THARCILLA ISABELLA RODRIGUES COSTA ALVARENGA

MANIPULATION OF FATTY ACIDS IN MUSCLE LAMB

LAVRAS – MG

2015

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Ruminantes, para a obtenção do título de Doutor.

Orientador

Dr. Juan Ramon Olalquiaga Perez

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APROVADA em 23 de junho de 2015.

Dr. David L. Hopkins	NSW DPI – Austrália
Dr. Eduardo Mendes Ramos	DCA – UFLA
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Dr. Juan Ramon Olalquiaga Perez

Orientador

LAVRAS – MG

2015

Ao meu maravilhoso marido Flávio Alvarenga pelo inestimável
incentivo e ajuda na realização deste trabalho,
Aos meus pais e minha irmã que plantaram as melhores sementes para
que este fruto fosse hoje colhido,
Sem vocês eu nada seria,

DEDICO

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'Se eu vi mais longe, foi por estar de pé sobre ombros de gigantes.'
'If I have seen further it is by standing on the shoulders of giants.'
Isaac Newton

'Para ser um membro imaculado de um rebanho de ovelhas, deve-se, antes de tudo, primeiro ser uma ovelha.'
'In order to form an immaculate member of a flock of sheep one must, above all, be a sheep.'
Albert Einstein

Muito obrigada!

Dreams,

... And just like that, after a long wait, a day like any else,
I decided to triumph, to look for the opportunities, not to wait.
I decided to see every problem as the opportunity to find a solution.
I decided to see every desert as the opportunity to find an oasis.
I decided to see every night as a mystery to solve.
I decided to see every day as a new opportunity to be happy.
That day I found that my only enemies were my own weaknesses,
That day I lost the fear of losing and I started to fear not winning,
I discovered that I was not the best and maybe never have been.
I stopped caring about who was the winner and who was the loser.
Now I care only about knowing more than yesterday.
I learned that the hard thing is to stop climbing to the top, instead of not reaching it.
I learned that the best triumph that I can have is to have the right of calling someone "my friend".
I discovered that love is more than a feeling of being in love, "love is a philosophy of life".
That day I stopped being a reflection of the few triumphs in my past and I started to be my own tenuous light of the present;
I learned that it does not matter if you are alight… if you are not going to illuminate another's road.
That day I decided to change so many things…
That day I learned that dreams only exist to be made to come true
Since that day I don't sleep to rest…
Now, I dream just for dreams.
(Walt Disney)

Sonhos,

... E assim, depois de muito esperar, num dia como outro qualquer, decidi triunfar... Decidi não esperar as oportunidades e sim, eu mesmo buscá-las. Decidi ver cada problema como uma oportunidade de encontrar uma solução. Decidi ver cada deserto como uma possibilidade de encontrar um oásis. Decidi ver cada noite como um mistério a resolver. Decidi ver cada dia como uma nova oportunidade de ser feliz. Naquele dia descobri que meu único rival não era mais que minhas próprias limitações e que enfrentá-las era a única e melhor forma de as superar. Descobri que eu não era o melhor e que talvez eu nunca tivesse sido. Deixei de me importar com quem ganha ou perde. Agora me importa simplesmente saber melhor o que fazer. Aprendi que o difícil não é chegar lá em cima, e sim deixar de subir. Aprendi que o melhor triunfo é poder chamar alguém de "amigo". Descobri que o amor é mais que um simples estado de enamoramento, "o amor é uma filosofia de vida". Naquele dia, deixei de ser um reflexo dos meus escassos triunfos passados e passei a ser uma tênue luz no presente. Aprendi que de nada serve ser luz... se não iluminar o caminho dos demais. Naquele dia, decidi trocar tantas coisas... Naquele dia, aprendi que os sonhos existem para tornar-se realidade. E desde aquele dia já não durmo para descansar... Agora, simplesmente durmo para sonhar."

(Walt Disney)

RESUMO GERAL

Ácidos graxos de sabões de cálcio (CSFA) podem aumentar a densidade energética dos alimentos e tem sido utilizado para melhorar o nível de ácidos graxos saudáveis na carne, no entanto, há uma escassez de conhecimento sobre o impacto na qualidade da carne, tais como características de cor, força de cisalhamento e colágeno. O objetivo do primeiro estudo foi determinar os efeitos do CSFA sobre características de qualidade da carne de cordeiros quando incluídos em uma dieta de confinamento. Sessenta e três cordeiros cruzados foram confinados individualmente aos 24 ± 2,5 kg e abatidos aos 44 ± 1,1 kg. Vinte e nove cordeiros foram alimentados com a dieta controle e 34 cordeiros foram alimentados com uma dieta contendo 5,4% de CSFA. Em 24h postmortem o músculo *longissimus lumborum* (LL) foi coletado do lado direito da carcaça. O músculo foi cortado em fatias de 2,54 cm e embalados a vácuo. Amostras foram armazenadas a -20°C (sem maturação) e outras armazenadas a 2ºC por 10 dias de maturação e então congeladas. Perda por cocção foi reduzida ($P < 0,05$) em cordeiros alimentados com CSFA alimentados, mas não houve efeito sobre quaisquer outros parâmetros ($P > 0,05$). A força de cisalhamento foi significativamente reduzida ($P < 0,05$) durante a maturação, e isto foi acompanhado por um aumento nos valores de índice de fragmentação miofibrilar. CSFA reduziu EPA e DHA no músculo *longissimus*. Alga é um candidato potencial para melhorar os níveis de ácidos graxos poliinsaturados de cadeia longa (LC-PUFA) na carne de ruminantes. No segundo trabalho foram estudados os efeitos da suplementação de cordeiros com alga sobre a expressão de genes que direcionam o acúmulo de LC-PUFA. As mães dos corderios foram alimentadas com dietas à base de silagem (SLG) ou aveia/algodão (OAT) por seis semanas antes e, três semanas após a concepção. Os níveis de mRNA de FADS1, FADS2, CPT1, SCD, ACC e FAD2 foram medidos no fígado, músculo e gordura subcutânea de cordeiros alimentados com uma dieta controle a base de aveia e grãos de lupin e alfafa picada (CTRL) ou a dieta CTRL com adição de alga (DHAgold ™) a 1,92% DM (ALG). A expressão de FADS1 no fígado não foi afetada ($P > 0,05$) pela interação entre nutrição da ovelha e suplementação com alga, no entanto, foi maior ($P < 0,05$) quando cordeiros receberam a dieta ALG em comparação com a CTRL, e quando suas mães foram alimentadas SLG em comparação com a dieta OAT. A expressão dos genes FADS1, FADS2, SCD e ACC no músculo dos cordeiros foi significamente afetada pela nutrição da ovelha, apresentando níveis mais elevados para o tratamento SLG + ALG ($P < 0,05$) em comparação com os demais tratamentos.

Palavras-chave: Alga. Ácido docosahexaenóico. Ácido eicosapentaenóico. Expressão gênica. Gordura protegida.

GENERAL ABSTRACT

Calcium soap fatty acids (CSFA) can increase the energy density of feeds and has been used to improve the level of healthy fatty acids in meat, however there is a paucity of knowledge about the impact on meat quality traits such as colour traits, shear force and collagen. The objective of first study was to determine the effects of CSFA on meat quality traits of lambs when included in a finishing diet. Sixty-three crossbreed lambs were feedlot individually at 24 ± 2.5 kg and slaughtered at 44 ± 1.1 kg. Twenty-nine lambs were fed the Control diet and 34 lambs were fed a diet containing 5.4% CSFA. At 24h postmortem the *longissimus lumborum* (LL) muscle was collected from the right side of the carcass. The muscle was cut into 2.54-cm thick slices and vacuum-packaged. Samples were stored at -20ºC (0 day ageing) and others stored at 2ºC for 10 days ageing and then frozen. Cooking loss was reduced ($P < 0.05$) in CSFA fed lambs, but there was no effect on any other traits ($P > 0.05$). Shear force was significantly reduced by ageing ($P < 0.05$), and this was matched by an increase in myofibrillar fragmentation index values with ageing. CSFA decreased EPA and DHA in the *longissimus* muscle. Algae is a potential candidate to improve the levels of long chain polyunsaturated fatty acids (LC-PUFA) in ruminant meat. In the second study the effect of the supplementation of lambs with algae on the expression of genes that direct the accumulation of LC-PUFA was investigated. The mothers of the lambs were fed with either silage (SLG) or oat/cottonseed (OAT) based diets for six weeks prior to and, three weeks following conception. mRNA levels of FADS1, FADS2, CPT1, SCD, ACC and Fad2 were measured in the liver, muscle and subcutaneous fat from lambs fed a control diet consisting of oat and lupin grains and chopped lucerne (CTRL) or the CTRL diet with algae (DHAgold™) added at 1.92% DM (ALG). The expression of FADS1 in liver tissue was not affected ($P > 0.05$) by the interaction between dam nutrition and algae supplementation, however it was higher ($P < 0.05$) when lambs received the ALG ration compared with the CTRL and when their dams were fed SLG compared with OAT diet. The expression of FADS1, FADS2, SCD and ACC genes in lamb muscle was differentially affected by dam nutrition with the highest levels for the SLG+ALG treatment ($P < 0.05$) compared with other treatments.

Keywords: Algae. Docosahexaenoic acid. Eicosapentaenoic acid. Gene expression. Protected fat.

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LIST OF PUBLICATIONS

During the course of this study a number of publications and public presentation have been made which are based on the work presented in this thesis. They are listed below for future reference.

Journal Article

Alvarenga, T.I.R.C., Chen, Y., Lewandowski, P., Ponnampalam, E.N., Sediq, S., Clayton, E.H., van de Ven, R.J., Perez, J.R.O., Hopkins, D.L. (2015). The expression of genes encoding enzymes regulating fat metabolism is affected by maternal nutrition when lambs are fed algae high in omega-3. British Journal of Nutrition (submitted).

Review Paper

Alvarenga, T.I.R.C., Chen, Y., Furusho-Garcia, I.F., Perez, J.R.O., Hopkins, D.L. (2015). Manipulation of omega-3 PUFAs in lamb: phenotypic and genotypic views. Compreehensive Reviews in Food Science and Food Safety, 14 (3): 189-204. DOI: 10.1111/1541-4337.12131.

Short Paper

Alvarenga, T.I.R.C., Furusho Garcia, I.F., Perez, J.R.O., Ramos, E.M., Faria, P.B., Alvarenga, F.A.P., Leopoldino Junior, I., Chizzotti, M.L. and Hopkins, D.L. (2014). Effect on lamb meat of supplementing with calcium soap fatty acids. In: The 60th International Congress of Meat Science and Technology (ICoMST); 2014 August 17-22; Punta del Este, Uruguay. Archivos Latinoamericanos de Produccion Animal, 22 (5): 125-129.

Conference Presentation

Alvarenga, T.I.R.C., Furusho Garcia, I.F., Perez, J.R.O., Ramos, E.M., Faria, P.B., Alvarenga, F.A.P., Leopoldino Junior, I., Chizzotti, M.L. and Hopkins, D.L. (2014). Effect on lamb meat of supplementing with calcium soap fatty acids. In Proceedings of the 60th International Congress of Meat Science and Technology – ICoMST, 17-22th August, Conrad Hotel, Punta del Este, Uruguay.

SUMMARY

FIRST PART

GENERAL INTRODUCTION

Several studies have shown that dietary omega-6 and omega-3 long chain fatty acids can be incorporated into muscle tissue of ruminants despite the biohydrogenation of dietary fatty acids in the rumen. The main focus is on eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids because health departments around world consider the sum of these fatty acids as the basis of classifying a food as a source or good source of omega-3 polyunsaturated fatty acids.

A high proportion of polyunsaturated fatty acids are hydrogenated in the rumen, leading to the higher concentration of 18:0, but a significant amount escapes from the rumen to be absorbed intact in the small intestine.

Feeding strategies in ruminants have been successfully applied to increase the absorption of polyunsaturated fatty acid (PUFA) in the small intestine and therefore increase the levels in muscle. Protected fats and algaes are strong candidates to improve the nutritional value of red meat in ruminants.

Efforts to understand the genetic basis of fatty acid metabolism have been underway. The knowledge of the main genes which control the output of omega-3 fatty acids is still lacking, gene expression and RNA sequencing have helped to explain the deposition of these acids in muscle, liver and subcutaneous tissue of ruminants.

The hypothesis is assess how a source of protected fat, such as calcium soap fatty acid, can alter meat traits and fatty acid profile of lamb meat in the first experiment and in the second experiment to compare the expression of genes related with long chain omega-3 fatty acid when the lamb meat shows

high levels of DHA and EPA under algae feeding. Therefore, this thesis addresses these issues as individual chapters to assess the effect of protected fat on the meat quality of lambs, and the effect of the inclusion of the algae in a lamb diet on the genetic expression of fatty acid metabolism in muscle, liver and subcutaneous fat.

Review Paper One explores existing published literature to formulate a brief and comprehensive literature review discussing omega-3 long chain fatty acid metabolism, strategies to increase the DHA and EPA levels in lamb meat and tools to explore the deposition in muscle as gene expression.

Paper Two evaluates the effect on lamb meat of supplementing with calcium soap fatty acid at 5.4% when fed individually. *Longissimus lumborum* (LL) was collected and vacuum-packaged and evaluated at day 1 pm and day 10 aging at 2^{o}C the follows traits: pH, colour, water holding capacity, thaw loss, cooking loss, shear force, myofibrillar fragmentation index, sarcomere length, collagen and fatty acid composition.

Paper Three investigates the gene expression related with fatty acid deposition when lambs are fed with algae DHA-Gold at 1.92% DM. The RNA was extracted from muscle, liver and subcutaneous fat to the qPCR analysis and SCD, FADS1, FADS2, CPT1, ACC and fad2 genes were assessed.

REVIEW PAPER 1

MANIPULATION OF OMEGA-3 PUFAS IN LAMB: PHENOTYPIC AND GENOTYPIC VIEWS

Paper according guidelines of Comprehensive Reviews in Food Science and Food Safety – published version.

Manipulation of omega-3 PUFAs in lamb: phenotypic and genotypic views

Abstract: A number of studies have shown that dietary omega-6 and omega-3 long-chain fatty acids can be incorporated into muscle tissue of ruminants despite the biohydrogenation of dietary fatty acids in the rumen. The main focus of this review is on eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) fatty acids because health authorities around the world consider the sum of these fatty acids as the basis of classifying a food as a source or good source of omega-3 polyunsaturated fatty acids (PUFAs). A high proportion of polyunsaturated fatty acids are hydrogenated in the rumen, leading to a higher concentration of 18:0, but some escapes the rumen to be absorbed intact by the small intestine. Feeding strategies for ruminants have been successfully applied to increase the absorption of PUFAs in the small intestine and therefore to increase the levels of PUFAs in muscle tissue. Protected fats and algae are strong candidates to improve the nutritional value of red meat in ruminants in terms of health-claimable omega-3 fatty acids. Efforts to understand the genetic basis of fatty acid metabolism have been underway. The knowledge of the main genes which control the output of omega-3 fatty acids is still lacking, but gene expression studies have helped to explain the deposition of these acids in muscle, liver, and subcutaneous fat.

Keywords: docosahexaenoic acid, eicosapentaenoic acid, fatty acid, gene expression, sheep

Introduction

The major roles of complex lipids in the body are as structural components, mainly in cell membranes (phospholipids), or as energy reserves (triacylglycerols), from which the excess energy is converted to long chain fatty acids which, after esterification to form triacylglycerols, are deposited as adipose tissue (Annison 1993). Strategies to minimize fat deposition in carcasses and increase the levels of the omega-3 (n-3) fatty acids in meat have been developed in response to consumer demands due to their substantial effect on human health. Emphasis has been placed on reducing the intake of saturated fatty acids (SFAs) because they are considered to be associated with increased cholesterol levels (Scollan and others 2014). Public health policies in most developed countries recommend population-wide decreases in the intake of *trans*-fatty acids and increases in the consumption of the long chain n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA) (Shingfield and others 2013). Dietary n-3 PUFAs are derived from plants (for example, linolenic acid [ALA]) and marine products (for example, EPA and DHA) (Welch and others 2010).

To maintain high levels of health-claimable fatty acids in lamb, the overall nutritional status of the flock and the nutritional status of individual animals are important (Ponnampalam and others 2014a). Strategies to increase lean meat production (genetics) and decrease fat deposition (nutritional) result in improved feed conversion efficiency (Cockett and others 2005), but can also reduce the nutritional value (Mortimer and others 2014) and eating quality of meat (Hopkins and others 2007). Feeding systems (for example, grain feed compared with grazing) are an important strategy to alter the fatty acid profile of meat. Grass feeding improves EPA, DPA (22:5n-3, docosapentaenoic acid), and

DHA levels in muscle tissue (Fisher and others 2000) as pasture species increase the content of ALA (18:3n-3) in the diet, which is the precursor for DHA and EPA production (Kitessa and others 2010). Supplementation with algae can also contribute to maximizing the levels of health-claimable fatty acids with recent work providing encouraging findings (Hopkins and others 2014).

Identifying the key genes that control the synthesis and deposition of n-3 fatty acids will contribute to our understanding of the regulation and thus phenotypic expression of these fatty acids. One approach to studying gene expression is to use RNA sequencing which provides quantitative expression measurements and sequence information (Ramayo-Caldas and others 2012). New knowledge on gene regulation of n-3 levels in lamb meat may help to accelerate the production of healthier meat and satisfy consumer demand for such foods. Therefore, in this review the important relationships between feeding lambs and lipid composition and gene expression in lamb meat are discussed with a focus on DHA and EPA fatty acids in the context of their role in human health.

Requirements for human health

Given consumer pressure to reduce the fat content of meat, researchers have been encouraged to develop strategies which minimize fat deposition in animals used for human consumption (Annison 1993) in order to revise the quantitative and qualitative view of dietary sources of lipids. In terms of qualitative aspects, the fatty acid composition of animal products is currently focused on EPA and DHA levels. Of particular focus is the sum of EPA and DHA content in meat. For example, Food Standards Australia New Zealand (FSANZ) consider a source and a good source of EPA + DHA as 22 and 44 mg per 100g serving (FSANZ 2012), respectively. European standards for source

and good source are 40 and 80 mg/100 g, respectively (Commission Regulation of European Union 2010). The population worldwide should consume no more than 7% to 10% of calories from SFAs, less than 300 mg/d of cholesterol, and maintain *trans*-fatty acid consumption as low as possible (Russo 2009). Recommendations by the Dept. of Health (1994), cited by Stockley (1996) are that the intake of the omega-6 (n-6) PUFA series should not be altered, while the intake of the n-3 fatty acids such as ALA, EPA, and DHA should be increased to approximately twice their current levels. The dietary intake of n-3, ALA, EPA, DHA, and linoleic acid (LA) estimated by Welch and others (2010) is about 1.50, 1.23, 0.11, 0.16, and 12.35 g/d for men and 1.22, 0.99, 0.09, 0.13, and 9.42 for woman, respectively. Furthermore, Wijendran and Hayes (2004) recommended that the absolute weight of n-6 and n-3 should be 1st considered and they suggest a n-6:n-3 ratio of 6:1. The ratio of these fatty acids is the more important consideration in terms of cardiovascular health according to Wijendran and Hayes (2004), whereas others have considered recommendations on a mass basis (g/d) (Kris-Etherton and others 2000). Harnack and others (2009) showed that the administration of LA and ALA at a ratio of 1:1 led to the highest formation of EPA and DHA using incubated human hepatoma cells.

Studies show that EPA, at a dose of 1800 mg/d, is a very promising regimen for prevention of major coronary events (Yokoyama and others 2007). The recommendation for patients without documented coronary heart disease is to eat fish at least twice a week and include oils and foods rich in α-linolenic acid (Kris-Etherton and others 2003). For patients with documented coronary heart disease, they should consume approximately 1 g of EPA + DHA per day, preferably from oily fish (Kris-Etherton and others 2003).

Omega-3 and omega-6 fatty acids can compensate for growth retardation (Russo 2009) and low intakes of n-3 fatty acids from DHA alone may increase the length of gestation in women and the birth weight of infants

(Smuts and others 2003). The consumption of DHA is also linked to optical and brain developmental health as it is found in high concentrations in neural tissue including brain cortex and the retina (Blank and others 2002).

By contrast, studies report that adults who consume a vegetarian diet are in a poorer state of health. This includes showing higher levels of impairment from disorders, chronic diseases, suffering from allergies, cancer, and mental health ailments (anxiety, or depression) compared to those who consume another diet (containing meat, fruits, and vegetables in variable proportions) (Burkert and others 2014). Efforts to increase the levels of n-3 fatty acids in lamb meat have focused on feeding oils/oilseeds, soybean, fish oil, protected fat, and algae which have higher levels of n-3 PUFA (Table 2). This is designed to improve the recognition of red meat as an essential food by reducing the health risk of consuming such meat.

Healthy fatty acids and good sources

The properties of fatty acids, and the complex lipids synthesized from them, are markedly dependent on chain length and the degree of saturation, and, for example, the unsaturated fatty acids have lower melting points than SFAs of the same length (Berg and others 2002). The most important PUFAs, for positive effects on human health, are n-6 and n-3. The main n-6 fatty acids are LA (C18:2) and arachidonic acid (C20:4 – ARA). Dietary sources of n-6 are abundantly present in liquid vegetable oils, including those from soybean, corn, sunflower, safflower, and cotton seed, while linseed and canola oils are rich in n-3 (Russo 2009) (Table 1).

The main n-3 fatty acids in terms of a positive effect on human health are ALA, EPA, and DHA. Fish lipids are well known to be rich in EPA and DHA and studies show that wild fish have higher levels of these fatty acids than

farmed fish. This, it is suggested, reflects the diets rich in n-3 fatty acids which marine fish consume, whereas farmed fish are fed diets containing higher proportions of lipids rich in SFAs and monounsaturated fatty acids (MUFAs) (Alasalvar and others 2002). Fish such as salmon, trout, and herring are higher in EPA and DHA than cod, haddock, and catfish (KrisEtherton and others 2003). Fish-oil supplements typically contain 30% to 50% n-3 fatty acids (Russo 2009). Najafi and others (2012) showed the composition of fish oil to be 25.8% C18:1, 3.6% C18:2, 1.3% C18:3, 8.3% EPA, and 17.6% DHA, but as shown in Table 1 the composition can vary widely.

Table 1. Sources of oleic, linoleic and linolenic fatty acids in animal feeds.

	Unit	Oleic	Linoleic	Linolenic	Reference
Concentrate					
Soybean	%	23.3	52.2	5.6	Chowdhury and others (2007)
Soybean	%	23.1	54.5	8.0	Najafi and others (2012)
Corn	%	37.0	47.2	1.3	Dauqan and others (2011)
Sunflower	%	45.4	46.0	0.1	Chowdhury and others (2007)
Sunflower	%	61.8	27.9	0.1	AbuGhazaleh and others (2007)
Mustard	%	38.2	25.3	11.3	Chowdhury and others (2007)
Rapeseed	%	46.8	19.5	8.7	Velasco and Becker (1998)
Canola	%	61.8	18.7	10.4	Seberry and others (2012)
Linseed	%	18.5	17.3	53.2	Popa and others (2012)
Extruded linseed	%	15.1	18.2	54.3	Gómez-Cortés and others (2014)
Protected linseed	g/100g	56.1	25.	7.7	Kitessa and others (2009)
Palm	%	41.9	11.0	-	Chowdhury and others (2007)
Palm	%	18.0	4.0	-	Najafi and others (2012)
Palm olein	%	49.5	11.7	0.5	Dauqan and others (2011)

Table 1 (continuation)

	Unit	Oleic	Linoleic	Linolenic	Reference
Red palm olein	%	44.6	10.4	0.3	Dauqan and others (2011)
Coconut	%	7.2	1.7	-	Dauqan and others (2011)
Coconut	%	5.8	1.3	-	Chowdhury and others (2007)
Cotton seed	%	17.0	53.3	-	Cherry and others (1986)
Oats	g/100g	38.8	38.3	1.2	Kitessa and others (2009)
Barley	g/100g	14.2	57.8	5.4	Kitessa and others (2009)
Lupins	g/100g	31.3	42.1	5.2	Kitessa and others (2009)
Flax seeds	%	22.0	18.3	48.2	El-Beltagi and others (2007)
Roughage					
Green maize fodder	g/100g	4.7	17.1	38.9	Shelke and others (2012)
Wheat straw	g/100g	14.9	-	-	Shelke and others (2012)
Perennial ryegrass	mg/g DW	0.4	2.73	15.0	Hegarty and others (2013)
Fresh grass perennial ryegrass	%	1.7	10.6	68.4	Elgersma and others (2003)
Fresh ryegrass	g/100g	1.5	12.6	55.3	Alves and others (2011)
Silage perennial ryegrass	%	1.2	11.8	64.7	Ortiz and others (2006)

Table 1 (continuation)

	Unit	Oleic	Linoleic	Linolenic	Reference
Silage ryegrass	g/100g	1.6	11.2	51.3	Alves and others (2011)
Fresh corn (whole plant)	g/100g	16.4	47.5	12.0	Alves and others (2011)
Silage corn	g/100g	14.4	41.4	10.6	Alves and others (2011)
Lucerne hay	%	8.0	24.4	23.2	AbuGhazaleh and others (2007)
Corn silage	%	18.8	48.5	11.1	AbuGhazaleh and others (2007)
Supplementation					
Algae *U. lactuca* (flour)	%	27.4	8.3	4.4	Ortiz and others (2006)
Algae *D. antarctica* (leaves)	%	25.4	10.8	3.9	Ortiz and others (2006)
Algae *D. antarctica* (stem)	%	25.8	15.7	1.1	Ortiz and others (2006)
DHA Gold™ algae	%	0.0	0.01	0.1	unpublished data
Protected fat	g/100g	26.4	32.7	2.0	Shelke and others (2012)
Megalac®	%	34.4	-	-	Wynn and others (2006)
Calcium soap of palm oil	%	9.7	-	-	Gómez-Cortés and others (2014)
Fish oil	%	25.8	3.6	1.3	Najafi and others (2012)
Fish oil	%	9.2	1.1	1.5	AbuGhazaleh and others (2007)

LA and ALA are 2 essential fatty acids, given the lack of Δ12 and Δ15 fatty acid desaturases (FADS) in mammals, which are responsible for the conversion of oleic acid to LA and LA to ALA, respectively (Zhu and others 2014). The term essential means that they are required by an organism (Berg and others 2002), and they cannot be synthesized by the body and must be supplied from the diet (Elliot and Elliot 2005). Even though forages contain relatively low levels of fatty acids bound in lipids (on a fresh weight basis), they are the cheapest and often the major sources of unsaturated fatty acids in ruminant diets (Kalac and Samkova 2010), mainly as LA and ALA forms.

Pasture is not a source of EPA and DHA, but is rich in ALA, the precursor of these n-3 long chain PUFAs (Table 1). Pasture is a richer source of other unsaturated fatty acids than silage (Alves and others 2011), especially the C18:1 and C18:3 forms (Elgersma and others 2003). The alterations in fatty acid composition of grass during ensiling may be due to microbial intervention during the process of ensiling, which may result in isomerization and hydrogenation similar to that which occurs by microbial action in the rumen (Alves and others 2011). Lactic acid bacteria can be isolated from forage crops to be used as silage additives (Cai and others 1999) and this can lead to the biohydrogenation of LA and ALA (Ogawa and others 2005). Another roughage source often used is hay. However, losses of PUFAs also occur during the drying of hay due to oxidation (Kalac and Samkova 2010).

Some algal supplements are not a source of ALA (Table 1); however, almost half of the fatty acids contained in the ether extract (EE) are in the form of DHA. Even with ruminal biohydrogenation supplementation with algae has provided large increases in EPA and DHA levels in muscle, increasing levels by 1.6- and 6.5-fold in the intramuscular fat (IMF) when lambs are fed with marine-based algae compared with a control diet (Hopkins and others 2014). By contrast, the potential to increase EPA and DHA in milk is extremely limited,

even with postruminal infusions of fatty acids (Shingfield and others 2013), since C18 are the longest fatty acids entering in the mammary gland (Jensen 2002).

Considering that concentrate-based diets are higher in LA than fibrous forage diets, have a small particle size and a shorter rumen transit time, they contribute to restricting opportunities for microbial biohydrogenation (Wood and others 2008). An accelerated rate of passage may also impact on tissue uptake, leading to lower utilization of consumed food. ALA is the major dietary fatty acid for pasture-fed ruminants since it constitutes over 50% of total fatty acids in grass and grass products (Wood and others 2008). The incorporation of LA from concentrate diets and ALA from pasture diets into lamb fat deposits in the study of Bas and Morand-Fehr (2000) led to higher synthesis of ALA and lower levels of oleic and LA in lambs fed the pasture system, compared with lambs fed the concentrate diet. Suffolk lambs obtained higher levels of EPA, DHA, and their precursor ALA when produced on grass compared with concentrate feeding (Fisher and others 2000), providing evidence of the benefits of pasture feeding for increasing long chain n-3 fatty acid levels in muscle. Caparra and others (2007) also confirmed this conclusion by showing a 2-fold increase in the ALA level in muscle from lambs fed pasture which lead to an increase in EPA and DPA levels, although the DHA level was not altered.

The source of protein and energy in the diet also has an effect on fatty acid deposition in lamb tissue. Bas and Morand-Fehr (2000) reviewed 108 papers and observed that lucerne (alfalfa) (*Medicago sativa*) meal, rich in ALA, induced a higher level of ALA in tissues than soybean, cotton, and fish meal; and when the proportion of lucerne was increased in the diet oleic and LA were generally lower in tissues. The values reported for ALA as a proportion of the fatty acid composition of lamb fat deposits were 1.4%, 0.8%, 0.4%, and 0.6%, respectively, when lucerne meal, soybean meal, cotton meal, and fish meal were

used as the main sources of nitrogen in a concentrate diet. According to the NRC (2007), soybean meal, cotton meal, and fish meal contain 1.6%, 5%, and 8% EE while lucerne meal was reported by Chen and others (2013) to contain 2.3% EE. It demonstrates that even in lucerne, which contains smaller amounts of EE, the deposition of ALA in tissues can be elevated. Lucerne as an option for finishing lambs in a grazing system provides good concentrations of EPA + DHA in muscle—23 mg/100 g (Ponnampalam and others 2014b).

Compared to forage, grains are energy-dense due to their high starch content and are, consequently, an important component in feedlot rations (Seabrook and others 2011). However, special attention should be paid to high-starch concentrate diets because they lead to alteration in the fermentation pattern and bacterial populations and biohydrogenation of unsaturated fatty acids (Berthelot and others 2014), and as such these alterations may affect the absorption of nutrients in the small intestine and, consequently, the fatty acid deposition in the tissue.

Conversion of ALA to EPA and DHA

Ruminants are capable of synthesizing large quantities of long chain fatty acids. Most of the starch-based and other glucose in the diet is fermented to volatile fatty acids in the rumen, while acetate and β-hydroxylbutyrate are the precursors for fat synthesis. The 1st step in synthesis of fatty acids is the carboxylation of acetyl-CoA to form malonyl-CoA in the liver. Synthesis of fatty acids requires large amounts of nicotinamide adenine dinucleotide phosphate (NADPH), which is derived from different sources (Church 1979). Microbial fatty acids originate from biohydrogenation and utilization of dietary fatty acids, and fatty acid synthesis *de novo* (Shingfield and others 2013). The major substrates of the biohydrogenation are LA and ALA fatty acids and the

rate of rumen biohydrogenation of fatty acids is typically faster with increasing unsaturated fatty acid dietary proportions (Bauman and others 2003).

The degree to which EPA and DHA are biohydrogenated in the rumen is not well understood. However, *in vivo* studies involving dietary supplements of fish oil indicate that much of the EPA and DHA is biohydrogenated, although to a lesser extent than typically observed for LA and ALA fatty acids (Bauman and others 2003).

Intermediates are produced during ruminal biohydrogenation of PUFAs from the diet, mainly LA and ALA (Alves and others 2013). The level of ALA reached 6.7% of total C18 fatty acids in the rumen of lambs fed a pelleted lucerne diet, whereas with concentrate diets it only averaged 0.4% of the total C18 fatty acids (Alves and others 2013). Soybean oil supplementation increased the concentration of 18:1 *trans* isomers in the rumen content of lambs fed concentrates from 9.89 mg/g DM to 17.7 mg/g (Alves and others 2013).

Several hypotheses have been proposed about how to reduce ruminal biohydrogenation such as the use of secondary plant metabolites (Willems and others 2014), feeding high levels of dietary PUFAs (Toral and others 2012), or adoption of specific feeding systems as concentrate or herbage (Vasta and others 2009b). Maia and others (2007) reported toxicity in microbial ecology caused by PUFAs, particularly from the Butyrivibrio group, and the authors suggest that multiple double bonds increase toxicity with 2 possible explanations: due to sterically disruptive effects or because it becomes more difficult to biohydrogenate the entire molecule. Furthermore, no effect on the concentration of the EPA and DHA was observed due to the microorganisms tested.

The lipids of postruminal digestion are mainly composed of the highly saturated palmitic acid, stearic acid, and small and variable amounts of microbial phospholipids, and along with triglycerides of protected fat diets are absorbed in the jejunum (Bauchart 1993; Bauman and others 2003). In animals

phospholipids (or polar lipids) and triacylglycerols (or neutral lipids) are the 2 major classes of lipids. The phospholipids are located in the cell membranes and triacylglycerols in the adipocytes that are located along the muscle fibers and in the interfascicular area (De Smet and others 2004), although small amounts of triacylglycerols are located in lipid droplets within the fiber (Spangenburg and others 2011). The bioavailability of EPA and DHA has been reported to be dependent on triacylglyceride, ethyl-ester, or phospholipid forms (Schuchardt and others 2011). In addition, the source of phospholipids impacts on fatty acid composition. Phospholipids from marine organisms contain n-3 long chain PUFAs while phospholipids from vegetables do not contain these fatty acids (Burri and others 2012).

Fatty acyl-CoA is subjected to a complex series of elongation/desaturation reactions to generate long chain PUFAs in the endoplasmic reticulum (Russo 2009). In Figure 1, the steps to transform ALA to the higher unsaturated derivatives (EPA, DPA, and DHA) by the activities of consecutive desaturation and elongation reactions in eukaryotes are shown. The Δ6 desaturase recognizes and metabolizes LA and ALA. However, the affinity of this enzyme toward LA and ALA differs. For example, mammals convert LA to ARA more efficiently than ALA to EPA/DHA (Kang and Weylandt 2008). The rates of conversion of oleic into octadeca-6,9-dienoic, linoleic into γ - linolenic, and linolenic into octadeca-6,9,12,15-tetraenoic acids are variable; they are not interconvertible, but the amount of PUFA of each pathway accumulated in animal tissues depends on the presence and amount of the acids of the other pathway (Brenner and Peluffo 1966). The conversion steps of LA and ALA to EPA and DHA is dependent on the ratio of ingested n-6 and n-3 fatty acids (Harnack and others 2009). In addition, large amounts of linoleic may prevent hydrogenation of oleic to stearic acid (Harfoot and others 1973).

Thus, ALA is desaturated to stearidonic acid, elongated to eicosatetraenoic acid (20:4 n-3, ETA) and desaturated by Δ5 desaturase generating EPA. There is controversy about the mechanism of the formation of DHA. Some postulate that in mammals the conversion of EPA to DHA occurs via the Sprecher pathway, which is independent of Δ4 desaturase and involves 2 consecutive elongations, desaturation, and an β-oxidation step (Voss and others 1991; Sprecher and others 1999). The other view on the conversion of EPA to DHA in lower eukaryotes is that it occurs via elongation of EPA to DPA followed by desaturation by Δ4 desaturase to form DHA (Qiu and others 2001). Mammals can also convert DHA into EPA, but humans struggle to make this conversion which is not a very efficient process (Russo 2009).

Pereira and others (2004) isolated genes involved in DHA production from marine microalgal species, *Pavlova* and *Isochrys*is. They identified a higher overall amino acid sequence matching with mammalian PUFA elongases than with C18-PUFA elongases from the Sprecher pathway. In other words, more steps to form DHA including β-oxidation. *Pavlova*, the marine microalgal species, may function in DHA biosynthesis because *pav*ELO (a full-length gene) encodes an elongase that is specific for the C20- PUFA, EPA, and ARA. The IgD4 gene is capable of recognizing DPA as a substrate, converting about 28% of it to DHA, indicating that the gene encodes an enzyme with Δ4 desaturase activity. In other words, the microalga *Isochrysis galbana* takes the lower eucaryote pathway to convert EPA to DHA. Li and others (2010) related, for the 1st time, the Δ4 desaturase activity in vertebrate (fish) to convert DPA to DHA by a simpler pathway. These results support the view that genes are capable of functioning together to carry out the 2-step conversion of EPA to DHA (Figure 1).

How to manipulate IMF and fatty acid composition on muscle

The nutritional value of the meat can be influenced by the type of diet consumed by animals and by genetic effects (Fisher and others 2000; Wachira and others 2002; Scollan and others 2014; Ponnampalam and others 2014a). In this regard, Ponnampalam and others (2014a) showed that environmental effects such as site of production and slaughter date had a much greater impact on EPA and DHA levels than sire or breed type. Late-maturing animals contribute to the strategy to minimize carcass fat (Annison 1993), but so does nutritional manipulation, genetic selection, and exogenous agents. Research shows that increasing the energy concentration of a diet does not necessarily increase the subcutaneous or IMF content of a carcase (Wynn and others 2006). In ruminants, it is possible to manipulate intramuscular fatty acid composition of muscle without large increases in fatness once the long chain n-3 PUFAs are incorporated mainly into membrane phospholipids instead of into triacylglycerols (Ponnampalam and others 2001a; Scollan and others 2014).

IMF factor

The level of IMF has an effect on lamb meat flavor (Pannier and others 2014) and Hopkins and others (2006) reported that 3% of the variation in overall liking was explained by IMF. Despite this low number to achieve a mean overall liking score of 63, to give a consumer failure rate of about 10%, analysis suggested an IMF level of 5% was required. However, more recent research suggested that a level of 3.9% is sufficient to ensure the “good every day” eating quality grade is achieved (Pannier and others 2014).

IMF in lamb muscle consists proportionately on average of 46% to 50% SFA, 38% MUFA, and 12% to 16% PUFA (Cooper and others 2004; Hopkins

and others 2014). Palmitic (16:0), stearic (18:0), and oleic (*cis*-9 18:1) acids are the major fatty acid fractions in phospholipids and triacylglycerols, but the phospholipids contain higher proportions of PUFAs (Shingfield and others 2013). The desirable polyunsaturated:saturated fatty acid (P:S) ratio for lamb muscle is above 0.45 and it is calculated by adding the level of LA and ALA and dividing by the summation of the level of C12:0, C14:0, C16:0, and C18:0 (Demirel and others 2004). The n-6:n-3 ratio for lamb is beneficially low and it is desirable for the ratio to be below a maximum recommended level of 4.0 (Demirel and others 2004).

Diets that promote glucose supply to the muscle might increase IMF deposition, while limiting fat deposition in external fat tissues of the carcass (Scollan and others 2014). A higher glucose supply to muscles may be achieved by maximizing fermentation in the rumen to produce gluconeogenic precursors (propionate) or by increasing starch digestion (releasing glucose) in the small intestine (Scollan and others 2014).

Some genotypes, for example, callipyge genotypes, have been characterized by muscle hypertrophy associated with extensive muscling in the loin and hind quarters of sheep. This alters the carcass and meat composition leading to reduced IMF content (Carpenter and others 1996) reflected in changes to muscle fiber metabolism. In a study reported by Carpenter and others (1996), the hypertrophy in callipyge muscle was strongly associated with changes in the fast twitch glycolytic fibers, the only fiber type that increased in both size and percentage of total. Differences in EPA + DHA levels occur between muscles associated with different activities, due to differences in the ratio of type I and type II muscle fibers (Ponnampalam and others 2014b). De Smet and others (2004) suggested that oxidative and glycolytic muscles may contain similar amounts of IMF, but the phospholipid fraction and the distribution of classes vary according the fiber type, although it is not the strongest explanation for the

differences in fatty acid composition between muscles. From the viewpoint of production, callipyge lambs are more desirable than normal lambs as all measures of fatness, subcutaneous, intermuscular, intramuscular, and perinephric are decreased in callipyge lamb carcasses (Cockett and others 1999); however, there is no information about the callipyge effect on the fatty acid profile. Furthermore, the changes due to the callipyge genotype are not the same across different muscle types (Carpenter and others 1996; Ducket and others 2000).

IMF has a significant impact on eating quality traits with a negative correlation with shear force in lambs. An increase of 3.5% in IMF provided a reduction in shear force at 5 d postslaughter by over 10 N (Newtons) (Warner and others 2010). There is a detrimental effect on palatability when the IMF is less than 3% (Warner and others 2010) and an IMF of 4% to 5% is required to maximize eating quality in lambs (Hopkins and others 2006). It is known that the polar fraction (phospholipids) in the muscle is independent of the total fat content, and the neutral fraction (triacylglycerols) is strongly related to the total fat content (De Smet and others 2004). In terms of fatty acid composition and eating quality, n-6 and n-3 contribute to the odor and flavor of meat when ruminants are fed with grain or forage, respectively (Sanudo and others 2000).

Fatty acid composition

Studies with lambs have examined the potential of grains, dried, ensiled or fresh forage, dietary plant oils, oilseeds, fish oil, and marine algal supplements to alter the fatty acid composition of IMF (Table 2).

In a study analyzing the nutritional value of retail meat, Williams (2007) demonstrated that, although white fish muscle has a higher level of EPA than lamb muscle, the latter is higher in total and DPA fatty acids. Lamb muscle

tends to also have a higher DPA level than EPA (Table 2). Although DPA fatty acid is not considered in the classification of foods as a source of health-claimable n-3, the beneficial effects of DPA for lipid metabolism (triglyceride, total cholesterol, and phospholipid [mg/liver]) in mice were considered intermediate between EPA and DHA (Gotoh and others 2009). Some benefits are unique to DPA in human health nutrition: i) DPA inhibits platelet aggregation more than EPA and DHA, ii) DPA is the precursor for oxylipins, anti-inflammatory, and neuroprotective compounds, iii) it stimulates endothelial cell migration much more efficiently than EPA, and iv) it is incorporated into phospholipids faster than EPA, being utilized by the body more efficiently (Byelashov 2013). However, pure DPA has not been readily available for testing compared to EPA and DHA, and its role in human health has not been systematically examined (Kaur and others 2011). The beneficial effects of pure DPA in animal nutrition are scarcely known.

In general, grain feeding provides higher levels of LA than pasture feeding, and pasture is higher in ALA than concentrates. Soybean oil provides more ALA than fish oil, but the latter provides higher levels of EPA and DHA fatty acids. Silage is rich in n-3, and oats and cottonseed meal in n-6, and dams fed with these ingredients have been shown to affect EPA and DHA levels in lamb muscle when associated with algal supplementation (Hopkins and others 2014).

Accordingly, Wood and others (2008) suggest that the capacity for incorporation of PUFAs into phospholipids is limited and that LA competes for incorporation much more effectively than ALA. Feeding lambs a diet rich in PUFAs promotes fat deposition in the phospholipid fraction, and SFAs, when in excess, are deposited and stored in the triglyceride fraction (Ponnampalam and others 2001a). Studies have shown differences in the proportion of PUFAs between phospholipid and triacylglycerol fractions. Ponnampalam and others

(2001b) showed that the fatty acid composition of the phospholipid fraction was less for 18:0 and greater for LA when compared with the triglyceride fraction in mg/100 g of *longissimus thoracis* muscle (64 to 559 and 62 to 47, respectively). The same outcome was observed by Demirel and others (2004) in *semimembranosus* muscle and by Ponnampalam and others (2001a) and Cooper and others (2004) in *longissimus* muscle.

Table 2. Diet supplementation effect on the level of *n*-6 and *n*-3 fatty acids in lamb and fish muscle.

Description	Unit	OA	LA	ALA	CLA	EPA	DPA	DHA	Reference
Fish									
White fish	mg/100g muscle	110	10	0	-	48	21	111	Williams (2007)
Lamb									
Grain and forage	mg/100g muscle	1830	294	40	-	30	38	13	Hopkins and others (2014)
Grain and forage + algae (2% DHA-Gold)	mg/100g muscle	1488	300	38	-	46	36	82	Hopkins and others (2014)
0% DHA	mg/100g muscle	6691	605	36.5	140	8.4	29.0	9	Meale and others (2014)
1% DHA	mg/100g muscle	5171	418	27.5	205	9.1	40.1	50	Meale and others (2014)
2% DHA	mg/100g muscle	4799	398	24.6	100	17.9	46.1	58	Meale and others (2014)
3% DHA	mg/100g muscle	4885	451	24.9	120	32.1	61.3	114	Meale and others (2014)

Table 2 (continuation)

Description	Unit	OA	LA	ALA	CLA	EPA	DPA	DHA	Reference
Grass	mg/100g muscle	593	119	41	-	24	27	10	Fisher and others (2000)
Concentrate	mg/100g muscle	725	188	14	-	8.4	16	5.5	Fisher and others (2000)
No Tannin (concentrate + herbage)	mg/100g muscle	577	140	23.1	9.77	13.5	-	5.31	Vasta and others (2009a)
Tannin (concentrate + herbage)	mg/100g muscle	464	176	26.2	11.1	11.5	-	4.27	Vasta and others (2009a)
Lupin	mg/100g muscle	1297	159	34	-	12.1	15.6	7.1	Ponnampalam and others (2002)
Fish meal	mg/100g muscle	969	107	30	-	33.4	23.4	17.0	Ponnampalam and others (2002)
Barley	mg/100g muscle	984	129	30	-	16.4	17.7	6.8	Ponnampalam and others (2002)
Control (no antioxidant, 34g palm oil)	mg/100 muscle	569	136	13.7	5.93	10.6	13.9	5.66	Andrés and others (2014)

Table 2 (continuation)

Description	Unit	OA	LA	ALA	CLA	EPA	DPA	DHA	Reference
Quercetin (Control + 2g quercetin)	mg/100g muscle	447	125	10.9	4.5	9.08	12.4	4.60	Andrés and others (2014)
Linseed (85g/kg)	mg/100g muscle	592	141	24.6	5.03	15.8	17.6	7.18	Andrés and others (2014)
High forage quality and low phenol	g/100 g FAME	28.2	5.71	2.18	0.96	0.98	0.84	0.24	Willems and others (2014)
Moderate forage quality and high phenol	g/100 g FAME	23.3	8.69	3.44	0.75	1.14	1.02	0.31	Willems and others (2014)
Megalac	% total fatty acid	34.9	4.87	1.44	0.99	0.68	0.66	0.28	Wachira and others (2002)
Linseed	% total fatty acid	30.86	3.98	3.09	1.55	1.03	0.74	0.40	Wachira and others (2002)
Fish oil	% total fatty acid	25.85	3.36	1.37	1.10	2.32	1.29	0.79	Wachira and others (2002)

Table 2 (continuation)

Description	Unit	OA	LA	ALA	CLA	EPA	DPA	DHA	Reference
Linseed + fish oil	% total fatty acid	27.4	3.48	1.84	1.66	1.61	1.02	0.63	Wachira and others (2002)
CSFA (3%)	% FAME	31.95	8.41	0.11	0.36	0.44	1.02	0.62	Gomez-Cortes and others (2014)
Extruded linseed (9%)	% FAME	25.43	9.16	0.12	1.25	1.42	1.60	1.25	Gomez-Cortes and others (2014)
Control (none palm oil, none CSFA)	% total fatty acid	38.72	10.16	0.56	-	-	-	-	Castro and others (2005)
Low palm oil	% total fatty acid	36.99	10.46	0.47	-	-	-	-	Castro and others (2005)
High palm oil	% total fatty acid	34.61	11.07	0.45	-	-	-	-	Castro and others (2005)
Low CSFA	% total fatty acid	35.89	10.68	0.58	-	-	-	-	Castro and others (2005)
High CSFA	% total fatty acid	37.76	9.53	0.43	-	-	-	-	Castro and others (2005)

Table 2 (continuation)

Description	Unit	OA	LA	ALA	CLA	EPA	DPA	DHA	Reference
Basal diet[1]	mg/100 g muscle	1394	118	35.7	-	14.14	18.04	5.5	Ponnampalam and others (2001a)
Fish meal[1]	mg/100 g muscle	1113	99	18.3	-	16.42	18.47	11.5	Ponnampalam and others (2001a)
Fish oil + protected sunflower meal[1]	mg/100 g muscle	1046	105	24.5	-	33.21	21.8	18.8	Ponnampalam and others (2001a)
Fish oil[1]	mg/100 g muscle	919	102	27.3	-	36.2	22.69	18.93	Ponnampalam and others (2001a)
Protected sunflower meal[1]	mg/100 g muscle	1589	123	26.5	-	13.5	17.3	4.8	Ponnampalam and others (2001a)
Basal[1-Exp1]	mg/100 g muscle	1086	111	40	-	18	19	7.5	Ponnampalam and others (2001b)
Fish meal[1-Exp1]	mg/100 g muscle	777	83	28	-	21	21	15	Ponnampalam and others (2001b)
Canola meal[1-Exp1]	mg/100 g muscle	994	113	40	-	15.2	17	5	Ponnampalam and others (2001b)

Table 2 (continuation)

Description	Unit	OA	LA	ALA	CLA	EPA	DPA	DHA	Reference
Soy meal[1-Exp1]	mg/100 g muscle	1055	127	42	-	21.2	24	8	Ponnampalam and others (2001b)
Basal diet[1-Exp2]	mg/100 g muscle	953	81	24.2	-	12.8	16	4.1	Ponnampalam and others (2001b)
Fish meal 9% DM[1-Exp2]	mg/100 g muscle	1163	75	24	-	16	16.8	10.2	Ponnampalam and others (2001b)
Unprotected rapeseed oil 7% DM[1-Exp2]	mg/100 g muscle	1176	91	28.9	-	13.1	14.1	4.5	Ponnampalam and others (2001b)
Protected canolaseed oil 6% DM[1-Exp2]	mg/100 g muscle	1046	132	34.6	-	12.8	15	4.8	Ponnampalam and others (2001b)
Megalac[1]	g/100g total fatty acid	66	14.02	5.78	-	4.1	3.8	1.2	Demirel and others (2004)
Protected whole linseed[1]	g/100g total fatty acid	59.6	12.05	10.74	-	5.6	5	1.1	Demirel and others (2004)
Protected whole linseed + fish oil[1]	g/100g total fatty acid	53.7	10.85	6.82	-	7.7	5.6	1.8	Demirel and others (2004)

Table 2 (continuation)

Description	Unit	OA	LA	ALA	CLA	EPA	DPA	DHA	Reference
Linseed oil[1]	% total fatty acid	51.8	19.11	8.7	1.77	3.88	3.39	1.14	Cooper and others (2004)
Fish oil[1]	% total fatty acid	49	13.01	3.24	1.18	6.1	3.45	2.26	Cooper and others (2004)
Protected linseed and soybean[1]	% total fatty acid	43.51	43.8	6.88	0.97	2.68	2.98	0.67	Cooper and others (2004)
Fish oil + algae[1]	% total fatty acid	44.3	15.16	2.12	1.13	9.5	3.88	7.22	Cooper and others (2004)
Protected linseed and soybean + algae[1]	% total fatty acid	42.6	33.85	5.42	1.21	5.13	2.55	6.85	Cooper and others (2004)

OA: oleic acid. LA: linoleic acid. ALA: linolenic acid. CLA: conjugated linoleic acid, *cis*9*trans*11 C18:2. EPA: eicosapentaenoic acid. DPA: docosapentaenoic acid. DHA: docosahexaenoic acid. [1]Phospholipid + triglyceride fraction.

The nutrition of the animal is the main source of variation in the fatty acid profile in lamb meat and it has been strongly correlated with the ruminal biohydrogenation. Forages rich in tannins may partially inhibit ruminal biohydrogenation and thus reduce the loss of n-3 and n-6 fatty acids from the diet. Willems and others (2014) reveal that the highest levels of LA, ALA, and the sum of PUFAs in either total lipids and phospholipids of lamb muscle was found in a diet with the largest amount of tannins. In the study by Willems and others (2014), the highest levels of LA and ALA found in the muscle were not from animals fed with the highest levels of those fatty acids in the diet. This suggests that factors that alter ruminal biohydrogenation may affect the fatty acid deposition in muscle more than the dietary profile, including content and composition of phenolic compounds. Similarly, Vasta and others (2009b) found the highest unsaturated fatty acid concentrations in ruminal fluid from lambs supplemented with tannins, following on to an increase in PUFAs and a reduction in SFA levels in IMF.

Some studies indicate microalgae as sources of phenols (Abd ElBaky and others 2008, 2009) and this could be one of the causes of the high levels of PUFAs in IMF due to reduction in biohydrogenation in the rumen and a large amount of fatty acids reaching the small intestine. A recent study investigated the variation in long chain n-3 PUFAs in lamb muscle, and on-farm factors such as genetics, gender, rearing status, and age of dam had a relatively small impact on EPA and DHA levels (Ponnampalam and others 2014a). By contrast, Ponnampalam and others (2014a) describe a massive effect on EPA and DHA levels in lamb muscle due to production site. When a large part of the diet was high-quality green pasture compared to lambs run on dry pasture and supplemented with pellets and grain, then the levels of EPA and DHA were increased. For example, at sites where lambs were grazing on green pasture the EPA + DHA level was above 30 mg/100 g muscle. At sites where the feeding

changed from green pasture to dry pasture supplemented with grains, the EPA + DHA level was lowered from 33 to 10 mg/100 g muscle. The diet effect is related to seasonal conditions with summer and autumn resulting in less green pasture, which influences the EPA + DHA level in muscle. Very low levels of EPA + DHA in the muscle of lambs were found during the autumn season when the pasture-fed lambs were supplemented with triticale, hay, and feedlot grains (Ponnampalam and others 2014b).

The fat source in the diet of suckling lambs has been shown to affect PUFA levels, with higher levels when extruded linseed was fed compared to calcium soap fatty acid (CSFA) (Gomez-Cortes and others 2014). Other factors that affect fatty acid deposition include the form of the grain, with whole cottonseed in a lamb diet leading to lower deposition of ALA, EPA, and DHA in IMF, than when it is fed as a by-product meal (Paim and others 2014). Cottonseed contains also malvalic and sterculic acid, potent inhibitors of Δ9 desaturase which has impact in the PUFA synthesis (Griinari and others 2000). The oil source in lamb diets affects the fatty acids in the phospholipid fraction of the muscle, so that the inclusion of linseed oil increases the LA and ALA levels and decreases the EPA and DHA levels when compared with fish oil (Cooper and others 2004).

Another potential pathway to increase PUFAs in ruminant tissues is to exploit breeds with an increased capacity to deposit these fatty acids or deposit n-3 PUFAs in preference to those of the n-6 fatty acid series (Demirel and others 2004). Wachira and others (2002) found that Suffolk and Soay lambs contained more ALA than the Friesland lambs, and Soay lambs had higher intramuscular levels for all the major n-6 PUFAs and CLA than Suffolk or Friesland lambs. The LA and ALA were higher in the Suffolk × Lleyn lambs than Scottish Blackface, as measured in the polar lipids of the *semimembranosus* muscle (Demirel and others 2004). Lambs from Merino dams had about 2 mg/100 g

higher levels of EPA + DHA than lambs from cross-breed dams, when the sire breed was Poll Dorset (Ponnampalam and others 2014a).

Hopkins and Mortimer (2014) list EPA and DHA as heritable traits (h2: 0.17 to 0.29 EPA, 0.16 to 0.25 DHA), enabling genetic manipulation of these n-3 fatty acids. However, nongenetic approaches such as nutritional manipulation can be more easily and quickly applied in animal production systems. Further selection for EPA and DHA would require development of genomic breeding values as these traits cannot be measured in live animals practically or cost-effectively.

Radzik-Rant and others (2012) found 7%, 39%, and 29% more LA, ALA, EPA, respectively, for lambs slaughtered at 60 d of age than 90 d, although the diet changed from suckling feeding (until 60 d) to grass hay and concentrate diets (61 to 90 d). Diaz and others (2003) did not find an effect of live weight (10, 12, and 14 kg) on fatty acid composition. Likewise, the age at slaughter was not shown as a source of variation for muscle EPA + DHA level by Ponnampalam and others (2014a), even when this variation was large (150 to 500 d).

Protected fat and algae as sources of n-3 fatty acids

Protected fat

To avoid the inhibitory effect of added fat on the ruminal ecosystem, protected fats can be fed (Haddad and Younis 2004). Protected fat is the commercial name and one of the forms marketed is calcium soap from palm oil (Castro and others 2005; Casals and others 2006) or calcium salts from palm oil (Demirel and others 2004). Calcium salts of fatty acids consist of fatty acids associated with calcium ions instead of a glycerol backbone (Block and others

2005). They consist of mainly palmitic acid (16:0) and oleic acid (18:1n-9), 48.8% and 34.4%, respectively, as reported by Wynn and others (2006) and 0.26%, 1.20%, 46.9%, 40.7%, and 9.70% of C12:0, C14:0, C16:0, C18:0, and C18:1, respectively, for calcium soap of palm oil (Gomez-Cortes and others 2014). When calcium is associated with unsaturated fatty acids, the fat supplement has different physical properties, similar to SFAs. It is solid at high temperatures and does not melt (Block and others 2005). At normal rumen pH, 6 to 7 (Church 1979), more than 60% to 90% of the calcium salts pass through the rumen as inert; but this depends on the pH range and rate of feed passage (Block and others 2005).

In addition, the digestibility of all fatty acids is not same. Palmitic and stearic acids are about 5% more digestible than other SFAs (Ferlay and others 1993). Stearic acid has lower availability in the intestine than unsaturated fatty acids (Block and others 2005). The differences in the intestinal absorption of saturated and unsaturated fatty acids may be due to many factors such as carbon length chain, solubility, esterification, fatty acid-binding protein, melting point, and bile salt concentration as described by Ockner and others (1972). Therefore, CSFA is less susceptible to biohydrogenation (Zinn and others 2000) and can be used successfully to increase the unsaturated fatty acid content in adipose depots in muscle. The effects of rumen-protected lipid supplementation vary with breed, dietary composition, and tissue depot (Wachira and others 2002; Castro and others 2005; Seabrook and others 2011).

High levels of CSFA (11% DM) result in a voluntary reduction in daily feed intake in lambs and this is matched by a reduction in average daily gain (Seabrook and others 2011). However, these authors found the same feed efficiency and final body weight, but lower daily dry matter intake by including 4% of the CSFA in the diet compared to a control diet. Demirel and others (2004) reported no effect on feed intake, growth rate, and feed conversion

according to the type of dietary fat (Megalac® , protected whole linseed and protected whole linseed + fish oil), but mean carcass conformation scores were higher for lambs fed Megalac compared with those fed the other diets. There was no significant effect of dietary fat source on the food conversion ratio (kg DM intake/kg gain) or carcass weight (kg) in lambs fed with Megalac, linseed, fish oil, or linseed plus fish oil (Wachira and others 2002) in agreement with the findings of Demirel and others (2004).

Fatty acids in excess of 5% to 6% of the diet are absorbed less efficiently (Palmquist and Jenkins 1980), and thus, to avoid impairment of rumen function, the capacity to manipulate the fatty acid composition by use of ruminally available fatty acids is limited (Scollan and others 2014). The substitution of corn with CSFA may require a greater number of days on feed to achieve the same finishing weight (Seabrook and others 2011), but when the price of the corn is high such a strategy can be favorable. Thus, the potential to alter the fatty acid composition is determined, to a large extent, by ruminal biohydrogenation of dietary lipids. Demirel and others (2004) reported an increase in the C18:1n-7 concentration (g/100 g total fatty acids) in *semimembranosus* muscle from 0.81 to 1.18 in the lambs fed with protected whole linseed compared to those fed Megalac. They also found a large increase in EPA, DPA, and DHA concentrations from 4.4 to 9.1, 3.9 to 5.6, and 1.5 to 2.1 g/100 g of the total fatty acids, respectively, when they added protected whole linseed + fish oil as a fat source to replace Megalac. The *trans*-10, *cis*-12 CLA content of lamb *longissimus* muscle increased 16.7% when 86.6 g of Megalac/kg of diet DM was fed for 10 wk (Wynn and others 2006).

Wachira and others (2002) kept the LA level high in the *longissimus* muscle of sheep fed with Megalac, compared with those fed linseed, fish oil, and linseed plus fish oil, but it was not the same for EPA, DPA, and DHA when fish oil was added to the diet of the animals. Fish oil contributes the long chain

PUFAs (EPA and DHA) in a diet and increases their levels in muscle (Demirel and others 2004). Cooper and others (2004) also found higher levels of EPA and DHA in muscle by feeding lambs with fish oil, compared with protected linseed and soybean.

Dietary inclusion of protected fat generally increases the concentration of n-3 and n-6 fatty acids in muscle in some, but not all, studies (Castro and others 2005; Seabrook and others 2011). In these latter studies (Castro and others 2005; Seabrook and others 2011), there was a higher amount of corn in the control diet instead of in the protected fat diet and thus more LA (Table 1). It should also be noted that it is not clear if the 18:3 shown is n-3, n-6, or the sum of both, in the study of Castro and others (2005). By contrast, Gomez-Cortes and others (2014) report the fatty acid composition of lamb muscle with a significant difference among diets (CSFA compared with extruded linseed) for 18:3n-3 (α-linolenic acid, ALA), but with no effect on 18:3n-6 (γ -linolenic acid, GLA). Andres and others (2014) also found a significant difference among diets for the muscle level of ALA and no effect on GLA.

A recent study showed the ALA, EPA, DPA, DHA, and the sum of PUFA levels, in IMF were higher in lambs fed with extruded linseed than calcium soap of palm oil (Gomez-Cortes and others 2014). This could be due to the higher level of ALA in extruded linseed than in calcium soap of palm oil (see Table 1), which is the precursor of the n-3 long chain PUFA.

Algae

Macro-algae or “seaweeds” are multicellular plants and microalgae are microscopic organisms that both grow in either salt or fresh water. Macro-algae are classified into 3 broad groups based on their pigmentation: brown seaweed (Phaeophyceae), red seaweed (Rhodophyceae), and green seaweed

(Chlorophyceae). The 3 most important classes of micro-algae in terms of abundance are the diatoms (Bacillariophyceae), green algae (Chlorophyceae), and the golden algae (Chrysophyceae). Algae are mainly utilized for the production of food, food additives, and animal feed. The main storage compound is starch and oils (Carlsson and others 2007; Sivakumar and Rajendran 2013).

Algae have several advantages over conventional plants such as a high DM output per hectare low water usage per unit of biomass, the whole plant can be utilized and it has high protein, lipid, and vitamin concentration (Sivakumar and Rajendran 2013). In addition, microalgae are a new source of biofuel which, along with application as an animal feed, can help alleviate the greenhouse effects associated with current energy and food production (Lum and others 2013). The chemical composition of many micro-and macro-algae have been reported in the literature (Ventura and Castanon 1998; Becker 2007; Sivakumar and Rajendran 2013), and these exhibit wide variation.

Macro-algae (seaweed) are a potential source of natural antioxidants and, given the vast range of compounds present in such algae, seaweed can be considered a functional food (Moroney and others 2012). However, the inclusion of algae as a human food is limited due to the fishy smell, dark green colour, and dried biomass (Becker 2007). For animal feed these aspects are not important and such algae can be a source of protein and long chain n-3 fatty acids, although minimal research has been carried out to evaluate their nutritional value for lambs.

The protein content of *Ulva lactuca* a macro-alga is greater than lucerne hay (Ventura and Castanon 1998). The average quality of most algal amino acids is equal or even superior to that of conventional plant amino acids. The amounts in g/100 g protein for isoleucine, leucine, valine, and methionine in soybean are 5.3, 7.7, 5.3, and 1.3, and in *Spirulina platensis* are 6.7, 9.8, 7.1, and

2.5, respectively (Becker 2007). Where corn and soybean are the base of food crops for humans, algae may be a viable replacement of these grains representing a source of protein, minerals, vitamins, and DHA and EPA fatty acids (Lum and others 2013).

Studies have reported the negative effect of the intake of dietary phenolic compounds in terms of ruminal biohydrogenation (Vasta and others 2009b), but it can be positive due to the higher bypass of C18 PUFAs arriving in the small intestine. For example, lambs supplemented with high tannin levels have greater percentages of PUFAs and reduction of SFAs compared with those fed with plants containing lower tannin levels (Willems and others 2014). Algae are a source of phenolic compounds which possess antioxidant and antibacterial activity (Abd El-Bakey and others 2008). The interaction between dietary algae and ruminal biohydrogenation in lactating sheep was reported by Toral and others (2012). In their study, the inclusion of marine algae (DHA Gold Animal Feed Ingredient) increased the sum of *trans* 18:1 and decreased 18:0 concentrations in rumen fluid, thus reflecting changes in ruminal biohydrogenation pathways. Experiments *in vitro* show the incomplete biohydrogenation of LA and ALA acids (Boeckaert and others 2007) and inhibition of bacteria and enzymes involved in the biohydrogenation of nonconjugated 18:2 isomers in response to algal supplementation (Vlaeminck and others 2008).

Several studies have demonstrated that the inclusion of algae could be a useful strategy to improve the nutritional properties of meat with regard to DHA and EPA fatty acid composition. Cooper and others (2004) showed dietary microalgae increased the n-3 PUFAs in lamb muscle. Hopkins and others (2014) found the total of EPA + DHA from lambs fed with an algal supplement at 1.92% of the daily intake (DHA Gold^{TM} algae) when dams were fed silage at conception period, was 140 mg/100 g muscle. This would provide 1.75 times

more than European standards require to claim the food as a “good source” of n-3. Meale and others (2014) showed 3.8 and 12.6 times more EPA and DHA, respectively, in IMF from lambs fed with 3% DM of DHA Gold compared to those fed a diet without algae. Dib (2012) did not find any effects on the fatty acid composition of *longissimus* in wethers fed with algal meal, and this could be due to the fact the animals were fed for only 28 d (21 adjustment period plus 7 d testing period) and the percentage of EE of the algal meal (*Chlorella sp.*) was very low (1.96% DM), and the algae meal might not have had adequate levels of EPA and DHA available for bypassing rumen biohydrogenation. Despite biohydrogenation in the rumen, a significant proportion of dietary PUFAs bypasses the rumen intact and is absorbed and deposited in IMF when lambs are fed with marine algae as part of the diet.

Hopkins and others (2014) showed a negative effect of a high PUFA diet on the lipid oxidation of muscle from lambs fed with algae, which could lead to rancidity and may cause the development of other off-flavors. Effective antioxidants in the diet could overcome this, and perennial pasture (Ponnampalam and others 2012a), saltbush (Pearce and others 2005), and vitamin E supplementation (Berthelot and others 2014) have been shown to be effective in raising the level of α-tocopherol in lamb muscle. This would ensure that diets with high PUFA levels will not lead to excessive lipid oxidation and off-flavor development. The interrelationship between muscle biochemical composition of PUFA, vitamin E, and iron components in relation to lipid oxidation and off-flavor development has been reported in detail in a recent publication (Ponnampalam and others 2014c).

Gene expression of the long chain PUFAs

This review has shown there are many reasons to improve the level of long chain n-3 in red meat. However, it is also important to understand the mechanisms which impact on the level and profile of fatty acid deposition in farm animals. Such knowledge can be used to manipulate this deposition and, therefore, produce meat which can satisfy consumer concerns about the healthfulness of the red meat they consume.

The molecular mechanisms and which genes are responsible for phenotypic variation of fatty acid deposition are not clear. The identification of genes which affect adiposity may be helpful to explain the function of these genes in fatty acid metabolism and fat deposition. Fatty acid composition of animal tissue is related to dietary fatty acids which are directly incorporated into animal tissue, or to *in vivo* fatty acid biosynthesis through the action of enzymes and gene regulation (Vasta and others 2009a).

The following section summarizes the characteristics of genes involved in fatty acid metabolism in livestock and elucidates the correlation between fat deposition and n-3 uptake. The fatty acid deposition depends on nutritional factors, as discussed earlier, and also on gene expression regulation. Related genes can be identified by correlated expression patterns in time or space (Lim and others 2010).

Genes related to the long chain PUFA metabolism

Lipoprotein lipase (LPL), fatty acid synthase (FASN), stearoyl coA desaturase (SCD), fatty acid desaturases (FADS), acetyl CoA carboxylase (ACACA or ACC), and carnitine palmitoyltransferase (CPT) are the most frequently studied genes shown to influence fat traits in sheep.

Lipoprotein lipase

LPL is a rate-limiting enzyme in the hydrolysis of triglycerides circulating in the form of chylomicrons and very low density lipoproteins into free fatty acids and 2-monoacylglycerols (Dervishi and others 2012b). This enzyme plays an important role in the differentiation and maturation of adipose cells (Dervishi and others 2011). The LPL gene is expressed in adipose tissue, heart muscle, and skeletal muscles (Ren and others 2002). The LPL enzyme is synthesized by adipocytes and migrates in its active form toward the capillary endothelium. This enzyme also hydrolyzes triglycerides in lipoproteins and phospholipid complex of chylomicrons and very low-density lipoprotein (VLDL) (Faulconnier and others 1994). LPL has a role in partitioning triglyceride fatty acids between adipose tissue and oxidative muscles according to the needs of the organism (Bonnet and others 2000).

Fatty acid synthase

FASN is the central enzyme in the lipoprotein pathway that catalyzes all the reactions involved in the last step of the fatty acid biosynthetic pathway (Laliotis and others 2010). SFAs are derived from dietary sources or production by *de novo* biosynthesis with the aid of FASN (Chorna and others 2013). In the presence of NADPH, FASN catalyzes the synthesis of long chain SFAs from palmitate derived from acetyl-CoA and malonyl-CoA (Dervishi and others 2012b).

In bovines, the FASN gene is expressed in all tissues (Laliotis and others 2010). It is highly conserved between mammals (goat, horse, cow, and human, 93% of homology) and mRNA sequences are available for different species in the GeneBank (Braglia and others 2014). The expression pattern of FASN is

different across tissues and species (Bakhtiarizadeh and others 2013). In sheep, this gene has been found expressed in muscle and adipose tissue (Qiao and others 2007; Bakhtiarizadeh and others 2013). Qiao and others (2007) reported that the FASN expression level negatively correlated with IMF content in Kazak sheep muscle.

Stearoyl coenzyme A desaturase

SCD enzyme (or Δ9 desaturase) desaturases *trans*-11 vaccenic acid to *cis*-9, *trans*-11 CLA (Mao and others 2012) and stearic acid to oleic acid (Nakamura and Nara 2004). SCD activity is important for maintaining membrane fluidity (Wongwathanarat and others 1999), lipid metabolism, and adiposity (Kim and others 2002), and its expression may improve the functional food attributes by increasing CLA content in lamb tissue (Mao and others 2012). SCD has been identified as a candidate for genetic variation in fatty acid composition (Mannen 2012). Expression of the SCD gene is highly regulated by dietary factors (Kim and others 2002), and the inhibition of SCD activity is by n-6 and n-3 PUFA (Nakamura and Nara 2002). SCD is mainly expressed in liver and adipose tissue, upregulated by high carbohydrate diets, and downregulated with PUFA-rich diets (Estany and others 2014).

SCD1 and SCD5 are in the SCD gene family in vertebrates (Castro and others 2011). SCD2, SCD3, and SCD4 are SCD1 homologs (Castro and others 2011). In sheep, the mRNA expression of SCD5 and SCD1 is greatest in the brain and adipose tissue, respectively (Lengi and Corl 2008).

Fatty acid desaturases

PUFA desaturation activity in mammals is encoded by the FADS genes. Fatty acid desaturase 1 (FADS1) catalyzes the conversion of ETA to EPA by the Δ5 desaturase enzyme (Nakamura and Nara 2004). Fatty acid desaturase 2 (FADS2) catalyzes the desaturation of ALA to stearidonic acid (C18:4n-3) and LA to GLA by Δ6 desaturase enzyme (Matsumoto and others 2014). FADS3 is a putative PUFA desaturase gene with no known function. It has a high degree of sequence homology with FADS1 (52%) and FADS2 (62%) (Reardon and others 2013) and 7 alternative transcripts expressed in at least 12 organs in an apparently constitutive manner (Brenna and others 2010).

Fatty acyl desaturase 2 (fad2) has been reported as responsible for Δ4 desaturase activity. Fad2 is commonly found in protozoan trypanosomes (Tripodi and others 2006), photosynthetic freshwater protists (Meyer and others 2003), and marine microalgae (Qiu and others 2001). The 1st report was in marine herbivorous fish (Li and others 2010) and more recently in marine carnivorous fish (Morais and others 2012). Li and others (2010) isolated cDNA of fad2 from the fish *Siganus canaliculatus* and by heterologous expression in the yeast *Saccharomyces cerevisiae* showed an ability to convert DPA to DHA, indicating that the fad2 gene encoded an enzyme having 4Δ fad activity. Morais and others (2012) showed the development and nutritional regulation of a 4Δ fad in a vertebrate species (*Solea senegalensis*). It indicates that both pathways to DHA biosynthesis (Figure 1) can be carried out in vertebrates. However, phylogenetic analysis described *Siganus canaliculatus* 4Δ Fad closer to marine teleost 6Δ Fads and more distant to the lower eukaryotes Fads, including other 4Δ Fads (Morais and others 2012).

In addition, Fonseca-Madrigal and others (2014) have characterized a fads2a gene acting on Δ4 desaturase and fadsb on Δ6 in fish. Thus, it is

considered important to understand the possibility of the conversion of DPA to DHA by Δ4 desaturase in ruminants, since it can be a shorter and more efficient reaction. Plants insert additional double bounds by Δ12 and Δ15 FADS, and mammals use Δ9, Δ6, and Δ5 desaturases activities. However, direct evidence for Δ4 desaturase activity in mammals still remains elusive.

Acetyl coenzyme A carboxylase

ACC produces malonyl-CoA and is the 1st intermediate in the synthesis of long chain fatty acids in lipogenic adipose and liver tissues (Rasmussen and others 2002), and it is a potent inhibitor of CPT1 (McGarry and Brown 1997), hindering the mechanism of the mitochondrial transport of long chain fatty acids. This system of regulation discourages fatty acid oxidation when fatty acids are being synthesized (Grummer 1993). The accumulation of malonyl-CoA in liver and adipose tissue stimulates long chain fatty acid synthesis, while concomitantly inhibiting long chain fatty acid oxidation (Rasmussen and others 2002).

The highest levels of ACC enzyme are found in lipogenic tissues such as liver, adipose tissue and the mammary gland during lactation (Garcia-Fernandez and others 2010). Two isoforms of ACC have been identified in sheep. ACC-α (or ACACA), which is the rate-limiting enzyme in the biogenesis of long chain fatty acid, converts acetyl-CoA to malonyl-CoA and ACC-β (or ACACB). It also stimulates the process to generate malonyl-CoA and control mitochondrial fatty acid oxidation (Moibi and others 2000; Najafpanah and others 2014). ACC affects the biosynthesis of palmitic acid and long chain fatty acids, being one of the most important enzymes involved in the synthesis of SFAs (Garcia-Fernandez and others 2010).

Carnitine palmitoyltransferase

Carnitine palmitoyltransferase 1 (CPT1) enzyme is responsible for a mitochondrial transport system and is a key in the control of long chain fatty acid oxidation (Clarke 2000), stimulating mitochondrial β-oxidation (Rasmussen and others 2002; Dervishi and others 2011). It converts the long chain acyl-CoA to long chain acylcarnitine allowing the fatty acid moiety to be transported across the inner mitochondrial membrane via carnitine translocase, which exchanges long chain acylcarnitines for carnitine (Fillmore and others 2011). An inner mitochondrial membrane CPT2 then converts the long chain acylcarnitine back to long chain acylCoA (Fillmore and others 2011).

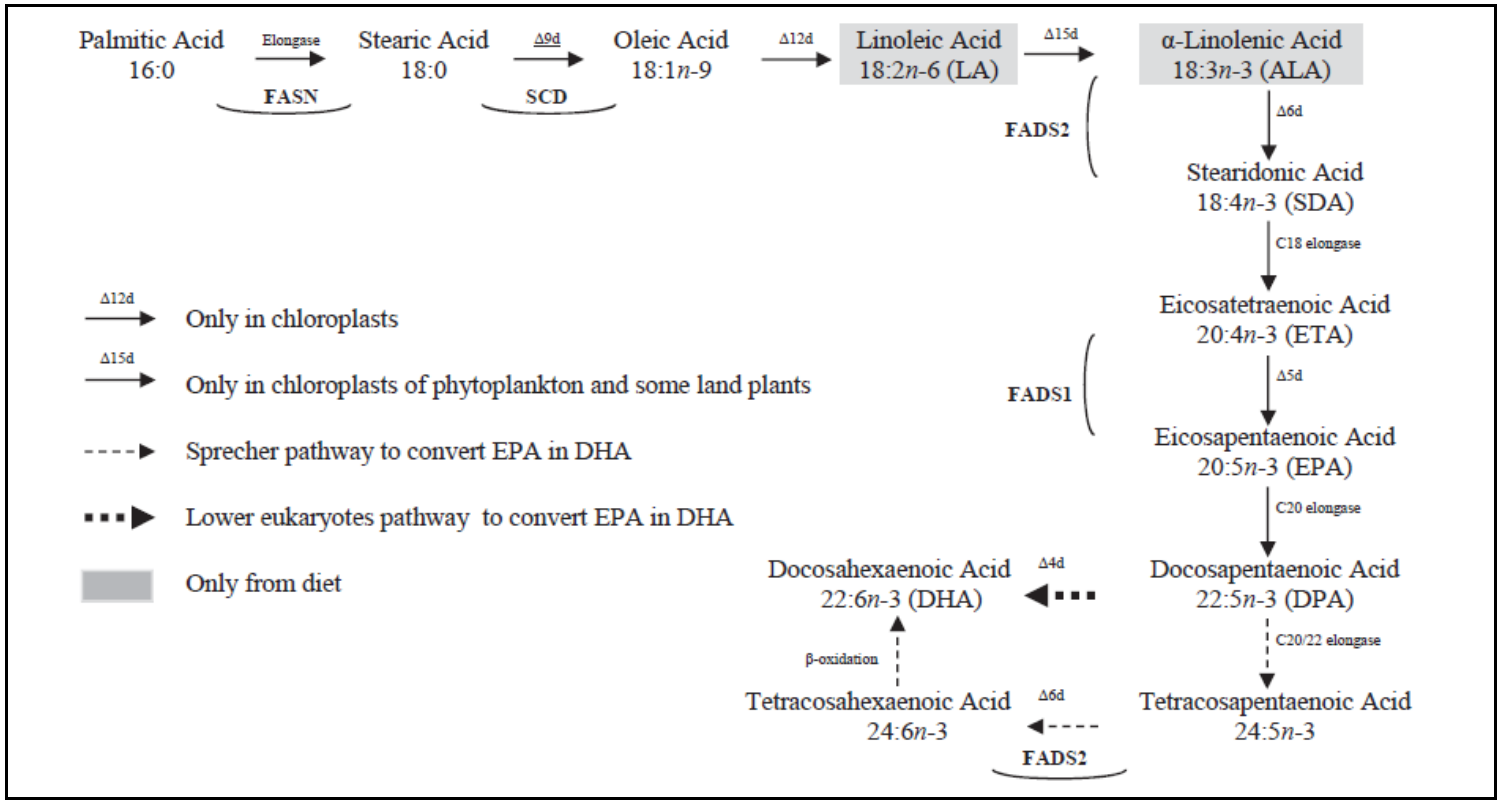

Figure 1. PUFA biosynthesis via lower eukaryotes (dashed bold arrow) and Sprecher pathway (dashed arrow). FASN = fatty acid synthase. SCD = stearoyl CoA desaturase. FADS2 = fatty acid desaturase 2. FADS1 = fatty acid desaturase 1.

Gene expression in sheep

van Harten and others (2013) showed a reduction of FASN expression by a factor of 4.47 and 6.33 for Australian Merino and Dorper sheep (respectively) under restricted feeding. There is a strong relationship between nutritional energy input and fatty acid synthesis and this relationship is independent of animal breed under normal feeding levels.

Bakhtiarizadeh and others (2013) selected Zel and Lori-Bakhtiari breeds as models to understand fat tissue deposition in sheep. Zel is characterized as a lean breed which has a thin tail and Lori-Bakhtiari is considered a fat-tailed breed. No differences in relative expression levels of LPL, SCD, and FASN were found between breeds and adipose tissue depots (fat-tail or visceral). However, FABP4 showed higher expression in Lori-Bakhtiari sheep than Zel sheep in both tail and visceral adipose tissues.

Feeding lambs with algae increased liver mRNA levels of the Δ6 desaturase gene associated with an increase of muscle DHA (Ponnampalam and others 2012b). However, the liver CPT1 mRNA level decreased for lambs fed the algal diet and the authors suggested that these lambs might be using glycogen as an energy source. On the other hand, a reduction of the fatty acid β-oxidation pathway when associated with high levels of DHA in muscle may suggest that this long chain fatty acid (DHA) might be produced by an alternative pathway (Δ4 desaturase activity) instead of by β-oxidation. In addition, the CPT1B gene was expressed in the mammary gland of the Churra Tensina sheep breed at lower levels in a hay indoor feeding system, compared with outdoor grazing plus lucerne pellets (Dervishi and others 2012b).

Soybean oil supplementation increases SCD expression in lamb muscle providing an effect on *cis*-9, *trans*-11 CLA levels (Mao and others 2012). Since soybean oil is a rich source of LA (see Table 1), this fatty acid undergoes

ruminal biohydrogenation, and endogenous SCD converts *trans*-11 vaccenic acid to *cis*-9, *trans*-11 CLA.

A positive correlation was observed between SCD gene expression and oleic acid content in *semitendinosu*s muscle (Dervishi and others 2010). The increase of SCD activity in sheep adipose tissue can decrease the SFA content and increase the oleic acid content (Daniel and others 2004a). Furthermore, an interaction between diet (concentrate diet freely intake (HI), dehydrated grass pellets (GP), and a concentrate diet gives a growth rate similar to GP (LO)) and adipose tissue depots (subcutaneous, omental, and perirenal) have been shown by Daniel and others (2004b) for SCD mRNA. Lambs fed HI had greater levels of SCD than GP or LO for subcutaneous and omental adipose tissue, while in the perirenal depot the HI was greater than GP and neither were different from LO. In lamb liver, there was an effect of diet on SCD expression. However, ACC remained constant (Daniel and others 2004b). ACC has been positively correlated with total intramuscular fatty acid content (Jeong and others 2012). Underfeeding was reported to alter LPL expression by Bonnet and others (2000), who demonstrated that LPL activity is downregulated by underfeeding and upregulated by refeeding. Furthermore, the data of Bonnet and others (2000) showed a tissue-specific LPL gene expression without nutritional status modulation.

In intensive feeding systems, genes related to adipogenesis are upregulated, such as LPL, ACACA, FASN, and SCD, and genes related with oxidation such as CPT1 gene are downregulated (Dervishi and others 2011). Exercise induces a decline of malonyl CoA levels in skeletal muscles (Rasmussen and Winder 1997), thus in the grazing system the fall of malonyl CoA would also decrease fatty acid biosynthesis and then the expression of ACACA and FASN genes is lower (Dervishi and others 2011). CPT1B gene expression is associated with fatty acid oxidation for energy production and has

shown higher levels in a grazing system compared with an indoor system (Dervishi and others 2011), indicating that animals under extensive systems use more fat reserves as fuel through β-oxidation.

Gender was reported to affect SCD gene expression associated with dam nutrition (Dervishi and others 2012a). Male lambs kept with dams fed pasture hay freely had greater SCD gene expression in *longissimus* muscle compared to those where the dam grazed mountain pasture. For female lambs there were no significant differences, although the same tendency was found. In addition, higher expression of LPL, ACACA, FASN, and SCD was shown in females compared to males. This suggests that females have the predisposition to develop fat by upregulating key adipogenic genes. However, for perirenal and subcutaneous adipose depots no difference in mRNA expression was found for most of the genes related with fat deposition, such as PPARγ , adiponectin, G3PDH, and LPL between gender and birth type in lambs (Muhlhausler and others 2008).

Nevertheless, a drawback of these studies is that they did not assess the fatty acid gene expression in IMF. Although it is a challenge to extract RNA for this specific component, this shortfall cannot be dismissed due to the association with the fatty acid profile of meat.

Conclusions

Research that is based on developing animal feeds rich in EPA and DHA may offer potential for interventions to production systems in regions where the levels of n-3 in feed sources are low. Such studies are important to ensure that the meat reaching consumers provides the health requirements in terms of n-3 fatty acids. It is also important to determine the health benefits of diets rich in DPA which currently is not a health-claimable n-3 fatty acid, yet the levels in lamb meat are relatively high and this would further strengthen the health benefits of consuming lamb meat. Although fish is the main source of EPA and DHA, high levels of these n-3 fatty acids have been found in lamb meat under certain feeding conditions and this can contribute to improvements in the marketing of lamb meat by optimizing its status as a "healthy" food. New knowledge of DHA and EPA metabolism in terms of physiological and gene expression will contribute to the development of technological approaches for the purpose of maximizing metabolism so as to achieve higher levels of these fatty acids. It is necessary to accelerate studies that focus on the characterization of the desaturases and elongases responsible for the production of PUFAs to enable the manipulation of the enzymes that enhance the production of DHA and EPA. In addition, the use of DPA in a pure form may reveal benefits for human health as has been shown for DHA and EPA. Nutrigenomics can help our understanding of how nutrition alters the action of genes and the functions of genes and their interactions, supporting strategies to increase desirable fatty acid levels in meat by using alternative feeding systems. The role of Δ6 desaturase to produce DHA is known, but the presence of Δ4 desaturase in ruminants has still not been clarified. The shorter alternative pathway to produce DHA (by Δ4 desaturase) may bring more efficiency in DHA production. The superfeeding with diets rich in DHA has shown great results on tissue levels of DHA and

EPA. The maximized outcome may be a result of enzymatic conversion (desaturases and elongases) or due to the increased bypass straight to the small intestine without biohydrogenation, or even greater interaction between environment (diet) and genotype (genetic material). Examination of the gene expression of tissues and rumen bacteria will help to understand if the substrate is undergoing biohydrogenation or passing through the rumen or whether it is genetic potential being expressed through nutrition.

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SECOND PART

PAPER 2

EFFECT ON LAMB MEAT OF SUPPLEMENTING WITH CALCIUM SOAP FATTY ACIDS

Paper according guidelines of Archivos Latinoamericanos de Producción Animal

Effect on lamb meat of supplementing with calcium soap fatty acids

Abstract – The objective of this study was to determine the effects of calcium soap of fatty acids (CSFA) on meat quality traits of lambs when included in a finishing diet. Sixty-three crossbreed lambs were feedlot individually at 24 ± 2.5 kg and slaughtered at 44 ± 1.1 kg. Twenty-nine lambs were fed the Control diet and 34 lambs were fed a diet containing 5.4% CSFA. At 24h postmortem the *Longissimus lumborum* (LL) muscle was collected from the right side of the carcass. The muscle was cut into 2.54-cm thick slices and vacuum-packaged. Samples were stored at -20ºC (0 day ageing) and others stored at 2ºC for 10 days ageing, then frozen at -20ºC for subsequent analysis. There was no significant interaction ($P > 0.05$) between both fixed effects (diet and ageing) for any traits. There were no significant effects on shear force, collagen, sarcomere length and myofibrillar fragmentation index due to diet effect. Cooking loss was reduced ($P < 0.05$) in CSFA fed lambs, but there was no significant effect on any other traits. Samples of *longissimus* muscle were evaluated at 0 and 10 days ageing. Shear force was significantly reduced ($P < 0.01$) by ageing, and this was matched by an increase ($P < 0.01$) in myofibrillar fragmentation index values with ageing. L^*, b^* and pH values increased ($P < 0.05$) with ageing. CSFA increased ($P < 0.05$) the level of saturated fatty acids and decreased ($P < 0.05$) EPA and DHA fatty acids in lamb meat.

Keywords: aged, collagen, protected fat, shear force

1. Introduction

At the sheep production level there is pressure for productivity increases and using supplements can increase the energy density of feeds so that lambs can store more energy with less dry matter consumption. On such potential supplement are calcium soaps fatty acids (CSFA). These are produced in a granulated-solid form and are easily incorporated into ruminant diets; affordability will depend largely upon duration and amount of supplementation (Seabrook *et al*., 2011). Protected lipids as CSFA are present in an inert form in the rumen; and apparently do not interfere with rumen metabolism, but they are efficiently digested in the lower tract (Salinas *et al*., 2006) and subject to minimal biohydrogenation (Zinn *et al*., 2000). Previous research found no effect on carcass characteristics from including calcium soap fatty acid in the diet of lambs (Castro *et al*., 2005). When a high level of CSFA from palm oil was fed to lambs it was found to improve lipid digestibility and the feed conversion ratio without affecting carcass yield and muscle chemical composition (Manso *et al*., 2006). Arana *et al*. (2006), working with lambs fed 5% protected fat over a 35 day period reported increased accumulation of adipose tissue in internal depots by adipocyte hypertrophy, whereas carcass depots were not affected.

Calcium soap from soybean oil contains a greater proportion of polyunsaturated fatty acids (PUFA) and a lower proportion of saturated fatty acids (SFA) than calcium soap from palm oil (51.6 vs 0.31 PUFA and 25.52 vs 53.81 SFA) (Souza, 2014) which when used in animal production feeding may improve the health status of ruminant meat. Several studies have evaluated the impact of CSFA in the diet of ruminants on the composition of ewe milk (Lurueña-Martínez *et al*., 2010; Casals *et al*., 2006; Gargouri *et al*., 2006; Dobarganes Garcia *et al*., 2005; Holter *et al*., 1993), but there is a paucity of knowledge, about the impact on meat quality traits such as colour traits, shear

force, collagen concentration and long chain PUFA deposition in muscle lamb, especially when the CFSA comes from soybean oil. In addition the replacement of CSFA for carbohydrates, such as corn, in a lamb feedlot diet at 4% gave a decrease in intake without a reduction in gain daily, feed efficiency and carcass quality (Seabrook *et al*., 2011). This provides an option to lower meat production costs.

Numerous studies have evaluated the effect of ageing on the tenderness of meat post-mortem (Martinez-Cerezo *et al*., 2005; Wheeler and Koohmaraie, 1999; McDonagh *et al*., 1999), but whether there is any interaction of CSFA and ageing is unknown. Therefore, the objective of this study was to determine the effects of CSFA on the meat quality of lambs finished in a feedlot, where the subsequent meat was subjected to different ageing times.

2. Materials and Methods

2.1. Lambs

Sixty-three crossbreed male lambs (hair breed over Santa Inês, Black Dorper, White Dorper, Texel, Lacaune and East Friesan sires) were used in this study. The feeding period started with lambs weighing on average 24 ± 2.5 kg at 92 ± 13.9 days of age. Twenty-nine lambs were fed diets containing 85% concentrate and 15% roughage, with a control diet based on oat hay, coffee hulls, corn, soybean meal, limestone, mineral supplement and Rumensin®. The remaining 34 lambs were fed the same diet with calcium soap fatty acids from soybean oil at 5.4% of the ration, while maintaining an isonitrogenous balance. The metabolizable energy (ME) MJ/kg and crude protein (%) levels were 12.30 and 12.14 for the control diet and 13.18 and 12.42 for the CSFA diet, respectively (Table 1).

Table 1. Ingredients of the experimental diets.

	Control (%)	CSFA (%)
Ingredient (%)		
Oat hay	12.3	12.3
Coffee hulls	2.5	2.5
Corn	69.8	64.8
Soybean meal	12.7	13.7
CSFA	-	5.4
Limestone	1.7	0.3
Mineral supplementation	0.9	0.9
Rumensin® (g/ Kg diet)	0.2	0.2
Chemical composition		
Dry matter (%)	90.30	90.68
Crude protein (%)	12.14	12.42
Ether extracted (%)	2.76	8.11
Metabolisable energy (MJ/kg DM)	12.30	13.18
Neutral detergent fiber (%)	21.39	20.87
Fatty acid composition (% total fatty acids in the ether extracted)		
C16:0 (palmitic acid)	16.06	22.34
C18:0 (stearic acid)	2.20	5.15
C18:1 n-9 (oleic acid)	29.20	23.89
C18:2 n-6 (linoleic acid)	40.12	31.52
C18:3 n-3 (linolenic acid)	0.00	3.01
C20:4 n-6 (arachidonic acid)	0.00	0.00
C20:5 n-3 (eicosapentaenoic acid)	0.09	0.002
C22:5 n-3 (docosapentaenoic acid)	0.00	0.00
C22:6 n-3 (docosahexaenoic acid)	0.003	0.001

CSFA: Calcium soap fatty acid from soybean oil. Dry matter, crude protein and extracted ethereo were determinated according AOAC (1990). Metabolisable energy and neutral detergent fiber were estimated according NRC (2007).

Each lamb was fed individually. The lambs were slaughtered at 44 ± 1.1 kg of liveweight after 72 ± 18.2 days on feed (172 ± 30.4 days of age). The carcasses were kept at room temperature for approximately 6 h and then chilled at 2-4ºC for 18h. At 24h postmortem (pm) the carcasses were split down the midline and from the right side of each carcass the *Longissimus lumborum* (LL) muscle was collected. The muscle was cut into 2.54-cm thick slices and vacuum-packaged. Day 1 pm samples were stored immediately at -20ºC for subsequent analysis. Other samples were vaccum packed and stored at 2ºC for 10 d ageing, then frozen at -20ºC for subsequent analysis. Fatty acid was measured in the sample at day 1 pm and all others traits at days 1 and 10 pm.

2.2. Cooking loss, shear force and water holding capacity measurement

The samples were weighed before and after thawing for determination of thaw loss. The slices were thawed overnight at 4ºC and weighed individually for determination of cooking loss before and after broiling on a grill (Mega Grill; Britânia, Curitiba, PR, Brazil). One slice was cooked for measurement of shear force on a grill to an internal temperature of 71ºC, monitored using copper constatan thermocouples. After cooking, the slices were cooled at room temperature and about four or five 1.0 x 1.0 x 2.5 cm cross-sectional square cores were taken from each cooked slice running parallel to the longitudinal axis of the muscle fibers. The cores were sheared with a Warner-Bratzler V-shaped cutting blade on a TA-XT2 Texture Analyser (Stable Micro Systems Ltd., London, UK). The crosshead speed was set at 200 mm/min and the shear force was recorded in Newtons (N). Water holding capacity (WHC) was determined according to methodology described previously by Hamn (1961) with slight modifications. Briefly, samples of 200-400 mg were placed between filter papers and a 5 kg-weight exerted pressure for 5 min. The pressed meat area

(MA) and the fluid area (FA) were estimated using the Universal Desktop Rule software and the WHC expressed in terms of the ratio of meat to total area, i.e., WHC = MA/(MA + FA).

2.3. Colour and pH measurement

The pH of the sample LL was measured using a Jenco 6009 meter with temperature compensation and an Ionode IJ42 spear electrode, with the electrode inserted into the muscle at 0 and 10 days ageing. The electrode was calibrated in buffers at pH 4.0 and 7.0. One steak from the LL was allowed to bloom for 30 min at room temperature before colour measurement at 0 and 10 days ageing. Colour measurements were collected using a Minolta CM-700 (Konica Minolta, Japan). Illuminant A, 10° standard observer and a specular component excluded (SCE) mode were used when collecting values. A total of 5 readings were taken on each steak and averaged. CIE lightness (L^*), redness (a^*) and yellowness (b^*) were recorded.

2.4. Myofibrillar fragmentation index and sarcomere length measurement

In duplicate, at 0 and 10 d ageing, LL samples were taken for determination of the myofibrillar fragmentation index (MFI) as described by Hopkins *et al*. (2000). The mean of the absorbance readings was multiplied by 200 to give index values for myofibrillar fragmentation (MFI). Sarcomere length was determined on cooked cores samples prior to measurement for shear force as described previously by Wheeler and Koohmaraie (1999). From each core about eighteen fiber samples were used to determine sarcomere length by helium neon laser diffraction (model 05-LHR-073, Melles Griot, Carlsbad, CA) as described previously by Cross *et al*. (1981).

2.5. Soluble and insoluble collagen determination

Collagen content and heat solubility were determined by Hill's method (Hill, 1966) with slight modifications. Four grams of homogenized muscle sample was placed in 50 ml disposable centrifuge test tubes, to which 12 mL of 1/4 Ringer's solution (0.86% NaCl, 0.30% KCl and 0.33% CaCl2) was added and mixed with a vortex stirrer. The tubes were kept in a water bath at 77°C for 70 min and, after cooling to room temperature, centrifuged for 10 min at 3,000 x *g*. The supernatant solution was transferred to a new tube and the pellet was suspended in 8 mL of 1/4 Ringer's solution and centrifuged again for 10 min at 3,000 x g. The supernatant solutions were joined with the first supernatant and 8 mL of distilled water was used to rinse the remaining pellet from the test tube to a residual tube. Sixteen mL of 6 N HCl was added to supernatant tubes and 10 mL of the same acid to the residual tubes and then both were hydrolyzed for 18 h at 105°C. The supernatant and residual hydrolysates were mixed with 1 g of carbon decolorizing agent and filtered. The pH of the filtered hydrolysates was adjusted to 6.4-7.0 with 2 N NaOH and diluted (1:10 for supernatant and 1:25 for residual) in distilled water. The amount of hydroxyproline was determined using a procedure described previously Bergman and Loxley (1963). To determine the collagen content, hydroxyproline amount was multiplied by 7.52 for the supernatant and 7.25 for the residual (Cross *et al*., 1973). The supernatant was considered the soluble collagen (mg collagen/g meat) and the residual as insoluble collagen (mg collagen/g meat).

2.6. Fatty acid analysis

The LL from the left side of each carcass at 24 h pm was collected to fatty acids analysis. The muscle lipids were extracted according to Hara and

Radin (1987) and the methylation according to Christie (1982). The transmethylated analysis were performed similar to Oliveira *et al*. (2014). The data were expressed as the percentanage of each fatty acid relative to the total fatty acids content. The fatty acid intake was estimated using % EE diet, % DM diet, total diet intake (grams) and % fatty acid in the diet.

2.7. Statistical analysis

REML Linear mixed models were generated through Genstat 16th Edition (GENSTAT, 2013) using the factors diet (treatment), ageing and the diet x ageing interaction as fixed effects, and genotype, slaughter day and animal identification as random terms. pH was tested as a covariate for colour traits, cooking loss and shear force. Correlation analysis was applied to examine the strength of association between intake level of individual fatty acids and levels in muscle. Ageing was not included as fixed effect for fatty acid analysis.

2. Results and Discussion

In our study, there was no significant interaction between both fixed effects (diet and ageing) for any traits. In Table 2, summary results for pH, *L**, *a**, *b**, thaw loss (TL) and water holding capacity (WHC) are given. Ageing was the main factor that affected the meat characteristics, pH, *b**, *L**. The pH ranged from 5.65 to 5.69, considered normal for lamb meat and indicating that animals were not stressed at the time of slaughter. According to previous research (Pardi *et al*., 2001), pH values ranging from 5.5 to 5.8 are normal 24 h after slaughter. The pH value was higher 10 days after the slaughter. These results may have been due to microbial proteolytic activity in the vacuum packaging during

ageing, resulting in an elevated pH due to the release of amine compounds (Rhee *et al*., 1997).

There was no significant effect of the covariate pH on colour measures. Meat colour was influenced by ageing, with the meat being lighter (*L**) and higher *b** 10 days post mortem. This can be explained by higher blooming ability (Vitale *et al*., 2014), increased oxygen penetration depth and decreased oxygen consumption rate in aged meat (McKenna *et al*., 2005). Others have shown a positive correlation between ageing time for *L** and *b** (Boakye and Mittal, 1996) and similar to our study these authors didn't find a significant change in *a** values until after 12 days of ageing, attributing this to the short time to bloom (only 20 min), which may not be enough time for maximum red colour development, which is similar to the 30 min bloom time applied in our study.

Table 2. Predicted means (± standard error) for pH, colour (*L**, *a**, *b**), thaw loss (TL) and water holding capacity (WHC) in the *longissimus* muscle of lambs fed with calcium soap fatty acid (CSFA).

Trait	Control		CSFA		P value	
	Ageing (d)		Ageing (d)			
	0	10	0	10	Diet	Ageing
pH	5.66 ± 0.02	5.67 ± 0.02	5.65 ± 0.02	5.69 ± 0.02	0.727	0.044
*L**	47.2 ± 0.91	48.55 ± 0.91	47.59 ± 0.90	48.86 ± 0.90	0.356	<0.001
*a**	11.43 ± 0.61	11.17 ± 0.61	11.16 ± 0.61	10.70 ± 0.61	0.136	0.164
*b**	9.47 ± 0.42	10.35 ± 0.42	9.15 ± 0.41	9.90 ± 0.41	0.106	<0.001
TL (%)	4.73 ± 0.46	4.30 ± 0.46	4.82 ± 0.42	4.13 ± 0.43	0.944	0.189
WHC (%)	0.187 ± 0.005	0.186 ± 0.005	0.190 ± 0.004	0.197 ± 0.004	0.117	0.485

In Table 3, summary results for shear force (SF), cooking loss (CL), myofibrillar fragmentation index (MFI), collagen concentration(s) and sarcomere length (SL) are given. There was no significant effect of the covariate pH on cooking loss or shear force. There were no significant effects on these traits due to diet, except for cooking loss with lower values in lambs fed CSFA ($P < 0.05$). In general, a more energetic diet produces rapid gains and fatter carcasses (Ferrel *et al*., 1978). The degree of cooking losses from muscles tends to decrease with increasing marbling scores (Li *et al*., 2006) and this is a plausible explanation for the effect found in this study.

At 10 days post mortem, shear force measurements were lower than the unaged meat as expected. It is well established that myofibrillar degradation increases during post-mortem meat storage, which can be seen in our study with an increase of 36% in MFI values and a 26% reduction in shear force with ageing for 10 days. The calpains have established effects on muscle during post-mortem storage and play an important physiological role in intracellular protein degradation (McDonagh *et al*., 1999). There was however, no effect of diet on these traits, although it has been found in previous work that a 22% improvement in tenderness was achieved based on sensory tests when CSFA was an added to the diet (Partida *et al*., 2007). The shear force values achieved would in the past be considered acceptably tender for Australian consumers (< 50 N) after the 10 days of ageing, according to Shorthose *et al*. (1986). However, to achieve the classification as good every day (3 star) as proposed by Hopkins *et al*. (2006), a shear force level less than 27 N is required to give a failure rate of 10%. In our study 23.8% of the samples unaged achieved this requirement and it increased to 57.2% with ageing. In addition, Hopkins *et al*. (2006) worked with crossbred lambs which have faster weight gains and consequently likely better tenderness values when compared with the Santa Ines breed. Also the Santa Inês breed has a high potential to produce lean carcass

(Furusho-Garcia *et al*., 2006), and this could impact on chilling rates and thus shear force, such as in the current study where the Santa Inês breed was the major genetic make up of the lambs. Neither diet nor ageing had an impact on collagen.

Martinez-Cerezo *et al*. (2005) reported an effect of slaughter weight on colour traits and collagen properties in Rasa Aragonesa and Churra lamb meat. The redness increased and lightness and collagen solubility decreased when slaughter weight ranged from 11kg to 21kg. No differences in meat colour and collagen due to diet were found in the current study and this may have been due to the similar slaughter weight for all animals (44.0 ± 1.14 kg).

The sarcomere length values ranged from 1.37 to 1.45 µm for cooked samples. Although Wheeler and Koohmaraie (1999) found a correlation between raw and cooked sarcomere length of the 0.97 in their study, the sarcomere length was shorter in cooked samples by about 0.2 and 0.4 µm (Wheeler and Koohmaraie, 1999, Koohmaraie *et al*., 1996). The values in the current study are similar to the results previously reported for cooked samples (Wheeler and Koohmaraie, 1999; Lewis *et al*., 1997). The effect of cooking is a plausible explanation for the lower levels than seen in fresh meat given that sarcomeres length < 1.6 µm are considered as indicative of short sarcomeres (Devine *et al*., 2002).

Table 3. Predicted means (± standard error) for meat shear force (SF), cooking loss (CL), myofibrillar fragmentation index (MFI), sarcomere length (SL), soluble and insoluble collagen in the *longissimus* muscle from lambs fed with calcium soap fatty acid (CSFA).

Trait	Control		CSFA		P value	
	Ageing (d)		Ageing (d)			
	0	10	0	10	Diet	Ageing
SF (N)	45.2 ± 2.9	33.0 ± 2.9	43.0 ± 2.7	32.0 ± 2.7	0.634	<0.001
CL (%)	24.5 ± 0.55	24.8 ± 0.55	23.0 ± 0.52	23.2 ± 0.52	0.018	0.799
MFI (%)	36.2 ± 2.55	47.3 ± 2.55	37.3 ± 2.43	52.4 ± 2.43	0.149	<0.001
SL (µm)	1.37 ± 0.03	1.43 ± 0.03	1.45 ± 0.03	1.45 ± 0.03	0.160	0.393
Soluble collagen (mg/g muscle)	0.59 ± 0.04	0.60 ± 0.04	0.61 ± 0.04	0.62 ± 0.04	0.469	0.634
Insoluble collagen (mg/g muscle)	1.62 ± 0.08	1.50 ± 0.08	1.58 ± 0.08	1.57 ± 0.08	0.805	0.291

The intake of fatty acids was higher when lambs were fed CFSA diet due the higher amount of EE in this diet (Table 1). The lowest proportion of oleic acid in the *longissimus* muscle was found when lambs received CSFA compared with Control diet ($P < 0.01$), however the oleic intake was higher in lambs fed with CSFA (Figure 1), even with lower proportions of oleic in the CSFA ratio (Table 1). The negative correlation between oleic intake and oleic deposition (Table 4) is explained by the higher deposition of stearic acid in the muscle when lambs were fed with CSFA because oleic acid is precursor to stearic acid by ruminal biohydrogenation. It also explains the positive correlation between oleic intake and SFA deposition (Table 4).

De novo fatty acid synthesis is catalyzed by Acetyl-CoA carboxylase and Fatty acid synthase enzymes (Alvarenga *et al*., 2015). C18:1 decreases *de novo* fatty acid synthesis by inhibition of ACC activity (Natali *et al*., 2007). Thus, the lower monounsaturated fatty acids (MUFA) proportion in the *longissimus* when lambs were fed CSFA diet may be attributed to increase of *de novo* synthesis (palmitic biosynthesis).

Similarly, Chouinard *et al*. (1998) showed that the proportion of stearic acid in milk increased with the addition of CSFA in the diet of cow's due to CSFA dissociation in the rumen and consequently fatty acid saturation by ruminal microorganisms. In contrast to the current study, Chouinard *et al*. (1998) showed that the inclusion of CSFA in the diet resulted in increase of oleic acid. The lower proportion of oleic observed suggested that linoleic and linolenic dietary were fully hydrogenated by ruminal bacteria instead of an increase in the production of oleic acid as result of partially hydrogenation in the rumen.

Linoleic acid (LA), linolenic acid (ALA) and PUFA in lamb muscle were not affected by CSFA supplementation ($P > 0.05$) (Figure 1). These results are in agreement with others studies in which protected fat did not alter LA,

ALA and PUFA composition in *longissimus* muscle of lambs (Castro *et al*., 2005) and yong bulls (Partida *et al*., 2007). Even when the vegetable oils used to prepare calcium salts were high in linoleic or linolenic, the feeding with CSFA to lactating cows failed to enhance LA and ALA milk concentration (Chouinard *et al*., 1998).

EPA, DPA and DHA level in the *longissimus* muscle decreased ($P < 0.05$) with the inclusion of CSFA in the lamb's diet. Contrasting this, a higher proportion of SFA in the muscle was found when lambs were fed the CSFA diet and this may be due to the higher amount of SFA provided by CSFA ration compared with Control ration (Figure 1). The intake of ALA, PUFA and unsaturated fatty acids (UFA) showed a negative correlation ($P < 0.05$) with EPA and DPA in muscle deposition followed by the positive correlation ($P < 0.01$) between SFA intake and SFA deposition in the *longissimus* muscle (Table 4).

There are many possible explanations for the higher proportion of SFA and lower proportion of UFA in the *longissimus* muscle observed in lambs fed CSFA diet. Firstly, the major fatty acids present in protected fat are palmitic (8.29 to 48.8%) and stearic (40.7 to 43.83%) (Wynn *et al*., 2006, Gomez-Cortes *et al*., 2014, Andrade *et al*., 2014) that may be directly incorporated in intramuscular fat. Second, the oleic acid from protected fat (9.7 to 34.4%) (Wynn *et al*., 2006, Gomez-Cortes *et al*., 2014, Andrade *et al*., 2014) is hydrogenated directly to stearic acid in the rumen.

Hence, protected fat may be a strategy to increase the PUFA uptake in the small intestine, however as shown in other studies (Wachira *et al*., 2002, Andrade *et al*., 2014) this protection was not enough to save the dietary lipid from ruminal biohydrogenation as occurred in the current study. The degree of biohydrogenation in CSFA is related to chain fatty acid, unsaturated calcium soaps (eg. from soybean oil) are more dissociated in the rumen to produce 18:0

than saturated calcium soaps (eg. from palm oil) (Sukhija and Palmquist, 1990). At pH 5.0 about 80% of soaps were dissociated when soya was tested and just less than 10% for all others soaps (tallow, stearic acid and palm fatty acid distillate) analysed in the Sukhija and Palmquist (1990) study. The saturation stability is attributed to melting point, as unsaturated vegetable oils are liquid at room temperature and unstable (Klonoff, 2007). In addition, the high concentrate proportion in the diet (>80%) may has been another factor to decrease ruminal pH. These characteristcs may have contributed to make the calcium soap from soybean oil used in the currect study more susceptible to dissociation in rumen fluid increasing the saturated fatty acid proportion in the *longissimus* muscle.

High proportion of saturated fat is found in animal products. The human intake of these fatty acids is recommended to be limited due risk for developing chronic health conditions. Studies in ruminant nutrition have focused in high consumption of long-chain omega-3 and omega-6, however strategies to decrease fat ruminal biohydrogenation needs to be clearer.

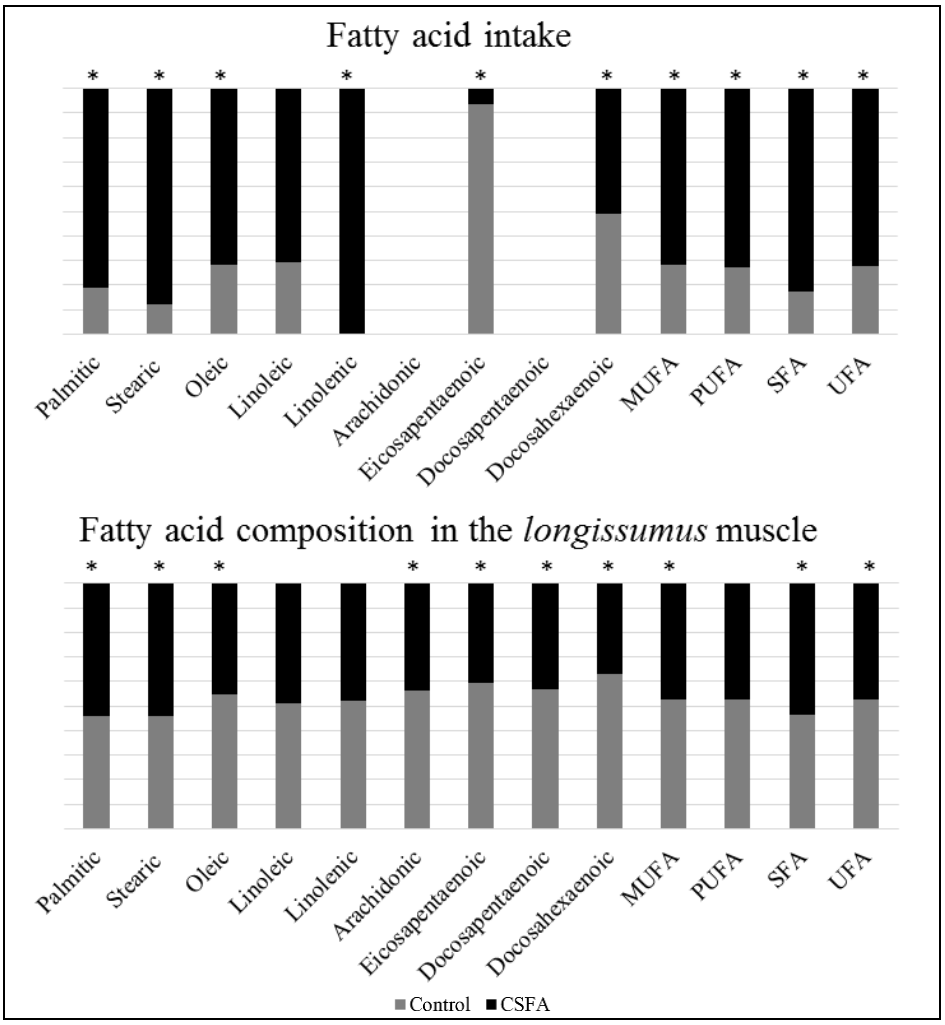

Figure 1. Feeding relationship for fatty acid intake and depositition in the *longissimus* muscle of lambs. The fatty acids are the relative values of the sum between Control and CSFA content. SFA = saturated fatty acid. MUFA = monounsaturated fatty acid. PUFA = polyunsaturated fatty acid. UFA = unsaturated fatty acid. $^{*}P < 0.05$.

Table 4. Correlation and P-value for each pair of traits (fatty acids from muscle and fatty acid intake).

Intake	Fatty acid in muscle	Correlation	*P*-value
Oleic acid	Oleic acid	-0.559	**<0.001**
	Stearic acid	0.387	**0.003**
	SFA	0.459	**<0.001**
Linolenic acid	Eicosapentaenoic acid	-0.328	**0.015**
	Docosapentaenoic acid	-0.272	**0.044**
	Docosahexaenoic acid	-0.255	**0.060**
PUFA	Linoleic acid	0.016	0.907
	Linolenic acid	-0.304	**0.024**
	Arachidonic acid	-0.125	0.363
	Eicosapentaenoic acid	-0.313	**0.020**
	Docosapentaenoic acid	-0.240	**0.078**
	Docosahexaenoic acid	-0.223	0.101
SFA	SFA	0.478	**<0.001**
	MUFA	-0.462	**<0.001**
	PUFA	-0.145	0.281
	UFA	-0.472	**<0.001**
UFA	PUFA	-0.065	0.637
	Arachidonic acid	-0.123	0.371
	Eicosapentaenoic acid	-0.312	**0.020**
	Docosapentaenoic acid	-0.239	**0.079**
	Docosahexaenoic acid	-0.222	0.103

SFA = saturated fatty acid. MUFA = monounsaturated fatty acid. PUFA = polyunsaturated fatty acid. UFA = unsaturated fatty acid.

3. Conclusion

There was no detrimental effect of feeding CSFA at 5.4% in the diet of lambs on traits associated with meat tenderness, and there was a small beneficial reduction in cooking loss. CSFA increased saturated and decreased the unsaturated proportion of fatty acids in the *longissiumus* muscle. Ageing for 10 days improved the meat tenderness and colour measurements, without any adverse effects in the traits measured in this study.

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PAPER 3

THE EXPRESSION OF GENES ENCODING ENZYMES REGULATING FAT METABOLISM IS AFFECTED BY MATERNAL NUTRITION WHEN LAMBS ARE FED ALGAE HIGH IN OMEGA-3

Paper according guidelines of British Journal of Nutrition – submitted version

The expression of genes encoding enzymes regulating fat metabolism is affected by maternal nutrition when lambs are fed algae high in omega-3

Abstract: The current study investigated the effects of supplementation of lambs with algae on the expression of genes that direct the accumulation of long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA). The nutrition of the lamb's dam around mating was also considered. The dams were fed with either silage (SLG) or oat/cottonseed (OAT) based diets for six weeks prior to and, three weeks following conception. mRNA levels of FADS1, FADS2, CPT1, SCD, ACC and fad2 were measured in the liver, muscle and subcutaneous fat from lambs fed a control diet consisting of oat and lupin grains and chopped lucerne (CTRL) or the CTRL diet with algae (DHAgold™) added at 1.92% DM (ALG). The expression of FADS1 in liver tissue was not affected ($P > 0.05$) by the interaction between dam nutrition and algae supplementation, however it was higher ($P < 0.05$) when lambs received the ALG ration compared with the CTRL and when their dams were fed SLG compared with OAT diet. The expression of FADS1 in muscle was negatively correlated ($P < 0.05$) with the concentration of 20:4n-6, 20:5n-3 and 22:4n-6. The expression of FADS1, FADS2, SCD and ACC genes in lamb muscle was differentially affected by dam nutrition with the highest levels for the SLG+ALG treatment ($P < 0.05$) compared with other treatments. The expression of SCD gene was not affected ($P > 0.05$) by algae supplementation, but it was higher ($P < 0.05$) when dams were fed SLG compared with OAT, however ACC was not affected ($P > 0.05$).

Keywords: dam nutrition, eicosapentaenoic acid, docosahexaenoic acid, fatty acid desaturase, sheep, stearoyl-CoA desaturase

1. Introduction

Algae derived supplements high in long chain omega-3 (n-3) polyunsaturated fatty acids (LCn-3PUFA) can significantly increase the level of those fatty acids in animal products. The deposition of LCn-3PUFA, specifically eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), in muscle[1, 2] and milk[3, 4] is increased when sheep are fed with marine algae.

There are reports that dam nutrition around conception and offspring nutrition post weaning can interact to alter fat metabolism and the accumulation of LCn-3PUFA. Such reports have been published in rats[5] and humans[6]. In the work of Hopkins *et al.*[1] an interaction between two distinct stages of feeding, around conception time (six weeks prior to and, three weeks following conception) and post weaning in the progeny was found in muscle LCn-3PUFA concentration when lambs were fed with algae. This raises the question as to whether gene expression was altered by the nutritional interaction, however, in sheep there is a paucity of reports on the expression of genes responsible for the accumulation of LCn-3PUFA. Study of gene expression has enabled clarification of the mode of fatty acid metabolism in muscle and the accumulation of intramuscular fat or marbling[7] and the role of the genes that promote fatty acid oxidation and mitochondrial respiration in the liver, muscle and adipose tissue[8]. Given this, it is important to identify key genes and elucidate how they are regulated and to establish isoforms that encode for proteins[9] when LCn-3PUFA are manipulated in different tissues by dietary supplementation.

Fatty acid composition of lamb muscle is affected by phenotypic factors such as diet, age and breed[10-16], however, our understanding of the mechanisms involved has benefitted from study at the nucleic acid level. The altered metabolism of n-3 observed in previous studies could have been associated with

differences in the expression of important genes for fatty acid metabolism as several genes encode enzymes associated with fatty acid metabolism in lamb, including fatty acid desaturase (FADS), acetyl-coenzyme A carboxylase (ACC), stearoyl-CoA desaturase (SCD) and carnitine palmitoyltransferase 1 (CPT1)[17]. For example, the desaturation of eicosatetraenoic acid (ETA, 20:4n-3) to eicosapentaenoic acid is catalysed by the Δ5 desaturase enzyme, which is encoded by the fatty acid desaturase 1 (FADS1) gene[18]; while the desaturation of linolenic acid (ALA, 18:3n-3) to stearidonic acid (SDA, 18:4n-3) and linoleic acid (LA, 18:2n-6) to γ-linolenic acid (GLA, 18:3n-6) are catalysed by the Δ6 desaturase enzyme, which is encoded by the FADS2 gene[19]. The effect of maternal nutrition on the expression of these genes when lambs are fed algae high in *n*-3 has not previously been reported.

In addition, Fatty acyl desaturase 2 (fad2) has been reported as responsible for Δ4 desaturase activity. Albeit fad2 is commonly found in protozoan trypanosomes[20], photosynthetic freshwater protists[21], and marine microalgae[22], it has been reported in marine herbivorous fish[23] and more recently in marine carnivorous fish[24], however the activity in ruminants is still unknown.

Therefore, the aim of the current study was to determine whether the differential accumulation of LCn-3PUFA in lamb fed with algae associated with maternal nutrition is also reflected in the expression of genes involved with fat metabolism in muscle, liver and subcutaneous fat.

2. Materials and Methods

2.1. Animal background and diet

Forty, Poll Dorset x Border Leicester x Merino wether lambs were used in a study to examine the effect of algae supplementation on the concentration of LCn-3PUFA in meat. Management details of the lambs and the fatty acid profile in the muscle of the lambs was described previously in Hopkins *et al.*[1]. The dams of the lambs were fed a ration based on either silage (SLG, high in n-3 fatty acids) or oats-cottonseed meal (OAT, high in omega-6 (n-6) fatty acids) for six weeks prior to conception and, three weeks following, conception (Table 1) in one of eight outdoor pens for feeding (four 12 x 7 m pens per treatment, n = 38 ewes per pen) All ewes grazed improved pastures as one group from three weeks post-conception until lambing and weaning according to methods described previously[25]. The lambs were 117.4 ± 2.4 days of age and weighed 37.3 ± 3.2 kg at the beginning of the experimental period. Within each group the lambs were allocated to one of 4 pens (5 lambs per pen), with water provided via troughs. One group of lambs (n = 20) were fed the control ration (CTRL) and the other group (n = 20) the CTRL ration with DHAgold™ algae (DSM Nutritional Products Australia Pty Limited) included at 1.92% dry matter (DM) (ALG, Table 1) as described previously[1, 26]. The combination of treatments is described below:

SLG+CTRL: lambs fed the control diet from dams fed the silage diet (n=9);
OAT+CTRL: lambs fed the control diet from dams fed the oat diet (n=11);
SLG+ALG: lambs fed the algae diet from dams fed the silage diet (n=9) and;
OAT+ALG: lambs fed the algae diet from dams fed the oat diet (n=11).

The lambs were fed daily over the 6 weeks of the study. The metabolizable energy (ME) and crude protein (CP) concentrations were 11.5 MJ/kg DM and 18.0% DM, respectively, for the CTRL ration and 11.6 MJ/kg DM and 17.9% DM, respectively, for the ALG ration. The rations contained 6.82 mg of Vitamin E/kg DM with measurement of Vitamin E as previously described[27]. Blood samples were collected from lambs at two (Day 14) and six

weeks (Day 42) after the introduction of the ALG ration. The collection of plasma and analysis of fatty acids was described previously[26]. The lambs were slaughtered at 160.4 ± 2.4 days of age with a final live weight of 50.1 ± 4.4 kg also as described previously[1].

A sample of liver from the lateral lobes (~3 g) was taken immediately after the liver was inspected by abattoir health inspectors and frozen immediately in liquid nitrogen. A sample of *longissumus lumborum* (~3 g) muscle was taken from the caudal region over the lumbar-sacral junction once the carcases were in the chiller and also a subcutaneous fat sample (~3 g) was taken from around the tail area within 1 hour post-slaughter. Samples were packed in micro tubes and frozen immediately in liquid nitrogen. The samples were held at -80°C until RNA extraction. A muscle sample was removed from the *longissumus lumborum* muscle (left side loin), between the 6th lumbar vertebrae and the 12th rib for testing of intramuscular fat and the full array of fatty acids as described by Hopkins *et al.*[1].

2.2. Purification of total RNA

Total RNA was extracted from liver, muscle and subcutaneous fat tissues by TRI Reagent® (Sigma Aldrich, Australia) and RNeasy MiniElute kit (Qiagen, Australia) using a modified protocol. In brief, approximately 30 mg of liver tissue (100 mg for muscle and subcutaneous fat tissue) held on dry ice was finely minced and mixed with 500 µL Tri-Reagent in a 2 ml screw top tube (500 µL and 1000 µL of Tri-Reagent for muscle and subcutaneous fat, respectively). The mix were immediately homogenized for approximately 45-50 sec by dispersing devices (IKA) and incubated at room temperature for 5 min. The resulting RLT lysate was centrifuged at 10,000 x g for 15 min at 4^{o}C and the supernatant was transferred to a new microfuge tube. The supernatant was mixed

well with 100 µL BCP (1-Bromo-3-chloropropane (BCP), Sigma Aldrich, Australia) incubated at room temperature for 10 min, followed by centrifugation at 10,000 x g at 4^{o}C for 15 min. The top aqueous layer was transferred to a new microfuge tube and mixed with an equal volume of 75% ethanol. The resulting lysate was then loaded onto the QiagenRNeasyMinElute column and RNA was purified per protocol with on column DNA digestion. RNA was eluted in a total of 30µl RNase-free water and RNA quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Australia) at 260 nm, and purity was assessed using the 260 to 280 nm ratios. Integrity of the RNA was checked by electrophoresis in a 1% agarose gel.

2.3. Reverse transcriptase and RT-PCR

Total RNA (1 µg) was reverse transcribed into cDNA using a AMV reverse transcriptase first strand cDNA synthesis kit according to the manufacturer's protocol, that included oligo$(dT)_{20}$ primers (Marligen Biosciences, Sydney, Australia). Real-Time PCR was performed using a Bio-Rad IQ5 detection system, with reactions performed using SYBR Green Supermix (Bio-Rad, Sydney, Australia). Primers were designed using Primer 3, and obtained from GeneWorks (Hindmarsh, Australia; Table 2). The amplification of cDNA samples (0.5 ± 0.009 ng) was carried out in triplicate using IQ SYBR green™ following the manufacturer's protocols (BioRad, Sydney, Australia). Fluorescent emission data was captured and mRNA levels were analyzed using the critical threshold (CT) value[28]. Thermal cycling and fluorescence detection were conducted using the BioRad IQ5 sequence detection system (BioRad, Sydney, Australia). Samples were normalized for the cDNA concentration determined with OliGreen (Invitrogen, Mulgrave, Australia)[29].

2.6. Statistical analysis

The experiment was conducted as a split-plot design with the two treatment groups (CTRL or ALG) each randomly assigned to four pens. Animals within each pen were randomly sourced from dams feed one of two peri-conceptional diets (SLG or OAT). Analysis of the data included the 2 x 2 treatment structure; a source of variance associated with pen; and random error. Data were $\log_e$ transformed prior to analysis to improve variance homogeneity. Six genes within each tissue were analysed separately. Each tissue x gene was analysed using the model: $\log_e$(Trait) = mean + algae effect + dam nutrition effect + algae:dam nutrition + pen + *error*. If the interaction effect (algae x dam nutrition) was significant ($P < 0.05$) the results for the main effects were not given. The predicted means (and standard errors) for each algae x dam nutrition combination are given together with a least significant difference (LSD) ranking of the means within each trait. The model was fitted using the software package asreml[30] under R[31].

For selected pairs of muscle fatty acids and gene expression traits, the pairwise correlations, on the $\log_e$ scale and after adjustment for algae x dam nutrition average effects, were estimated and tested for difference (statistically) from zero using a Likelihood Ratio Test. No adjustment for Pen effects were not made for these correlation analyses as all previous univariate analyses of traits indicated no statistically significant pen variation.

To analyse the correlation between blood fatty acids with gene expression, two results for each fatty acid were used (blood collected on day 14 or 42). For each pair of variables a three component vector was formed, comprising the gene expression trait and the two blood sample measures (day 14 and 42). This vector was $\log_e$ transformed and a tri-variate linear regression analysis was undertaken for results across the 40 animals. In the model each trait was allowed

to differ on average across the four algae x dam nutrition combinations. The residuals were modelled as correlated tri-variate normal random variables.

2. Results

Impact of dam nutrition

The expression of the FADS1 and SCD genes in lamb liver was higher ($P < 0.01$) when dams were fed silage compared with oats (Table 3). The expression of fad2 in muscle was not ($P = 0.054$) increased when dams were fed oats compared with silage. The expression of SCD was higher ($P = 0.04$) in subcutaneous fat when dams were fed silage compared with oats, however, the expression of ACC in subcutaneous fat was not affected by dam nutrition (Table 3). The expression of CPT1 was not affected by dam nutrition in muscle or subcutaneous fat ($P > 0.05$).

Impact of algae supplementation

The expression of FADS1 in liver was higher ($P < 0.01$) when lambs were fed the ALG compared with the CTRL ration. The expression of the SCD gene in liver was not higher ($P = 0.06$) when lambs were fed the ALG compared the CTRL ration (Table 3). The expression of CPT1 in muscle and subcutaneous fat, the expression of fad2 in muscle and liver and the expression of SCD and ACC in subcutaneous fat was not significantly ($P > 0.05$) affected by algae supplementation. The expression of SCD and ACC genes in subcutaneous fat was also not affected by algae supplementation ($P > 0.05$) as shown in Table 3.

Interaction between dam nutrition and algae supplementation

The expression of several genes in muscle, liver and subcutaneous fat was affected by the interaction between dam nutrition and algae supplementation (Table 3). There was an interaction between dam nutrition and algae supplementation for FADS1, FADS2, SCD and ACC expression in the *longissumus* muscle ($P < 0.01$) and for FADS1, FADS2 in the subcutaneous fat ($P < 0.01$) and FADS2 and ACC in liver ($P < 0.05$). All of these genes were higher when lambs received the SLG+ALG treatment compared with all other combinations (Table 3).

The expression of FADS2 in liver was higher, however, the expression of CPT1 was lower, when lambs were fed algae and their dams were fed silage compared with all other treatments (Table 3).

The expression of ACC in lamb liver was not affected ($P > 0.05$) when ewes received the oat diet independent of the lambs diet, however those lambs from the SLG+ALG treatment had a significantly ($P < 0.05$) higher level of expression (Table 3) compared with all other treatments.

The expression of fad2 gene in subcutaneous fat was not significantly ($P < 0.05$) affected by dam nutrition for lambs receiving the control diet, however when lambs were fed algae the level of expression of fad2 was higher ($P > 0.05$) when dams were previously fed SLG compared with OAT.

Correlation between gene expression and the concentration of fatty acids in muscle

In Table 4 the estimated residual correlations, after adjustment for the algae x dam nutrition combination for each pair of traits on the $\log_e$ scale are given. FADS1 was negatively ($P < 0.05$) correlated with 20:4n-6 (AA,

arachidonic acid), 20:5n-3 and 22:4n-6 (adrenic acid) (Table 4). FADS2 was negatively correlated with 18:3n-6 and 20:3n-6 (DGLA, dihomo-γ-linolenic acid). Illustrated in Figure 1 is the declining trend in log (Muscle FADS1) with increasing log (20:5n-3) within each algae x dam nutrition combination.

Correlation of gene expression with fatty acid of blood

Blood samples were taken from each animal on two separate occasions (Day 14 and Day 42). The correlation between the gene expression of FADS2 in liver and the 18:3n-6 in blood at Day 14 was -0.364 and at Day 42 was 0.177 (*P* = 0.029) (Figure 2).

3. Discussion

The expression of SCD in the liver and subcutaneous fat of lambs was enhanced when their dams were fed the diet based on silage (high in n-3) compared with oats (high in n-6). The increase in SCD activity is related to the increase of oleic acid, which is synthesized by desaturation of stearic acid via the SCD enzyme[32]. As in the current study, the expression of SCD has been reported as more abundant in subcutaneous fat than the remaining tissues evaluated[33]. Elevated activity of SCD is related to low levels of oleic acid available in the diet[34], and our results are in agreement with this expectation as the silage diet provided lower levels of oleic acid than oat diet (3.84 vs 17.34 g/kg DM) for the ewes and this may have influenced the concentration of fatty acids in the lambs. Keating et *al.*[35] showed that the SCD gene promoter was down-regulated by oleic acid in bovine muscle and they associated this with a negative feedback effect of oleic acid on SCD expression which regulates the desaturase index and maintains membrane fluidity to ensure normal cell

function. Lim *et al.*[36] suggested that oleic acid could act as a fatty acid sensor to maintain cellular and lipid homeostasis.

Liver plays an important role in the biosynthesis of LCn-3PUFA and this is controlled mainly by the enzymes encoded by FADS1 and FADS2[37]. The higher expression of FADS1 in the liver of lambs born to dams fed the SLG diet compared with OAT around conception could be due to the higher levels of 18:2n-6 in the latter diet which promotes the expression of genes encoding for Δ5 desaturase in order to incorporate an adequate level of LC-PUFA in membrane phospholipids[37].

In terms of the dam nutrition effect, PUFAs are feedback inhibitors of their own synthesis[38]. Dams fed with the oat diet received considerably higher levels of PUFA (n-3 + n-6) than silage (17.7 vs 10.2, Table 1), thus the inhibitory potential in FADS1 in the lambs liver when dams are fed oat diets might be higher than a silage diet. The relation of dam nutrition around conception and the gene expression of lambs post weaning may be due to environmental exposures during these critical and sensitive periods of life, that develop permanent changes in many physiological processes which is called "programing"[39]. In fact, the silage diet was lower in 18:2n-6 than the oat diet during the conception period of the ewes. However, the mechanism to explain how n-3 PUFA and n-6 PUFA feeding in these distinct stages, dam conception and growth-offspring, are directly interconnected to the expression of fat metabolism in later life is still an unanswered question.

The higher expression of the FADS1 gene in liver when lambs were fed the ALG diet compared with the CTRL diet is consistent with the elevated concentration of EPA in muscle previously observed when the lambs were fed algae[1], as FADS1 encodes the Δ5 desaturase enzyme which catalyses the conversion of 20:4n-3 to 20:5n-3[18]. The higher concentration of FADS2 in the muscle observed in the current study when lambs were fed ALG and their dams

were previously fed SLG high in n-3 compared with OAT high in n-6 agrees with previous observations that muscle DHA was also differentially affected by maternal periconceptional nutrition[1]. However, FADS1 showed a negative correlation with 20:4n-6, 20:5n-3 and 22:4n-6, such as FADS2 did with 18:3n-6 and 20:3n-6 (Table 4). This may be due the negative effect of the intake of dietary phenolic compounds contained in algae under ruminal biohydrogenation[40], associated with a higher bypass of C18 PUFA arriving in the small intestine. Algae are a source of phenolic compounds that possess antioxidant and antibacterial activity[41].

Moreover, DHA supplementation increases EPA deposition by the biohydrogenation process in the rumen or retroconversion of DHA to EPA, which involves the removal of the double bond at the Δ4 position[42]. The end product of PUFA n-6 is the conversion of 22:4n-6 to 22:5n-6 (DPA, osbond acid) by fad2 (Δ4 desaturase)[43]. Li *et al.*[23] reported for the first time the presence of Δ4 Fad activity in a vertebrate species (fish), indicating an alternative simpler pathway for the production of DHA from EPA. There was no effect in liver and muscle, but in subcutaneous fat fad2 showed the highest levels for the SLG+ALG lambs, such as found for 22:5n-6 concentration in muscle (unpublished data).

Grønn *et al.*[44] found that the retroconversion to EPA was not stimulated by carnitine, which is codified by the CPT1 gene stimulating mitochondrial β-oxidation. Grønn *et al.*[44] suggested that this retroconversion is not mitochondrial, but a peroxisomal function. On the other hand, the presence of the Δ4 desaturase in lamb tissue could be associated with the retroconversion from DHA to EPA which may be acting by a lower eukaryotes pathway[22] instead of the Sprecher pathway[45,46] by a direct retroconversion of 22:6n-3 to 22:5n-3 (DPA, clupanodonic acid) by Δ4 desaturase.

Cord blood lipid concentrations are correlated with maternal blood lipids during the third trimester of pregnancy[47]. Shand *et al.*[48] showed that fatty acid composition of liver is highly related with Δ6 desaturase activity. They found that the conversion of LA to AA by Δ6 desaturase was 4 to 5 fold higher in lambs born from ewes that had received protected PUFA oil supplementation (for the last 8 weeks of pregnancy) than lambs born from ewes fed with the control diet. Linoleic acid is converted to GLA by Δ6 desaturase that is codified by FADS2. The algae diet provided a lower percentage of LA than the control diet (26.9 vs 32.7 % total fatty acids) and it decreased the GLA concentration in blood, however FADS2 expression in liver had a negative correlation with this fatty acid.

Acetyl coenzyme A carboxylase produces malonyl-CoA, being the first intermediate in the synthesis of long chain fatty acids in lipogenic adipose and liver tissues[49]. It is also a potent inhibitor of CPT1[50], hindering the mechanism of the mitochondrial transport of long chain fatty acids and it explains the lower levels of ACC in liver when lambs received OAT+ALG and OAT+CTRL treatments associated with higher levels of CPT1 found in these animals. The increased expression of the ACC gene suggests that there is improved capacity for *de novo* synthesis of fatty acids in liver and muscle of lambs fed with SLG+ALG. Kumamoto & Ide[51] showed that dietary α-linolenic acid induces hepatic β-oxidation activity and CPT1 is a key enzyme in the control of long chain fatty acid oxidation[49,52], being a candidate gene responsible for cellular mechanisms involved in muscle lipid oxidation[53]. Thus, the decrease in CPT1 expression in the liver of lambs fed with algae may be associated with lower amount of α-linoleic acid in the algae diet compared to the control diet (1.69 vs 2.03 % total fatty acids).

Maternal nutritional status during early pregnancy can have later health consequences in the progeny[5,6,54,55]. Epigenetic modifications by DNA

methylation and histone modifications may alter DNA transcription and gene expression in offspring with consequences in later life arising from maternal nutritional status[54]. Such alterations in early life are called programming and Lucas[56] defined a more general process whereby a stimulus or insult at a critical period of development has lasting or lifelong significance. In brief epigenetic regulation of gene expression could be described as a series of complex interactions between the genome and environmental factors[57]. Changes in fatty acid composition have been shown to be affected by maternal intake during pregnancy[58].

Dominguez-Salas *et al.*[6] confirmed for the first time in humans that the maternal blood biomarker status measured around the time of conception can be used to predict the methylation patterns of metastable epialleles in offspring, however, the phenotypic consequences of these variations in methylation are not yet known. Although we did not assess the epigenetic event, our results provide evidence that dam nutrition during conception altered the fatty acid profile of the lambs later in life. The oat diet of the ewes had 2.0 and 4.4 more times total lipids and 18:2n-6, respectively, than the silage diet[25], and the ewes had higher fat scores when fed oats instead of silage[25]. These effects might be associated with epigenetic events as Niculescu *et al.*[59] demonstrated that perinatal manipulation of n-3 and n-6 fatty acids in different ratios induced epigenetic changes to FADS2 in maternal and offspring livers as measured at the end of the lactation period. On this basis, the expression of FADS1 and FADS2 in muscle tissue of lambs would be expected to be higher when dams were fed an oat diet. However, the results indicated an opposite relationship when algae was added to the lambs diet and it can be explained by the higher amount of 20:5n-3 and 22:6n-3 available in the algae (Table 1). Chen *et al.*[60] suggest that the post-weaning environment is more powerful than any early nutritional programming during early life as suggested by the current study.

Conclusions

The supplementation with algae as fed in the current study led to an increase in the expression of genes associated with LCn-3PUFA. The dam nutrition during conception time interacted with post-weaning diets in the expression of FADS1, FADS2, SCD, ACC and CPT1 genes in lamb tissue. However, the mechanisms for these interactions during these distinct developmental stages on fatty acid metabolism are still unclear.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. Fatty acid and nutritional composition of dam and lamb diets.

	Ewe nutrition[1]		Lamb nutrition[2]	
	SLG	OAT	CTRL	ALG
Fatty acid composition [g/kg DM (% total fatty acids)]				
14:0	0.24 (1.3)	0.15 (0.3)	0.11 (0.4)	0.69 (2.2)
16:0	4.00 (21.2)	7.90 (17.8)	5.10 (19.5)	6.41 (20.6)
18:0	0.47 (2.5)	0.98 (2.2)	1.03 (4.0)	1.04 (3.3)
18:1*n*-9	3.84 (20.3)	17.34 (39.1)	10.56 (40.4)	10.35 (33.2)
18:1*n*-7	0.13 (0.7)	0.26 (0.6)	0.27 (1.0)	0.27 (0.9)
18:2*n*-6	3.58 (18.9)	15.64 (35.2)	8.56 (32.7)	8.40 (26.9)
18:3*n*-3	6.67 (35.2)	2.10 (4.7)	0.53 (2.0)	0.53 (1.7)
20:4*n*-6	-	-	0.00 (0.0)	0.03 (0.1)
20:5*n*-3	-	-	0.00 (0.0)	0.09 (0.3)
22:5*n*-6	-	-	0.00 (0.0)	0.85 (2.7)
22:6*n*-3	-	-	0.00 (0.0)	2.52 (8.1)
n-6:*n*-3[3]	0.54	7.44	16.14	2.96
Feed offered (per head)				
DM (kg/day)	1.49	0.97	1.24	1.26
ME (MJ/day)	14.1	11.1	14.4	14.6
CP (g/day)	152.7	142.3	227.1	227.5

[1]Six weeks prior to and, three weeks following, conception. [2]Six weeks from 117-160 days of age and approximately 37 - 50 kg. [3]Ratio of omega-6 to omega-3 fatty acids. SLG = dams were fed a diet based on silage. OAT = dams were fed a diet based on oat+cottonseed meal. CTRL = lambs were fed a control diet. ALG = lambs were fed a control diet with DHAgold™ algae.

Table 2. PCR primers for gene expression analysis.

Gene	Forward	Reverse
FADS1	5’-AGGAACAGTGTGACCCCTTG-3’	5’-AAAAACAGCTTCTCCCAGCA-3’
FADS2	5’-ATCTGCCCTACAACCACCAG-3’	5’-TGTGACCCACACAAACCAGT-3’
CPT1A	5’-CTGTATCGTCGCACATTAGACCGT-3’	5’-CAGACCTTGAAGTACCGCCCTCT-3’
ACC	5’-CATGTCTGGTTTGCACCTAGTCA-3’	5’-TCACTTTATTCCCACCAAAACGA-3’
SCD	5’-CGAACCTACAAAGCTCGGCT-3’	5’-TGGAACGCCATGGTGTTG-3’
Fad2	5’-CACCACTCCTGGGTGAAAGT-3’	5’-AACCTGGTCACTCCAACCTG-3’

FADS1: fatty acid desaturase 1 encoding Δ 6 desaturase. FADS2: fatty acid desaturase 2 encoding Δ 5 desaturase. CPT1A: carnitine palmitoyl transferase 1A. ACC: acetyl-CoA carboxylase. SCD: stearoyl-CoA desaturase encoding Δ 9 desaturase. Fad2: delta 4 desaturase/sphingolipid 2.

Table 3. Predicted means ± s.e. for algae, dam nutrition and interaction (algae x dam nutrition) effects on relative mRNA expression per tissue.

Tissue	Gene	CTRL		ALG		*P*-value		
		SLG	OAT	SLG	OAT	ALG	DAM	ALG x DAM
Liver	FADS1	4.31 ± 0.36	1.91 ± 0.14	61.91 ± 5.73	18.30 ± 1.32	0.000	0.000	0.071
	FADS2	9.37 ± 1.46 b	1.01 ± 0.14 c	35.66 ± 5.93 a	7.19 ± 1.01 b	-	-	0.042
	SCD	11.38 ± 2.09	4.57 ± 0.77	18.42 ± 3.55	7.09 ± 1.19	0.056	0.000	0.898
	ACC	5.06 ± 0.95 b	2.27 ± 0.39 c	32.08 ± 6.34 a	2.79 ± 0.48 c	-	-	0.000
	CPT1	20.40 ± 4.43 ab	26.03 ± 5.19 a	3.89 ± 0.89 c	13.00 ± 2.58 b	-	-	0.015
	fad2	2.26 ± 0.47	2.06 ± 0.38	1.51 ± 0.33	1.85 ± 0.34	0.263	0.800	0.457
Muscle	FADS1	3.94 ± 1.29 b	4.58 ± 1.29 b	30.95 ± 10.14 a	2.78 ± 0.81 b	-	-	0.000
	FADS2	4.34 ± 1.84 b	5.48 ± 2.11 b	44.69 ± 18.78 a	4.76 ± 1.87 b	-	-	0.000
	SCD	7.94 ± 2.81 b	10.27 ± 3.00 b	48.59 ± 17.06 a	6.58 ± 2.04 b	-	-	0.001
	ACC	4.97 ± 1.66 b	7.10 ± 2.08 b	58.80 ± 19.52 a	4.53 ± 1.37 b	-	-	0.000
	CPT1	26.18 ± 9.38	30.42 ± 9.70	9.02 ± 3.23	19.85 ± 6.50	0.101	0.082	0.227
	fad2	1.87 ± 0.48	4.67 ± 1.01	2.80 ± 0.72	2.80 ± 0.63	0.637	0.054	0.058

Table 3 (continuation)

Tissue	Gene	CTRL		ALG		*P*-value		
		SLG	OAT	SLG	OAT	ALG	DAM	ALG x DAM
Fat	FADS1	8.05 ± 2.64 b	7.70 ± 2.38 b	46.96 ± 16.37 a	6.71 ± 1.99 b	-	-	0.002
	FADS2	3.30 ± 0.99 b	4.32 ± 1.23 b	27.00 ± 8.76 a	2.95 ± 0.78 b	-	-	0.000
	SCD	55.20 ± 26.54	30.80 ± 14.24	74.94 ± 37.29	38.28 ± 17.33	0.631	0.042	0.882
	ACC	72.68 ± 31.79	67.50 ± 28.09	47.01 ± 21.19	54.36 ± 22.30	0.527	0.903	0.690
	CPT1	25.78 ± 11.62	33.99 ± 14.46	11.89 ± 5.57	17.41 ± 7.24	0.164	0.314	0.872
	fad2	8.50 ± 2.26 ab	9.34 ± 2.35 ab	14.13 ± 4.01 a	5.29 ± 1.27 b	-	-	0.035

LSD rankings are given for each row separately when $P < 0.05$, and based on this ranking, means not having a letter in common are significantly different at $P = 0.05$. SLG = dams were fed a diet based on silage. OAT = dams were fed a diet based on oat+cottonseed meal. CTRL = lambs were fed a control diet. ALG = lambs were fed a control diet with DHAgold™ algae.

Table 4. Estimated residual correlation and *P*-value for each pair of traits on the $\log_e$ scale (fatty acids from muscle and gene expression).

Gene expression	Fatty acid	Correlation	s.e.	*P*-value
Muscle				
FADS1	20:5*n*-3	-0.31	0.161	**0.036**
	22:5*n*-3	-0.14	0.173	0.214
	20:4*n*-6	-0.31	0.160	**0.029**
	22:4*n*-6	-0.35	0.155	**0.016**
FADS2	18:3*n*-6	-0.31	0.160	**0.030**
	20:3*n*-6	-0.34	0.156	**0.017**
	18:4*n*-3	-0.20	0.170	0.120
CPT1	22:5*n*-6	-0.08	0.176	0.380
	22:6*n*-3	-0.22	0.168	0.096
SCD	16:1*n*-7	-0.10	0.175	0.337
	18:1*n*-9	-0.10	0.175	0.335
ACC	22:5*n*-6	-0.21	0.169	0.105
	22:6*n*-3	-0.05	0.176	0.524
	PUFA	-0.14	0.173	0.227
	SFA	-0.16	0.172	0.181
fad2	22:6*n*-3	0.14	0.173	0.212
Liver				
SCD	IMF/100g[1]	0.18	0.166	0.137
Fat				
SCD	IMF/100g[1]	0.03	0.171	0.637

[1]IMF/100g muscle.

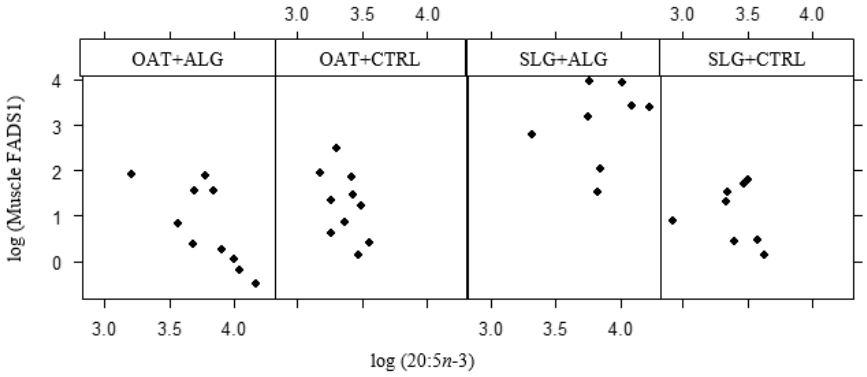

Figure 1. Relationship between the concentration of log (Muscle FADS1) and log (20:5n-3) levels in muscle when lambs were fed a control ration (CTRL) or the CTRL ration with DHA-GoldTM algae included at 1.92% (ALG) for 6 weeks prior to slaughter and their dams were previously fed a diet based in silage (SLG) or oat/cottonseed grain (OAT) around conception. R2 = -0.295, P = 0.036.

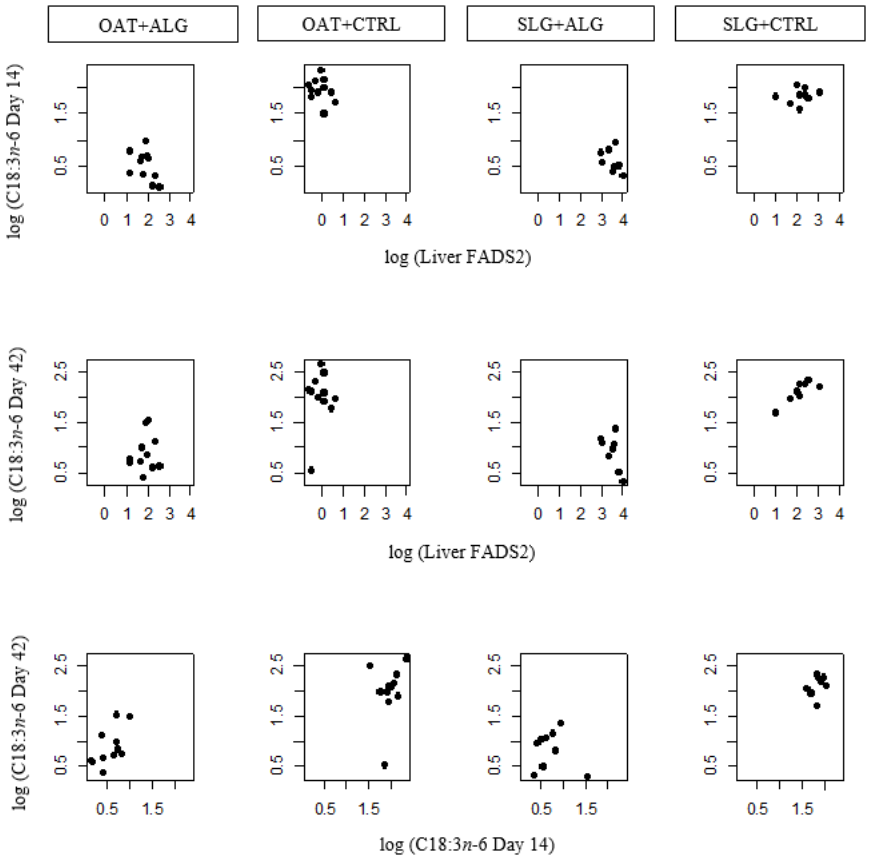

Figure 2. Pair-wise relationship between log (18:3n-6) levels in blood at 14 and 42 days of feeding and log (Liver FADS2 expression) of lambs fed a control ration (CTRL) or the CTRL ration with DHA-Gold™ algae included at 1.92% (ALG) for 6 weeks prior to slaughter and their dams were previously fed a diet based in silage (SLG) or oat/cottonseed grain (OAT) around conception. Rows correspond to pair-wise combinations of traits and columns to algae x dam nutrition combination.