0.476	0.53
ΣMUFA	28.3 ± 0.990	28.7 ± 0.723	0.71	28.9 ± 0.827	28.2 ± 0.840	0.49
ΣPUFA	28.6 ± 0.974	28.8 ± 0.700	0.89	28.8 ± 0.852	28.5 ± 0.747	0.84
PUFA:SFA*	0.67 ± 0.026	0.68 ± 0.018	0.84	0.68 ± 0.022	0.66 ± 0.020	0.67
Σn-6*	20.0 ± 0.691	20.1 ± 0.502	0.94	20.2 ± 0.619	19.9 ± 0.511	0.84
Σn-3	8.58 ± 0.318	8.72 ± 0.228	0.80	8.70 ± 0.257	8.60 ± 0.279	0.86
n-6:n-3*	2.31 ± 0.048	2.30 ± 0.038	0.95	2.31 ± 0.038	2.30 ± 0.049	0.93
h/H	1.52 ± 0.049	1.53 ± 0.043	0.91	1.56 ± 0.038	1.49 ± 0.054	0.35
Atherogenic index	0.63 ± 0.019	0.62 ± 0.015	0.90	0.61 ± 0.013	0.64 ± 0.021	0.63
Thrombogenic index	0.79 ± 0.025	0.78 ± 0.018	0.94	0.77 ± 0.021	0.80 ± 0.021	0.82

Ctrl: control; Qrc: quercetin-supplemented; ΣSFA: total saturated FA content; ΣMUFA: total monounsaturated FA content; ΣPUFA: total polyunsaturated FA content; Σn-6: total n-6 PUFA; Σn-3: total n-3 PUFA; h/H: hypocholesterolemic/hypercholesterolemic FA ratio.
* Winsorised means and standard errors of the means (SEM) reported

However, the results for the individual FAMEs and the n-6:n-3 ratio did not align with those of Simitzis *et al*. (2014), who found that dietary hesperidin supplementation increased levels of C14:0, but decreased C18:0, C16:1, C20:4n-6, C20:5n-3 and C22:5n-3, as well as the Σn-6 PUFA. It also contradicts previous findings for poultry and ruminants, which have generally found that flavonoid supplementation increased the PUFA:SFA and n-6:n-3 ratios (Andrés *et al*., 2014; Kamboh & Zhuh, 2013; Tan *et al*., 2011).

One suggested explanation for the increased prevalence of PUFA found in previous studies was that the antioxidant and antimicrobial effects of the flavonoids provided protection for these more vulnerable FA from oxidation and biohydrogenation (Kamboh & Zhuh, 2013; Tan *et al*., 2011). However, as there was no effect on the levels of these FAMEs in the caecotrophes, and all the FAMEs found to be affected by diet in this study were present at higher concentrations in the meat than in the feed or caecotrophes, it seems more likely that the effect was due to the modulation of endogenous FA synthesis or modification.

A study on broiler chicks fed a vitamin E-deficient diet found that both quercetin and morin affected the levels of unsaturated FA produced endogenously through elongase and desaturase reactions, specifically decreasing the n-9 series, and increasing the n-6 and n-3 series PUFA (Jenkins & Atwal, 1995). While Jenkins and Atwal (1995) also found that the flavonoids increased the n-6:n-3 ratio, they concluded that the limited increase in n-3 PUFA was due to the very low dietary content of n-3 PUFA. It may thus be possible that the results found for the PUFA in this study were due to the combined effect of the interaction of quercetin with elongase and desaturase enzymes, and the high dietary content of C18:3n-3, relative to Andrés *et al*. (2014), Jenkins and Atwal (1995) and Kamboh and Zhuh (2013). This combination may have favoured the activity of the desaturase and elongase enzymes on the n-3 series PUFA. Dal Bosco *et al*. (2014) found that supplementation with fresh alfalfa, which contains high levels of both flavonoids and C18:3n-3, increased the Δ^5- plus Δ^6-desaturase activity, as well as decreasing the n-6:n-3 ratio, as found in this study. While Dal Bosco *et al*. (2014) concluded that the C18:3n-3 content of the supplemented alfalfa was responsible for this effect, the results of the current study suggest that the flavonoids may have also played a role. In contrast, Andrés *et al*. (2014) supplemented the diets of lambs with quercetin and linseed, either separately or in combination, and while the combined linseed and quercetin treatment decreased the n-6:n-3 ratio relative to the control, it increased the ratio relative to that of the linseed treatment alone. This may suggest that the ratio of C18:2n-6 to C18:3n-3 provided by the linseed diet was still too high to allow the desaturation and elongation process to favour the n-3 series PUFA, whereas the control diet used in the current study provided a much greater proportion of C18:3n-3.

The cause of the higher proportions of C20:0, C15:1 and C20:1, and tendencies for higher levels of C10:0, C12:0 and C14:1 in Qrc rabbits is unclear, as this effect does not appear to have been reported previously. Tan *et al*. (2011) found that supplementing tea catechins to goats decreased the levels of C20:1 in the loin meat, whereas other studies did not report any effect on these specific SFA and MUFA. However, it seems likely that these FAMEs, like the PUFA, are indicative of an effect on endogenous FA metabolism. Considering the low levels of these FAMEs in the meat, and the lack of significant changes in the nutritional indexes, despite the tendencies for

differences in the ΣMUFA and ΣPUFA, the implications for the nutritional value of the meat are likely to be minimal. Nonetheless, these effects do suggest that more research on the effects of quercetin on lipid metabolism is needed.

Table 6.5
The effects of dietary quercetin supplementation at 2 g/kg feed and sex (male and female) on the fatty acid (FA) composition (% total FAME) of rabbit loin meat (LSMeans ± SEM).

	Diet			Sex		
	Ctrl	**Qrc**	***P*-value**	**Male**	**Female**	***P*-value**
N	13	9		9	13	
C6:0	1.32 ± 0.132	1.65 ± 0.092	0.53	1.43 ± 0.123	1.55 ± 0.128	0.82
C10:0	1.69 ± 0.190	1.97 ± 0.133	0.06	1.73 ± 0.168	1.93 ± 0.175	0.17
C12:0	1.82 ± 0.188	2.10 ± 0.120	0.07	1.87 ± 0.176	2.05 ± 0.167	0.24
C14:0	2.43 ± 0.155	2.42 ± 0.142	0.96	2.28 ± 0.168	2.57 ± 0.130	0.13
C15:0	0.63 ± 0.039	0.64 ± 0.028	0.96	0.61 ± 0.047	0.67 ± 0.026	0.76
C16:0	26.6 ± 1.019	25.9 ± 0.920	0.45	25.9 ± 1.141	26.7 ± 0.919	0.44
C18:0	9.00 ± 0.293	9.90 ± 0.261	0.38	9.43 ± 0.418	9.47 ± 0.253	0.97
C20:0	0.68 ± 0.087	0.88 ± 0.036	0.003	0.73 ± 0.100	0.83 ± 0.064	0.10
C14:1	1.00 ± 0.113	1.19 ± 0.176	0.09	1.21 ± 0.158	0.98 ± 0.122	0.04
C15:1	0.37 ± 0.091	0.63 ± 0.122	0.002	0.49 ± 0.128	0.50 ± 0.099	0.88
C16:1	4.74 ± 0.459	5.20 ± 0.325	0.38	4.52 ± 0.352	5.42 ± 0.430	0.09
C17:1	0.33 ± 0.113	0.22 ± 0.142	0.26	0.25 ± 0.131	0.29 ± 0.121	0.69
C18:1n-9	16.7 ± 0.760	17.7 ± 0.509	0.19	18.0 ± 0.792	16.4 ± 0.576	0.04
C20:1	0.78 ± 0.082	0.92 ± 0.026	0.01	0.82 ± 0.104	0.87 ± 0.049	0.38
C18:2n-6	14.3 ± 0.783	12.8 ± 0.505	0.14	13.6 ± 0.825	13.4 ± 0.698	0.82
C18:3n-6	2.49 ± 0.379	3.40 ± 0.152	0.001	2.80 ± 0.386	3.09 ± 0.324	0.28
C18:3n-3	4.52 ± 0.391	4.59 ± 0.228	0.91	4.45 ± 0.230	4.67 ± 0.387	0.71
C20:2n-6*	0.91 ± 0.076	0.69 ± 0.070	0.004	0.80 ± 0.089	0.79 ± 0.078	0.95
C20:3n-6	1.06 ± 0.134	1.40 ± 0.067	<0.001	1.15 ± 0.158	1.31 ± 0.101	0.10
C20:3n-3	3.71 ± 0.348	4.70 ± 0.337	0.001	4.14 ± 0.301	4.26 ± 0.400	0.69
C20:4n-6	0.70 ± 0.109	0.90 ± 0.033	0.005	0.79 ± 0.105	0.82 ± 0.092	0.67
ΣSFA	44.5 ± 1.330	45.6 ± 0.950	0.44	44.2 ± 1.821	45.9 ± 0.749	0.25
ΣMUFA	23.9 ± 1.116	25.8 ± 0.591	0.09	25.2 ± 1.094	24.5 ± 0.994	0.52
ΣPUFA	31.8 ± 1.989	28.5 ± 0.983	0.08	30.7 ± 2.525	29.6 ± 1.331	0.58
PUFA:SFA*	0.72 ± 0.060	0.63 ± 0.035	0.10	0.70 ± 0.078	0.65 ± 0.038	0.40
Σn-6*	22.4 ± 1.496	19.2 ± 0.583	0.02	20.9 ± 1.614	20.7 ± 1.241	0.85
Σn-3	8.31 ± 0.395	9.32 ± 0.421	0.11	8.71 ± 0.389	8.93 ± 0.440	0.72
n-6:n-3*	2.68 ± 0.198	2.07 ± 0.043	<0.001	2.43 ± 0.233	2.31 ± 0.161	0.47
h/H	1.38 ± 0.050	1.46 ± 0.087	0.30	1.46 ± 0.066	1.38 ± 0.064	0.29
Atherogenic index	0.70 ± 0.035	0.70 ± 0.036	0.99	0.68 ± 0.046	0.73 ± 0.027	0.38
Thrombogenic index	0.79 ± 0.034	0.76 ± 0.036	0.81	0.77 ± 0.043	0.79 ± 0.030	0.85

Ctrl: control; Qrc: quercetin-supplemented; ΣSFA: total saturated FA content; ΣMUFA: total monounsaturated FA content; ΣPUFA: total polyunsaturated FA content; Σn-6: total n-6 PUFA; Σn-3: total n-3 PUFA; h/H: hypocholesterolemic/hypercholesterolemic FA ratio.
* Winsorised means and standard errors of the means (SEM) reported

It is interesting to note that all the FAMEs found to be affected by diet in the LTL were present at much higher levels in the LTL than in the HL, where no diet effects were found. This may suggest a muscle-specific effect of

the flavonoids, as found for basic meat quality parameters in goats supplemented with tea catechins by Tan *et al*. (2011) and Zhong *et al*. (2009). Interactions between the effects of diet and muscle have also been previously found for rabbits fed diets supplemented with soybean oil, with C15:1, C18:3n-6 and C20:3n-6 being three of the FA specifically effected (Papadomichelakis, Karagiannidou, Anastasopoulos & Fegeros, 2010a).

In view of the apparent health benefits of decreasing the n-6:n-3 ratio of the human diet to 1:1 – 4:1, the results of this study are highly promising. They suggest that the supplementation of quercetin to rabbits tends to increase the proportion of n-3 FA, decreasing the n-6:n-3 ratio and therefore improving the nutritional quality of the meat. However, the comparison with results of other studies seems to suggest that this effect may be dependent on having a relatively high level of n-3 FA in the diet. Further research comparing the effects of quercetin supplementation to rabbits on diets containing variable levels of C18:2n-6 and C18:3n-3 should be done to clarify the interaction between the FA composition and the flavonoid.

6.4.3 Effect of sex

The most distinctive sex-effects were seen in the caecotrophes (Table 6.2), with females having more C15:0 and C18:0, and less C18:2n-6, C18:3n-3 and ΣPUFA, resulting in a higher ΣSFA content, TI and AI. Females also had a lower Σn-3 content and higher n-6:n-3 ratio than males. This sex-effect on caecal FAMEs does not appear to have been examined previously. However, considering the lack of concomitant differences in the tissue samples, as well as the nature of the specific FAMEs impacted, it seems likely to be related to a sex-effect on caecal microbial metabolism. More specifically, it seems that biohydrogenation took place to a greater extent in female (F) than male (M) rabbits. It is therefore notable that female rabbits were found to have considerably higher levels of a number of bacterial families in the caecal content, with this including *Eubacteriaceae* (F = 4.7 %; M = 2.9 %), *Flavobacteriaceae* (F = 0.8 %; M = 0.3 %) and *Geobacteraceae* (F = 0.3 %; M = 0.1 %), as discussed in Chapter 4. Research correlating caecal bacterial families to lipid metabolism in rabbits does not appear to have been done, and the results of this study do not align with the results of studies on rumen or human intestinal biohydrogenation. Devillard *et al*. (2009) linked more rapid biohydrogenation of C18:2n-6 to levels of *Lachnospiraceae* in the human gut, whereas Petri, Mapiye, Dugan and McAllister (2014) found that the genera *Clostridium IV* and *Ethanoligenens* correlated negatively with levels of n-3 PUFA in the subcutaneous fat of steers. *Butyrivibrio*, *Pseudobutyrivibrio*, *Howardella*, *Oribacterium*, *Succinovibrio*, *Roseburia*, *Prevotella* and *Lachnospiraceae Incertae Sedis*, as well as unclassified members of *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* have also been linked to the biohydrogenation process in previous studies on cattle (Huws *et al*., 2015; Huws *et al*., 2011). It is therefore clear that while some relationship does exist, the identification of influential bacterial groups is still far from complete. Considerable further research is thus needed both to confirm the sex effects on the caecotrophe FAMEs found in this study, as well as to investigate the links between the caecal microbial profile and FAME profile.

However, none of the sex effects on the caecotrophes carried over into the meat and fat samples. This supports the conclusion that the caecotrophe FAMEs had a relatively limited effect on the tissue FAME composition. Nonetheless, other sex effects were found, with female rabbits having higher levels of C16:1 in the fat (Table 6.3)

and lower levels of C18:1n-9 in the loin meat (Table 6.5), with these sex effects being significant across the tissue-types for both FA. Females also had lower levels of C14:1 in the LTL meat and C20:1 in the HL meat. Gašperlin, Polak, Rajar, SkvarÈa and Zlender (2006) also reported higher levels of C16:1 in female than male rabbits; however, they did not offer any explanation for this finding. Lazzaroni, Biagini and Lussiana (2009) reported a similar effect of sex on C16:1 in the LTL, as well as on C14:0, C14:1 and C17:1, and suggested that these differences were linked to the fatness level. However, there were no differences between the sexes in the ΣSFA, ΣMUFA and ΣPUFA contents in this study, despite the tendency ($P \leq 0.10$) for the HL meat to have a higher total lipid content in females (F = 7.0 %, M = 5.9 %). The contrasting effects on C16:1, and C14:1, C18:1n-9 and C20:1, all MUFA, also cast doubts on this explanation. Alternatively, these sex differences may be related to the effects of sex hormones on FA metabolism, despite the relatively young age at which the rabbits were slaughtered. Oestrogen administration has been found to impact a wide range of factors involved in lipid absorption, lipogenesis and triglyceride synthesis, including the synthesis and turnover rate of FA-binding proteins, and the activities of a number of enzymes (Bass *et al*., 1985). However, considering the lack of effect on nutritional quality parameters such as the PUFA:SFA and n-6:n-3 ratios, or the AI or TI indexes, these sex effects do not appear to be of great practical importance.

6.5 Conclusion

Overall, the results for the different sample types supported previous suggestions that while biohydrogenation certainly takes place in the caecum, this process has a limited influence on the FA profile of the carcass fat. Furthermore, the considerable differences in the FA composition of the HL and LTL support the analysis of samples from more than one muscle in future studies.

Dietary quercetin supplementation had no effect on the FAMEs in the caecotrophes, which aligned with the limited effect on the caecal microbiome, as discussed in Chapter 4. However, Qrc rabbits had a lower n-6:n-3 ratio in the LTL, which may have been due to the combined effects of the high C18:3n-3 content of the feed and the effects of quercetin on the elongase and desaturase enzyme systems involved in endogenous lipid metabolism. Further research measuring the effects of dietary quercetin on the activities of these enzymes is needed. It is notable that there was no effect on the HL FAME composition, suggesting a diet-muscle interaction. Nonetheless, considering the possible health implications, the beneficial effect of Qrc on the n-6:n-3 ratio is worth further study.

The higher C18:0 and lower C18:3n-3 levels in the caecotrophes of female than male rabbits suggested differences in caecal biohydrogenation, which coincided with previous findings that the composition of the caecal microbiome differs between the sexes. However, these differences did not result in a similar effect on the carcass components; instead, females had higher levels of C16:1 and lower levels of C18:1 across the sample types. This may reflect differences in the fatness level of the sexes, or direct effects of sex hormones on enzymes involved in FA metabolism. However, these differences did not have any effect on the nutritional quality parameters.

6.6 References

Andrés, S., Morán, L., Aldai, N., Tejido, M. L., Prieto, N., Bodas, R. & Giráldez, F. J. (2014). Effects of linseed and quercetin added to the diet of fattening lambs on the fatty acid profile and lipid antioxidant status of meat samples. *Meat Science, 97*, 156 – 163.

Banskalieva, V., Sahlu, T. & Goetsch, A. L. (2000). Fatty acid composition of goat muscles and fat depots: a review. *Small Ruminant Research, 37*, 255 – 268.

Dal Bosco, A., Mugnai, C., Roscini, V., Mattioli, S., Ruggeri, S. & Castellini, C. (2014). Effect of dietary alfalfa on the fatty acid composition and indexes of lipid metabolism of rabbit meat. *Meat Science, 96*, 606 – 609.

Dalle Zotte, A. & Szendrő, Zs. (2011). The role of rabbit meat as functional food. *Meat Science, 88*, 319 – 331.

De Smet, S., Raes, K. & Demeyer, D. (2004). Meat fatty acid composition as affected by fatness and genetic factors: a review. *Animal Research, 53*, 81 – 89.

Devillard, E., McIntosh, F. M., Paillard, D., Thomas, N. A., Shingfield, K. J. & Wallace, R. J. (2009). Differences between human subjects in the composition of the faecal bacterial community and faecal metabolism of linoleic acid. *Microbiology, 155*, 513 – 520.

Folch J., Lees M. & Sloane-Stanley G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry, 226*, 495 – 509.

Gašperlin, L., Polak, T., Rajar, A., SkvarÈa, M. & Zlender, B. (2006). Effect of genotype, age at slaughter and sex on chemical composition and sensory profile of rabbit meat. *World Rabbit Science, 14*, 157 – 166.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67 – 202.

Hernández, P., Cesari, V. & Blasco, A. (2008). Effect of genetic rabbit lines on lipid content, lipolytic activities and fatty acid composition of hind leg meat and perirenal fat. *Meat Science, 78*, 485 – 491.

Hernández, P. & Dalle Zotte, A. (2010). Influence of diet on rabbit meat quality. In C. de Blas & J. Wiseman (Eds.), *Nutrition of the rabbit* (2nd edition, pp. 163 – 178). Oxfordshire, UK: CABI International.

Huws, S. A., Kim, E. J., Cameron, S. J. S., Girdwood, S. E., Davies, L., Tweed, J., Vallin, H. & Scollan, N. D. (2015). Characterization of the rumen lipidome and microbiome of steers fed a diet supplemented with flax and echium oil. *Microbial Biotechnology, 8*(2), 331 – 341.

Huws, S. A., Kim, E. J., Lee, M. R. F., Scott, M. B., Tweed, J. K. S., Pinloche, E., Wallace, R. J. & Scollan, N. D. (2011). As yet uncultured bacteria phylogenetically classified as *Prevotella*, *Lachnospiraceae* incertae sedis and unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* may play a predominant role in ruminal biohydrogenation. *Environmental Microbiology, 13*(6), 1500 – 1512.

Jenkins, K. J. & Atwal, A. S. (1995). Flavonoids increase tissue essential fatty acids in vitamin E-deficient chicks. *Nutritional Biochemistry, 6*, 97 – 103.

Kamboh, A. A. & Zhuh, W.-Y. (2013). Effect of increasing levels of bioflavonoids in broiler feed on plasma anti-oxidative potential, lipid metabolites, and fatty acid composition of meat. *Poultry Science, 92*, 454 – 461.

Lazzaroni, C., Biagini, D. & Lussiana, C. (2009). Fatty acid composition of meat and perirenal fat in rabbits from two different rearing systems. *Meat Science, 83*, 135 – 139.

Leiber, F., Meier, J. S., Burger, B., Wettstein, H. R., Kreuzer, M., Hatt, J. M. & Clauss, M. (2008). Significance of coprophagy for the fatty acid profile in body tissues of rabbits fed different diets. *Lipids, 43*, 853 – 865.

Mozaffarian, D., Micha, R. & Wallace, S. (2010). Effects on coronary heart disease of increasing polyunsaturated fat in place of saturated fat: a systematic review and meta-analysis of randomized controlled trials. *PLoS Medicine, 7*(3), e1000252.

Papadomichelakis, G., Karagiannidou, A., Anastasopoulos, V. & Fegeros, K. (2010a). Effect of dietary soybean oil addition on the odd-numbered and branched-chain fatty acids in rabbit meat. *Meat Science, 86*, 264 – 269.

Papadomichelakis, G., Karagiannidou, A, Anastasopoulos, V. & Fegeros, K. (2010b). Effect of high dietary digestible fibre content on the fatty acid composition of two muscles in fattening rabbits. *Livestock Science, 129*, 159 – 165.

Papadomichelakis, G., Mountzouris, K. C., Paraskevakis, N. & Fegeros, K. (2011). Caecum odd-numbered and branched-chain fatty acid composition in response to dietary changes in fattening rabbits. *Journal of Animal Physiology and Animal Nutrition, 95*, 707 – 716.

Patterson, E., Wall, R., Fitzgerald, G. F., Ross, R. P. & Stanton, C. (2012). Health implications of high dietary omega-6 polyunsaturated fatty acids. *Journal of Nutrition and Metabolism,* 539426, 1 – 16.

Petri, R. M., Mapiye, C., Dugan, M. E. R. & McAllister, T. A. (2014). Subcutaneous adipose fatty acid profiles and related rumen bacterial populations of steers fed red clover or grass hay diets containing flax or sunflower-seed. *PLoS One, 9*(8), e104167.

Santos-Silva J., Bessa R. J. B. & Santos-Silva F. (2002). Effect of genotype, feeding system and slaughter weight on the quality of light lambs. II. Fatty acid composition of meat. *Livestock Production Science, 77*,187 – 194.

Simitzis, P. E., Babaliaris, C., Charismiadou, M. A., Papadomichelakis, G., Goliomytis, M., Symeon, G. K. & Deligeorgis, S. G. (2014). Effect of hesperidin dietary supplementation on growth performance, carcass traits and meat quality of rabbits. *World Rabbit Science, 22*, 113 – 121.

Simopoulos, A. P. (2004). Omega-6/omega-3 essential fatty acid ratio and chronic diseases. *Food Reviews International, 20*(1), 77 – 90.

Sohaib, M., Butt, M. S., Shabbir, M. A. & Shahid, M. (2015). Lipid stability, antioxidant potential and fatty acid composition of broilers breast meat as influenced by quercetin in combination with α-tocopherol enriched diets. *Lipids in Health and Disease, 14*(61), 1 – 15.

Tan, C. Y., Zhong, R. Z., Tan, Z. L., Han, X. F., Tang, S. X., Xiao, W. J., Sun, Z. H. & Wang, M. (2011). Dietary inclusion of tea catechins changes fatty acid composition of muscle in goats. *Lipids, 46*(3), 239 – 247.

Ulbricht T.L.V. & Southgate D.A.T. (1991). Coronary heart disease: seven dietary factors. *Lancet, 338*, 985 – 992.

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Richardson, R. I. & Sheard, P. R. (1999). Manipulating meat quality and composition. *Proceedings of the Nutrition Society, 58*, 363 – 370.

Wood, J., Richardson, R., Nute, G., Fisher, A., Campo, M., Kasapidou, E., Sheard, P. & Enser, M. (2004). Effects of fatty acids on meat quality: a review. *Meat Science, 66*(1), 21 – 32.

Zhong, R. Z., Tan, C. Y., Han, X. F., Tang, S. X., Tan, Z. L. & Zeng, B. (2009). Effect of dietary tea catechins supplementation in goats on the quality of meat kept under refrigeration. *Small Ruminant Research, 87*, 122 – 125.

CHAPTER 7:

The effects of dietary quercetin supplementation on the changes during chilled storage in raw, minced loin meat from New Zealand White grower rabbits

Abstract

Thirty-four New Zealand White rabbits of both sexes were fed a control or supplemented (2 g/kg quercetin dihydrate) diet from weaning until slaughter (13 weeks). After *post-mortem* chilling, excised and minced loins were stored at 3.2 °C under oxygen-permeable wrapping for 1, 3 or 5 days. Colour, pH, lipid oxidation (TBARS), antioxidant capacity (FRAP), volatile compound profile and microbial count (at day 5) were determined. Quercetin reduced alkane and day 1 hexanal concentrations, but otherwise had minimal antioxidant effect and did not benefit microbial quality, suggesting that supplementation did not substantially improve shelf-life. The effect of sex was limited and was likely linked to fatty acid composition or muscle physiology. Overall, the pH increased and FRAP decreased during storage, but TBARS did not change and discolouration seemed delayed. The volatile compound profile was dominated by esters, alcohols and heterocyclic compounds, and while it changed during storage, lipid oxidation products did not increase as expected, suggesting that rabbit meat may have relatively active reducing mechanisms.

7.1 Introduction

The chilled storage of meat is essential for its commercial production, distribution and sale; however, many of the changes that can take place during storage are unwelcome, negatively impacting appearance, odour and flavour, and even nutritional value (Lambert, Smith & Dodds, 1991; Shahidi & Zhong, 2010). This is particularly problematic for processed meat products, such as lower-value cuts that are transformed into mince, as this processing increases both the risk of microbial contamination and the surface area available for oxidation (Ladikos & Lougovois, 1990). The development of methods to slow the rate of these changes and extend the shelf-life of chilled meat products is therefore a constant challenge, with a range of approaches being tested, including the addition of antioxidants (Gray, Gomaa & Buckley, 1996). While a number of synthetic antioxidants are highly effective and are used in the food industry, concerns have been raised about the safety of some of these products (Shahidi & Zhong, 2010). Moreover, consumers are becoming increasingly anxious about additives in food, even in the absence of any evidence of possible negative effects on health (Brewer, 2011). Researchers in food production therefore need to investigate and identify possible natural alternatives to synthetic antioxidants (Brewer, 2011). One chemical group that has attracted attention are the flavonoids.

Flavonoids, which are ubiquitous polyphenolic compounds produced by plants as secondary metabolites, have a wide range of pharmacological properties, including antioxidant and antimicrobial activity (Havsteen, 2002). This has been demonstrated practically through the addition of plant extracts rich in flavonoids to meat products during processing, which has been found to reduce oxidation and limit microbial growth (Irkin & Arslan, 2010; Kim, Cho & Han, 2013; Rodríguez Vaquero, Aredes Fernández & Manca de Nadra, 2011; Shah, Bosco & Mir, 2014). However, the incorporation of any additive into meat products has its challenges, including avoiding undesirable organoleptic effects and ensuring the even distribution of the additive within the product (Decker & Park, 2010; Mitsumoto, 2000). Furthermore, the *post-mortem* incorporation of antioxidants into raw meat is currently not permitted in many countries (Mitsumoto, 2000).

One possible solution is to add the antioxidant to the animal's diet, rather than to the end product, thereby taking advantage of the animal's own physiological systems to distribute the antioxidant within the muscle (Decker & Park, 2010; Falowo, Fayemi & Muchenje, 2014). The disadvantages of this system are that far more of the antioxidant has to be used, to make up for losses during ingestion, digestion, absorption and metabolism, and that these many variables can greatly impact the efficacy of the antioxidant in the final product (Surai, 2014). However, a possible benefit is the improvement of the live performance of the animal (Jiang & Xiong, 2016).

Studies have had varied success with the incorporation of plant extracts or pure flavonoids into livestock feeds, and there is still limited data and a clear lack of consensus on its effectiveness (Goliomytis *et al*., 2015; Koné *et al.*, 2016; Surai, 2014). Drawing conclusions based on these studies is also complicated by the wide variety of naturally-occurring flavonoids, and the effects of interactions between flavonoids and other phenolic compounds in mixed extracts. This study therefore examined the effects of purified quercetin dihydrate as a dietary supplement on the changes in rabbit meat during chilled storage. Rabbit, as a white meat containing a relatively

high proportion of unsaturated fatty acids (Dalle Zotte & Szendrő, 2011), is particularly vulnerable to oxidation during storage, which could result in a shortened shelf-life (Dalle Zotte, 2002). There is also a limited amount of information available on the effects of chilled storage on rabbit meat, particularly in terms of the volatile compound profile. An additional aim of this study was therefore to add to the existing knowledge of the changes in rabbit meat during storage.

7.2 Materials and methods

Ethical clearance for this study was obtained from the Stellenbosch University Animal Care and Use Committee (protocol number SU-ACUD16-00094).

7.2.1 Growth and slaughter

Full descriptions of the dietary treatments, rearing conditions and slaughter protocols can be found in North, Dalle Zotte and Hoffman (2018a, Chapter 3) and North, Dalle Zotte and Hoffman (2018b, Chapter 5). Briefly, 34 New Zealand White rabbits were fed either the control (Ctrl: 0 g quercetin/kg feed) or treatment (Qrc: 2 g quercetin/kg feed) diet from weaning at 5 weeks until slaughter at 13 weeks of age. Each treatment group was balanced for gender as far as possible, resulting in the following diet-sex treatment combinations: Ctrl males (N = 8), Ctrl females (N = 10), Qrc males (N = 7) and Qrc females (N = 9). The rabbits were housed individually and split evenly between two rearing rooms, following a randomised-block experimental design.

At 13 weeks of age the rabbits were slaughtered, with initial electrical stunning being followed by exsanguination via the carotid arteries and jugular veins, whereafter the skins, distal parts of the legs and gastrointestinal tracts were removed. The live weights and hot carcass weights were measured, prior to chilling the carcasses at 3.2 ± 0.4 °C for 24 hours to allow the resolution of rigor. The chilled carcass and reference carcass (RC) weights were recorded and the dressing out percentages and RC yields calculated. The head, liver, kidneys, lungs, oesophagus, trachea, thymus and heart were included in the hot and chilled carcasses, as recommended by Blasco and Ouhayoun (1996), but were removed to produce the reference carcasses.

7.2.2 Sample processing

After weighing the carcasses, both the left and right *longissimus thoracis et lumborum* (LTL) muscles were excised and coarsely homogenised, in order to allow their even distribution among the storage periods and to maximise potential oxidation. Samples collected for the determination of the proximate composition were vacuum-packed and frozen at -20 °C until further analysis. The remaining minced LTLs were divided into three portions (*ca*. 56 g each) per rabbit, which were placed onto polystyrene punnets (one per sampling period) lined with sterile stomacher bags (Curved 400, Grade Products Ltd, Leicestershire, England), and were overwrapped with low-density polyethylene (LDPE) film (moisture vapour transfer rate: 585 g/m^2/24 h/1 atm, O_2 permeability: 25 000 cm^3/ m^2/24 h/1 atm, CO_2 permeability: 180 000 cm^3/ m^2/24 h/1 atm; Freddy Hirsch, Cape Town, South Africa). The overwrapped punnets were placed in a cold-room with constant lighting (Philips TL-D 58W/33-640, cool

white, 4600 Lumen) at 3.2 ± 0.4 °C, with the punnets being randomly packed in a single layer and evenly distributed across the shelving area within the room in order to take into account any variation in temperature, lighting or ventilation.

7.2.3 Measurement and sampling

Measurement and sampling was done at 1, 3 and 5 days *post-mortem*, with a separate punnet being used for each sampling period.

The surface colour of the mince (L*, a* and b* values) and the pH were measured at every time point, with the pH being measured twice per sample, using a Crison PH25 portable pH meter with a 50 54 electrode (Crison Instruments S.A., Barcelona, Spain, sourced from Lasec SA Pty Ltd, Cape Town, South Africa), and the colour being measured three times per sample, using a Spectro-guide 45/0 gloss CIELab colour meter, with a D65 illuminant, 11 mm aperture and an observer angle of 10° (catalogue number 6801, BYK-Gardner, Geretsried, Germany). Day 1 samples were allowed to bloom for at least three hours at 3.2 ± 0.4 °C under LDPE film prior to removing the film and measuring the colour parameters, and the film was similarly removed before colour measurement on the other sampling days. Chroma (C*) values were calculated as $C^* = \sqrt{(a^*)^2 + (b^*)^2}$, and hue angle (H°) values as $H^\circ = \tan^{-1}\left(\frac{b^*}{a^*}\right)$, as specified by the American Meat Science Association (AMSA, 2012). However, when calculating the hue angle for samples with negative a* values, 180° was added to the calculated value in order to accommodate a 360° representation and remove negative hue angle values (McLellan, Lind & Kime, 1995).

Samples for the determination of the degree of lipid oxidation (thiobarbituric acid reactive substances: TBARS) and antioxidant capacity (ferric reducing antioxidant power: FRAP) were also collected at 1, 3 and 5 days *post-mortem*, and were vacuum-packed and frozen at -80 °C until analysis.

Samples for the determination of the meat volatile compound content were collected at 1 and 5 days *post-mortem*. Aliquots of raw meat (2 g) were weighed directly into solid-phase microextraction (SPME) headspace vials (P/N: VA201, 20 ml, clear precision screw-thread vial, round bottom, USP 1, expansion 33), which were sealed using screw-thread magnetic caps with polytetrafluoroethylene/silicone septa (P/N: SACA001), and were frozen at -80 °C until analysis (maximum storage period of one week).

Meat samples for microbial analysis were collected at 5 days *post-mortem*, prior to measuring the colour and pH, or collecting any other samples. The surface of the overwrapping was sterilised using 70 % ethanol, prior to cutting open the packaging using stainless-steel scissors that had been dipped in ethanol, flamed and briefly allowed to cool. Similarly-sterilised stainless-steal forceps were used to transfer *ca.* 10 g of the minced meat into a sterile stomacher bag (Curved 400, Grade Products Ltd, Leicestershire, England), with the precise weight being recorded. The stomacher bags were taped closed and stored overnight at 3.2 °C until further processing the following day.

7.2.4 Proximate chemical composition

The proximate chemical composition was determined according to the methods of the Association of Official Analytical Chemists (2002), as described in Chapter 5.

7.2.5 Lipid oxidation (TBARS)

The TBARS contents of the meat samples were determined using a modification of the method described by Lynch and Frei (1993). Briefly, 1 g meat samples were homogenised (T18 digital ULTRA TURRAX®, IKA®, Staufen im Breisgau, Germany) with 10 ml 0.15 M potassium chloride for 20 sec at 6400 rpm, whereafter 500 µl of the homogenate was transferred to a clean tube. To each tube was added 250 µl 1 % (w/v) 2-thiobarbituric acid in 50 mM sodium hydroxide, and 250 µl 2.8 % (w/v) trichloroacetic acid. The tubes were vortexed briefly and immediately placed in a water bath at 95 °C. After an incubation period of 1 hour the tubes were removed from the water bath and allowed to cool, whereafter 2 ml 1-butanol was added to each tube. After vortexing, the tubes were centrifuged for 30 minutes at 4 °C at 2383 × g (Sigma 2-16 K, Wirsam scientific, Cape Town, SA). A 200 µl aliquot of the supernatant from each tube was transferred to a 96 well clear microplate (Greiner Cellstar 96 well flatbottom plate, Sigma-Aldrich, St Louis, USA), and the absorbance was read at 532 nm (Spectrostar Nano, BMG Labtech, Ortenberg, Germany).

All samples were extracted and read in duplicate (four readings per sample). TBARS were quantified by comparison to a 1,1,3,3-tetramethoxypropane (TMP) standard curve (0 – 20 µM, $R^2 > 0.99$) and were expressed as mg malondialdehyde/kg meat (mg MDA/kg meat).

7.2.6 Ferric reducing antioxidant power (FRAP)

The ferric reducing ability of the meat samples was determined as described by Descalzo *et al*. (2007) and Rupasinghe, Wang, Huber and Pitts (2008), with some modifications. Briefly, 1 g samples of meat were homogenised (T18 digital ULTRA TURRAX®, IKA®, Staufen im Breisgau, Germany) at 9000 rpm for 2 minutes in 5 ml potassium phosphate buffer (pH 7.2), after which the homogenate was centrifuged at 4024 × g for 30 minutes at 20 °C. The FRAP reagent was prepared immediately before the assay by combining 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) and 20 mM ferric chloride in a 10:1:1 ratio. A 20 µl aliquot of the sample supernatant was combined with 180 µl FRAP reagent in a microplate well (Greiner Cellstar 96 well flatbottom plate, Sigma-Aldrich, St Louis, USA), and the absorbance was read at 593 nm after a 3 second shaking period (Spectrostar Nano, BMG Labtech, Ortenberg, Germany).

All samples were extracted and assayed in duplicate (four readings per sample). FRAP activity was quantified by comparison to a ferrous sulphate standard curve (0.1 – 0.8 mM, $R^2 > 0.99$) and is expressed as mmol ferrous equivalent/kg wet meat (mmol Fe^{2+} eq/kg wet meat).

7.2.7 Volatile compound profile

The volatile compound profile of the samples was determined using solid-phase microextraction, followed by gas chromatography–mass spectrometry (SPME-GC-MS).

Sample vials were defrosted and 100 µl internal standard solution (1 ppm 3-octanol and anisole-d_8 in methanol) was added. The vials were allowed to equilibrate for 10 minutes at 50 °C using a CombiPAL (CTC, Switzerland), before inserting a preconditioned fibre into the vial headspace above the sample. The fibre was preconditioned by heating in a gas chromatograph injection port at 270 °C for 60 minutes and was coated with a 50/30 µm thick divinylbenzene/carboxen/polydimethylsiloxane layer (Supelco 57298-U, Sigma). After a 20 minute extraction period (with agitation) the fibre was retracted from the vial and inserted into the gas chromatograph (GC) injection port. The SPME fibre was desorbed at 250 °C for 10 minutes, with the injection port operating in a splitless mode.

Separation of the volatile compounds was achieved using an Agilent 6890 N (Agilent Technologies, Palo Alto, CA, USA) GC with a polar Zebron 7HG-G009-11 ZB-FFAP capillary column (length 30 m, diameter 0.25 mm, film thickness 0.25 µm) from Separations, South Africa. A single-ramp temperature cycle was used, with an initial temperature of 40 °C being held for 5 min, followed by an increase to 240 °C at 5 °C/minutes and a final holding period of 2 minutes at 240 °C. The total run time per sample was 47 minutes.

The carrier gas (helium) had a constant flow rate of 1.0 ml/minute and the transfer line was maintained at 250 °C. The mass spectra for the separated compounds were obtained using an Agilent mass spectrometer detector (5975B, Palo Alto, California, USA), working with the electronic impact at 70 eV. The detector operated in full scan mode (35 – 450 m/z), and the ion source and quadrupole temperatures were maintained at 240 °C and 150 °C, respectively.

Qualitative and quantitative processing of the chromatograms was done using Xcalibur™ Software from Thermo Fisher Scientific (Massachusetts, USA). Values reported for each peak are in µg/kg meat, and were calculated using the area ratio (the area of the peak in question relative to the area of the peak of the internal standard) and the concentration of the internal standards. Anisole-d_8 was used for the quantification of all chemical groups apart from alcohols, for which 3-octanol was used. Compounds were tentatively identified by comparing their mass spectra to those contained in the NIST11 mass spectral library (National Institute of Standards and Technology, Gaithersburg), using NIST MS Search version 2.0. The identification of hexanal (RT 6.35) was additionally confirmed through comparison with an external standard.

Retention indices (RI) were calculated to provide additional verification by comparing the retention times of the identified compounds to that of a series of alkanes run on the same instrument and under the same conditions, according the following formula:

$$RI = 100\left[\frac{t_x - t_n}{t_{n+1} - t_n} + n\right]$$

Where: *RI* is the calculated retention index, t_x is the retention time (RT) of the compound of interest, t_n is the retention time of the alkane eluting before the compound of interest, t_{n+1} is the retention time of the alkane eluting after the compound of interest, and *n* is the carbon number of the alkane eluting before the compound of interest (McNaught & Wilkinson, 1997).

It must be emphasized that while the volatile compounds will henceforth be referred to by their IUPAC or common names, the identification of these compounds (apart from hexanal) is strictly tentative, as they have not been compared to pure standards for confirmation.

7.2.8 Microbial count

Meat samples in stomacher bags were diluted 1:10 with the addition of 9 volumes of quarter-strength Ringer's solution (P/N R5020FP, Minema chemicals Pty Ltd, Johannesburg, South Africa), and were homogenised in a stomacher for 30 seconds. Serial dilutions (10^{-2} – 10^{-4}) were prepared by adding 1 ml aliquots of homogenate or dilution to 9 ml Ringer's solution, and 1 ml aliquots of each dilution were pipetted onto Aerobic Count Plate (APC) Petrifilms™ (3M South Africa Pty Ltd, Cape Town, South Africa) and *E. coli*/Coliform Count Plate (EC) Petrifilms™ (3M South Africa Pty Ltd, Cape Town, South Africa). The APC petrifilms were incubated at 30 °C for 72 hours prior to counting, with the preferable counting range of 10 to 300 colonies per petrifilm being taken into account when counting and calculating the results. The EC petrifilms were incubated for 24 hours at 35 °C before counting. Results were reported as the log of the colony-forming units/g (log CFU/g).

7.2.9 Statistical analysis

A randomised block experimental design was used in order to take into account any possible environmental differences between the two rearing rooms. The main effects of interest (diet, sex and time *post-mortem*) were combined in a two-by-two-by-three factorial design.

Statistica version 13 software was used to analyse the data, with the normality first being tested using normal probability plots and outliers being removed where necessary. The significance of the blocks, main effects and interactions was determined using the R lm package when only diet and sex were factors (only data for a single time point, no repeated measures). The R lmer package (for mixed models) was used when diet, sex and time *post-mortem* were assessed as fixed effects, and animal nested in treatment as a random effect. Fisher's least significant differences (LSD) *post hoc* test was used to compare the individual values for the days *post-mortem* and second- and third-order interactions. Slaughter weight was included as a covariate.

In addition to the basic analysis of variance, principle component analysis (PCA) based on a Pearson's correlation matrix was also performed using XLStat software (Version 2018, Addinsoft, New York, USA), in order to visualise the relationships between the treatment groups and variables.

Main effects and interactions with $P \leq 0.05$ were considered significant, whereas those with $P \leq 0.10$ are reported as trends. Values are reported as LSMeans ± standard error of the mean (SEM).

7.3 Results

Minimal differences between the sex- and diet-treatments were seen for the carcass components and proximate chemical composition of the LTL muscle (Table 7.1), and their interaction was not significant for any of the variables. While male rabbits had heavier hot carcasses (2152 ± 44.58 g, vs. 2107 ± 64.94 g; $P = 0.04$), this effect diminished with cooling, with the chilled carcass weight ($P = 0.08$) and dressing out percentage ($P = 0.06$) being only slightly higher for males, and no difference between the sexes for the reference carcass weights and yields being found. The only effect of diet was a tendency for Ctrl rabbits to have a higher protein content in the LTL ($P = 0.07$).

With regards to the changes during storage, the initial examination of the PCA observations plot (Figure 7.1) indicated that the storage period had a greater effect on the meat than did sex or quercetin supplementation. The variables plot (Figure 7.2) displayed a similar pattern, with a bilateral grouping of most of the variables along the x-axis. The colour parameters did not form part of this grouping, correlating more strongly with the second principle component (F2), whereas the first principle component (F1) correlated predominantly with the volatile compounds.

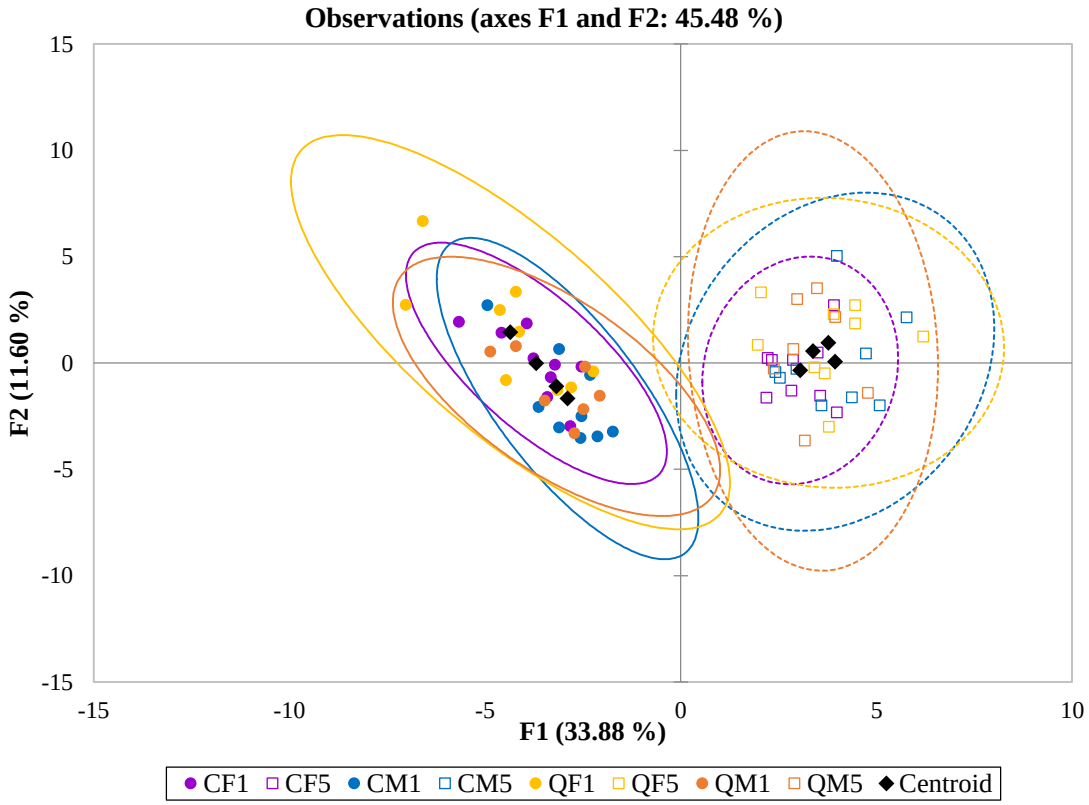

Figure 7.1 Principle component analysis (PCA) observations plot of minced New Zealand White rabbit *longissimus thoracis et lumborum* muscle samples stored for 1 or 5 days in oxygen-permeable packaging at 3.2 °C, classified according to diet (C: control, Q: quercetin supplemented at 2 g/kg feed), sex (M: male, F: female), and storage period (1 or 5 days). Ellipses indicate the 95 % confidence intervals of the treatment groups, with solid-lines indicating day 1 samples and dotted lines indicating day 5 samples.

The limited effects of sex and diet could also be seen for the shelf-life parameters in Table 7.2, with only the LTL pH being impacted by sex, and diet not causing any effect. The pH was higher in meat from males than females (5.76 vs. 5.68; $P = 0.001$), as well as increasing during storage ($P < 0.001$). There also tended to be some diet-sex-day interaction ($P = 0.08$), with all the treatment groups, with the exception of the Ctrl males, having an increase in pH *post-mortem*.

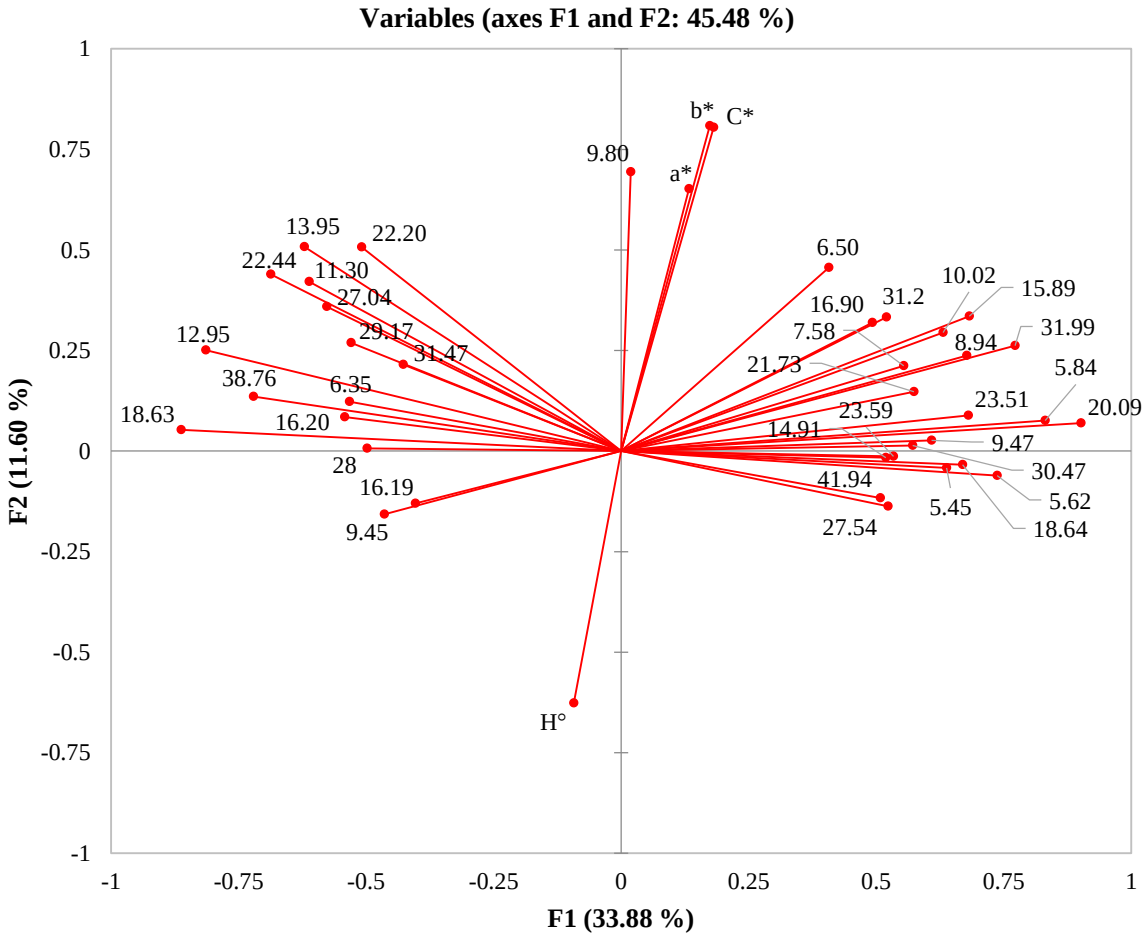

Figure 7.2 Principle component analysis (PCA) variables plot of shelf-life quality components and volatile compounds in minced New Zealand White rabbit *longissimus thoracis et lumborum* muscle samples stored for 1 or 5 days in oxygen-permeable packaging at 3.2 °C. Data labels identify the variables represented, with retention times given for volatile compounds (for tentative identifications see Tables 7.3 – 7.6).

Despite their low correlation with the first principle component, the colour parameters were impacted by storage, with redness ($P < 0.001$), yellowness ($P < 0.001$) and chroma ($P < 0.001$) values all peaking at three days *post-mortem*. The hue angle showed the opposite trend, with the lowest value being recorded after three days of storage ($P < 0.001$). The lightness increased from day 1 to day 5 ($P = 0.03$); however, this was solely due to an increase in lightness in the meat from male rabbits (Figure 7.3), with female LTL meat showing no change in lightness during storage ($P_{s\times d} = 0.01$). There were no significant effects of diet, sex or storage time on the degree

of lipid oxidation (TBARS) or on FRAP values. However, there was a tendency for FRAP values to decrease during storage ($P = 0.07$).

The volatile compounds contributing the most to the differences between the shelf-life periods, based on the PCA, were 2-methyldecane, 3-methyldecane, 3,7-dimethylocta-1,6-dien-3-yl 2-aminobenzoate, 2-chloroethenyl dimethyl phosphate, methyl heptanoate, methyl nonanoate and methyl octadeca-9,12-dienoate, with the first four correlating with the day 5 samples ($r \geq 0.70$), and the latter three correlating with the day 1 samples ($r \leq -0.70$). A number of these compounds were only detected in either the day 1 or day 5 samples (Table 7.3). In total, 30 volatile compounds were detected in day 1 samples, and 41 were detected in day 5 samples.

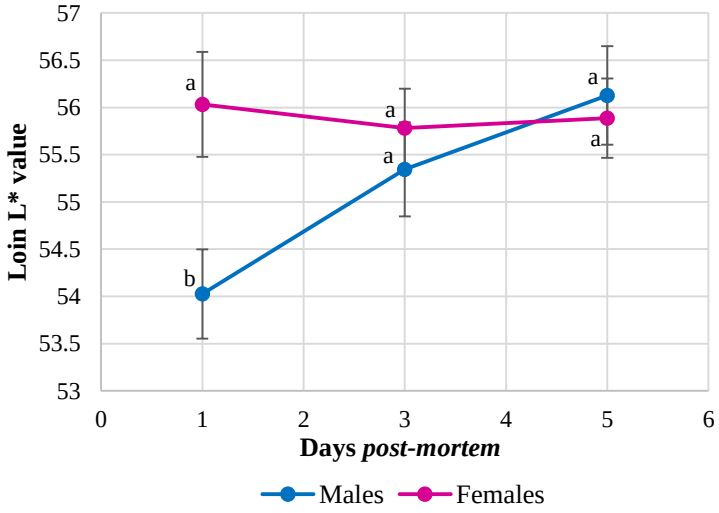

Figure 7.3 The effect of sex on the CIELab L* values of New Zealand White rabbit *longissimus thoracis et lumborum* muscle during refrigerated storage for 1, 3 or 5 days in oxygen-permeable packaging at 3.2 °C. Error bars indicate the standard error of the mean. Data points with different significance letters differ significantly ($P \leq 0.05$).

As can be seen in Table 7.3, the analysis of variance found that a number of the compounds that were detected in both day 1 and 5 samples differed significantly between the days. However, the interpretation of the results for some of these was complicated by the significance of second-order diet-day or sex-day interactions (Tables 7.4 and 7.5).

Five compounds showed diet-day interactions (Table 7.4), with one of these also being effected by the third-order interaction. As can be seen in Figure 7.4 ($P_{d\times s\times d} = 0.001$), the concentration of 2,6-dimethyloct-7-en-2-ol increased from day 1 to 5 for Qrc females, but decreased for Ctrl females and did not change for either of the male treatments. For hexanal ($P = 0.02$) and 4,7,7-trimethylbicyclo[2.2.1]heptan-3-one ($P = 0.04$) there was a decrease in concentration from day 1 to 5 in the meat from the Ctrl rabbits, but less or no change for the Qrc rabbits. In contrast, methyl octadeca-9,12-dienoate ($P = 0.02$) declined more in the Qrc meat, and methyl 12-methyltetradecanoate ($P = 0.06$) tended to decrease in the Qrc group but did not change during storage in Ctrl rabbits.

The sex-day interaction was significant for six compounds (Table 7.5), mostly esters. The one aldehyde (hexanal, $P = 0.002$), decreased from day 1 to 5 in females, but did not change in males. The opposite pattern was found for methyl pentanoate ($P = 0.001$) and methyl hexanoate ($P = 0.01$), for which females showed no change while males increased during storage. For methyl pentanoate, females had consistently lower values, whereas they had higher levels of methyl hexanoate. For the last three compounds the levels decreased more in meat from female than male rabbits, although males only showed no change in the concentration of ethyl decanoate ($P = 0.01$), showing some decline for the other two esters.

The majority of the compounds that changed during storage were tentatively identified as esters (Table 7.3), with 12 out of the 17 decreasing in concentration from day 1 to day 5. Ethyl hexanoate, methyl nonanoate and methyl (E)-dodec-9-enoate were only detected in day 1 samples, whereas methyl 2-hydroxypropanoate ($P < 0.001$), methyl 12-methyltridecanoate ($P = 0.001$), methyl 12-methyltetradecanoate ($P = 0.002$) and methyl pentadecanoate ($P = 0.002$) were detected in both day 1 and 5 samples but decreased during storage. Two esters were only found in day 5 samples, namely (1-hydroxy-2,4,4-trimethylpentan-3-yl)2-methylpropanoate and 2-chloroethenyl dimethyl phosphate, and methyl octanoate ($P < 0.001$) increased during storage.

Only two alcohols were detected in both day 1 and day 5 samples, with 2-butoxyethanol ($P = 0.003$) decreasing during storage. Of the other alcohols, four were only found in day 5 samples, namely pentan-1-ol, hexan-1-ol, oct-1-en-3-ol and 2-propylpentan-1-ol. Only one alcohol 2-(2-methylpropoxy)ethanol was only detected in day 1 samples.

Only four heterocyclic compounds were detected in both day 1 and day 5 samples, and of these, only two differed between the storage periods. While 3,7-dimethylocta-1,6-dien-3-yl 2-aminobenzoate increased during storage ($P < 0.001$), oxolan-2-one decreased ($P = 0.01$). A second non-aromatic heterocyclic compound, 4,7,7-trimethylbicyclo[2.2.1]heptan-3-one, showed the same tendency ($P = 0.10$). Of the other heterocyclic compounds, seven were only detected in day 5 samples, and five were only detected in day 1 samples.

The remaining chemical types (aldehydes, alkanes, carboxylic acids, ketones and sulphur-containing compounds) made only minor contributions to the volatile compound profile. The aldehydes (apart from hexanal), alkanes and carboxylic acids were only detected in day 5 samples, whereas the one ketone, 3-hydroxybutan-2-one was only detected in day 1 samples. Methylsulfonylmethane was found at both storage periods, but decreased ($P < 0.001$) from day 1 to 5.

Despite the lack of separation of the diets and sexes shown in the PCA plot, a few volatile compounds did display a diet-sex interaction (Table 7.6). There was no obvious pattern of effect across the different volatile compounds however, and the majority of the compounds were only detected in day 5 samples. The only peak effected that was found in both day 1 and 5 samples, hexanal, was the highest in Ctrl females ($P = 0.04$), followed by Qrc males, with Ctrl males and Qrc females having the lowest concentrations.

Table 7.1

The effects of dietary quercetin supplementation (0 or 2 g/kg) and sex on the carcass yields and proximate composition of New Zealand White rabbit *longissimus thoracis et lumborum* (LTL) muscle (LSMean ± SEM).

	Overall	Diet		Sex	
		Control	Quercetin	Male	Female
Slaughter weight (SW, g)	3476 ± 70.2	3552 ± 104.6	3392 ± 90.2	3483 ± 86.7	3461 ± 107.5
Hot carcass weight (g)	2126 ± 41.0	2125 ± 60.7	2133 ± 53.8	2152^{a} ± 44.58	2107^{b} ± 64.94
Chilled carcass (CC) weight (g)	2040 ± 39.6	2038 ± 59.4	2047 ± 51.0	2062^{α} ± 42.7	2023^{β} ± 63.0
Dressing out percentage (% SW)	58.8 ± 0.30	58.7 ± 0.42	59.0 ± 0.45	59.4^{α} ± 0.40	58.2^{β} ± 0.42
Reference carcass (RC) weight (g)	1720 ± 33.6	1715 ± 52.5	1726 ± 41.6	1726 ± 35.0	1715 ± 55.1
RC yield (% CC)	84.0 ± 0.72	83.4 ± 1.32	84.5 ± 0.32	83.9 ± 0.26	84.0 ± 1.29
LTL moisture (%)	73.8 ± 0.10	73.8 ± 0.12	73.9 ± 0.18	74.0 ± 0.15	73.7 ± 0.14
LTL protein (%)	22.9 ± 0.17	23.1^{α} ± 0.25	22.5^{β} ± 0.21	22.6 ± 0.29	23.0 ± 0.19
LTL fat (%)	2.70 ± 0.171	2.51 ± 0.212	2.93 ± 0.277	2.78 ± 0.317	2.67 ± 0.183
LTL ash (%)	1.17 ± 0.019	1.16 ± 0.027	1.17 ± 0.029	1.17 ± 0.028	1.17 ± 0.027

SEM: Standard error of the mean

Means with different superscript letters in the same row (within main effect) differ significantly (^{ab} $P \leq 0.05$), or tend to differ ($^{\alpha\beta}$ $P \leq 0.10$)

Table 7.2

The effects of dietary quercetin supplementation (0 or 2 g/kg) and sex on minced New Zealand White rabbit *longissimus thoracis et lumborum* (LTL) muscle pH, colour, and oxidative status during refrigerated storage for 1, 3 or 5 days in oxygen-permeable packaging at 3.2 °C (LSMean ± SEM).

		Overall	Diet		Sex		Days *post-mortem*		
			Control	Quercetin	Male	Female	1	3	5
pH		5.72 ± 0.008	5.71 ± 0.010	5.73 ± 0.013	5.76^{a} ± 0.012	5.68^{b} ± 0.008	5.70^{b} ± 0.015	5.72^{b} ± 0.012	5.74^{a} ± 0.013
Colour	L*	55.6 ± 0.20	55.5 ± 0.25	55.5 ± 0.33	55.2 ± 0.31	55.9 ± 0.27	*55.0^{b} ± 0.40*	*55.6^{ab} ± 0.32*	*56.0^{a} ± 0.33*
	a*	1.50 ± 0.137	1.37 ± 0.171	1.62 ± 0.215	1.26 ± 0.206	1.73 ± 0.181	0.86^{c} ± 0.219	2.30^{a} ± 0.191	1.33^{b} ± 0.238
	b*	11.0 ± 0.13	10.8 ± 0.16	11.3 ± 0.21	10.9 ± 0.22	11.2 ± 0.17	10.4^{c} ± 0.19	11.8^{a} ± 0.18	11.0^{b} ± 0.26
	H°	83.0 ± 0.66	83.4 ± 0.84	82.7 ± 1.04	84.3 ± 1.03	81.7 ± 0.83	85.8^{a} ± 1.16	79.2^{b} ± 0.78	84.0^{a} ± 1.18
	C*	11.2 ± 0.15	11.0 ± 0.18	11.5 ± 0.23	11.0 ± 0.23	11.4 ± 0.18	10.5^{c} ± 0.20	12.0^{a} ± 0.21	11.2^{b} ± 0.27
Lipid oxidation (mg MDA/kg meat)		25.3 ± 1.27	23.4 ± 1.52	27.5 ± 2.06	24.1 ± 1.61	26.8 ± 1.91	26.8 ± 1.74	22.3 ± 2.52	27.3 ± 2.25
FRAP (mmol Fe^{2+} eq/kg wet meat)		0.37 ± 0.007	0.37 ± 0.010	0.37 ± 0.009	0.38 ± 0.012	0.36 ± 0.008	0.39^{α} ± 0.013	$0.37^{\alpha\beta}$ ± 0.012	0.36^{β} ± 0.010

SEM: standard error of the mean; MDA: malondialdehyde; FRAP: ferric-reducing antioxidant power; Fe^{2+} eq: ferrous equivalent

Means with different superscript letters in the same row (within main effect) differ significantly (^{abc} $P \leq 0.05$), or tend to differ ($^{\alpha\beta}$ $P \leq 0.10$)

Of the alcohols, pentan-1-ol did not differ between the sexes for the Ctrl group, but had higher (P = 0.01) day 5 levels in Qrc males than in all the other groups. Conversely, 2-[(1S)-4-methylcyclohex-3-en-1 had the highest (P = 0.001) levels in Ctrl males, with the other groups not differing. The one alkane, 3-methyldecane (P = 0.02), showed the same pattern, with Ctrl males having the highest levels, whereas 2-methylocta-4,6-diyn-3-one (P = 0.01) was most prevalent in Qrc females.

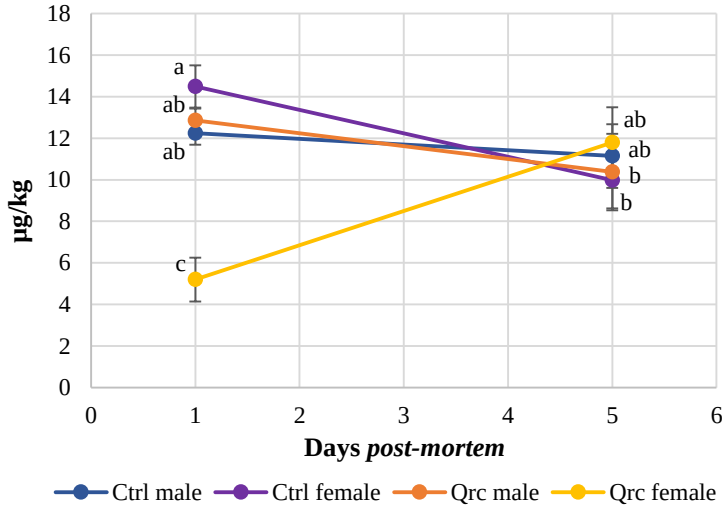

Figure 7.4 The effect of the interaction between dietary quercetin supplementation (Ctrl: 0 or Qrc: 2 g/kg) and sex on the 2,6-dimethyloct-7-en-2-ol (RT 18.08 minutes) content of New Zealand White rabbit *longissimus thoracis et lumborum* muscle during refrigerated storage for 1 or 5 days in oxygen-permeable wrapping at 3.2 °C. Error bars indicate the standard error of the mean. Data points with different significance letters differ significantly ($P \leq 0.05$).

Eight compounds showed a sex effect (Table 7.3), with all the esters being higher in meat from female rabbits, whereas the two heterocyclic compounds (1,4-xylene, P = 0.07, and 1,4-dichlorobenzene, P = 0.10) and the one terpenoid (4,7,7-trimethylbicyclo[2.2.1]heptan-3-one, P = 0.002) were higher in males. Diet also affected a few compounds, independent of storage time, with 2-methyldecane (P = 0.02) being higher for Ctrl rabbits, but 1,4-xylene (P = 0.03) and ethyl decanoate (P = 0.05) being higher in meat from Qrc rabbits.

With regards to the microbial status of the stored meat samples (Table 7.7), only five of the 34 samples had any growth on the *E. coli*/coliform petrifilms, and none of these were identified as *E. coli*. The coliform colony-forming unit (CFU) counts were also all below the minimum reliable count, even for the lowest dilution (10^{-1}), and statistical analysis was therefore not possible. As can be seen in Table 7.7, the distribution of those samples that tested positive for coliforms was relatively even across the treatment groups.

Statistical analysis was performed for the APC data, and the interaction between diet and sex was significant (P = 0.03). Qrc females had the highest APC levels (5.2 ± 0.18 log CFU) and Ctrl females had the lowest (4.4 ± 0.11 log CFU), with the meat from male rabbits fed either diet having intermediate levels.

7.4 Discussion

Prior to examining the changes in the meat during storage, and the possible effects of sex and quercetin supplementation on these, it was necessary to establish a baseline for the carcass and meat proximate composition (Table 7.1). As was found previously (North *et al.*, 2018b, Chapter 5), there were few treatment effects, with only the hot carcasses being heavier in male rabbits, and this having little effect on the dressing percentage. The somewhat greater effect of sex in this study than was found by North *et al*. (2018b) may have been due to the older age of the rabbits, and thus slightly greater maturity at slaughter. The only diet effect was a slightly lower protein content in Qrc rabbits. This was in contrast with the results of North *et al*. (2018b), and does not appear to have been measured in previous studies. However, this difference was extremely small.

As can be seen in the PCA observations plot (Figure 7.1), the majority of the variation in the samples for the shelf-life variables was as a result of the different storage periods. This was linked to both basic physical and chemical parameters and the more complex volatile compound profile of the meat (Figure 7.2).

7.4.1 Basic physicochemical parameters

The changes in the pH of the meat during storage (Table 7.2) were in agreement with the results of Lan, Shang, Song and Dong (2016), who found no change from day 0 to 2, followed by an increase from day 2 to 4 and onwards. This increase was attributed to the degradation of muscle proteins resulting in the accumulation of alkaline break-down products such as ammonia and amines (Rodríguez-Calleja, García-López, Santos & Otero, 2005).

Male rabbits had consistently higher pH values than females. This concurred with the findings of North *et al*. (2018b), Pla, Guerrero, Guardia, Oliver and Blasco (1998) and Yalçin, Onbaşilar and Onbaşilar (2006), but contradicted Carrilho, Campo, Olleta, Beltrán and López (2009), Dalle Zotte, Ouhayoun, Parigi Bini and Xiccato (1996) and Trocino, Xiccato, Queaque and Sartori (2003), who found no differences between the sexes. This difference in pH could have been linked to muscle fibre type and physiology, as sex effects on these have been reported previously (Dalle Zotte *et al*., 1996; Lawrie & Ledward, 2006). However, no such sex-effect on pH or muscle fibre composition was reported by Dalle Zotte and Rémignon (2005), although muscles from male rabbits had larger red, fast-twitch fibres than females.

The meat colour changes during storage were expected (Table 7.2); however, the patterns of change contradicted some previous reports of either consistent increases or decreases in colour parameters during storage (Lan *et al*., 2016; Mancini, Secci, Preziuso, Parisi & Paci, 2018). However, somewhat similar results were reported by Corino, Pastorelli, Pantaleo, Oriani and Salvatori (1999), for rabbits supplemented with dietary vitamin E. Considering the chemistry of meat colour, the initial increases in a*, b* and chroma values observed were likely due to an increase in the oxymyoglobin content, while the later declines in the a* and chroma values and increase in hue angle were due to increasing proportions of metmyoglobin, a product of oxidation during storage (Karamucki, Jakubowska, Rybarczyk & Gardzielewska 2013; Lindahl, Karlsson Lundström & Andersen, 2006). The apparent delay in the discolouration of the meat, relative to previous studies, may be indicative of oxidative

stability. Alternatively, it is possible that despite allowing at least 3 hours bloom time before measuring colour on day 1, the blooming process was not yet complete when the measurements were taken. Maj, Bieniek, Sternstein, Węglarz and Zapletal (2012) found that a*, b* and chroma values of rabbit LTL meat continued to increase from 45 minutes to 24 hours *post-mortem*.

Although the L* values also appeared to increase during the storage period (Table 7.2), the sex-day second-order interaction was significant, and this increase was in fact only found in samples from male rabbits (Figure 7.3). The sex effect was also only significant for the fresh meat samples, with no differences at 3 and 5 days *post-mortem*. This effect of sex on lightness, although not previously reported (Maj *et al*., 2012), was likely linked to the higher pH of meat from male rabbits, as a higher pH tends to correlate with a decrease in lightness values (Karamucki *et al.*, 2013). However, this relationship between pH and colour was not consistent throughout the storage period.

The lack of change in the TBARS content of the meat was surprising (Table 7.2), as many previous studies have reported increases during storage (Fernández-Esplá & O'Neill, 1993; Lan *et al*., 2016; Mancini *et al*., 2018), and lipid oxidation is an important component of the *post-mortem* changes in meat (Gray *et al*., 1996). However, while Corino *et al*. (1999) also reported an increase in TBARS during storage, it only occurred between 4 and 10 days *post-mortem*. Oxidation may therefore have simply been delayed in this study, as there does appear to be some tendency for an increase in TBARS from day 3 to day 5 (P_{LSD} = 0.08). This also concurs with the apparent lag phase in the discolouration process that was speculated on previously. A more extended sampling period would have provided clarification regarding the pattern of change in lipid oxidation levels**.**

Although there was a tendency for FRAP values to decrease during storage, this change was very small and non-significant. This concurred with the lack of increase in the TBARS content, and with the results of Mancini *et al.* (2018). Nonetheless, the FRAP assay results did appear to suggest that oxidation occurred during storage, although to a very minor extent.

7.4.2 Volatile profile

In contrast with the findings for the TBARS and FRAP assays, the volatile compound profile of the samples differed significantly between the two storage periods, with some compounds only being detected in samples from one of the storage times (Table 7.3). However, the interpretation of these results was complicated by the lack of previous volatile data for rabbit meat, and the predominance in literature of studies of the volatile composition of cooked, rather than raw, meat.

7.4.2.1 Esters

The most prevalent chemical group in raw rabbit meat was the esters, accounting for 67.04 % of the total volatile content (Table 7.3). This was unexpected, as most previously reported volatile profiles for raw meat samples have not had esters as a primary component (Saraiva *et al*., 2015; Schindler, Krings, Berger & Orlien, 2010; Soncin, Chiesa, Cantoni & Biondi, 2007).

Table 7.3

The effects of dietary quercetin supplementation (0 or 2 g/kg) and sex on the volatile composition (µg/kg) of raw, minced New Zealand White rabbit *longissimus thoracis et lumborum* meat, during refrigerated storage for 1 or 5 days in oxygen-permeable packaging at 3.2 °C (LSMean ± SEM).

RT	Volatile compounds*	% Prob	RI	Overall	Diet		Sex		Days *post-mortem*	
					Control	Quercetin	Male	Female	1	5
Alcohols 16.79 %										
14.91	hexan-1-ol	39.9	1286	10.6 ± 2.280	10.6 ± 3.445	11.0 ± 3.028	13.5 ± 4.288	8.13 ± 2.089	n.d	10.6 ± 2.280
16.19	2-butoxyethanol	59.7	1329	8.99 ± 1.429	10.1 ± 2.038	7.94 ± 1.998	9.27 ± 1.743	8.72 ± 2.234	13.5^{a} ± 2.638	4.54^{b} ± 0.317
16.20	2-(2-methylpropoxy)ethanol	41.9	1329	3.71 ± 0.685	2.79 ± 0.874	4.57 ± 1.031	2.71 ± 0.975	4.65 ± 0.933	3.71 ± 0.685	n.d
17.61	oct-1-en-3-ol	57.6	1379	11.8 ± 4.269	10.9 ± 2.799	14.7 ± 8.921	16.7 ± 8.961	8.90 ± 2.601	n.d	11.8 ± 4.269
18.64	2-propylpentan-1-ol	21.9	1416	56.9 ± 9.216	54.6 ± 13.09	58.6 ± 13.26	58.8 ± 13.50	54.3 ± 12.98	n.d	56.9 ± 9.216
Aldehydes 0.45 %										
16.90	3-(4-tert-butylphenyl)propanal	47.1	1354	1.34 ± 0.279	1.39 ± 0.396	1.21 ± 0.403	1.28 ± 0.396	1.33 ± 0.403	n.d	1.34 ± 0.279
Alkanes 0.97%										
5.45	4-ethylheptane	37.7	1024	2.29 ± 0.355	2.42 ± 0.516	2.05 ± 0.497	1.81 ± 0.537	2.66 ± 0.458	n.d	2.29 ± 0.355
5.62	2-methyldecane	25.9	1030	2.27 ± 0.259	2.84^{a} ± 0.345	1.61^{b} ± 0.346	2.37 ± 0.434	2.09 ± 0.309	n.d	2.27 ± 0.259
Carboxylic acids 0.13 %										
23.59	2-methylheptanoic acid	36.2	1612	0.51 ± 0.090	0.54 ± 0.120	0.45 ± 0.138	0.41 ± 0.123	0.58 ± 0.131	n.d	0.51 ± 0.090
31.20	2-methylhexanoic acid	40.2	1967	0.36 ± 0.066	0.28 ± 0.082	0.45 ± 0.103	0.38 ± 0.095	0.35 ± 0.094	n.d	0.36 ± 0.066
Esters 67.04 %										
6.50	methyl pentanoate	82.1	1061	2.78 ± 0.238	2.57 ± 0.326	3.10 ± 0.349	*3.32^{a} ± 0.460*	*2.35^{b} ± 0.157*	*1.98^{b} ± 0.238*	*3.69^{a} ± 0.363*
9.80	methyl hexanoate	75.4	1149	179 ± 14.76	165 ± 20.05	192 ± 22.00	*157 ± 21.55*	*200 ± 19.98*	*158^{β} ± 18.91*	*199^{α} ± 22.50*
11.30	ethyl hexanoate	61.9	1183	0.81 ± 0.186	0.75 ± 0.196	0.79 ± 0.336	0.36^{b} ± 0.213	1.19^{a} ± 0.273	0.81 ± 0.186	n.d
12.10	methyl 2-ethylhexanoate	60.5	1201	1.90 ± 0.220	2.00 ± 0.275	1.74 ± 0.354	1.53 ± 0.251	2.21 ± 0.343	2.27^{α} ± 0.408	1.47^{β} ± 0.137
12.95	methyl heptanoate	61.4	1227	3.07 ± 0.250	2.94 ± 0.324	3.20 ± 0.392	*2.87 ± 0.298*	*3.27 ± 0.392*	*4.58^{a} ± 0.298*	*1.56^{b} ± 0.148*
13.95	methyl 2-hydroxypropanoate	77.5	1257	158 ± 8.890	152 ± 9.551	162 ± 15.69	143^{β} ± 11.81	172^{α} ± 12.92	191^{a} ± 13.94	124^{b} ± 7.548
15.89	methyl octanoate	79.6	1319	72.1 ± 7.544	72.3 ± 10.73	70.7 ± 10.73	65.9 ± 10.99	77.2 ± 10.43	28.7^{b} ± 2.936	114^{a} ± 10.55
18.63	methyl nonanoate	74.3	1416	5.72 ± 0.487	5.33 ± 0.450	6.00 ± 0.916	4.69^{b} ± 0.570	6.64^{a} ± 0.713	5.72 ± 0.487	n.d
22.20	ethyl decanoate	57.6	1556	1.43 ± 0.209	1.00^{b} ± 0.190	1.84^{a} ± 0.376	*1.08 ± 0.171*	*1.76 ± 0.359*	*2.07^{a} ± 0.372*	*0.77^{b} ± 0.107*
22.44	methyl (Z)-dec-4-enoate	43.7	1565	5.14 ± 0.673	4.20 ± 0.769	5.91 ± 1.135	*3.32^{b} ± 0.718*	*6.78^{a} ± 1.046*	*8.15^{a} ± 1.083*	*1.95^{b} ± 0.257*

Table 7.3 *(continued)*

RT	Volatile compounds*	% Prob	RI	Overall	Diet		Sex		Days *post-mortem*	
					Control	Quercetin	Male	Female	1	5
Esters *(continued)*										
27.04	methyl (E)-dodec-9-enoate	45.5	1763	0.65 ± 0.143	0.54 ± 0.198	0.70 ± 0.210	0.32^{b} ± 0.196	0.93^{a} ± 0.190	0.65 ± 0.143	n.d
27.54	(1-hydroxy-2,4,4-trimethylpentan-3-yl) 2-methylpropanoate	42.3	1786	0.65 ± 0.113	0.66 ± 0.162	0.61 ± 0.160	0.60 ± 0.155	0.67 ± 0.164	n.d	0.65 ± 0.113
29.17	methyl 12-methyltridecanoate	35.7	1864	1.20 ± 0.150	1.01 ± 0.195	1.39 ± 0.228	1.01 ± 0.184	1.39 ± 0.231	1.72^{a} ± 0.246	0.68^{b} ± 0.126
31.47	methyl 12-methyltetradecanoate	63.7	1981	0.75 ± 0.084	0.76 ± 0.109	0.74 ± 0.131	0.71 ± 0.115	0.79 ± 0.122	0.98^{a} ± 0.135	0.52^{b} ± 0.084
31.99	2-chloroethenyl dimethyl phosphate	87.7	2001	0.72 ± 0.073	0.68 ± 0.116	0.77 ± 0.087	0.77 ± 0.121	0.68 ± 0.087	n.d	0.72 ± 0.073
32.13	methyl pentadecanoate	58.0	2002	2.40 ± 0.295	2.60 ± 0.406	2.16 ± 0.433	2.49 ± 0.378	2.27 ± 0.447	3.22^{a} ± 0.500	1.54^{b} ± 0.239
38.76	methyl octadeca-9,12-dienoate	32.2	2056	3.48 ± 0.520	*2.76^{b} ± 0.556*	*4.37^{a} ± 0.900*	3.70 ± 0.869	3.44 ± 0.619	*6.48^{a} ± 0.778*	*0.66^{b} ± 0.083*
Heterocyclic compounds 13.34 %										
Aromatic 11.57 %										
8.94	1-[1-(2,3,4,5-tetrahydropyridin-6-yl)cyclopropyl]ethanone	79.4	1130	0.88 ± 0.108	0.87 ± 0.160	0.89 ± 0.147	0.87 ± 0.185	0.90 ± 0.126	n.d	0.88 ± 0.108
9.45	pyridine	62.4	1141	36.6 ± 7.681	39.6 ± 13.30	33.8 ± 6.977	39.6 ± 10.46	33.8 ± 11.37	36.6 ± 7.681	n.d
9.47	1,4-xylene	51.1	1142	0.93 ± 0.156	0.64^{b} ± 0.117	1.32^{a} ± 0.287	1.25^{α} ± 0.306	0.71^{β} ± 0.096	n.d	0.93 ± 0.156
15.31	2-methoxy-3-methylpyrazine	92.9	1299	4.31 ± 1.098	5.21 ± 1.889	3.40 ± 0.981	4.74 ± 1.489	3.87 ± 1.628	4.31 ± 1.098	n.d
17.04	2-methoxy-3-propan-2-ylpyrazine	66.4	1359	4.94 ± 1.401	6.24 ± 2.373	3.68 ± 1.328	5.63 ± 1.915	4.29 ± 2.064	4.94 ± 1.401	n.d
17.25	1,4-dichlorobenzene	54.0	1366	0.61 ± 0.097	0.50 ± 0.134	0.77 ± 0.140	0.80^{α} ± 0.138	0.47^{β} ± 0.131	n.d	0.61 ± 0.097
20.09	3,7-dimethylocta-1,6-dien-3-yl 2-aminobenzoate *(linalyl anthranilate)*	59.2	1472	7.59 ± 0.928	7.47 ± 1.229	7.70 ± 1.424	7.58 ± 1.335	7.59 ± 1.307	0.74^{b} ± 0.146	14.4^{a} ± 0.800
21.73	dimethyl 2-(2-benzoylhydrazinyl)-2-hydroxypropanedioate	39.7	1537	0.95 ± 0.153	0.98 ± 0.218	0.89 ± 0.223	0.85 ± 0.240	1.02 ± 0.202	n.d	0.95 ± 0.153
23.14	(2-methylbenzoyl) 2-methylbenzoate	50.2	1593	0.98 ± 0.161	0.73 ± 0.169	1.23 ± 0.279	0.69^{β} ± 0.178	1.28^{α} ± 0.251	1.05 ± 0.271	0.91 ± 0.179

Table 7.3 *(continued)*

RT	Volatile compounds*	% Prob	RI	Overall	Diet		Sex		Days *post-mortem*	
					Control	Quercetin	Male	Female	1	5
Aromatic *(continued)*										
30.47	pentan-3-yl 2-chlorobenzoate	39.6	1929	0.53 ± 0.100	*0.60 ± 0.145*	*0.47 ± 0.140*	*0.53 ± 0.151*	*0.54 ± 0.137*	n.d	0.53 ± 0.100
35.46	dimethyl benzene-1,2-dicarboxylate	54.9	2030	10.9 ± 3.12	9.93 ± 4.67	12.2 ± 4.24	14.3 ± 5.65	7.83 ± 2.96	10.9 ± 3.12	n.d
36.71	diethyl benzene-1,2-dicarboxylate	46.5	2040	4.98 ± 1.30	4.59 ± 1.88	5.45 ± 1.84	6.16 ± 2.33	3.88 ± 1.27	4.98 ± 1.30	n.d
41.94	4-(2-methylbutan-2-yl)phenol	36.5	2082	1.70 ± 0.349	2.07 ± 0.543	1.37 ± 0.414	2.13 ± 0.564	1.31 ± 0.430	n.d	1.70 ± 0.349
Non-aromatic 1.77 %										
10.02	(4R)-1-methyl-4-prop-1-en-2-ylcyclohexene *(D-limonene)*	27.7	1154	6.09 ± 0.973	4.87 ± 1.187	7.51 ± 1.558	6.50 ± 1.405	5.88 ± 1.378	n.d	6.09 ± 0.973
18.91	4,7,7-trimethylbicyclo[2.2.1]heptan-3-one *(camphor)*	39.3	1427	0.15 ± 0.028	*0.21^{a} ± 0.047*	*0.09^{b} ± 0.020*	0.23^{a} ± 0.052	0.07^{b} ± 0.015	*0.19$^{\alpha}$ ± 0.051*	*0.11$^{\beta}$ ± 0.020*
21.87	oxolan-2-one *(butyrolactone)*	64.4	1542	2.16 ± 0.281	2.20 ± 0.459	2.14 ± 0.306	2.22 ± 0.338	2.13 ± 0.441	2.98^{a} ± 0.478	1.37^{b} ± 0.227
Ketones 0.77 %										
12.83	3-hydroxybutan-2-one *(acetoin)*	72.7	1223	1.68 ± 0.467	1.21 ± 0.420	2.32 ± 0.880	2.01 ± 0.866	1.51 ± 0.447	1.68 ± 0.467	n.d
Sulphur-containing compounds 0.61 %										
28.00	Methylsulfonylmethane	90.5	1807	4.00 ± 0.692	3.56 ± 0.749	4.59 ± 1.214	4.67 ± 1.217	3.47 ± 0.744	6.83^{a} ± 1.194	1.31^{b} ± 0.282

*Tentative identification by comparison of mass spectra to NIST 11 library, IUPAC name given, common name in italics for some compounds
SEM: Standard error of the mean; RT: Retention time; % Prob: percentage probability of identification, based on mass spectra; RI: Retention index , n.d.: not detected
^{ab} Means with different superscript letters in the same row (within main effect) differ significantly ($P \leq 0.05$)
$^{\alpha\beta}$ Means with different superscript Greek letters in the same row (within main effect) show a tendency towards significant differences ($P \leq 0.10$)
Italicised values: second- or third-order interactions of main effects significant (see Tables 7.4, 7.5 and 7.6)

One exception to this was raw foal meat, in which they were the dominant volatile chemical group (Domínguez, Gómez, Fonseca & Lorenzo, 2014a; Domínguez, Gómez, Fonseca & Lorenzo, 2014b; Lorenzo & Domínguez, 2014). This similarity between rabbit and foal meat may be related to their similar digestive systems and/or similar diets, which may have resulted in analogous flavour precursors in the meat.

Esters are formed in meat by the esterification of alcohols with carboxylic acids, through the action of either endogenous enzymes or microbial activity. They are thought to be degraded *post-mortem* during the lipid oxidation process, with a negative correlation between TBARS levels and the ester content being reported (Lorenzo & Domínguez, 2014**;** Schindler *et al*., 2010)**.** In this study, 12 of the 17 identified esters decreased or tended to decrease during storage (Table 7.3), which seems to support this theory, despite the absence of a significant increase in TBARS, which may have been related to the limited number of samples used.

The changes during storage of five of the identified esters differed between the sexes (Table 7.5). Methyl heptanoate, ethyl decanoate and methyl (Z)-dec-4-enoate all decreased in concentration during storage, with females having a greater decline than males, largely as a result of a higher content at day 1. This may be related to the effects of sex on the fatty acid composition, with the lower polyunsaturated fatty acids (PUFA) levels in females, as reported by Lazzaroni, Biagini and Lussiana (2009), perhaps reducing the rate of lipid oxidation, and thus allowing the higher ester level in day 1 samples from female rabbits. However, rabbits reared under the same conditions but slaughtered at 12 weeks of age showed no significant difference in the proportion of PUFA in the LTL between the sexes (Chapter 6), although the LTLs from male rabbits did contain higher levels of C18:1n-9.

Methyl pentanoate and methyl hexanoate both increased in concentration in meat from male rabbits during storage, but did not change in females (Table 7.5). Similarly, methyl octanoate, (1-hydroxy-2,4,4-trimethylpentan-3-yl)2-methylpropanoate and 2-chloroethenyl dimethyl phosphate increased during storage. This suggests that there is some balance between the esterification process and the degradation of the formed esters in the meat *post-mortem*, with, for some esters, this balance leaning in the favour of formation. Some studies have found that the ester content increased during the drying-ripening (Lorenzo, 2014) or seasoning (Lorenzo & Carballo, 2015) process of dry-cured foal meat.

For two of the esters detected, the change during storage was influenced by the dietary treatment (Table 7.4). In both cases, a greater decline from day 1 to day 5 was found for the samples from the Qrc rabbits, largely due to higher day 1 levels in treated rabbits. This may suggest that lipid oxidation occurred to a greater extent in the Qrc than the Ctrl samples, which contradicts previous findings for meat from lambs supplemented with quercetin (Andrés, Huerga, *et al.*, 2014; Andrés, Morán, *et al.*, 2014). However, it is possible that the initially higher concentrations of these esters in the Qrc samples was due to some protective effect of the quercetin reducing oxidation, and thus degradation, very early *post-mortem*.

.

Table 7.4

The effect of the interaction between dietary quercetin supplementation (0 or 2 g/kg) and storage period (1 or 5 days) on the volatile composition (µg/kg) of raw New Zealand White rabbit *longissimus thoracis et lumborum* muscle, minced and stored in oxygen-permeable packaging at 3.2 °C (LSMean ± SEM).

RT	Volatile compounds*	% Prob	RI	Overall	Control		Quercetin	
					1	5	1	5
Alcohols								
18.08	2,6-dimethyloct-7-en-2-ol	58.3	1395	11.0 ± 0.532	*13.4^{a} ± 0.626*	*10.6^{b} ± 1.001*	*9.03^{b} ± 1.147*	*11.1^{ab} ± 1.222*
Aldehydes								
6.35	hexanal	63.1	1056	1.63 ± 0.259	3.25^{a} ± 0.641	0.33^{c} ± 0.081	2.01^{b} ± 0.384	0.97^{bc} ± 0.474
Esters								
31.47	methyl 12-methyltetradecanoate	63.7	1981	0.75 ± 0.084	0.86$^{\alpha\beta}$ ± 0.187	0.66$^{\alpha\beta}$ ± 0.113	1.10$^{\alpha}$ ± 0.197	0.38$^{\beta}$ ± 0.121
38.76	methyl octadeca-9,12-dienoate	32.2	2056	3.48 ± 0.520	4.76^{b} ± 0.886	0.76^{c} ± 0.131	8.20^{a} ± 1.204	0.55^{c} ± 0.088
Heterocyclic compounds — Non-aromatic								
18.91	4,7,7-trimethylbicyclo[2.2.1]heptan-3-one *(camphor)*	39.3	1427	0.15 ± 0.028	0.30^{a} ± 0.086	0.12^{b} ± 0.028	0.08^{b} ± 0.028	0.10^{b} ± 0.028

*Tentative identification by comparison of mass spectra to NIST 11 library, IUPAC name given, common name in italics for some compounds
SEM: Standard error of the mean; RT: Retention time; % Prob: percentage probability of identification, based on mass spectra; RI: Retention index
^{abc} Means with different upper case superscript letters in the same row (within main effect) differ significantly ($P \leq 0.05$)
$^{\alpha\beta}$ Means with different superscript Greek letters in the same row (within main effect) show a tendency towards significant differences ($P \leq 0.10$)
Italicised values: third-order interaction of main effects significant (Figure 7.4)

While esters are important flavour components in cured meats, they appear to be largely degraded during the cooking process (Domínguez *et al*., 2014b), and may therefore not form an important component of the cooked rabbit meat flavour profile, despite their high content in the raw meat. The determination of the volatile profile of cooked rabbit meat would be necessary to confirm this speculation

7.4.2.2 Alcohols

The second most prevalent chemical group was the alcohols, contributing 16.79 % of the total volatiles (Table 7.3). Unlike the esters, alcohols were not a dominant chemical group in raw foal meat (Domínguez *et al*., 2014a; Domínguez *et al*., 2014b; Lorenzo & Domínguez, 2014); however, they were the most prevalent compounds in both raw pork and raw duck meat, although for a limited number of samples (Soncin *et al*., 2007). While some authors consider alcohols to be less important flavour components in meat, due to their relatively high odour thresholds (Drumm & Spanier, 1991; Morán *et al*., 2013), aliphatic alcohols are thought to have an impact on meat flavour (Soncin *et al*., 2007). However, it must again be emphasized that cooking could cause extensive changes in the concentrations of these volatile compounds, with pentan-1-ol increasing in foal meat during cooking (Domínguez *et al*., 2014a), but oct-1-en-3-ol being undetectable in cooked foals' meat, despite being present in the raw meat (Lorenzo & Domínguez, 2014).

The changes in the alcohols during storage consisted predominantly of increases in concentration (Table 7.3), with five of the compounds only being detected in day 5 samples. As alcohols are formed through the reduction of aldehydes, which are the products of the oxidation of lipids and amino acids (Morán *et al*., 2013; Soncin *et al*., 2007), this increase concurs with expectations. Proposed precursors for this process include PUFA such as C18:2n-6 and C20:4n-6, as well as the amino acid phenylalanine, which is broken down to an aldehyde via the Strecker reaction (Lorenzo & Domínguez, 2014; Soncin *et al.*, 2007). However, two alcohols (2-butoxyethanol and 2-(2-methylpropoxy)ethanol) decreased during storage. It is possible that these compounds were degraded to other chemical groups, and/or that the necessary precursor molecules had been depleted.

For one of the alcohols, 2,6-dimethyloct-7-en-2-ol, the change during storage was influenced by both diet and sex, tending to decrease in meat from Ctrl rabbits and Qrc males, but significantly increasing in Qrc female samples, which contained much lower concentrations than the other groups at day 1 (Figure 7.4). This could suggest that either the initial oxidation process, or the secondary reducing activity, was retarded in these samples, possibly due to a lower C18:1n-9 content (Chapter 6), which could have slowed lipid oxidation, and this may have interacted with some antioxidant effect of the quercetin supplementation (Andrés, Huerga, *et al*., 2014; Andrés, Morán, *et al.*, 2014). However, if this was the case, similar interactions for the other volatile compounds would be expected.

Pentan-1-ol was only detected in day 5 samples, but the level in these samples was affected by a diet-sex interaction, with Qrc males having much higher levels than the other treatment groups (Table 7.6). This may be related to differences in the fatty acid composition resulting in increased levels of aldehyde production through oxidation, in conjunction with some increase in reducing activity as a result of quercetin supplementation.

7.4.2.3 Heterocyclic compounds — aromatic

The third-most prevalent (13.34 %), and most heterogeneous, chemical group was the heterocyclic compounds, including both aromatic and non-aromatic volatiles (Table 7.3). Relatively large proportions of these compounds were also found in raw foal meat (Domínguez *et al.*, 2014a; Domínguez *et al*., 2014b; Lorenzo & Domínguez, 2014), and some were detected in raw beef (Saraiva *et al.*, 2015). Nonetheless, the concentration and diversity of structures found in rabbit meat was comparatively high. However, the cooking process does appear to decrease the amount of cyclic volatiles, suggesting that they may not play as large a role in the cooked flavour profile as the raw profile suggests (Domínguez *et al.*, 2014a).

The majority of the heterocyclic compounds tentatively identified were aromatic, containing a benzene-type ring structure (Table 7.3). The presence of the pyrazines and pyridines was somewhat unexpected as they were not found in raw foal meat (Domínguez *et al*., 2014a), and are generally thought to be products of the Maillard reaction taking place during cooking (Domínguez *et al.*, 2014a; Resconi, Escudero & Campo, 2013; Watanabe & Sato, 1971). However, these reactions can occur, if very slowly, in the absence of heat (Resconi *et al.*, 2013). It may thus be that rabbit meat provided conditions favourable for the formation of these compounds, as a result of precursor (reducing sugars and amino acids) concentrations and/or the reactivity of the available amino acids (Resconi *et al*., 2013).

The lack of pyrazines detected in the day 5 samples may have been due to aldehydes produced by lipid oxidation reacting with the necessary amino groups and thereby making them unavailable for the formation of aminocarbonyls, which are pyrazine precursors (Resconi *et al.*, 2013). Surprisingly, pyridine was only found in day 1 samples, whereas 1-[1-(2,3,4,5-tetrahydropyridin-6-yl)cyclopropyl]ethanone was only found in day 5 samples (Table 7.3). This may suggest that chemical interactions involving pyridine take place during storage, resulting in the formation of more complex chemical compounds.

Unlike most of the other aromatic compounds, 1,4-xylene was found in raw foal meat (Domínguez *et al*., 2014a; Domínguez *et al*., 2014b; Lorenzo & Domínguez, 2014) and raw beef (Saraiva *et al*., 2015) although it was not found in pork, duck or goose meat (Soncin *et al*., 2007), or in beef or chicken by Schindler *et al*. (2010). It has been suggested that 1,4-xylene present in meat is transferred from ingested plants or the environment (Meynier, Novelli, Chizzolini, Zanardi & Gandemer, 1999; Warinda Vejaphan, Hsieh & Williams, 1988); however, in this case it was only detected after 5 days chilled storage (Table 7.3). This may suggest that the 1,4-xylene developed in the meat during storage (Watanabe & Sato, 1971) or that there were changes in the release of the compound from the meat structure. 1,4-Xylene has been found to be relatively stable during cooking, and may therefore contribute to fruity and sweet aromas in cooked rabbit meat (Lorenzo & Domínguez, 2014).

In addition to only being detected in day 5 samples, 1,4-xylene was also affected by the dietary treatment, with samples from Qrc rabbits containing more than double the concentration of 1,4-xylene than those from Ctrl rabbits (Table 7.3). If this compound was ingested and absorbed directly from the feed, this would suggest that either the quercetin supplementation altered the gut environment, and thus the absorption of 1,4-xylene, or that the

quercetin supplement itself contained 1,4-xylene. However, both seem unlikely, as a purified (> 95.86 % quercetin dihydrate) extract was used, and 1,4-xylene was not detected in either feed. Alternatively, if the 1,4-xylene developed in the meat during storage, or the 1,4-xylene was released more from day 5 samples, the diet effect would suggest that a greater degree of change occurred in meat from the Qrc rabbits, suggesting that the quercetin was acting as a pro-oxidant rather than an antioxidant (Morán *et al*., 2013). In terms of the tendency for a difference between the sexes, the reason for the higher content in samples from male rabbits is unclear.

The benzene-compounds tentatively identified in this study do not appear to have been detected previously, and most of the complex alkylbenzenes, as well as the single phenol, increased during storage. This suggests that they formed as products of lipid oxidation, or carbohydrate or amino acid degradation, rather than being from an environmental source, as suggested by Warinda Vejaphan *et al*. (1988). The two exceptions to the increase in the alkylbenzenes during storage were dimethyl phthalate (dimethyl benzene-1,2-dicarboxylate) and diethyl phthalate (diethyl benzene-1,2-dicarboxylate), both of which were only detected in day 1 samples (Table 7.3). Diethyl phthalate was detected in raw foal meat by Lorenzo and Domínguez (2014), but not by Domínguez *et al*. (2014a) or Domínguez *et al.* (2014b), and was similarly not detected in other raw meat by Soncin *et al*. (2007), Saraiva *et al*. (2015) or Schindler *et al*. (2010). Phthalates are considered food contaminants, and are commercially used as plasticisers for packaging polymers. It is thus possible that the dimethyl and diethyl phthalates found in the samples in this study were contaminants from the polystyrene punnets or the sterile stomacher bags used to line the punnets for the shelf-life study (Fasano, Bono-Blay, Cirillo, Montuori & Lacorte, 2012). However, if this was the case, one could have reasonably expected higher concentrations in the day 5 samples, due to their longer exposure to the packaging.

The presence of 1,4-dichlorobenzene (Table 7.3), even at low concentrations, was also surprising, as it is used industrially as a disinfectant, deodorant and pesticide, and is considered a carcinogen (Pohanish, 2002). Soncin *et al.* (2007), and Watanabe and Sato (1971) found 1,4-dichlorobenzene in goose and crayfish tail meat samples, respectively, and concluded that it was most likely a contaminant, a decomposition product of a pesticide. As it was detected in the feeds used in this study, it was most likely transferred to the meat from pesticide residues on the feed material.

7.4.2.4 Heterocyclic compounds — non-aromatic

The non-aromatic heterocyclic compounds comprised three terpenes and a lactone (Table 7.3). Limonene and camphor are generally thought to be of dietary origin, as they are almost exclusively synthesized by plants (Calkins & Hodgen, 2007; Resconi *et al*., 2013; Warinda Vejaphan *et al*., 1988). Limonene ((4R)-1-methyl-4-prop-1-en-2-ylcyclohexene), in particular, has been previously detected in *Medicago sativa* flowers, and its presence in the rabbit meat samples is thus most likely related to the alfalfa content of the feed used (Loper, 1972; Tava & Pecetti, 1997). This is supported by its detection in the feeds. The lack of limonene detected in day 1 samples could have been related to changes in the structure of the meat during storage allowing the greater release of the compound from day 5 samples (Resconi *et al*., 2013).

Table 7.5

The effect of the interaction between sex (male or female) and storage period (1 or 5 days) on the volatile composition (µg/kg) of raw New Zealand White rabbit *longissimus thoracis et lumborum* muscle, minced and stored in oxygen-permeable packaging at 3.2 °C (LSMean ± SEM).

RT	Volatile compounds*	% Prob	RI	Overall	Male		Female	
					1	5	1	5
Aldehydes								
6.35	hexanal	63.1	1056	1.63 ± 0.259	1.77^{b} ± 0.307	1.10^{bc} ± 0.466	3.49^{a} ± 0.636	0.20^{c} ± 0.077
Esters								
6.50	methyl pentanoate	82.1	1061	2.78 ± 0.238	1.79^{b} ± 0.412	4.85^{a} ± 0.623	2.17^{b} ± 0.267	2.53^{b} ± 0.160
9.80	methyl hexanoate	75.4	1149	179 ± 14.76	107^{b} ± 19.90	207^{a} ± 34.65	209^{a} ± 27.01	191^{a} ± 30.10
12.95	methyl heptanoate	61.4	1227	3.07 ± 0.250	3.90^{b} ± 0.385	1.84^{c} ± 0.275	5.25^{a} ± 0.399	1.29^{c} ± 0.117
22.20	ethyl decanoate	57.6	1556	1.43 ± 0.209	1.33^{b} ± 0.309	0.84^{b} ± 0.137	2.81^{a} ± 0.610	0.70^{b} ± 0.163
22.44	methyl (Z)-dec-4-enoate	43.7	1565	5.14 ± 0.673	5.38^{b} ± 1.229	1.26^{c} ± 0.266	10.9^{a} ± 1.528	2.64^{bc} ± 0.392

*Tentative identification by comparison of mass spectra to NIST 11 library, IUPAC name given
SEM: Standard error of the mean; RT: Retention time; % Prob: percentage probability of identification, based on mass spectra; RI: Retention index
^{abc} Means with different upper case superscript letters in the same row (within main effect) differ significantly ($P \leq 0.05$)

Table 7.6

The effect of the interaction between dietary quercetin supplementation (0 or 2 g/kg) and sex on the volatile composition (µg/kg) of raw New Zealand White rabbit *longissimus thoracis et lumborum* muscle, minced and stored for 1 or 5 days in oxygen-permeable packaging at 3.2 °C (LSMean ± SEM).

RT	Volatile compounds*	% Prob	RI	Overall	Control		Quercetin	
					Male	Female	Male	Female
Alcohols								
11.89	pentan-1-ol[#]	40.2	1196	7.13 ± 2.037	3.17^{b} ± 0.992	6.01^{b} ± 1.265	19.7^{a} ± 8.316	2.48^{b} ± 1.303
Aldehydes								
6.35	hexanal	63.1	1056	1.63 ± 0.259	1.11^{b} ± 0.297	2.47^{a} ± 0.727	1.76^{ab} ± 0.515	1.22^{b} ± 0.406
Alkanes								
5.84	3-methyldecane[#]	28.1	1038	1.16 ± 0.089	1.61^{a} ± 0.148	1.00^{b} ± 0.158	0.87^{b} ± 0.181	1.08^{b} ± 0.147
Heterocyclic compounds — Non-aromatic								
23.51	2-[(1S)-4-methylcyclohex-3-en-1-yl]propan-2-ol[#] (*L-a-terpineol*)	50.4	1609	3.18 ± 0.487	5.86^{a} ± 1.003	1.57^{b} ± 0.179	1.67^{b} ± 0.169	3.29^{b} ± 1.112
Ketones								
7.58	2-methylocta-4,6-diyn-3-one[#]	63.8	1099	3.35 ± 0.706	2.42^{b} ± 0.593	1.78^{b} ± 0.401	1.47^{b} ± 0.560	7.32^{a} ± 1.995

*Tentative identification by comparison of mass spectra to NIST 11 library, IUPAC name given; [#] Only detected in day 5 samples
SEM: Standard error of the mean; RT: Retention time; % Prob: percentage probability of identification, based on mass spectra; RI: Retention index
^{ab} Means with different upper case superscript letters in the same row differ significantly ($P \leq 0.05$)

There was a diet-day interaction for camphor (4,7,7-trimethylbicyclo[2.2.1]heptan-3-one), with the Ctrl samples having higher levels than the Qrc samples at day 1, but not day 5 (Table 7.4). If the camphor was absorbed from the feed, this result suggests that the quercetin somehow reduced its absorption, and that it was broken down in the meat from the Ctrl rabbits *post-mortem*. However, in view of the low confidence with which this peak was identified, it is possible that this apparent effect was as a result of co-elution or misidentification. In addition, camphor was not detected in the feed, casting further doubt on its presence and origin.

One heterocyclic non-aromatic compound, L-a-terpineol (2-[(1S)-4-methylcyclohex-3-en-1-yl]propan-2-ol), was only found in day 5 samples, and showed a diet-sex interaction (Table 7.6). L-a-terpineol has typically been found in processed meat products, such as ham and salami, in previous studies (Lorenzo & Carballo, 2015; Meynier et al., 1999), and was thought to originate from the spice mixes used. Considering that it is a known plant secondary metabolite (Brewer, 2011), it would seem likely that it was of dietary origin in this case, despite only being detected in day 5 samples. However, L-a-terpineol was not detected in either the Qrc or Ctrl feed.

Butyrolactone was also found in raw foal meat (Domínguez *et al.*, 2014a), and is thought to be derived from products of lipid oxidation, specifically hydroxy acids (Morán *et al.*, 2013; Resconi *et al.*, 2013). However, this process may occur in the gastrointestinal tract and the living animal, rather than just taking place in the meat during storage (Resconi *et al.*, 2013). This may provide an explanation for the decrease in the butyrolactone content of the meat during storage (Table 7.3), as it could have been formed and deposited in the muscle prior to slaughter, and then subsequently broken down in the meat during storage.

7.4.2.5 Minor volatiles

Alkanes made a limited contribution to the total volatiles (0.97 %), far less than was found for raw foal meat (Domínguez *et al.*, 2014a; Domínguez *et al.*, 2014b; Lorenzo & Domínguez, 2014) but in agreement with reported volatile profiles for duck, goose and pork (Soncin *et al.*, 2007). They are typically products of lipid oxidation (Resconi *et al.*, 2013), which concurs with their detection in day 5 samples only. Alkanes have relatively high odour thresholds, and are consequently not considered to have a significant effect on meat flavour (Lorenzo & Domínguez, 2014), despite generally increasing in concentration during the cooking process (Domínguez *et al.*, 2014b).

The significant diet-sex interaction (Table 7.6) found for one of the alkanes, 3-methyldecane, may be linked to the higher levels of C18:1n-9 found in male rabbit meat (Chapter 6) resulting in a greater degree of oxidation, and thus formation of this alkane. The difference between the dietary treatments for male samples may thus indicate some degree of antioxidant activity by the quercetin supplementation. This would concur with the higher levels of 2-methyldecane found in Ctrl samples after 5 days of storage.

Acetoin, a ketone, has been detected in chicken (Ioannidis *et al.*, 2018), duck (Soncin *et al.*, 2007), beef (Saraiva *et al.*, 2015) and pork (Soncin *et al.*, 2007; Sun, Fu, Li & Peng, 2018), and is derived from the microbial degradation of aspartate or catabolism of glucose (Casaburi, Piombino, Nychas, Villani & Ercolini, 2015), which explains previous findings that the acetoin concentration increased with storage (Ioannidis *et al.*, 2018; Sun *et al.*,

2018). However, this was not the case for the rabbit meat samples, with only day 1 samples containing acetoin (Table 7.3), possibly due to further degradation during storage. While it is considered an important flavour component, producing creamy/buttery odours, the concentration was far below the reported odour threshold of 800 µg/L (Casaburi *et al*., 2015). It was also not influenced by sex or diet. Similarly, Morán *et al*. (2013) found that dietary carnosic acid did not influence acetoin levels in lamb meat.

A second ketone, 2-methylocta-4,6-diyn-3-one, was only present in day 5 samples, which concurs with literature. Day 5 levels differed for the four diet-sex treatment groups (Table 7.6), with Qrc females having higher levels than the other groups. As ketones are generally products of microbial activity, this may suggest a greater degree of microbial growth in the meat samples of this treatment, although why this should be the case is unclear.

The single sulphur-compound detected in raw rabbit meat was methylsulfonylmethane (MSM), or dimethyl sulfone (Table 7.3). Only two previous studies appear to have detected MSM in raw meat, in both cases foal meat (Domínguez *et al*., 2014b; Lorenzo & Domínguez, 2014). Dimethyl sulfone is naturally produced through the activity of marine microorganisms, oxidation and disproportionation, forming part of the sulphur cycle (Butawan, Benjamin & Bloomer, 2017). It is also a widely used alternative medicine, and has been demonstrated to have anti-inflammatory activity (Butawan *et al.*, 2017). In this case it seems likely that the MSM present in the meat was of dietary origin, as it was detected in both feeds, and this concurred with the decrease in its concentration during storage.

Somewhat surprisingly, the aldehydes made only a very small contribution to the total volatile compounds (0.48 %), with only two members of this chemical group being detected. Aldehydes usually form a major component of the meat volatile compound profile, being both indicators of oxidation and important odour components (Andrés, Huerga, *et al*., 2014; Morán *et al*., 2013). However, this may be more relevant for cooked than raw meat, as the aldehyde content of meat samples has been found to increase significantly during cooking (Lorenzo & Domínguez, 2014; Resconi *et al*., 2013). Nonetheless, the combined occurrence of a large alcohol component and a very limited aldehyde content may suggest that the reducing mechanisms in rabbit meat were extremely active (Soncin *et al*., 2007), converting aldehydes produced through lipid oxidation into alcohols before they could accumulate.

As products of the oxidation of PUFA, aldehydes also generally increase during storage (Morán *et al*., 2013; Ortuño, Serrano, Jordán & Bañón, 2014; Ortuño, Serrano, Jordán & Bañón, 2016). However, in this study, hexanal, which is thought to be derived from the oxidation of oleic (18:1n-9) and linoleic (18:2n-6) acid (Andrés, Huerga, *et al*., 2014), decreased from 1 to 5 days *post-mortem*. Descalzo *et al*. (2008), found a similar decline in hexanal in buffalo meat during storage, and overall very low levels of this aldehyde, and concluded that this was as a result of various genetic and environmental effects, including differences in fatty acid profile (PUFA specifically) and meat antioxidant content.

The decline in hexanal was also influenced by both diet and sex (Tables 7.4 and 7.5). While Qrc rabbits had lower levels than the Ctrls at 1 day *post-mortem*, which may indicate some antioxidant effect of the quercetin, this

difference was no longer present at 5 days *post-mortem*. This could suggest that the antioxidant effect of the quercetin was rapidly depleted during storage**,** concurring with its effect on the esters**.** The higher levels in samples from female rabbits at 1 day *post-mortem* may have been linked to differences between the sexes in the fatty acid content of the meat, although male rabbits were found to have higher levels of C18:1n-9 than females (Chapter 6). Once again, these differences were no longer present after 5 days of storage.

The interaction between diet and sex was also significant for hexanal concentrations (Table 7.6), with there being no effect of diet on meat from male rabbits, but Ctrl females having higher levels than Qrc females. This may be linked to some interaction between the intrinsic nature of the meat and the antioxidant effect of the supplemented quercetin.

The carboxylic acids made the lowest contribution to the volatile profile of rabbit meat (0.13 %). They are formed as products of lipid oxidation (Mottram, 1998), and their minimal concentration thus aligns with the results found for aldehydes and alkanes.

7.4.3 Microbial count

Although both the anaerobic plate count and coliform count were determined, coliforms were detected in too few samples to statistically compare the treatments, and none of the samples tested positive for *E. coli*. The distribution of those samples for which coliforms were detected was, however, relatively even across the treatments, with no trend for either sex or diet (Table 7.7).

Table 7.7
The effect of the interaction between dietary quercetin supplementation (0 or 2 g/kg) and sex on the microbial status of raw New Zealand White rabbit *longissimus thoracis et lumborum* muscle, minced and stored for 5 days in oxygen-permeable packaging at 3.2 °C (LSMean ± SEM).

	Overall	Control		Quercetin	
		Male	Female	Male	Female
APC log count	4.7 ± 0.11 (34)	4.7^{ab} ± 0.16 (9)	4.4^{b} ± 0.11 (9)	4.5^{ab} ± 0.36 (7)	5.2^{a} ± 0.18 (9)
Coliform log count	1.4 (5)	1 (1)	1.7 (2)	1.3 (1)	1.5 (1)

SEM: Standard error of the mean; APC: Aerobic plate count
^{ab} Means with different upper case superscript letters in the same row differ significantly ($P \leq 0.05$)
(The number of samples counted per treatment group is given in parentheses)

The diet-sex interaction for the APC indicated that while the dietary treatments did not differ for samples from male rabbits, Qrc female rabbits had higher counts than Ctrl females. This was in contrast to previous studies, which have generally found that flavonoids, including quercetin, have antimicrobial activity (Narayana, Reddy, Chaluvadi & Krishna, 2001). Furthermore, Koné *et al*. (2016) found that dietary supplementation with cranberry, strawberry or onion extracts reduced the microbial load on rabbit hindlegs while fresh and after aerobic storage for 15 days. The dietary supplementation of liquorice root extract, which contains isoflavones, among other bioactive components, also decreased the *Pseudomonas* species count after 3 and 6 days of storage (Dalle Zotte *et al*., 2017). However, after only 5 days of aerobic storage neither the onion nor strawberry extract had any significant effect on the total aerobic mesophilic count (Koné *et al*., 2016), and Dalle Zotte *et al*. (2017) found no

effect of dietary liquorice root extract on the total viable count of rabbit meat. It therefore does not appear that there is clear consensus on the antimicrobial effect of dietary flavonoids under practical conditions. Nonetheless, the higher APC for Qrc females was unexpected and difficult to explain, although it does concur with the pattern of differences found for 2-methylocta-4,6-diyn-3-one in day 5 samples. All the treatments had APC counts below 8 log CFU/g after 5 days of storage, indicating that they had not reached the end of their shelf-life by 5 days *post-mortem* (Rodríguez-Calleja *et al.*, 2005).

7.5 Conclusion

As can be seen in the PCA observations plot, extensive changes took place in the raw rabbit meat during storage. However, the changes in surface colour suggested a delay in discolouration, possibly indicating relatively high oxidative stability, with this being supported by the lack of change in TBARS and FRAP values during storage.

Overall, the volatile composition of the raw rabbit meat was quite unusual, being dominated by esters, alcohols and heterocyclic compounds, rather than by aldehydes and alkanes as often found for other species. However, it did seem to have some similarities to raw foal meat, possibly due to their similar feed resources and digestive systems. The volatile profile changed considerably during storage, with the ester content decreasing and alcohol content increasing. However, the overall concentrations of alkanes and aldehydes, both products of lipid oxidation, were extremely low, and hexanal, typically used as an indicator of oxidation, decreased during storage. This, along with the large proportions of esters and alcohols, the increase in the levels of some of the esters, and the lack of pyrazines in day 5 samples, suggested that while lipid oxidation did take place, it was only to a limited degree, and slow relative to the rates of the other chemical reactions taking place. The volatile profile found may therefore suggest that the reducing mechanisms in the rabbit meat samples were fairly active.

Relative to the changes during storage, the effects of sex and diet were very limited. However, meat from male rabbits did have a higher pH and darker meat on day 1, and a number of the volatile compounds differed between the sexes, both in overall levels and changes during storage. These differences may be linked to previous findings of sex effects on the fatty acid composition and muscle physiology of rabbit meat, although the effects on the former were found to be very limited for the LTL (Chapter 6). Higher ester and lower hexanal levels in fresh Qrc samples, and the higher alkane content of Ctrl samples, may suggest some decrease in oxidation due to supplementation. In some cases, the diet-effect also differed between the two sexes, suggesting an interaction between the intrinsic nature of the meat and the supplementation. However, these effects of diet were extremely minimal, and quercetin-supplementation did not improve the microbiological status of the meat.

In conclusion, it does not appear that the supplementation of quercetin provided any substantial benefits for rabbit meat shelf-life, during a storage period of up to 5 days.

7.6 References

AMSA. (2012). *Meat color measurement guidelines*. Illinois, USA: American Meat Science Association.

Andrés, S., Huerga, L., Mateo, J., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Rotola, L. & Giráldez, F. J. (2014). The effect of quercetin dietary supplementation on meat oxidation processes and texture of fattening lambs. *Meat Science, 96*, 806 – 811.

Andrés, S., Morán, L., Aldai, N., Tejido, M. L., Prieto, N., Bodas, R. & Giráldez, F. J. (2014). Effects of linseed and quercetin added to the diet of fattening lambs on the fatty acid profile and lipid antioxidant status of meat samples. *Meat Science, 97*, 156 – 163.

Blasco, A. & Ouhayoun, J. (1996). Harmonization of criteria and terminology in rabbit meat research. Revised proposal. *World Rabbit Science, 4*(2), 93 – 99.

Brewer, M. S. (2011). Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety, 10*, 221 – 247.

Butawan, M., Benjamin, R. L. & Bloomer, R. J. (2017). Methylsulfonylmethane: Applications and safety of a novel dietary supplement. *Nutrients, 9*, 1 – 21.

Calkins, C. R. & Hodgen, J. M. (2007). A fresh look at meat flavor. *Meat Science, 77*, 63 – 80.

Carrilho, M. C., Campo, M. M., Olleta, J. L., Beltrán, J. A. & López, M. (2009). Effect of diet, slaughter weight and sex on instrumental and sensory meat characteristics in rabbits. *Meat Science, 82*, 37 – 43.

Casaburi, A., Piombino, P., Nychas, G.-J., Villani, F. & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiology, 45*, 83 – 102.

Corino, C., Pastorelli, G., Pantaleo, L., Oriani, G. & Salvatori, G. (1999). Improvement of color and lipid stability of rabbit meat by dietary supplementation with vitamin E. *Meat Science, 52*, 285 – 289.

Dalle Zotte, A. (2002). Perception of rabbit meat quality and major factors influencing the rabbit carcass and meat quality. *Livestock Production Science, 75*, 11 – 32.

Dalle Zotte A., Cullere M., Giaccone V., Celia C., Szendrő Zs., Gerencsér Zs. & Matics Zs. (2017). Can liquorice extract administered as dietary and/or as food supplement improve rabbit meat shelf-life? *Proceedings of the 63rd International Congress of Meat Science and Technology — August 13 – 18, 2017, Cork, Ireland.* (pp. 498 – 499).

Dalle Zotte, A., Ouhayoun, J., Parigi Bini, R. & Xiccato, G. (1996). Effect of age, diet and sex on muscle energy metabolism and on related physicochemical traits in the rabbit. *Meat Science, 43*(1), 15 – 24.

Dalle Zotte A. & Rémignon H. (2005). Meat quality and muscle fibre traits in rabbits of different genetic origin and sex. *Proceedings of the 51st International Congress of Meat Science and Technology — August 7 – 12, 2005, Baltimore, Maryland, USA. Vol. Th42*, (pp. 1410 – 1415).

Dalle Zotte, A. & Szendrő, Zs. (2011). The role of rabbit meat as functional food. *Meat Science, 88*(3), 319 – 331.

Decker, E. A. & Park, Y. (2010). Healthier meat products as functional foods. *Meat Science, 86*, 49 – 55.

Descalzo, A. M., Rossetti, L., Grigioni, G., Irurueta, M., Sancho, A. M., Carrete, J. & Pensel, N. A. (2007). Antioxidant status and odour profile in fresh beef from pasture or grain-fed cattle. *Meat Science, 75*, 299 – 307.

Descalzo, A. M., Rossetti, L., Sancho, A. M., García, P. T., Biolatto, A., Carduza, F. & Grigioni, G. M. (2008). Antioxidant consumption and development of oxidation during ageing of buffalo meat produced in Argentina. *Meat Science, 79*, 582 – 588.

Domínguez, R., Gómez, M., Fonseca, S. & Lorenzo, J. M. (2014a). Effect of different cooking methods on lipid oxidation and formation of volatile compounds in foal meat. *Meat Science, 97*, 223 – 230.

Domínguez, R., Gómez, M., Fonseca, S. & Lorenzo, J. M. (2014b). Influence of thermal treatment on formation of volatile compounds, cooking loss and lipid oxidation in foal meat. *LWT — Food Science and Technology, 58*, 439 – 445.

Drumm, T. D. & Spanier, A. M. (1991). Changes in the content of lipid autoxidation and sulfur-containing compounds in cooked beef during storage. *Journal of Agricultural and Food Chemistry, 39*, 336 – 343.

Falowo, A. B., Fayemi, P. O. & Muchenje, V. (2014). Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food Research International, 64*, 171 – 181.

Fasano, E., Bono-Blay, F., Cirillo, T., Montuori, P. & Lacorte, S. (2012). Migration of phthalates, alkylphenols, bisphenol A and di(2-ethylhexyl)adipate from food packaging. *Food Control, 27*, 132 – 138.

Fernández-Esplá, M. D. & O'Neill, E. (1993). Lipid oxidation in rabbit meat under different storage conditions. *Journal of Food Science, 58*(6), 1262 – 1264.

Goliomytis, M., Kartsonas, N., Charismiadou, M. A., Symeon, G. K., Simitzis, P. E. & Deligeorgis, S. G. (2015). The influence of naringin and hesperidin dietary supplementation on broiler meat quality and oxidative stability. *PLoS One, 10*(10), 1 – 11.

Gray, J. I., Gomaa, E. A. & Buckley, D. J. (1996). Oxidative quality and shelf life of meats. *Meat Science, 43* (S), S111 – S123.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67 – 202.

Ioannidis, A.-G., Walgraeve, C., Vanderroost, M., Van Langenhove, H., Devlieghere, F. & De Irkin, R. & Arslan, M. (2010). Effect of onion (*Allium cepa* L.) extract on microbiological quality of refrigerated beef meat. *Journal of Muscle Foods, 21*, 308 – 316.

Jiang, J. & Xiong, Y. L. (2016). Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. *Meat Science, 120*, 107 – 117.

Karamucki, T., Jakubowska, M., Rybarczyk, A. & Gardzielewska, J. (2013). The influence of myoglobin on the colour of minced pork loin. *Meat Science, 94*(2), 234 – 8.

Kim, A.-J., Cho, A. R. & Han, J. (2013). Antioxidant and antimicrobial activities of leafy green vegetable extracts and their applications to meat product preservation. *Food Control, 29*, 112 – 120.

Koné, A. P., Cinq-Mars, D., Desjardins, Y., Guay, F., Gosselin, A. & Saucier, L. (2016). Effects of plant extracts and essential oils as feed supplements on the quality and microbial traits of rabbit meat. *World Rabbit Science, 24*, 107 – 119.

Ladikos, D. & Lougovois, V. (1990). Lipid oxidation in muscle foods: A review. *Food Chemistry, 35*, 295 – 314.

Lambert, A. D., Smith, J. P. & Dodds, K. L. (1991). Shelf life extension and microbiological safety of fresh meat — a review. *Food Microbiology, 8*, 267 – 297.

Lan, Y., Shang, Y., Song, Y. & Dong, Q. (2016). Changes in the quality of superchilled rabbit meat stored at different temperatures. *Meat Science, 117*, 173 – 181.

Lawrie, R. A. & Ledward, D. A. (2006). *Lawrie's meat science* (7th ed.). Cambridge, England: Woodhead Publishing Limited.

Lazzaroni, C., Biagini, D. & Lussiana, C. (2009). Fatty acid composition of meat and perirenal fat in rabbits from two different rearing systems. *Meat Science, 83*, 135 – 139.

Lindahl, G., Karlsson, A. H., Lundström, K. & Andersen, H. J. (2006). Significance of storage time on degree of blooming and colour stability of pork loin from different crossbreeds. *Meat Science, 72*(4), 603 – 12.

Loper, G. M. (1972). *Medicago sativa* and *Citrus depressa* flower volatiles. *Phytochemistry, 11*, 1865.

Lorenzo, J. M. (2014). Changes on physico-chemical, textural, lipolysis and volatile compounds during the manufacture of dry-cured foal "cecina". *Meat Science*, *96*(1), 256 – 63.

Lorenzo, J. M. & Carballo, J. (2015). Changes in physico-chemical properties and volatile compounds throughout the manufacturing process of dry-cured foal loin. *Meat Science*, *99*, 44 – 51.

Lorenzo, J. M. & Domínguez, R. (2014). Cooking losses, lipid oxidation and formation of volatile compounds in foal meat as affected by cooking procedure. *Flavour and Fragrance Journal*, *29*, 240 – 248.

Lynch, S. M. & Frei, B. (1993). Mechanism of copper- and iron-dependent oxidative modification of human low density lipoprotein. *Journal of Lipid Research*, *34*, 1745 – 1753.

Maj, D., Bieniek, J., Sternstein, I., Węglarz, A. & Zapletal, P. (2012). Effect of genotype and sex on meat colour changes in rabbit. *Archiv Tierzucht*, *55*(4), 385 – 390.

Mancini, S., Secci, G., Preziuso, G., Parisi, G. & Paci, G. (2018). Ginger (*Zingiber officinale* Roscoe) powder as dietary supplementation in rabbit: life performances, carcass characteristics and meat quality. *Italian Journal of Animal Science*, doi: 10.1080/1828051X.2018.1427007.

McLellan, M. R., Lind, L. R. & Kime, R. W. (1995). Hue angle determinations and statistical analysis for multiquadrant Hunter L, a, b data. *Journal of Food Quality*, *18*, 235 – 240.

McNaught, A. D. & Wilkinson, A. (1997). *Compendium of chemical terminology, IUPAC recommendations* (2nd ed.). Oxford, UK: Blackwell Science.

Mitsumoto, M. (2000). Dietary delivery versus exogenous addition of antioxidants. In Decker, E. A., Faustman, C. and Lopez-Bote, C. J. (Ed.), *Antioxidants in muscle foods: Nutritional strategies to improve quality* (pp. 315 – 343). New York, USA: John Wiley & Sons, Inc.

Morán, L., Giráldez, F. J., Panseri, S., Aldai, N., Jordán, M. J., Chiesa, L. M. & Andrés, S. (2013). Effect of dietary carnosic acid on the fatty acid profile and flavour stability of meat from fattening lambs. *Food Chemistry*, *138*, 2407 – 2414.

Mottram, D. S. (1998). Flavour formation in meat and meat products: a review. *Food Chemistry*, *62*(4), 415 – 424.

Narayana, K. R., Reddy, M. S., Chaluvadi, M. R. & Krishna, D. R. (2001). Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Indian Journal of Pharmacology*, *33*, 2 – 16.

North, M. K., Dalle Zotte, A. & Hoffman, L. C. (2018a). The effect of quercetin supplementation on the growth, feed efficiency and serum hormone levels of New Zealand White grower rabbits. *South African Journal of Animal Science*, *48*(6), 1128 – 1139.

North, M. K., Dalle Zotte, A., & Hoffman, L. C. (2018b). The effects of quercetin supplementation on New Zealand White grower rabbit carcass and meat quality — A short communication. *Meat Science, 145*, 363 – 366.

Ortuño, J., Serrano, R., Jordán, M. J. & Bañón, S. (2014). Shelf life of meat from lambs given essential oil-free rosemary extract containing carnosic acid plus carnosol at 200 or 400 mg kg^{-1}. *Meat Science*, *96*, 1452 – 1459.

Ortuño, J., Serrano, R., Jordán, M. J. & Bañón, S. (2016). Relationship between antioxidant status and oxidative stability in lamb meat reinforced with dietary rosemary diterpenes. *Food Chemistry*, *190*, 1056 – 1063.

Pla, M., Guerrero, L., Guardia, D., Oliver, M. A. & Blasco, A. (1998). Carcass characteristics and meat quality of rabbit lines selected for different objectives: I. Between lines comparison. *Livestock Production Science*, *54*, 115 – 123.

Pohanish, R. P. (2002). *Dichlorobenzenes. Sittig's Handbook of Toxic and Hazardous Chemicals and Carcinogens* (4th ed). New York, USA: William Andrew Publishing/Noyes Publications.

Resconi, V. C., Escudero, A. & Campo, M. M. (2013). The development of aromas in ruminant meat. *Molecules, 18*, 6748 – 6781.

Rodríguez-Calleja, J. M., García-López, M.-L., Santos, J. A. & Otero, A. (2005). Development of the aerobic spoilage flora of chilled rabbit meat. *Meat Science, 70*, 389 – 394.

Rodríguez Vaquero, M. J., Aredes Fernández, P. A. & Manca de Nadra, M. C. (2011). Effect of phenolic compound mixtures on the viability of *Listeria monocytogenes* in meat model. *Food Techonology and Biotechnology, 49*(1), 83 – 88.

Rupasinghe, H. P. V., Wang, L., Huber, G. M. & Pitts, N. L. (2008). Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder. *Food Chemistry, 107*, 1217 – 1224.

Saraiva, C., Oliveira, I., Silva, J. A., Martins, C., Ventanas, J. & García, C. (2015). Implementation of multivariate techniques for the selection of volatile compounds as indicators of sensory quality of raw beef. *Journal of Food Science and Technology, 52*(6).

Schindler, A., Krings, U., Berger, R. G. & Orlien, V. (2010). Aroma development in high pressure treated beef and chicken meat compared to raw and heat treated. *Meat Science, 86*, 317 – 323.

Shah, M. A., Bosco, S. J. D. & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science, 98*, 21 – 33.

Shahidi, F. & Zhong, Y. (2010). Novel antioxidants in food quality preservation and health promotion. *European Journal of Lipid Science and Technology, 112*, 930 – 940.

Soncin, S., Chiesa, L. M., Cantoni, C. & Biondi, P. A. (2007). Preliminary study of the volatile fraction in the raw meat of pork, duck and goose. *Journal of Food Composition and Analysis, 20*, 436 – 439.

Sun, Y., Fu, M., Li, Z. & Peng, X. (2018). Evaluation of freshness in determination of volatile organic compounds released from pork by HS-SPME-GC-MS. *Food Analytical Methods, 11*, 1321 – 1329.

Surai, P. F. (2014). Polyphenol compounds in the chicken/animal diet: from the past to the future. *Journal of Animal Physiology and Animal Nutrition, 98*, 19 – 31.

Tava, A. & Pecetti, L. (1997). Volatiles from *Medicago sativa* complex flowers. *Phytochemistry, 45*(6), 1145 – 1148.

Trocino, A., Xiccato, G., Queaque, P. I. & Sartori, A. (2003). Effect of transport duration and gender on rabbit carcass and meat quality. *World Rabbit Science, 11*(1), 23 – 32.

Warinda Vejaphan, T. C., Hsieh, Y. & Williams, S. S. (1988). Volatile flavor components from boiled crayfish (*Procambarus clarkii*) tail meat. *Journal of Food Science, 53*(6), 1666 – 1670.

Watanabe, K. & Sato, Y. (1971). Some alkyl-substituted pyrazines and pyridines in the flavor components of shallow fried beef. *Journal of Agricultural and Food Chemistry, 19*(5), 1017 – 1019.

Yalçin, S., Onbaşilar, E. E. & Onbaşilar, I. (2006). Effect of sex on carcass and meat characteristics of New Zealand White rabbits aged 11 weeks. *Asian-Australian Journal of Animal Science, 19*(8), 1212 – 1216.

CHAPTER 8:

General discussion and conclusions

Flavonoids are secondary metabolites produced by plants that have been found to possess a wide range of pharmacological properties (Havsteen, 2002). They have consequently been considered a possible alternative to products such as hormone- and antibiotic-based growth promoters for improving production efficiency in livestock (Kamboh *et al*., 2015), or synthetic antioxidants for increasing meat shelf-life (Surai, 2014).

Unfortunately, research on their effects under practical conditions when provided as a dietary supplement to livestock is still limited, and has had conflicting results (Chapter 2). Nonetheless, studies thus far have largely reported little impact on live performance, with the effects generally being more substantial when the animals used were under some sort of stress, such as heat or disease (Alhidary & Abdelrahman, 2014; Greiner, Stahly & Stabel, 2001; Onderci *et al*., 2004; Tuzcu *et al*., 2008). The impact on basic carcass and meat quality parameters (physical and proximate chemical composition) has also been found to be limited, whereas several studies have reported effects on the cholesterol content and fatty acid composition (Andrés, Morán, *et al*., 2014; Jenkins & Atwal, 1995; Kamboh & Zhuh, 2013; Simitzis *et al*., 2014), as well as on the oxidative shelf-life of the meat (Andrés, Huerga, *et al*., 2014; Andrés, Morán, *et al*., 2014; Goliomytis *et al*., 2015; Goliomytis *et al*., 2014; Kamboh & Zhu, 2013; Simitzis *et al*., 2019; Simitzis *et al*., 2013; Simitzis *et al*., 2011; Sohaib *et al*., 2015). However, these generalisations have been based predominantly on results for poultry or ruminant livestock, as research on other species, such as rabbits, is still very limited. The aim of this collection of studies was therefore to add to the understanding of the effects of dietary flavonoid supplementation to growing meat rabbits, as well as to help guide future research in this field.

The studies examined the effects of quercetin dihydrate supplemented to New Zealand White rabbits of both sexes at 2 g/kg feed from weaning (5 weeks) until slaughter (12 or 13 weeks). The parameters investigated included live performance traits, the caecal microbiome, basic carcass and meat quality, caecotrophe and meat fatty acid composition and the shelf life of the meat. Overall, the results appeared to concur with the conclusions of existing literature, with little beneficial effects on live performance or basic carcass and meat quality being found, but considerable effects on the fatty acid composition. In contrast with previous research, the results of the shelf-life study did not indicate any improvement in oxidative or microbial stability as a result of the dietary quercetin supplementation, and there was a surprising lack of effect on the composition of the caecal microbiome.

The limited impact of quercetin-supplementation on the live performance (Chapter 3) included a tendency ($P \leq 0.10$) for a higher feed conversion ratio (FCR) and serum free triiodothyronine concentration, and reduced sex-effects on the growth and FCR, in the supplemented rabbits. Digestibility studies could determine whether the lower feed efficiency was due to a decrease in nutrient digestibility, as previously reported for flavonoids (Świeca, Gawlik-Dziki, Dziki, Baraniak & Czyż, 2013), and it may be interesting to examine the effects of dietary quercetin

on the serum levels of sex-related hormones. Nonetheless, in this study supplementing quercetin failed to improve the growth performance of rabbits. This could have been due to the relatively high flavonoid content of the basal diet used, or the low-stress rearing environment (as indicated by the high growth rate), and these factors should be kept in mind for future research. Female rabbits had much higher serum cortisol levels than males, which concurred with previous findings for rats and humans (Green & McCormick, 2016; Gunn, Middleton, Davies, Revell & Skene, 2016), but this had no meaningful effect on performance parameters.

The composition of the caecal microbiome was investigated because it plays an important role in the nutrition and health of rabbits (Combes, Fortun-Lamothe, Cauquil & Gidenne, 2013), and flavonoids have well-demonstrated antimicrobial properties, which suggests that they may impact the composition of this population (Cushnie & Lamb, 2005). It was therefore somewhat surprising that the effects of diet were relatively limited (Chapter 4), with only a few families in the phylum *Firmicutes*, as well as the genus *Anaerofustis,* tending to be more abundant in the supplemented rabbits, and control rabbits tending to have higher proportions of *Roseburia, Oscillibacter* and *Ruminococcus albus.* As *R. albus* plays an important role in the fermentation of fibrous cell-wall components (McAllister, Cheng, Okine & Mathison, 1996), this could be linked to the lower FCR found for the control rabbits. However, the large amount of variation in the caecal microbial profile found both between the individual rabbits in this study and between this study and previous results (Badiola, de Rozas, Gonzalez, Aloy & Carabaño, 2016; Bäuerl, Collado, Zúñiga, Blas & Martínez, 2014; Massip, Combes, Cauquil, Zemb & Gidenne, 2012; Monteils, Cauquil, Combes, Godon & Gidenne, 2008; Zhu, Wang & Li, 2015), emphasizes the difficulty of fully understanding the implications of differences in individual microbial species or families. One possible explanation for the minimal effects of the quercetin supplementation was the use of the aglycone, rather than glycosylated flavonoid. It has been suggested that it is the binding of flavonoids to sugars that reduces their absorption in the small intestine, consequently resulting in their accumulation in the caecum (Oteiza *et al*., 2018). It may therefore be possible that most of the ingested quercetin was absorbed prior to the caecum, reducing its effect on the microbiome. A study comparing the effects of quercetin to its glycosylated forms, such as rutin or quercitrin, could test this theory.

Several families in the microbiome were affected by sex, with the most abundant of these, *Eubacteraceae*, being more prevalent in females than males. This suggested that the interactions between the intrinsic physiology of the rabbit and its caecal microbiome were extensive, which was also supported by the strong correlations found between the proportions of various families and the live performance traits and serum hormone levels. Further research characterizing the caecal microbiome and investigating the functional roles of different members of the population is necessary before any viable attempt to improve health and production through the manipulation of this population can be made.

The results for the basic carcass and meat quality traits (Chapter 5) generally continued the trend of limited effects of quercetin supplementation; however, there were exceptions. The supplemented rabbits had significantly higher meat to bone ratios for the hindlegs than the controls, with this being as a result of lighter hindleg bones.

From a meat yield perspective, an increase in the proportion of meat is beneficial, and it would be interesting to test whether this effect extended beyond the hindleg. However, if the lighter bones indicated a loss of bone strength and structural integrity, it could prove problematic for farmers, resulting in morbidities and mortalities, and/or bone breakage during slaughter. The effect on the bone weight is also interesting in the context of the previous literature, as the clear consensus from many physiological studies has been that dietary flavonoids improve skeletal health by decreasing bone demineralisation (Putnam, Scutt, Bicknell, Priestley & Williamson, 2007; Teixeira, 2002; Weaver, Alekel, Ward & Ronis, 2012). However, it is possible that both the lower bone weight and higher skin weight in the supplemented rabbits may have been related to the previously reported effects of flavonoids on the connective tissue. Further research investigating the bone histology and strength, and the pelt quality, could be of interest.

While sex did effect a number of traits, particularly the spleen weight, head weight, reference carcass yield and pH_u, the comparison of the age, slaughter weight and meat proximate composition to the results of previous studies suggested that these sex-effects were linked to the relatively mature nature of the rabbits used in this study. As most commercially farmed rabbits are slaughtered at a lighter live weight than was used in this case, it does not seem likely that the limited effects found would have commercial implications.

As found in previous literature, the most distinctive effect of the dietary quercetin supplementation was on the fatty acid composition of the meat (Chapter 6), with supplementation impacting the proportions of a number of different fatty acids, resulting in a lower n-6:n-3 ratio in the loin. As there was no effect on the caecotrophe fatty acid composition (which concurred with the results of Chapter 4), it seemed likely that the effect on the meat was due to some interaction between the flavonoid and the enzymes involved in endogenous lipid metabolism, as has been found previously (Dal Bosco *et al.*, 2014; Jenkins & Atwal, 1995). Moreover, the comparison of the results of this study to previous reports (Andrés, Morán, *et al*., 2014; Jenkins & Atwal, 1995; Kamboh & Zhu, 2013) suggested that there may also be some interaction between the effects of the flavonoid and the fatty acid composition of the diet. It is also notable that despite its extensive effects on the loin, the quercetin supplementation had no effect on the fatty acid composition of the deboned hindleg. This may suggest a muscle-specific effect, as reported in previous studies (Tan *et al.*, 2011; Zhong *et al.*, 2009). Overall, the effects on the fatty acid composition constituted the most promising in this compilation of studies, and, considering the health benefits of reducing the n-6:n-3 ratio (Simopoulos, 2004), further research on this is definitely warranted. Future studies should investigate the effects of a variety of combinations of flavonoid inclusion rates and dietary fatty acid compositions, and should keep in mind the possibility of different muscles responding differently to the dietary treatments.

The most distinctive effect of sex was on the fatty acid composition of the caecotrophes, which was surprising, but concurred with the effects of sex on the caecal microbiome. These effects did not carry over to the meat and fat samples, which supported the conclusion that caecal biohydrogenation had a limited impact on the fatty acid profile of the carcass. Although there were other effects of sex on the carcass fatty acids, which may suggest an

interaction between sex-hormones and lipid metabolism, these did not result in differences in the nutritional quality of the meat, as indicated by the reported indexes and ratios.

Unfortunately, the effects of quercetin-supplementation on the shelf-life of the meat were not as encouraging as those for the fatty acid composition. The meat colour, lipid oxidation (TBARS content) and antioxidant capacity (FRAP) showed no effect of the quercetin supplementation during 1, 3 or 5 days of storage, nor did the aerobic plate count. The only suggestion that the quercetin may have improved the oxidative stability of the meat was a higher concentration of some of the esters, and lower concentration of alkanes and hexanal in samples from the supplemented rabbits. Aldehydes and alkanes are products of the oxidation of polyunsaturated fats (Andrés, Huerga, *et al*., 2014; Morán *et al.*, 2013; Resconi, Escudero & Campo, 2013), whereas esters are thought to be degraded during the lipid oxidation process (Lorenzo & Domínguez, 2014**;** Schindler, Krings, Berger & Orlien, 2010). Further studies testing longer storage times, the flavonoid content of the meat and the volatile composition of the cooked meat may be worthwhile; however, the results of this trial did not indicate any benefit of dietary quercetin at 2 g/kg feed on the shelf-life of rabbit meat.

The sexes did differ in some aspects of meat quality and changes during storage, with loin meat from male rabbits having a higher pH_u than that from females, as was found in Chapter 5, and several esters differing in concentration in day 1 and/or day 5 samples. These effects were likely due to sex-differences in muscle physiology and/or fatty acid composition. However, as the volatile composition was determined for the raw meat, these differences cannot be linked conclusively to possible effects on meat flavour. It would be interesting to check whether these differences are also present in rabbits slaughtered at a younger age.

Based on the results of this collection of studies, the most promising avenues for further research into the use of flavonoids as dietary supplements for meat rabbits are the effects on the carcass meat yields and bone strength, and on the fatty acid composition of the meat. Both of these represent economically important factors for meat producers, and were found to be significantly impacted by quercetin supplementation. However, despite the minimal effects found on other aspects of production, such as the live performance and shelf-life, they should nonetheless not be completely neglected by future researchers, as these studies did have several limitations that should be taken into account when interpreting the results.

Possibly the greatest limitation across all the studies was the testing of only a single inclusion rate. It would have been preferable to include quercetin in the diet at a range of different levels, to determine the dose-response curve of quercetin *in vivo*. Flavonoids have demonstrated non-linear dose-response curves in previous studies (Almstrup *et al*., 2002), so it may be that performance traits not effected by the dietary supplementation in these studies could be impacted by higher or lower inclusion rates. A study testing the effects of a range of inclusion levels on basic production and meat quality parameters should ideally be done prior to further in-depth investigations. In addition, the effect on the live performance was unfortunately only determined under relatively low-stress environmental conditions in this study, whereas previous research suggests that more extensive effects may be seen when the animals used are under stress. Considering how farming in developed countries is moving

away from intensive systems in climate-controlled structures, and more towards extensive, organic methods, research on ways to ameliorate the negative effects of stressful environmental conditions on production is highly necessary. This is even more the case for small-scale subsistence farmers in developing countries, who are particularly at the mercy of the elements, and who will likely bear the brunt of the effects of climate change (Thornton, 2010). Future research testing the impact of dietary flavonoids on the growth performance and meat quality of rabbits under heat stress conditions would be extremely valuable. Other general limitations of the studies in this compilation included the number of replications used, and restrictions in the scope of some of the investigations, such as the number of serum hormones determined (Chapter 3). However, research will always be limited by infrastructure and costs, and these studies do, nonetheless, provide a useful starting point for the identification of aspects of production worth further investigation.

In conclusion, these studies provide valuable initial information on the effects of dietary quercetin on rabbits, as well as novel information on the rabbit caecal microbiome and the volatile composition of raw rabbit meat. While it cannot be said that they provide a definite answer to the question of whether quercetin can aid in the production and improvement of rabbit meat, they certainly pose a number of interesting questions that will have to be addressed by further studies. This research was based on and guided by the results and speculation of previous literature, and will hopefully inspire and guide yet more research into the fascinating field of flavonoids.

References

Alhidary, I. A. & Abdelrahman, M. M. (2014). Effect of naringin supplementation on performance and physiological responses of heat stressed lambs. *Journal of Animal and Veterinary Advances, 13*(19), 1126 – 1130.

Almstrup, K., Fernández, M. F., Petersen, J. H., Olea, N., Skakkebaek, N. E. & Leffers, H. (2002). Dual effects of phytoestrogens result in u-shaped dose-response curves. *Environmental Health Perspectives, 110*, 743 – 748.

Andrés, S., Huerga, L., Mateo, J., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Rotolo, L. & Giráldez, F. J. (2014). The effect of quercetin dietary supplementation on meat oxidation processes and texture of fattening lambs. *Meat Science, 96*, 806 – 811.

Andrés, S., Morán, L., Aldai, N., Tejido, M. L., Prieto, N., Bodas, R. & Giráldez, F. J. (2014). Effects of linseed and quercetin added to the diet of fattening lambs on the fatty acid profile and lipid antioxidant status of meat samples. *Meat Science, 97*, 156 – 163.

Badiola, I., de Rozas, P., Gonzalez, J., Aloy, N. and Carabaño, R. (2016). Recent advances in ERE in growing rabbits. *Proceedings of the 11th World Rabbit Congress — 15 – 18 June, 2016, Qingdao, China* (pp. 491 – 502).

Bäuerl, C., Collado, M. C., Zúñiga, M., Blas, E. & Martínez, G. P. (2014). Changes in cecal microbiota and mucosal gene expression revealed new aspects of epizootic rabbit enteropathy. *PLoS One, 9*(8), 1 – 12.

Combes, S., Fortun-Lamothe, L., Cauquil, L. & Gidenne, T. (2013). Engineering the rabbit digestive ecosystem to improve digestive health and efficacy. *Animal, 7*(9), 1429 – 1439.

Cushnie, T. P. T. & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents, 26*, 343 – 356.

Dal Bosco, A., Mugnai, C., Roscini, V., Mattioli, S., Ruggeri, S. & Castellini, C. (2014). Effect of dietary alfalfa on the fatty acid composition and indexes of lipid metabolism of rabbit meat. *Meat Science, 96*, 606 – 609.

Goliomytis, M., Kartsonas, N., Charismiadou, M. A., Symeon, G. K., Simitzis, P. E. & Deligeorgis, S. G. (2015). The influence of naringin and hesperidin dietary supplementation on broiler meat quality and oxidative stability. *PLoS One, 10*(10), 1 – 11.

Goliomytis, M., Tsoureki, D., Simitzis, P. E., Charismiadou, M. A., Hager-Theodorides, A. L. & Deligeorgis, S. G. (2014). The effects of quercetin dietary supplementation on broiler growth performance, meat quality, and oxidative stability. *Poultry Science, 93*, 1957 – 1962.

Green, M. R. & McCormick, C. M. (2016). Sex and stress steroids in adolescence: Gonadal regulation of the hypothalamic-pituitary-adrenal axis in the rat. *General and Comparative Endocrinology, 234*, 110 – 116.

Greiner, L. L., Stahly, T. S. & Stabel, T. J. (2001). The effect of dietary soy daidzein on pig growth and viral replication during a viral challenge. *Journal of Animal Science, 79*, 3113 – 3119.

Gunn, P.J., Middleton, B., Davies, S.K., Revell, V.L. & Skene, D.J. (2016). Sex differences in the circadian profiles of melatonin and cortisol in plasma and urine matrices under constant routine conditions. *Chronobiology International, 33*, 39 – 50.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67 – 202.

Jenkins, K. J. & Atwal, A. S. (1995). Flavonoids increase tissue essential fatty acids in vitamin E-deficient chicks. *Nutritional Biochemistry, 6*, 97 – 103.

Kamboh, A. A., Arain, M. A., Mughal, M. J., Zaman, A., Arain, Z. M. & Soomro, A. H. (2015). Flavonoids: health promoting phytochemicals for animal production-a review. *Journal of Animal Health and Production, 3*(1), 6 – 13.

Kamboh, A. A. & Zhu, W.-Y. (2013). Individual and combined effects of genistein and hesperidin supplementation on meat quality in meat-type broiler chickens. *Journal of the Science of Food and Agriculture, 93*, 3362 – 3367.

Lorenzo, J. M. & Domínguez, R. (2014). Cooking losses, lipid oxidation and formation of volatile compounds in foal meat as affected by cooking procedure. *Flavour and Fragrance Journal, 29*, 240 – 248.

Massip, K., Combes, S., Cauquil, L., Zemb, O. & Gidenne, T. (2012). High throughput 16S-DNA sequencing for phylogenetic affiliation of the caecal bacterial community in the rabbit : Impact of the hygiene of housing and of the intake level. *Proceedings of the VIIIth INRA-RRI Symposium on Gut Microbiology — 18 – 21 June, 2012, Clermont-Ferrand, France*, (pp. 57).

McAllister, T. A., Cheng, K. J., Okine, E. K. & Mathison, G. W. (1996). Dietary, environmental and microbiological aspects of methane production in ruminants. *Canadian Journal of Animal Science, 76*, 231 – 243.

Monteils, V., Cauquil, L., Combes, S., Godon, J.-J. & Gidenne, T. (2008). Potential core species and satellite species in the bacterial community within the rabbit caecum. *FEMS Microbiology Ecology, 66*, 620 – 629.

Morán, L., Giráldez, F. J., Panseri, S., Aldai, N., Jordán, M. J., Chiesa, L. M. & Andrés, S. (2013). Effect of dietary carnosic acid on the fatty acid profile and flavour stability of meat from fattening lambs. *Food Chemistry, 138*, 2407 – 2414.

Onderci, M., Sahin, K., Sahin, N., Gursu, M. F., Doerge, D., Sarkar, F. H. & Kucuk, O. (2004). The effect of genistein supplementation on performance and antioxidant status of Japanese quail under heat stress. *Archives of Animal Nutrition, 58*(6), 463 – 471.

Oteiza, P. I., Fraga, C. G., Mills, D. A. & Taft, D. H. (2018). Flavonoids and the gastrointestinal tract: Local and systemic effects. *Molecular Aspects of Medicine, 61*, 41 – 49.

Putnam, S. E., Scutt, A. M., Bicknell, K., Priestley, C. M. & Williamson, E. M. (2007). Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytotherapy Research, 21*, 99 – 112.

Resconi, V. C., Escudero, A. & Campo, M. M. (2013). The development of aromas in ruminant meat. *Molecules, 18*, 6748 – 6781.

Schindler, A., Krings, U., Berger, R. G. & Orlien, V. (2010). Aroma development in high pressure treated beef and chicken meat compared to raw and heat treated. *Meat Science, 86*, 317 – 323.

Simitzis, P. E., Babaliaris, C., Charismiadou, M. A., Papadomichelakis, G., Goliomytis, M., Symeon, G. K. & Deligeorgis, S. G. (2014). Effect of hesperidin dietary supplementation on growth performance, carcass traits and meat quality of rabbits. *World Rabbit Science, 22*, 113 – 121.

Simitzis, P. E., Charismiadou, M. A., Goliomytis, M., Charalambous, A., Ntetska, I., Giamouri, E. & Deligeorgis, S. G. (2019). Antioxidant status, meat oxidative stability and quality characteristics of lambs fed with hesperidin, naringin or α-tocopherol acetate supplemented diets. *Journal of the Science of Food and Agriculture, 99*(1), 343 – 349.

Simitzis, P. E., Ilias-Dimopoulos, V., Charismiadou, M. A., Biniari, E. E. & Deligeorgis, S. G. (2013). Effects of dietary hesperidin supplementation on lamb performance and meat characteristics. *Animal Science Journal, 84*, 136 – 143.

Simitzis, P. E., Symeon, G. K., Charismiadou, M. A., Ayoutanti, A. G. & Deligeorgis, S. G. (2011). The effects of dietary hesperidin supplementation on broiler performance and chicken meat characteristics. *Canadian Journal of Animal Science, 91*, 275 – 282.

Simopoulos, A. P. (2004). Omega-6/omega-3 essential fatty acid ratio and chronic diseases. *Food Reviews International, 20*(1), 77 – 90.

Sohaib, M., Butt, M. S., Shabbir, M. A. & Shahid, M. (2015). Lipid stability, antioxidant potential and fatty acid composition of broilers breast meat as influenced by quercetin in combination with α-tocopherol enriched diets. *Lipids in Health and Disease, 14*(61), 1 – 15.

Surai, P. F. (2014). Polyphenol compounds in the chicken/animal diet: from the past to the future. *Journal of Animal Physiology and Animal Nutrition, 98*, 19 – 31.

Świeca, M., Gawlik-Dziki, U., Dziki, D., Baraniak, B. & Czyż, J. (2013). The influence of protein-flavonoid interactions on protein digestibility in vitro and the antioxidant quality of breads enriched with onion skin. *Food Chemistry, 141*, 451 – 458.

Tan, C. Y., Zhong, R. Z., Tan, Z. L., Han, X. F., Tang, S. X., Xiao, W. J., Sun, Z. H. & Wang, M. (2011). Dietary inclusion of tea catechins changes fatty acid composition of muscle in goats. *Lipids, 46*(3), 239 – 247.

Teixeira, S. (2002). Bioflavonoids: Proanthocyanidins and quercetin and their potential roles in treating musculoskeletal conditions. *Journal of Orthopaedic and Sports Physical Therapy, 32*, 357 – 363.

Thornton, P. K. (2010). Livestock production: recent trends, future prospects. *Philosophical Transactions of the Royal Society, 365*, 2853 – 2867.

Tuzcu, M., Sahin, N., Karatepe, M., Cikim, G., Kilinc, U. & Sahin, K. (2008). Epigallocatechin-3-gallate supplementation can improve antioxidant status in stressed quail. *British Poultry Science, 49*(5), 643 – 648.

Weaver, C. M., Alekel, D. L., Ward, W. E. & Ronis, M. J. (2012). Flavonoid intake and bone health. *Journal of Nutrition in Gerontology and Geriatrics, 31*, 239 – 253.

Zhong, R. Z., Tan, C. Y., Han, X. F., Tang, S. X., Tan, Z. L. & Zeng, B. (2009). Effect of dietary tea catechins supplementation in goats on the quality of meat kept under refrigeration. *Small Ruminant Research, 87*, 122 – 125.

Zhu, Y., Wang, C. & Li, F. (2015). Impact of dietary fiber/starch ratio in shaping caecal microbiota in rabbits. *Canadian Journal of Microbiology, 61*, 771 – 784.