Production performance, milk composition and cheese quality of crossbred dairy sheep supplemented with dietary omega-3 oils

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Declaration

I hereby declare that:

- The research presented and reported in this thesis was conducted in accordance with the University of Tasmania Animal Ethics Committee Guidelines, the 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Animal Ethics Permit Number A0015657).
- To the best of my knowledge and belief, this thesis contains no material which has been accepted for the award of a degree or diploma by the University or any other tertiary institution. The thesis contains no material previously published or written by any other person (s) except where background information duly acknowledged is made in the text of the thesis.

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Abstract

This thesis primarily investigated the effect of supplementing pasture-based dairy sheep with different plant oil-infused and rumen-protected pellets containing eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) on lactation performance, milk and cheese fatty acid composition and cheese eating quality. It was hypothesised that plant oil supplements would interact with sire breed to influence feed intake, body condition score, milk yield and composition, milk and cheese fatty acid contents and sensory attributes of ripened cheese.

Sixty Awassi and Awassi × East Friesian crossbred ewes in mid-lactation, balanced by liveweight (59 ± 5.9 kg), milk yield (657 ± 100 g/day), parity (2.8 ± 0.5), and sire breed were randomly allocated into 6 treatment groups of 10 ewes each, that were: (1) supplemented with on-farm existing commercial wheat-based pellets without oil inclusion (control) or supplemented with wheat-based pellets infused with 50 mL/kg dry matter of oils from (2) canola, (3) rice bran, (4) flaxseed, (5) safflower, and (6) rumen-protected EPA + DHA in a 10-week supplementary feeding trial including a 2-week adjustment period. All supplementary diets included the same level of 50 mL/kg DM of oil except for the control group, and all diets were isocaloric and isonitrogenous. Experimental animals were grazed in the same paddock with *ad libitum* access to pasture, hay, and water. During milking time, each ewe was fed 1 kg/day of the supplemented pellets individually in the milking parlour.

Data on weekly body condition score, daily feed intake, feed composition, weekly bulked fresh milk, raw milk, and ripened cheese samples were collected. Feed intake, body condition score, milk yield, milk composition, fatty acid composition of milk and cheese, and cheese sensory attributes were analysed in SAS with sire breed, diet, and week of supplementation and their second-order interactions as fixed effects.

It was demonstrated that oil supplementation and sire breed affected animal performance, productivity and quality of milk and its processed product, in that:

1) Rumen-protected oil pellet containing EPA + DHA was the most effective diet that improved milk production, n-3 long-chain (C≥20) polyunsaturated fatty acids (n-3 LC-PUFA) in fresh milk and ripened cheese without any negative effect on animal performance and cheese eating quality. A serving of milk and cheese reached the "good source" and "source" levels of n-3 LC-PUFA, respectively;
2) Flaxseed oil supplementation elicited not only the highest concentration of α-linolenic acid (ALA, 18:3n-3) in both fresh milk and ripened cheese, but also improved all cheese eating sensory traits. Flaxseed oil also significantly increased n-3 LC-PUFA in milk because a serving of fresh milk met the claimed "source" of n-3 LC-PUFA;
3) Safflower oil diet considerably improved milk, fat and protein yields. More importantly, this diet also had the most efficiency at enhancing the level of linoleic acid (18:2n-6) in milk and cheese. Safflower oil inclusion had no effect on cheese eating quality;
4) Rice bran oil was the sole diet that improved milk yield with an increase in protein content. However, adding rice bran oil to the diet of grazing dairy ewes had only minor effects on altering milk and cheese PUFA composition. Together with flaxseed oil, rice bran oil significantly enhanced consumer acceptability of ripened cheese;
5) Canola oil was found to have minor but statistically significant effect on milk yield, body condition score, and docosapentaenoic acid (DPA, 22:5n-3) content in milk;
6) Sire breed and its interaction with diet mainly affected milk production, but not milk quality, in which crossbred Awassi x East Friesian had higher milk yield than pure-bred Awassi.

Taken together, these outcomes suggest that oil supplements and crossbreeding can be utilised by Australian sheep milk producers in pasture-based systems to improve production traits and cheese eating quality to increase farm-gate value.

Thesis Publications

Peer-reviewed Journal Papers with 2018 Impact Factor (IF)

1. Nguyen QV, Malau-Aduli BS, Cavalieri J, Nichols PD, Malau-Aduli AEO 2019. Enhancing omega-3 long-chain polyunsaturated fatty acid content of dairy-derived foods for human consumption. *Nutrients* 11(4): 743 **(IF 4.171)** https://doi.org/10.3390/nu11040743

2. Nguyen QV, Le HV, Nguyen DV, Malau-Aduli BS, Nichols PD, Malau-Aduli AEO 2019. Enhancement of dairy sheep cheese eating quality with increased omega-3 long-chain polyunsaturated fatty acids. *Journal of Dairy Science* 102(1): 211-222 **(IF 3.082)** https://doi.org/10.3168/jds.2018-15215

3. Nguyen QV, Le VH, Nguyen DV, Malau-Aduli BS, Nichols PD, Malau-Aduli AEO 2018. Supplementing grazing dairy ewes with oil and rumen-protected EPA+DHA pellets enhances health-beneficial n-3 long-chain polyunsaturated fatty acids in sheep milk. *European Journal of Lipid Science and Technology* 120 (6): 1700256 **(IF 2.200)** https://doi.org/10.1002/ejlt.201700256

4. Nguyen QV, Le HV, Nguyen DV, Nish P, Otto JR, Malau-Aduli BS, Nichols PD, Malau-Aduli AEO 2018. Supplementing dairy ewes grazing low quality pastures with plant-derived and rumen-protected oils containing Eicosapentaenoic Acid and Docosahexaenoic Acid pellets increases body condition score and milk, fat, and protein yields. *Animals* 8 (12): 241 **(IF 1.832)** https://doi.org/10.3390/ani8120241

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List of Abbreviations

ADF = acid detergent fibre

ALA = alpha-linolenic acid

AOAC = Association of Official Analytical Chemists

AW = Awassi

BCS = body condition score

BH = biohydrogenation

CHD = coronary heart disease

CLA = conjugated linoleic acid

CO= canola oil

CP = crude protein

CSIRO = Commonwealth Scientific and Industrial Research Organisation

CVD = cardiovascular diseases

DHA = docosahexaenoic acid

DM = dry matter

DMI = dry matter intake

DPA = docosapentaenoic acid

EB = energy balance

EE = ether extract

EF = East Friesian

EPA = eicosapentaenoic acid

FA = fatty acids

FADS = fatty acid desaturase

FAME: fatty acid methyl esters

FAO = Food and Agriculture Organisation

FCM = fat-corrected milk

FSANZ = Food Standards Australia and New Zealand

FSO = flaxseed oil

FY = fat yield

GC = gas chromatography

GC-MS = GC-mass spectrophotometry

IA = atherogenic index

IT = thrombogenic index

LDL = low-density lipoprotein

ME = metabolisable energy

MUFA = monounsaturated fatty acids

MY = milk yield

n-3 LC-PUFA = omega-3 long-chain polyunsaturated fatty acids

n-3 PUFA = omega-3 polyunsaturated fatty acids

n-6 PUFA = omega-6 polyunsaturated fatty acids

NDF = neutral detergent fibre

NHMRC = National Health and Medical Research Council

OM = organic matter

PUFA = polyunsaturated fatty acids

PY = protein yield

RBO = rice bran oil

REC = recovery

RPO = rumen-protected oil

SAS = Statistical Analysis System

SCC = somatic cell count

SFA = saturated fatty acids

SFO = safflower oil

SNF = solids-non-fat

SNP = single nucleotide polymorphism

TDN = total digestible nutrients

UFA = unsaturated fatty acids

WHO = World Health Organisation

Chapter 1: General Introduction

The Australian dairy sheep industry is small and mostly based on a natural grass grazing system (AgriFutures Australia, 2013). Therefore, the contribution of milk derived from sheep to national milk production in the country is low, with an annual milk production of 500 thousand litres (AgriFutures Australia, 2013) compared to 9 billion litres of milk produced by dairy cows nationwide (Dairy Australia, 2018). The lack of specialised breeds and typical Australian sheep milk products, in addition to pre-weaning lamb mortality, are the main challenges that limit the growth of the industry (Bencini, 1999). It was estimated that 250,000 ewes would have to be milked to meet the domestic market demand of 8000 tons of sheep milk products (Bencini and Murray, 2012). Currently, only 5,500 sheep are milked in Australia (AgriFutures Australia, 2013). Therefore, the market demand for sheep milk products is increasing over and above the supply from local producers (Bencini and Murray, 2012). This could be the basis and justification for expanding the dairy sheep industry in Australia.

Chronic or non-communicable diseases have remained the most leading cause of death worldwide, with 41 million deaths accounting for 71% of reported global deaths (57 million) (WHO, 2018). This report also indicated that an unhealthy diet with low intake of omega-3 long-chain polyunsaturated fatty acids (*n-3* LC-PUFA), continues to be one of the main factors that either directly or indirectly induce chronic diseases. Omega-3 polyunsaturated fatty acids (*n-3* PUFA) contain two or more double bonds with the first double bond on the third carbon atom from the methyl end of the molecule. The common types of *n-3* PUFA are: Short chain (SC, ≤C18) *n-3* PUFA including α-linolenic acid (ALA, 18:3n-3) and stearidonic acid (SDA, 18:4n-3), and long-chain (≥C20) *n-3* PUFA (*n-3* LC-PUFA) including eicosapentaenoic (EPA, 20:5n-3); docosahexaenoic (DHA, 22:6n-3); and the less studied docosapentaenoic (DPA, 22:5n-3) acids (Nichols *et al.*, 2010). Previous studies on *n-3* LC-PUFA focused mainly on

EPA and DHA, but not DPA, despite its structural and beneficial effects on human health being similar to those of EPA and DHA (Byelashov *et al.*, 2015). The unavailability of pure DPA as a commercial product for performing clinical and nutritional trials is one possible explanation for this shortcoming. The term *n-3* LC-PUFA in this study includes EPA, DHA, and DPA.

Although there is a general awareness that fish and seafood are the dominant sources of *n-3* LC-PUFA, seafood consumption is still insufficient, thus the human diet persists with low *n-3* PUFA intake (Simopoulos, 2011). The traditional diet often does not contain regular consumption of fish and marine products, especially in Western countries (Cordain *et al.*, 2005). When taken together with the often high cost of seafood (Kennedy *et al.*, 2012), these combined factors probably have been the major grounds for the insufficiency of seafood consumption. In contrast, milk and its processed products are known as poor sources of *n-3* LC-PUFA content (Shingfield *et al.*, 2013), although they have played an important role in human diets for more than 8000 years (Rozenberg *et al.*, 2016). This is because dairy foods are important sources of energy, protein, fat, and vital microelements including calcium, vitamin D and potassium for humans (Burgess, 2013; Kardas *et al.*, 2016). According to the OECD/FAO report (2017), the 2015 global consumption of milk and dairy products was 111.3 kg per capita, and is expected to increase by approximately 12.5% by 2025. This fact has led to a number of studies focusing on enhancing the beneficial n-3 PUFA and n-3 LC-PUFA in milk and its processed products, mostly from cows and sheep, for human consumption (Shingfield et al., 2013).

Nutritional manipulation to date has been the main approach for altering milk fatty acids (FA) due to public health concerns of lactose intolerance in some people for milk from dairy cows (Chilliard *et al.*, 2001) and high levels of short to medium chain fats in milk from small ruminants (Sanz Sampelayo *et al.*, 2007). According to Chilliard *et al.* (2007), FA profiles in milk are derived from four different sources including: *de novo* synthesis in the mammary

gland, diet, ruminal biohydrogenation, and body reserves in which dietary FA contribute half of the C16 and all of the long-chain FA that includes all n-3 PUFA and n-3 LC-PUFA. Lipid supplementation is an effective tool for altering milk fat composition (Chilliard *et al.*, 2003; Kennelly *et al.,* 2005) and increasing milk production (Palmquist and Jenkins, 2017).

The inclusion of oil seeds and vegetable oil in dairy animal diets significantly elevates ALA content (Glasser *et al.,* 2008a), while the addition of rumen-protected marine-derived oil is the most effective way to increase the concentration of EPA, DHA, and DPA in dairy products (Shingfield *et al.,* 2013). The main challenge of this nutritional manipulation approach is ruminal biohydrogenation in which dietary PUFA are hydrogenated into monounsaturated FA and/or ultimately, saturated FA, due to rumen microbial activities (Bauman and Griinari, 2003). In addition, a comprehensive review by Nguyen *et al.* (2018) reported that the biosynthetic pathway of n-3 LC-PUFA from the precursor ALA seems to be limited.

To our current knowledge, there is a dearth of studies investigating the effect of dietary supplementation with pellets infused with oils from canola, flaxseed, rice bran, and safflower on milk production and FA composition of grazing dairy ewes, particularly under Australian on-farm pasture-based production system. Canola is the largest oilseed crop in Australia (Seymour *et al.*, 2012). It contains an abundance of ALA together with an ideal ratio of n-6 PUFA to n-3 PUFA at 2:1 (Sakhno, 2010). Therefore, the utilisation of canola oil as infused pellets as supplements for lambs (Flakemore *et al.*, 2017; Nguyen *et al.*, 2017; Le *et al.*, 2018; Malau-Aduli *et al.*, 2019) and dairy cows (Otto *et al.*, 2015) was investigated under both pasture and feedlot systems. Flaxseed oil is well-known as the richest source of ALA among plant oil sources, and contains up to 59.3% ALA in total fatty acid composition (Teh and Birch, 2013). Thus, feeding flaxseed to ruminants either as whole or extruded grain, is a common strategy for improving FA profile of dairy products (Glasser *et al.,* 2008a). Rice bran and safflower oil on the other hand, are more abundant in linoleic acid (18:2n-6, LA) (Gopala

Krishna *et al.*, 2006; Matthaus *et al.*, 2015) than ALA. The LA content of rice bran oil varies widely from 28.0-53.4% depending on the refining process (physical or chemical) (Gopala Krishna *et al.*, 2006). The effect of supplementing ruminants with rice bran on animal performance and FA composition has been investigated in lambs (Bhatt *et al.,* 2013; Flakemore *et al.,* 2017; Le *et al.,* 2018) and dairy cattle (Lunsin *et al.,* 2012a,b), but not to a large extent in dairy sheep.

Grown in over 60 countries (Glibert and Porter, 2008), safflower is a very highly sustainable annual oilseed crop (Singh and Nimbkar, 2016). Therefore, it has been used widely as a supplement for ruminants (Alizadeh *et al.,* 2012) because of its rich poly and monounsaturated FA contents, particularly LA, which constitutes up to 77% of total FA (Matthaus *et al.*, 2015). However, there is relatively little information on its efficacy for improving milk production, composition and FA content as a supplement in lactating sheep diets.

Therefore, the series of studies in this thesis were conducted to answer the following primary research question: What is the impact of diverse dietary omega-3 oil supplements on animal performance, yield, composition, fatty acid profile and quality of milk and cheese from dairy ewes in a pasture-based system? The major objectives were to investigate the impact of supplementing grazing dairy ewes with rumen-protected oil pellets or pellets infused with oils from canola, rice bran, flaxseed, or safflower independently and their interactions with sire breed on:

- Animal performance traits of feed intake, lactation and body condition score.
- Enhancing the concentration of milk and cheese n-3 LC-PUFA.
- Enhancing cheese eating quality and consumer acceptability.

The thesis has been structured into the following chapters:

Chapter 1: General Introduction

Chapter 2: Literature Review: Comprehensively reviews existing published literature on the background of Australian dairy sheep industry, nutritional value of sheep milk, the role of body condition score in dairy sheep management, parameters that drive lactation and cheese flavour, and recent research aimed at elevating n-3 LC-PUFA content of dairy products. Knowledge gaps were identified after critical analysis of existing literature that in turn, informed the formulation of research objectives investigated in this thesis.

Chapter 3: Evaluates the impact of canola, rice bran, flaxseed, safflower, and rumen protected oil-infused supplements and their interactions with sire breed on lactation performance, milk composition and body condition score of dairy ewes in mid-lactation grazing low quality pastures.

Chapter 4: Uncovers the relationship between canola, rice bran, flaxseed, safflower, and rumen protected oil-infused diets and the concentration of n-3 LC-PUFA in milk from grazing ewes.

Chapter 5: Examines the hypothesis that supplementing grazing dairy ewes with different plant oil infused and rumen-protected EPA+DHA pellets would affect the concentration and recovery of n-3 LC-PUFA and alter cheese eating quality.

Chapter 6: Presents a general discussion and summary of the main findings from the study and suggests possible areas requiring further investigation.

Appendices: Contains copies of peer-reviewed publications from this thesis and all supplementary materials that were excluded from thesis chapters on the basis of direct relevance.

Chapter 2: Literature Review

2.1. Dairy sheep industry background

According to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2018), there are approximately 200 million dairy sheep worldwide, accounting for 21.3% of the total sheep population. In 2017, 10 million tonnes of sheep milk were produced (FAOSTAT, 2018) representing 1.3 % of the total milk production in the world. Asia is dominant over all regions for the number of dairy sheep and total milk yield (Table 2.1). Due to the growing trend during the last 50 years, it is expected that worldwide dairy sheep production will increase by 26% (approximately 2.7 million tonnes) in the next decade (Pulina *et al.*, 2018). Cheese is the major processed product manufactured from sheep milk, with worldwide production of 680 thousand tonnes in 2014 (Table 2.1).

Table 2.1. Worldwide sheep milk products (Source: FAOSTA (2018)

Items	Unit	World	Asia	Africa	Europe	America	Year
Fresh milk	Million tonnes	10.4	5.02	2.44	2.85	0.097	2017
Cheese	Thousand tonnes	680.3	273.3	57.4	34.2	7.84	2014
Butter and ghee	Thousand tonnes	63.3	61.0	2.3	-	-	2014

Although milking sheep have been kept in Australia since 1906, the Australian dairy sheep industry remains small compared to meat and wool sheep (Cameron, 2014). The lack of specialised breeds and typical Australian sheep milk products compared to lamb meat and wool industries is probably the main reason for this (Bencini, 1999). It was expected that 250,000

milking ewes producing 8,000 tonnes of milk products could meet the demand of the Australian domestic market (Bencini and Murray, 2012). However, of the 72 million sheep in Australia (FAOSTAT, 2018), only 5,500 head are used for milking in 13 commercial farms producing 550,000 litres of milk annually (AgriFutures Australia, 2013). Sixty percent of fresh milk is processed into yoghurt, and the rest is used for cheese production for the domestic market (AgriFutures Australia, 2013). Similar to most of the Australian meat and wool sheep, dairy ewes are raised in extensive grazing systems that may include dryland, senesced, green and irrigated pastures (Ponnampalam *et al.* 2014). As extensive production systems mainly depend on local environmental conditions that affect pasture quality and availability, animals raised on these systems generally have low production efficiency (De Brito *et al.,* 2017). The effort to establish a stable sheep milking industry was made by introducing the East Friesian (EF) and Awassi breeds into the country in the 1990s (Bencini and Murray, 2012). However, statistics on milk production of these productive breeds that might help the producers improve their farm gate value remain inadequate.

2.2. Sheep milk

2.2.1. Nutritional value

In comparison to milk from cows and goats, sheep milk is a richer source of fat and protein (Figure 2.1), and other vital micro and macro elements, particularly calcium (Park *et al.*, 2007; Balthazar *et al.*, 2017) (Figure 2.1).

2.2.1.1. Milk fat

Fat is the most important component of milk defining its nutritional and energetic values. Fat in milk from sheep is two times higher than fat in milk from goats and cows (Figure 2.1 a). It contributes a noticeable higher energy value of 105 calories/100 ml compared to 69 and 70 calories/100 ml in cow and goat milk (Park *et al.*, 2007). Moreover, milk fat globule size from goats (3.0 μm) and sheep (3.6 μm) are smaller than from cows (4 μm) (Gantner *et al.*, 2015).

Small size and high dispersion state results in easier access of lipolytic enzymes to fat globules, enabling easier digestibility for humans consuming sheep and goat milk than cow milk (Tomotake *et al.*, 2006). These fat globule characteristics also have technical advantages in reducing phase separation under frozen storage conditions used for cheese production. In terms of fatty acid profile, similar to goat and cow, sheep milk contains mainly saturated fatty acids (SFA) varying from 57-75% (of total fatty acids) (Gantner *et al.*, 2015). However, citing a number of authors, Markiewicz-Keszycka *et al.* (2013) concluded that the proportion of PUFA at 4.82% in sheep milk, is higher than in cows and goats (4.05 and 3.70%, respectively). Similarly, sheep milk has a higher concentration of conjugated linoleic acid (CLA) compared to goat and cow milk (1.08, 1.01, and 0.65%, respectively). A significantly lower ratio of n-6 PUFA to n-3 PUFA (Markiewicz-Keszycka *et al.*, 2013) makes sheep milk more desirable than cow and goat milk in inhibiting the risk of chronic diseases (Simopoulos, 2002; Zymon *et al.*, 2014).

2.2.1.2. Milk protein

In comparison with cow milk, sheep milk contains a higher percentage of protein (5.8 vs 3.3%) (Park *et al.*, 2007), of which 80% are casein complexes and the rest are whey protein fractions (Balthazar *et al.*, 2017). This high concentration of casein is of benefit to cheesemakers due to a positive relationship between casein content of raw milk and cheese yield (Colin *et al.*, 1992; Hurtaud *et al.*, 1995). Another structural advantage of sheep milk protein is that casein micelles have a rich calcium content that serves as a catalyst for rennet coagulation (Kethireddipalli and Hill, 2015), thus adding $CaCl_2$ is not required in sheep cheese making. Higher mineralization of casein micelles support cheesemakers to produce adequate curd from sheep milk using less rennet or chymosin and still achieve the same coagulation time compared to cow milk (Kalantzopoulos, 1993). With regards to nutritional value, a higher protein concentration with lower allergic sensitization (Masoodi and Shafi, 2010) are attributes of sheep milk that make it an ideal alternative protein source for consumers who have allergy to cow milk (Scintu and Piredda, 2007).

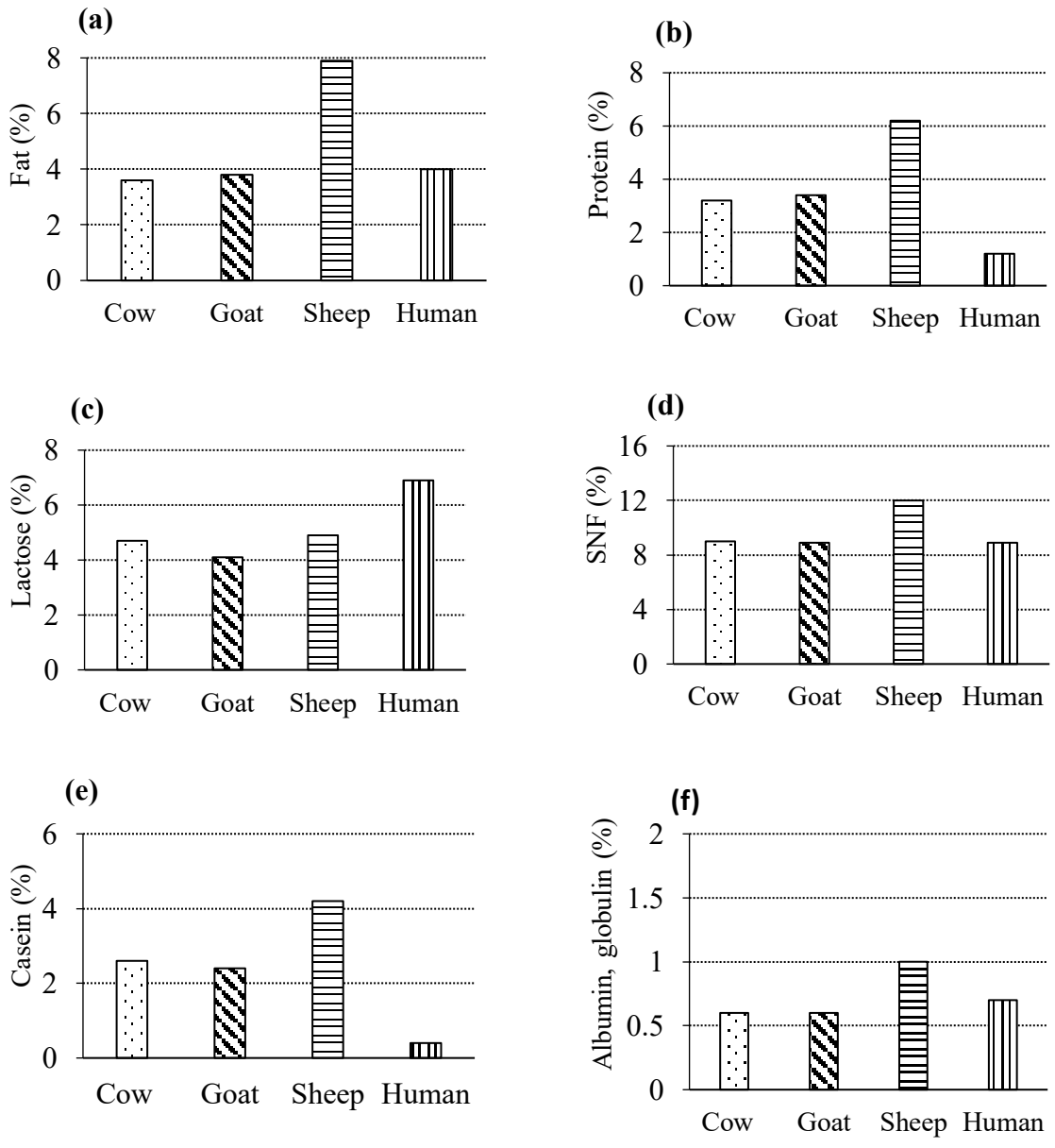

Figure 2.1. Fat (a), protein (b), lactose (c), solids-non-fat (SNF) (d), casein (e), albumin and globulin (f) percentage of milks from cow, goat, sheep and human (Park et al., 2007)

2.2.1.3. Minerals and Vitamins

Sheep milk is the richest source of vitamins and some critical minerals compared to goat and cow milk (Table 2.2). Calcium and phosphorus, the main macrominerals that affect growth and maintenance of skeletal structure, are much higher in sheep milk than in cow and goat milk.

According to NHMRC (2013), 494 mg of calcium in one standard serving (494mg/250 ml) contributes nearly half of the daily recommended intake of 1000 mg of calcium for adults (NHMRC, 2006). In humans, high protein intake was shown to elevate calcium absorption (Kerstetter *et al.*, 2011), thus sheep milk that contains high concentration of calcium and protein is considered as an effective dietary calcium supplement.

Table 2.2. Mineral and vitamin contents in sheep, goat and cow milk (Sources: Park et al., 2007; Balthazar et al., 2017)

Items	Unit	Sheep	Goat	Cow
Mineral				
Ca	mg/100 g	197.5	130	112
P	mg/100 g	141	109	91
Mg	mg/100 g	18	16	12
K	mg/100 g	138	185.5	145
Na	mg/100 g	39	39.5	42
Cl	mg/100 g	160	150	100
S	mg/100 g	29	28	32
Fe	mg/100 g	0.08	0.07	0.08
Cu	mg/100 g	0.04	0.05	0.06
Mn	mg/100 g	0.02	0.03	0.007
Zn	mg/100 g	0.6	0.43	0.4
Se	µg/100 g	1.0	1.33	0.96
Vitamin				
Vitamin A	µgRE/100 g	64	54.3	37
Vitamin B_6	mg/100 g	0.08	0.046	0.042
Vitamin B_{12}	µg/100 g	0.712	0.065	0.375
Vitamin C	mg/100 g	4.16	1.29	0.94
Vitamin D	µg/100 g	0.2	0.2	0.15
Biotin	µg/100 g	2.5	1.75	2.0
Niacin	mg/100 g	0.416	0.27	0.08
Riboflavin	mg/100 g	0.376	0.21	0.16
Pantothenic acid	mg/100 g	0.408	0.31	0.32
Folic acid	µg/100 g	5.0	1.0	5.0
Thiamine	mg/100 g	0.08	0.068	0.045

2.2.2. Factors affecting milk yield and composition

2.2.2.1. Genetic parameters

Although approximately 180 different sheep breeds produce milk for human consumption, only a few of these breeds are considered as primary "dairy" breeds (Table 2.3), with East Friesian (EF) and Awassi being probably the most popular (Park *et al.*, 2017). Developed in northern Germany and the Netherlands and known as the world's most productive dairy sheep (Haenlein, 2007), the use of EF as purebred animals in unfavourable environmental conditions such as excessive heat and humidity is limited (Gootwine and Goot, 1996). Thus, EF has been used widely in crossbreeding systems to improve milk production and prolificacy of local breeds. For example, EF rams were mated with Dorset-cross, Polypay, and Rambouillet ewes to improve the productivity of local dairy sheep under North American production conditions (Berger and Thomas, 1997; Thomas *et al.*, 1998; Thomas *et al.*, 2000). Following EF regarding milk production capacity, the Awassi is the most numerous and widespread breed of dairy sheep in the world because of its ability to adapt to diverse environmental conditions (Galal *et al.*, 2008).

Table 2.3. Average lactation length, milk yield and composition of common sheep breeds used for milk production (Sources: Haenlein, 2007; Park *et al.*, 2017)

Breed	Original country	Lactation length (days)	Milk Yield (kg)	Fat (%)	Protein (%)	Total solids (%)	Ash (%)	Lactose (%)
East Friesian	Germany	300	632	6.5	5.25	17.0	0.9	4.9
Awassi	Israel	270	495	6.61	5.74	18.24	0.93	4.96
Lacaune	France	165	270	7.40	5.63	18.63	0.93	4.67
Chios	Greece	210	218	7.90	6.20	19.08	0.92	4.06
Sarda	Italy	200	158	6.99	5.60	18.14	0.95	4.60
Manchega	Spain	210	300	7.78	6.01	18.98	0.90	4.29
Churra	Spain	150	150	7.30	5.98	18.30	0.95	4.25

Estimates of heritabilities and genetic correlations for major milk traits portrayed in Table 2.4 range from moderate for milk, fat and protein yields (approximately 0.25-0.30), to high for fat and protein content (0.50 to 0.60) (Park *et al.*, 2017). Genetic improvement programmes have been based on purebred selection and mainly implemented in Europe (Barillet, 2007) where milk, fat and protein yields are major selection criteria (Carta *et al.*, 2009) used by breeders. For instance, genetic programmes employed for Lacaune in France contributed to the annual increases of 0.12 and 0.14% in fat and protein contents, respectively, with an annual genetic gain of 5 litres in milk production (Astruc *et al.*, 2002).

Table 2.4. Heritabilities and genetic correlations for lactating traits of different breeds (Source: Park et al., 2017)

Trait	Milk yield	Fat yield	Protein yield	Fat (%)	Protein (%)
Milk yield	0.20 to 0.32	0.77 to 0.89	0.88 to 0.94	-0.43 to -0.56	-0.46 to -0.64
Fat yield		0.16 to 0.26	0.82 to 0.93	0.02 to 0.25	-0.36 to -0.12
Protein yield			0.18 to 0.28	-0.18 to -0.28	0.01 to -0.15
Fat (%)				0.10 to 0.61	0.41 to 0.85
Protein (%)					0.31 to 0.69

2.2.2.2. Dietary nutrients for improving milk production and composition

The synthesis of milk components is principally driven by secretory cells in the mammary gland from precursors derived directly or indirectly from circulating dietary nutrients (Pulina and Nudda, 2004). Therefore, the most effective approach to improve milk production and milk components is to alter dietary nutrition regimes (Bocquier and Caja, 2001; Kennelly *et al.*, 2005). As most sheep milk is used for cheese making (Balthazar *et al.*, 2017), with cheese yield

depending mainly on milk fat and protein concentrations (Pellegrini *et al.*, 1997), this review will lay emphasis on the effect of dietary nutrients on the yields and contents of milk fat and protein.

Dietary nutrient composition and milk yield

Milk production by dairy ewes is mainly affected by voluntary feed intake or more accurately, the level of energy intake to support the high energy content of sheep milk (Park *et al.*, 2017). Increasing the energy and nutritional value of the diet for lactating ewes is considered as one of the most critical strategies for improving milk production (Palmquist, 1994; Bocquier and Caja, 2001; Mikolayunas *et al.*, 2008; Mikolayunas *et al.*, 2011; Vazirigohar *et al.*, 2014). Fat supplementation has been demonstrated as an effective tool for not only improving milk yield (Palmquist, 1994; Vazirigohar *et al.*, 2014), but also for altering milk composition for human health benefits (Kennelly *et al.*, 2005).Various types and dosages of oil supplemented to dairy ewes have resulted in significant variation in animal performance (Table 2.5).

Dietary nutrient composition and milk protein content

Milk protein content is influenced by many nutritional factors with a lesser magnitude of changes than that of milk fat concentration in both dairy cows (Kennelly *et al.*, 2005) and sheep (Pulina *et al.*, 2006). According to Bocquier and Caja (2001), dietary energy concentration is positively correlated with milk protein content, especially when the energy sources are soluble carbohydrates (Gerson *et al.*, 1985). This is because carbohydrates are energy sources for most rumen microbes including bacterial protein that control nitrogen utilisation in the rumen (Russell *et al.*, 1992). Similar to dairy cows (Huhtanen and Hristov, 2009), dietary crude protein (CP) content has a negative influence on milk protein percentage in dairy sheep (Bocquier and Caja, 2001). This can be explain as the CP content of the diet increases, it may exceed microbial needs which induces excessive urinary N (Broderick, 2003) together with a decrease in microbial protein synthesis (Broderick and Clayton, 1997).

Table 2.5. Effect of lipid supplementation on milk yield and composition[a] of dairy ewes

Diet	MY	Fat	FY	Protein	PY	References
Palm oil	1242	8.43	103.8	5.18	64.0	Bodas *et al.* (2010)
Olive oil	1288	9.55	120.3	5.35	67.9	
Soybean oil	1321	8.37	111.9	5.23	68.2	
Linseed oil	974	8.77	96.5	5.29	55.2	
Control	3280	6.15	125.9	5.22	107.1	Toral *et al.* (2010)
Sunflower oil (SO)	3585	6.51	140.9	5.16	110.1	
SO + 8 g/ kg DM of Marine Algae	3608	5.75	115.7	4.93	98.7	
SO + 16 g/ kg DM of Marine Algae	3436	5.76	118.4	4.95	103.4	
SO + 24 g/ kg DM of Marine Algae	3459	5.29	101.7	4.96	95.5	
Control	1362	5.91	80.4	4.81	65.47	Mughetti *et al.* (2012)
100 g extruded linseed	1404	6.07	85.15	4.81	67.48	
200 g extruded linseed	1217	6.10	74.19	4.89	59.56	
Control	825	5.80	47.8	5.56	45.8	Buccioni *et al.* (2015)
Chestnut tannin	978	5.78	56.5	5.12	50.1	
Control	283.8	6.94	19.49	5.74	16.46	Caroprese *et al.* (2016)
Seaweed	324.2	7.21	22.96	5.85	19.09	
Whole flaxseed	341.5	6.84	22.75	5.88	19.81	
Seaweed + Whole flaxseed	346.3	6.85	22.36	5.89	19.48	
Control	484	7.4	36	5.4	26	Nguyen *et al.* (2018a)
Canola oil	525	7.2	38	5.5	29	
Rice bran oil	527	7.2	38	5.9	31	
Flaxseed oil	489	6.9	34	5.4	26	
Safflower oil	562	6.6	37	5.6	31	
Rumen-protected oil	628	6.6	41	5.4	34	
Control	782	6.42	50	5.69	50	Antonacci *et al.* (2018)
Soybean oil (SO)	963	5.96	60	5.67	50	
Linseed oil (LO)	862	6.59	60	5.18	50	
75% SO + 25% LO	854	6.56	60	5.79	50	
50% SO+ 50% LO	805	6.75	60	6.10	50	
25% SO + 75% LO	902	7.09	60	6.10	50	

[a] Milk yield (MY, g/day), fat (g/100 g milk), fat yield (FY, g/day), protein (g/100 g milk), protein yield (PY, g/day)

In addition, results from Gonzalez *et al.*, (1982) and Purroy and Jaime (1995) showed that milk protein content and yield can be influenced by different protein sources, probably due to the variation in rumen undegraded CP in dietary protein (Pulina *et al.*, 2006). In response to lipid supplementation, inconsistent results in milk protein concentration have been reported (Table

2.5). The wide range of inclusion rates, dietary components, feeding regimes might have led to these contrasting outcomes.

Dietary nutrients and milk fat content

Among all components of milk, fat content is the most amenable to change by altering dietary composition (Kennelly *et al.*, 2005). Energy balance (EB), neutral detergent fiber (NDF) intake and source, as well as dietary fat supplements are the most important factors influencing both milk fat yield and concentration (Bocquier and Caja, 2001; Pulina *et al.*, 2006).

Generally, milk fat content has a negative correlation with level of nutrition in dairy cows (Palmquist *et al.*, 1993) and sheep (Caja and Bocquier, 2000). Undernutrition is often observed in typical extensive or semi-intensive dairy ewe grazing systems, resulting in negative EB, and inducing an increase in milk fat concentration (Bocquier and Caja, 2001), probably due to lower milk volumes and/or high body fat mobilization into milk. Moreover, the relationship between fat content and EB is stronger for higher milk production ewes and become weaker for lower milk production ewes (Pulina *et al.*, 2006).

A positive correlation between fat content and dietary NDF have been confirmed consistently in dairy cows, but inconsistently in dairy sheep (Pulina *et al.*, 2006). Examining the relationship between NDF content and milk fat yield of 10 different dairy ewe breeds, Mele *et al.* (2005) conducted a meta-analysis and observed the highest fat yield in milk from ewes fed diets that contained 35% NDF on dry matter basis. These authors also noticed that when NDF level was either higher than 35% or lower than 30%, daily milk fat yield decreased. In contrast, Nudda *et al.* (2004) reported a weak positive correlation of +0.38 between dietary NDF and fat yield. However, Natel *et al.*, (2013) found that an increase in the levels of dietary NDF did not affect the composition of milk, although it significantly decreased milk yield. Thus, the positive effect of NDF on milk fat content may be largely contributed by a strong negative association between milk yield and NDF (Pulina *et al.*, 2006). Variable responses were also observed for milk

fat content when different sources of fat were included in the diet of dairy sheep (Table 2.5).

2.2.2.3. Other factors affecting milk yield and composition

Beside genetics and nutrition, other factors including parity, lambing season, milking frequency and stage of lactation also influence milk content and yield in ewes (Sevi *et al.*, 2000; Bocquier and Caja, 2001; Abd Allah *et al.*, 2011). According to Novotná *et al.* (2009) and Nudda *et al.* (2003), the highest daily milk yield is observed in parities 2 and 3. This high production normally remains up until the sixth lactation (6 years of age) (Pugliese *et al.*, 2000) and then decreases. Differences in udder glandular tissue (Sevi *et al.*, 2000) also significantly contribute to changes in milk protein and fat contents between ewes in their first and second lactations (Novotná *et al.*, 2009).

In terms of milking frequency, ewes milked twice a day tend to produce higher milk yield with lower percentage of fat and protein than those milked once daily (Nudda *et al.*, 2002). This is due to the autocrine regulation or local feedback mechanism of milk secretion which is defined as a self-regulated ability of mammary gland largely without the impact from systemic hormones or signals (Wilde *et al.*, 1998; Weaver and Hernandez, 2016) presented in sheep (Bencini *et al.*, 2003). Positive local feedback that cause greater milk secretion, can be induced by several factors such as increasing milking frequency (Wall and McFadden, 2012), cell proliferation (Collier *et al.*, 1993), cell differentiation (Lykos *et al.*, 2000), serotonin and parathyroid hormone activity (Laporta *et al.*, 2014), somatostatin (Bauman and Vernon, 1993), and prolactin (Chen *et al.*, 2012). The completeness of milk removal could affect milk secretion through changes in mammary blood flow together with the number and activity of secretory cells (Wilde and Peaker, 1990; Wall and McFadden, 2012). In addition, responses to milking frequency changes were not consistent across different breeds, probably due to the difference of the udder storage capacity (Pulina *et al.*, 2007).

The influences of lambing season and stage of lactation on lactation traits on the other hand, are generally attributed to nutrient value of available pastures in the grazing system (CappioBorlino *et al.*, 1997). Day length (hours of light) in different lambing seasons also causes changes in milk production (Cannas and Pulina, 2002). Increase in daylight for a long period (more than 30 days) resulted in an increase in feed intake and consequently improved milk production, but the opposite effect was observed with a short-term increment in day length (Pulina *et al.*, 2007).

2.3. Body condition score as an essential management tool for dairy sheep producers

Body condition score (BCS) has been employed as a health management tool for estimating body fat or energy reserves (Caldeira *et al.*, 2007) as well as animal welfare status (Morgan-Davies *et al.*, 2008; Caroprese *et al.*, 2009; Phythian *et al.*, 2011). BCS in dairy sheep was first standardized in the 1960s (Russel *et al.*, 1969). The technique uses subjective palpation along the backbone and ribs to evaluate bone sharpness or muscle roundness with the score varying from 1 to 5 (Table 2.6). Ewes with BCS scores lower than 2 are identified as being thin and emaciated; an indication of sub-optimal nutrition during early lactation, while ewes with BCS 4 and above are considered obese and probably over-fed (Caroprese *et al.*, 2009).

According to Cannas and Boe (2003), the body weight of any sheep breed can be predicted by BCS which may assist producers in terms of estimating the volume and quality of feed needed to meet the nutrient requirements of ewes. A comprehensive review by Kenyon *et al.* (2014) confirmed the positive association between BCS and ewe reproductive traits in different sheep breeds (Kenyon *et al.*, 2004; Abdel-Mageed, 2009 ; Yilmaz *et al.*, 2011; Corner-Thomas *et al.*, 2015) and suggested that the optimum ewe BCS at breeding is between 2.5 - 3.0 in order to have the highest pregnancy rate. Morgan-Davies *et al.* (2008) found an increased lamb survival rate in subsequent winters of ewes which had higher BCS scores in their mid-pregnancy. Relationship between BCS and milk production of dairy animal was determined mostly in cows (Domecq *et al.*, 1997; Berry *et al.*, 2003; Jilek *et al.*, 2008). Domecq *et al.* (1997) demonstrated that increasing one-point BCS of dairy cows during the dry period resulted in 545.5 kg more milk in the first 120 days of lactation period. Therefore, knowledge of BCS and its correlation

with animal performance traits would help sheep producers to improve productivity through appropriate nutritional management of feed intake at different stages of production.

Table 2.6. Description of body condition scoring of sheep (Source: Western Australian Department of Agriculture, 2018)

Grade and illustration	**Description**	
	Backbone	Short ribs
 Score 1	The bones form a sharp narrow ridge. Each vertebra can be easily felt as a bone under the skin. There is only a very small eye muscle.	The ends of the short ribs are very obvious. It is easy to feel the squarish shape of the ends. Using fingers spread 1 cm apart, it feels like the fingernail under the skin with practically no covering.
 Score 2	The bones form a narrow ridge but the points are rounded with muscle. It is easy to press between each bone.	The ends of short ribs are well rounded but it is easy to press between them. Using fingers spread 0.5 cm apart, the ends feel rounded like finger ends. They are covered with flesh but it is easy to press under and between them.
 Score 3	The vertebrae are only slightly elevated above a full eye muscle. It is possible to feel each rounded bone but not to press between them.	The ends of the short ribs are well rounded and filled with muscle. Using 4 fingers pressed tightly together, it is possible to feel the rounded ends but not between them. They are well covered and filled in with muscle.
 Score 4	It is possible to feel most vertebrae with pressure. The back bone is a smooth slightly raised ridge above full eye muscles and the skin floats over it.	It is only possible to feel or sense one or two short ribs and only possible to press under them with difficulty. It feels like the side of the palm, where maybe one end can be sensed.
 Score 5	The spine may only be felt (if at all) by pressing down firmly between the fat covered eye muscles. A bustle of fat may appear over the tail.	It is virtually impossible to feel under the ends as the triangle formed by the long ribs and hip bone is filled with meat and fat. The short rib ends cannot be felt.

2.4. Enhancing omega-3 long-chain polyunsaturated fatty acid content of dairy-derived foods for human consumption

2.4.1. Role of Omega 3 long chain polyunsaturated fatty acid

Omega-3 polyunsaturated fatty acids (n-3 PUFA) are termed essential fatty acids because they cannot be synthesized *de novo* by humans due to the lack of delta-12 and delta-15 desaturase enzymes and must therefore be acquired from the diet (Lee *et al.*, 2016). n-3 PUFA include α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic (EPA, 20:5n-3), docosahexaenoic (DHA, 22:6n-3), and the less recognized docosapentaenoic acid (DPA, 22:5n-3) (Nichols *et al.*, 2010). The three long-chain (≥C20) n-3 PUFA (n-3 LC-PUFA), EPA, DHA, and DPA play an important role in human health by reducing the risk of chronic diseases. Up to the present time, seafood, and in particular, fish oil-derived products, have been the richest sources of n-3 LC-PUFA (Nichols *et al.*, 2010). The human diet generally contains insufficient amounts of these essential FA due largely to the low consumption of seafood. This issue provides opportunities to enrich the content of *n-3* PUFA in other common food groups. Milk and milk products have traditionally been a major component of human diets, but are also among some of the poorest sources of *n-3* PUFA. Consideration of the high consumption of milk and its processed products worldwide and the human health benefits has led to a large number of studies targeting the enhancement of *n-3* PUFA content in dairy products. Nutritional manipulation to date has been the main approach for altering milk fatty acids (FA) in ruminants (Kennelly *et al.*, 2005). However, the main challenge is ruminal biohydrogenation in which dietary PUFA are hydrogenated into monounsaturated FA and/or ultimately, saturated FA, due to rumen microbial activities. The inclusion of oil seed and vegetable oil in dairy animal diets significantly elevates ALA content (Bayat *et al.*, 2018), while the addition of rumen-protected marine-derived supplements is the most effective way to increase the concentration of EPA, DHA, and DPA in dairy products (Shingfield *et al.*, 2013). The mechanisms of *n-3* LC-PUFA

biosynthesis pathway from ALA and the biohydrogenation of individual *n-3* LC-PUFA in ruminants need to be better elucidated. Identified knowledge gaps regarding the activities of candidate genes regulating the concentrations of *n-3* PUFA and the responses of ruminants to specific lipid supplementation regimes are also critical to a greater understanding of nutrition-genetics interactions driving lipid metabolism.

2.4.2. Structure of omega-3 LC-PUFA

Omega-3 polyunsaturated fatty acids (*n-3* PUFA) contain more than two double bonds with the first double bond on the third carbon atom from the methyl end of the molecule. The common types of n-3 PUFA are: Shorter chain (SC, ≤C18) n-3 PUFA including α-linolenic acid (ALA, 18:3n-3) and stearidonic acid (SDA, 18:4n-3), and long-chain (≥C20) n-3 PUFA (n-3 LC-PUFA) including eicosapentaenoic (EPA, 20:5n-3); docosahexaenoic (DHA, 22:6n-3); and the less studied docosapentaenoic (DPA, 22:5n-3) acids (Nichols *et al.*, 2010) (Figure 2.2).

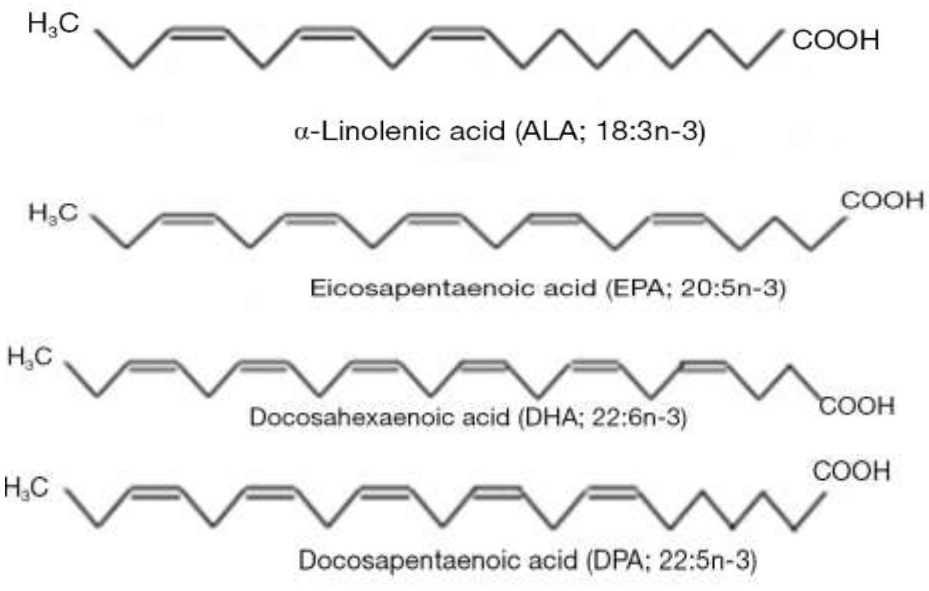

Figure 2.2. The structure of common omega-3 polyunsaturated fatty acids. Adapted from (Calder, 2017).

2.4.3. Metabolic pathways, human health benefits and recommended intake of n-3 PUFA

2.4.3.1. Dietary n-3 PUFA intake recommendations

Dietary intake recommendations of *n-3* LC-PUFA from different organisations vary largely and also depend on many factors including age, gender, and consumption purposes of consumers (Nichols *et al.*, 2010; Nguyen *et al.*, 2018). Adhering to National Health and Medical Research Council (NHMRC) recommendations (NHMRC, 2006), the daily intakes of ALA and total EPA+DPA+DHA considered adequate for men are 1.3 g/day, and 160 mg/day, and for women, 0.8 g/day and 90 mg/day, respectively. These dietary requirements of *n-3* PUFA are not optimal, but are seen as sufficient to prevent deficiency symptoms for adults. However, with the aim at reducing chronic disease risk, the NHMRC suggested that dietary intakes of total *n-3* LC-PUFA of 430 mg/day for women, and 610 mg/day for men should be adequate to meet requirement levels. In order to prevent the risk of coronary heart disease, FAO and WHO (FAO/WHO, 2008) recommended sufficient daily intake of EPA + DHA at 250 mg for adult males and non-pregnant or/and non-lactating adult females, and at 300 mg for lactating and pregnant women. In the case of disease treatment, such as for hypertriglyceridemia patients who have high triglyceride level symptoms, a much higher intake of total EPA + DHA from 2 - 4 g/day is recommended by the American Heart Association (Miller *et al.*, 2011). A recent review by Nguyen *et al.* (2018) stated that the intake recommendation of *n-3* LC-PUFA for primary prevention of cardiovascular disease across all organisations is about 500 mg/day, which is equivalent to two or three servings of fish per week.

2.4.3.2. Metabolic pathways for the biosynthesis and dietary sources of n-3 PUFA

Due to the lack of delta-12 and delta-15 desaturase enzymes, mammals (including humans) cannot synthesize *n-3* PUFA *de novo*, thus these essential FA must be acquired via foods or

nutritional supplements (Lee *et al.*, 2016). The first step in the *n-3* LC-PUFA synthesis pathway for the human body is the conversion of ALA to SDA, with ALA mostly acquired from green plant tissues and plant-derived oils, especially flaxseed/linseed and canola oil (Baker *et al.*, 2016) (Table 2.7).

Table 2.7. Common food sources of ALA (18:3n-3, as gram per serving)

Item	Unit	ALA
Flaxseed oil	g/ tbsp	7.26
Chia seed	g/ ounce	5.06
English walnuts	g/ ounce	2.57
Whole flaxseed	g/ tbsp	2.35
Canola oil	g/ tbsp	1.28
Soybean oil	g/ tbsp	0.92
Black walnut	g/ ounce	0.76

Data from Office of Dietary Supplements, National Institute of Health (NIH), USA
Tbsp denotes tablespoon.

There are two recognised biosynthesis pathways for *n-3* LC-PUFA (Figure 2.3), including the presently accepted pathway (Sprecher, 2002) and conventional metabolic pathway (Park *et al.*, 2009). In the former pathway, DHA was produced from DPA via sequential desaturation and elongation combined with a final β-oxidation where tetracosapentaenoic acid (24:5n-3) is chain-shortened by two carbons. The latter conventional metabolic pathway, in contrast, consists of direct conversion of DHA from DPA under the catalysis of delta-4 desaturase enzyme. The molecular evidence for delta-4 desaturase that supported the conventional metabolic pathway for *n-3* LC-PUFA biosynthesis was first demonstrated by Park *et al.* (2015). Further research is needed to clarify the specific pathway for *n-3* LC-PUFA biosynthesis in the

human body, but most studies have confirmed a very low rate of conversion of ALA to *n-3* LC-PUFA, in particular, to DHA (0.05% or less) (Burdge and Calder, 2006). The specific mechanism(s) by which biosynthesis of these essential FA occurs is limited in man and is still largely unknown. Calder (2014) suggested that a possible cause for this limitation is the competition between biosynthetic pathways of ALA conversion to *n-3* LC-PUFA and linoleic acid (18:2n-6) conversion to *n-6* LC-PUFA as the two pathways employ the same set of enzymes. In addition, based on previous animal studies, deficiencies of insulin (Brenner, 1977), protein (Narce *et al.*, 1988) and microminerals (Johnson *et al.*, 1989) might lead to lower delta-6 desaturase enzyme activity, thus contributing to the low efficiency of this pathway.

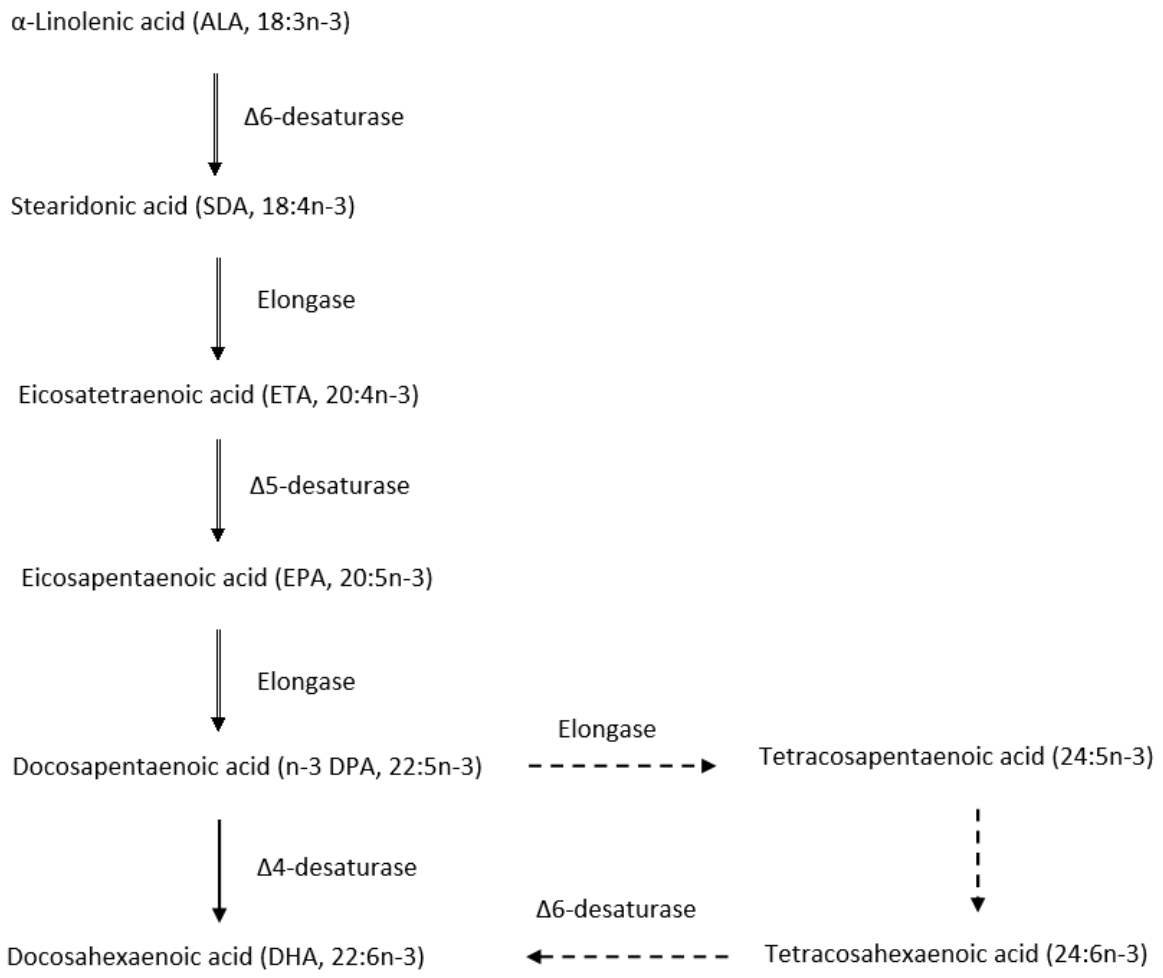

Figure 2.3. Possible biosynthesis and metabolic pathway of n-3 LC-PUFA. Thick arrows represent the conventional metabolic pathway; dotted lines with arrows represent newly proposed pathway (Source: Park *et al.*, 2009 and Sprecher, 2002).

Due to the limitation of *n-3* LC-PUFA biosynthesis in the human body from ALA, the best way of acquiring these essential FA is from dietary sources (Simopoulos, 2016). Fish and seafood currently are the major sources of *n-3* LC-PUFA with high concentration ranges (Nichols *et al.*, 2010; Tur *et al.*, 2012). The average content of total *n-3* LC-PUFA in 150 g wet weight of wild caught Australian fish, shellfish, prawns, and lobsters are 350, 250, 180, and 160 mg respectively, with a range of species also having markedly higher contents than these average values (Nichols *et al.*, 2010). The level of these FA for the two common fish species farmed in Australia - Atlantic salmon, and barramundi - examined by Nichols et al. (Nichols *et al.*, 2014) are 980 and 790 mg/100 g, respectively. Compared to the previous results (Nichols *et al.*, 2010), the concentration of *n-3* LC-PUFA for these farmed fish had decreased significantly by 50% or more. Changes in feed ingredients for farmed fish, in which fish meal and fish oils have been substituted by non-traditional oil sources such as plant and/or chicken oils were the reasons for this trend (Nichols *et al.*, 2014). Foods derived from animals have much lower *n-3* LC-PUFA content in comparison to marine products (Table 2.8).

Table 2.8. Content of n-3 LC-PUFA in common seafood and other animal sources

Item	Unit	EPA	DHA	DPA	Total *n-3* LC-PUFA	Reference
Wild seafood						Nichols *et al.* (2010)
Fish	mg/150 g	-	-	-	350	
Shellfish	mg/150 g	-	-	-	225	
Prawns	mg/150g	-	-	-	180	
Lobster	mg/150 g	-	-	-	160	
Farmed fish						
Atlantic salmon	mg/100 g	-	-	-	980	Nichols *et al.* (2014)
Barramundi	mg/100 g	-	-	-	790	
Other animal sources						
Beef	mg/100 g	15	12	20	47	Garcia *et al.* (2008)
Chicken breast	mg/100 g	-	-	-	62.04	Konieczka *et al.* (2017)
Pork	mg/100 g	23.3	3.9	21.1	48.3	Dugan *et al.* (2015)
Feedlot lamb meat	mg/100 g	17.9	4.9	15.6	38.4	Nguyen *et al.* (2017)
		28.9	13.3	19.6	61.8	Le *et al.* (2019)
Grazing lamb meat	mg/100 g	25	7.1	23.7	55.8	Le *et al.* (2018)
Sheep milk	mg/250 ml	17.8	19.8	24.1	61.7	Nguyen *et al.* (2018b)
Sheep cheese	mg/40 g	14.3	12.8	17.1	44.2	Nguyen *et al.* (2019)
Cow milk	mg/100 g	3.3	-	4.4	-	Benbrook *et al.* (2013)

2.4.3.3. n-3 LC-PUFA consumption and chronic diseases

The biological functions of *n-3* LC-PUFA are firstly represented by their occurrence in all cellular membranes in all tissues of the body, and in particular, at high content levels in the retina, brain, and myocardium (Surette, 2008; Li and Hu, 2009). For example, due to a high concentration of DHA in the membranes of the human retina and brain, it plays an important role in regulating membrane receptors, membrane-bound enzymes and transduction signals (Li and Hu, 2009). In addition, *n-3* LC-PUFA have the potential to transform into a group of mediators such as the E-series and D-series resolvins at the expense of inflammation mediators from arachidonic acid (20:4n-6, ARA) which is the primary cause of various chronic disease treatments (Calder, 2006; Surette, 2008). Chronic inflammation that persists for a long time has a strong link with the development of many chronic diseases including cancer, cardiovascular (CVD), neurodegenerative, and respiratory diseases (Calder, 2014; Kunnumakkara *et al.*, 2018). Moreover, there is a positive correlation between *n-3* PUFA dietary consumption and incorporation of these FA into cell membranes (Zuijdgeest-Van Leeuwen *et al.*, 1999; Surette *et al.*, 2003) that explains a positive effect of adequate dietary *n-3* PUFA consumption on inhibiting chronic diseases.

Cardiovascular diseases refer to a collective term for heart and/or blood vessels related diseases that are by far, the most leading cause of mortality worldwide with 17.9 million deaths reported in 2018 (WHO, 2018). Therefore, the effects of *n-3* PUFA on major CVD including coronary heart disease (CHD) and stroke have been reported in numerous studies (Bu *et al.*, 2016; Mozaffarian *et al.*, 2016; Alexander *et al.*, 2017). One of the potential roles of *n-3* PUFA in reducing the risk of CHD is by counteracting many steps of atherosclerosis (Colussi *et al.*, 2017), the major cause of CHD (Frostegard, 2013). Novel findings demonstrated that enriched-DHA canola oil supplementation could reduce the risk of CHD by improving high-density lipoprotein cholesterol, triglycerides, and blood pressure (Jones *et al.*, 2014). In addition,

previous meta-analyses established the link between increasing intakes of *n-3* LC-PUFA and reducing the risk of CHD death by 10-30% (Alexander *et al.*, 2017). In terms of stroke, dietary consumption of *n-3* PUFA can reduce the volume of ischemic stroke (Shirley *et al.*, 2014) by promoting antioxidant enzyme activities or partly acting as an antioxidant. n-3 PUFA can provide further benefits relating to stroke post-treatments (Bu *et al.*, 2016), by generating other important responses such as neuranagenesis and revascularization. The latest meta-analysis of prospective cohort studies (Zhao *et al.*, 2019) supported a strong inverse relationship between daily fish intake and the risk of stroke.

Following CVD, cancer is the second most common cause of death (WHO, 2018). Clinical and epidemiological studies have demonstrated the role of *n-3* LC-PUFA in either reducing the risk of developing cancer or improving chemotherapy outcomes in existing cancer patients of several common types of cancer (Calder, 2014; Shahidi and Ambigaipalan, 2018). Long-term studies by Kato *et al.* (1997), Terry *et al.* (2001) and Takezaki *et al.* (2003) concluded that increased consumption of dietary *n-3* LC-PUFA lowered the risk of colorectal, prostate and lung cancer, respectively. Van Blarigan *et al.* (2017) also reported that higher intake of *n-3* LC-PUFA improved disease-free survival by 28% in colon cancer patients. The effect of these PUFA is more varied. While Holmes *et al.* (Holmes *et al.*, 2003) showed no relation between fish consumption and breast cancer, recent studies confirmed the positive impact of *n-3* fat on not only inhibiting (Sczaniecka *et al.*, 2012; Makarem *et al.*, 2013), but also reducing fatigue (Pereira *et al.*, 2018), in breast cancer patients. In contrast to the large number of studies that confirmed the positive effects of n-3 PUFA on these two major chronic diseases, other research findings reported neutral, inconclusive or even possible negative effects (Shahidi and Ambigaipalan, 2018). For instance, there was no statistically significant association between major CVD events and n-3 PUFA supplementation based on a meta-analysis of previous randomized clinical trials (Rizos *et al.*, 2012). Similarly, results from a large prospective cohort

study by Rhee *et al.* (2017) reported a neutral effect of n-3 PUFA intake on the risk of major CVD in healthy women aged ≥45 years. With respect to cancer, Holmes *et al.* (2003) showed that there was no relationship between fish consumption and breast cancer, while in one case, the intake of n-3 PUFA was associated with higher risk of basal cell carcinoma on skin cancer (Park *et al.*, 2017).

Apart from CVD and cancer, large studies have recognised the role of *n-3* LC-PUFA in regards to brain related cognitive treatments and other common chronic diseases such as rheumatoid arthritis, type-2 diabetes and obesity. Relating to brain issues in humans, bioactivities of *n-3* LC-PUFA, particularly DHA, play an important role in neural membrane structure, neurotransmission, and signal transduction (Salem *et al.*, 2001), and positive effects on treatment of different neurodegenerative and neurological disorders (Dyall, 2015). Lower *n-3* PUFA intakes have been reported to induce the risk of Alzheimer's disease (Cole *et al.*, 2009), while increased fish oil intakes for Parkinson's disease patients resulted in a significant reduction in depressive symptoms (da Silva *et al.*, 2008). Examining rheumatoid arthritis, Abdulrazaq *et al.* (2017) reported that a majority of studies confirmed the beneficial effect of utilising *n-3* LC-PUFA at doses of 3-6 g/day on pain relief in patients. Findings on the benefits of *n-3* PUFA consumption in type-2 diabetes and obesity remain inconsistent. While some authors have recognised that *n-3* PUFA intake can reduce the incidence of diabetes (Wang *et al.*, 2003; Tsitouras *et al.*, 2008), the findings from a systematic review and meta-analysis reported by Wu *et al.* (2012) suggested a neutral effect of EPA + DHA and seafood consumption on the development of diabetes. Similarly, no significant relationship between *n-3* PUFA and obesity was reported in the review by Albracht-Schulte *et al.* (Albracht-Schulte *et al.*, 2018). In contrast, high fish intake in men could lower the risk of being overweight (He *et al.*, 2002), although an opposite result was observed in women with higher fish consumption (Iso *et al.*, 2001).

The controversies regarding the role of n-3 PUFA in chronic diseases may be explained by many factors such as dose, duration, baseline intake (Thota *et al.*, 2018), specific type of the chronic disease and risk group (O'Connell *et al.*, 2017). Due to this continuous debate and variations in experimental design, it has not been very evident from current scientific literature and medical opinion confirming or rejecting the beneficial effects of n-3 PUFA in reducing the risk of human chronic diseases (Shahidi and Ambigaipalan, 2018). Therefore, large and unified clinical trials need to be conducted to conclusively identify the exact role of n-3 PUFA as independent or supplementary factors in specific chronic diseases.

2.4.4. Lipid metabolism in ruminants: Obstacles to enriching milk fat with n-3 PUFA

Since all of the long-chain FA in milk fat are derived from the absorption of fatty acids from the small intestine and body fat reserves that have both originated from dietary FA (Chilliard *et al.*, 2007; Shingfield *et al.*, 2013), manipulating the diet or feeding regime is the most popular way to alter milk fat composition. However, the efficiency of this approach in ruminants is still limited due to rumen microbial fermentation (Buccioni *et al.*, 2012a).

Dietary lipid sources for ruminants are mainly from forages, supplements or concentrates including cereal grains, oilseeds and animal fats. Lipids derived from forages contain largely glycolipids and phospholipids, while triglycerides are found primarily in concentrates or supplements (Harfoot and Hazlewood, 1988). Once dietary lipids enter the rumen, lipolysis occurs and it involves hydrolysis of ester linkages to release free fatty acids for the next biohydrogenation (BH) process (Buccioni *et al.*, 2012a) (Figure 2.4).

Under the activity of rumen microbes, unsaturated fatty acids (UFA) including PUFA are hydrogenated to monounsaturated FA (MUFA) and ultimately, saturated FA (SFA) through the addition of two hydrogen atoms to a double bond. The principal role of this process is to maintain a stable rumen environment by reducing the toxic effects of free UFA on bacterial

growth in the rumen (Harfoot and Hazlewood, 1988). Due to the high rate of hydrolysis and BH, only small amounts of PUFA from the diet can pass through the rumen into the duodenum for absorption (Bauman and Lock, 2006). According to Shingfield *et al.* (Shingfield *et al.*, 2010), dietary ALA in the rumen can be hydrogenated into 18:0 (Figure 2.5) at the rate of 85 to 100%. Both *in vivo* (Shingfield *et al.*, 2012) and *in vitro* (Kairenius *et al.*, 2011) studies have confirmed an extensive BH of dietary EPA and DHA that was greater than 90%. In contrast to ALA, these PUFA are not completely hydrogenated into SFA, but numerous intermediates are produced including a majority of UFA and much lesser amounts of SFA (Chilliard *et al.*, 2000).

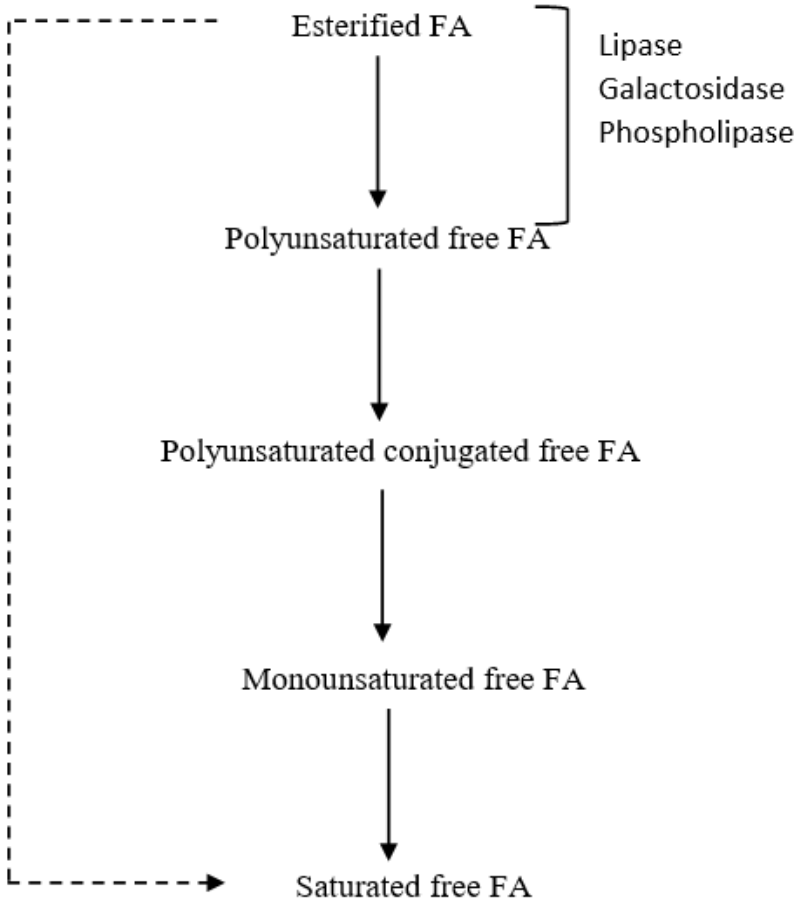

Figure 2.4. The scheme of lipolysis and biohydrogenation (adapted from Buccioni *et al.* (2012a)

The most recent *in vitro* study (Toral *et al.*, 2018) suggests that while the reduction of the double bond at the closest position to the carboxyl group is the main BH pathway of EPA and

DPA (Figure 2.6), this process is much less important for DHA. In addition, these authors stated that the possible interspecies differences between bovine and ovine BH of *n-3* LC-PUFA is directly correlated with slower and less complete BH observed in cattle, especially for EPA and DPA. However, the specific pathways for BH of individual *n-3* LC-PUFA still remain unclear.

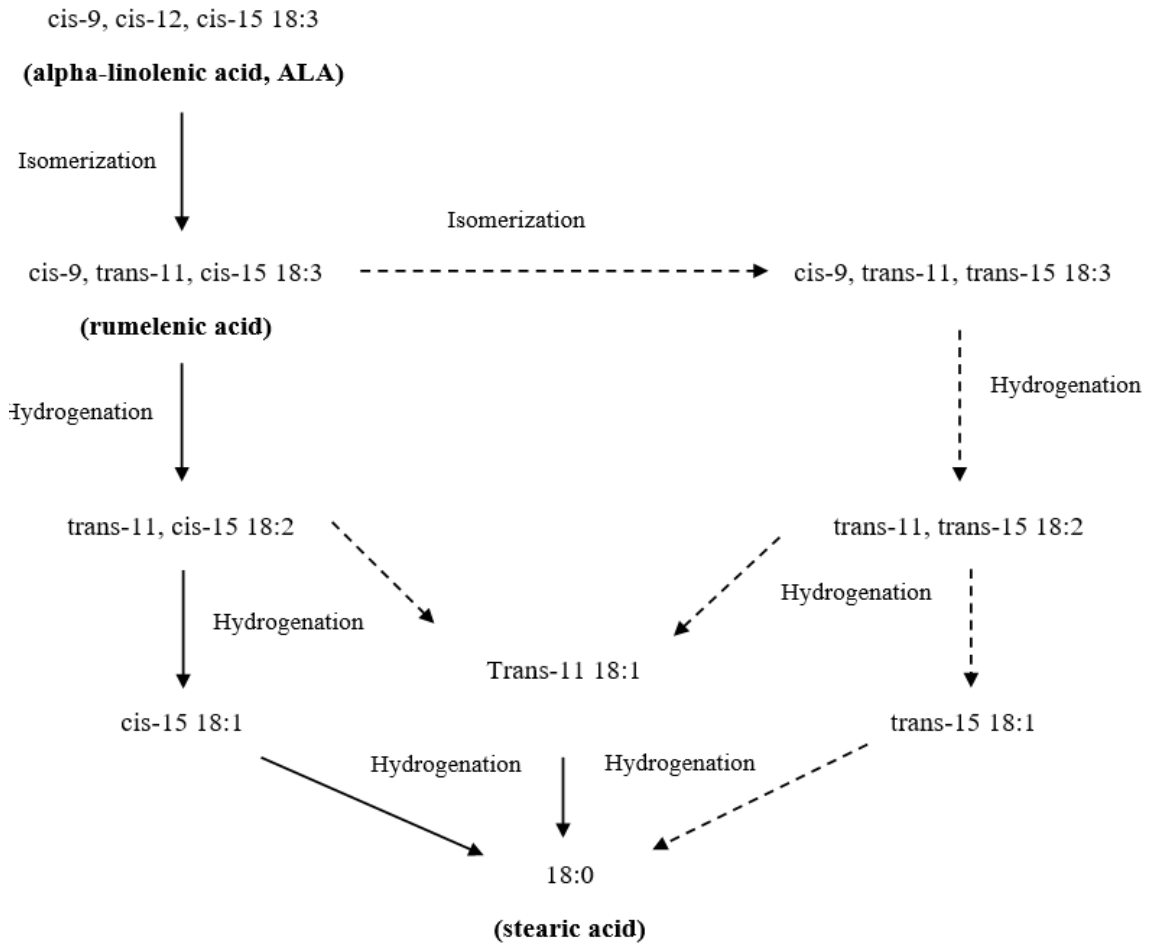

Figure 2.5. Ruminal biohydrogenation of α-linolenic acid. Thick arrows represent the major pathway; dotted lines with arrows represent putative pathway (adapted from Gomez-Cortes *et al.* (2009b).

Apart from ruminal BH, given the relatively low absorption rate from the small intestine into the mammary gland at 49% for ALA, and ranging from 14 to 33% for EPA, and from 13 to 25% for DHA (Shingfield *et al.*, 2013), it is not surprising that the proportion of these PUFA in dairy products is generally very low. Principal strategies for increasing *n-3* PUFA in milk

and milk products, therefore, have been to minimize the biohydrogenation effects of ruminal microbes and/or improving the absorption rate of these FA into the mammary gland.

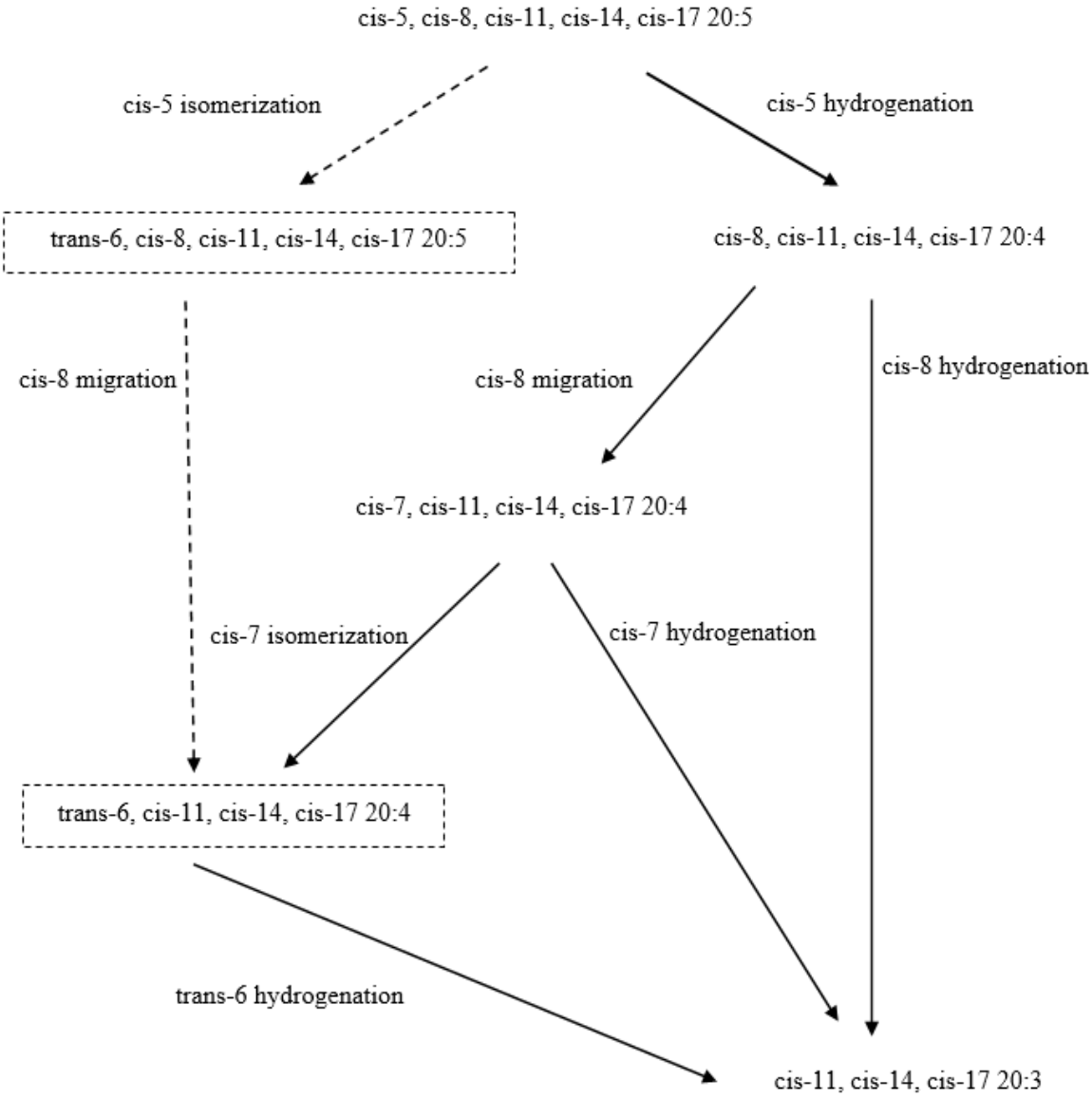

Figure 2.6. Possible biohydrogenation pathways of 20:5n-3. Solid arrows represent possible major pathway; dotted lines with arrows represents hypothetical pathway (adapted from Toral *et al.* (2018).

2.4.5. Recent attempts to increase n-3 PUFA content in dairy-derived products

Up to the present time, the nutritional manipulation of feeding regimes and supplementation with lipid sources containing high amounts of *n-3* PUFA (Shingfield *et al.*, 2013; Manso *et al.*, 2016) are the major approaches to improving *n-3* PUFA content in dairy products. In contrast, current efforts to employ genetic programmes in this theme have not yet yielded significant enhancement because the FA profile of milk processed products primarily depends on the FA composition of raw milk (Collomb *et al.*, 2006; Bisig *et al.*, 2007; Prandini *et al.*, 2011). Therefore, current studies mostly focus on milk content as the principal route of increasing *n-3* PUFA in other processed products.

2.4.5.1. Feeding regime

Previous studies had demonstrated that feeding regime, particularly changes in forage sources and feeding systems, had significant effects on short chain *n-3* PUFA, but minor effects on *n-3* LC-PUFA concentrations in both dairy ewes and cows (Table 2.9). This is because lipids from pasture sources contain abundant amounts of ALA (Dewhurst *et al.*, 2006; Woods and Fearon, 2009), but not EPA, DHA and DPA. For example, ALA content of fresh ryegrass varieties, a popular pasture used for ruminants worldwide, ranges from 62 to 74% of total fatty acids (Gilliland *et al.*, 2002). However, the pasture conservation processes, particularly grass wilting in the field, generally cause the oxidative loss of forage PUFA, subsequently and markedly reducing the content of ALA in hay or silage (Dewhurst *et al.*, 2006). Wilting ryegrass 24 h in glasshouse, for instance, reduced the percentage of ALA by 33% compared to unwilted grass (Elgersma *et al.*, 2003). Therefore, dairy ruminants that are kept in grazing systems or have free access to fresh grass produced much higher proportions of ALA in milk compared with animals fed conserved grass (hay and silage) (Leiber *et al.*, 2005; Gomez-Cortes *et al.*, 2009a; Mohammed *et al.*, 2009; Mierlita *et al.*, 2018). These results appear to be supported by the higher ALA intake of animals fed or grazed on fresh pastures.

Table 2.9. Effect of pasture feeding regimes on n-3 PUFA content of milk (g/100g fatty acids)

Forage source/ feeding system	Species	ALA	EPA	DHA	DPA	References
Ryegrass-dominated pastures	Bovine	0.703	0.083	0.009	0.109	Leiber *et al.* (2005)
Freshly harvested ryegrass		0.619	0.073	0.009	0.113	
Alpine pastures		1.146	0.083	0.009	0.120	
Freshly harvested Alpine		0.950	0.083	0.010	0.118	
Silage-concentrate diet (control)[1]		0.516	0.063	ND	0.082	
Ryegrass pasture	Bovine	0.68	0.05	0.02	0.07	Mohammed *et al.* (2009)
Freshly harvested ryegrass		0.82	0.07	0.02	0.08	
Ryegrass silage		0.34	0.05	0.02	0.09	
Indoor hay based diet	Bovine	0.72	0.08	-	0.147	Coppa *et al.* (2011)
Rotational grazing system		0.727	0.070	-	0.137	
Continuous grazing system		0.940	0.087	-	0.150	
Indoor conventional system	Bovine	0.579	0.072	-	0.118	Stergiadis *et al.* (2014)
Indoor organic system		1.199	0.098	-	0.098	
Mixed forage[2]	Bovine	0.47	-	-	-	Liu *et al.* (2016)
Corn stalk1 diet (35%)		0.58	-	-	-	
Corn stalk2 diet (53.8%)		0.63	-	-	-	
Daisy forb - winter	Ovine	1.62	-	-	-	Addis *et al.* (2005)
Ryegrass - winter		1.47	-	-	-	
Burr medic - winter		2.19	-	-	-	
Sulla - winter		2.98	-	-	-	
Daisy forb - spring		1.26	-	-	-	
Ryegrass - spring		1.44	-	-	-	
Burr medic - spring		1.84	-	-	-	
Sulla - spring		3.15	-	-	-	
Pasture	Ovine	1.07	0.06	-	0.13	Gomez-Cortes *et al.* (2009a)
Pasture + oat grain		0.59	0.05	-	0.12	
Total mixed ration[3]		0.33	0.03	-	0.06	
Grass hay (in door)	Ovine	1.31	0.19	0.30	-	Mierlita (Mierlita, 2016)
Part-time grazing		2.06	0.28	0.39	-	
Pasture	Ovine	2.09	0.30	0.37	-	Mierlita *et al.* (2018)
Pasture + standard concentrate		1.04	0.11	0.18	-	
Pasture	Ovine	0.44	0.01	0.07	0.13	Mohamed *et al.* (2018)
Pasture + concentrate		0.24	0.00	0.12	0.07	
Concentrate		0.21	0.00	0.00	0.08	
Red clover silage	Ovine	0.92	0.05	-	0.09	Guzatti *et al.* (2018)
Lucerne silage		0.70	0.05	-	0.09	

[1] The control diet contained 60% ryegrass silage, 30% maize silage and 10% grass hay on dry matter basis.

[2] Mixed forage contained 26.7% corn silage, 23.4% alfalfa hay and 3.7% Chinese wild rye on dry matter basis.

[3] Total mixed ration contained concentrate and forage in proportion of 80:20.

The transfer of *n-3* PUFA from forage into milk and milk products is also influenced by forage species (Table 2.9). Grazing dairy cows on diverse alpine pastures produced more ALA in their milk than on ryegrass-dominated paddocks (1.15 vs 0.70 g/100g FA) (Leiber *et al.*, 2005). Both Addis *et al.* (2005) and Bonanno *et al.* (2016) reported the greatest concentration of ALA in sheep milk and cheese from ewes grazed on Sulla pasture, versus other common forages including ryegrass, burr medic and daisy forb. Guzatti *et al.* (2018) showed higher levels of ALA in ewe milk for animals fed on clover silage compared with lucerne silage (0.92 vs 0.70 g/100g FA). Disparities observed between forage species in the transfer of *n-3* PUFA into milk in these studies were not correlated with ALA intake, but were associated with variation in condensed tannin content in the forages. The most possible mechanisms and effects of the condensed tannins were explained by Cabiddu *et al.* (Cabiddu *et al.*, 2009), in which tannins inhibited rumen microbial activities, thus ultimately lowering the PUFA biohydrogenation process in the rumen. The attempt to reduce microbial species involved in biohydrogenation such as B. proteoclasticus has been implemented with limited success due to many factors. See comprehensive coverage by Lourenco *et al.* (Lourenco *et al.*, 2010).

2.4.5.2. Lipid supplementation

Lipid supplementation has been used as an effective tool to improve animal performance due to its significant energy contribution (Woods and Fearon, 2009), and it can also alter FA composition of milk fat (Hristov *et al.*, 2004; Kennelly *et al.*, 2005). Fish oils and marine products, oilseeds and vegetable oils are the main sources that have been employed in ruminant diets to enhance the concentrations of health beneficial *n-3* PUFA and *n-3* LC-PUFA in milk and milk products (Woods and Fearon, 2009).

Oil seed and vegetable oil

Plant-derived fat is the most common fat source in ruminant supplements, and includes both oilseeds and extracted vegetable oils. This is because these materials not only contain a high concentration of PUFA (Dubois *et al.*, 2007), protein and energy (Petit, 2010), but are also more readily available and cheaper than other (marine) sources (Nguyen *et al.*, 2018). Therefore, a number of studies have examined the effects of oilseed and vegetable oils on the concentration of health beneficial *n-3* FA in both bovine and ovine milk products (Table 2.10). Based on previously reported results, the addition of flaxseed or linseed supplements in ruminant diets is a more effective strategy to enrich milk *n-3* PUFA compared to other plant fat supplementation methods (Table 2.10). Due to its very high content in ALA at approximately 53% of all FA (Bernacchia *et al.*, 2014), cows or sheep supplemented with flaxseed had substantial enhancement of this short chain *n-3* PUFA in milk products (Table 2.10).

Oil infusion is also considered an effective form of providing plant oil supplements that increases the escape rate of UFA from the BH of rumen microbes, thus enhancing the availability of *n-3* PUFA for absorption (Shingfield *et al.*, 2013). Khas *et al.* (Khas *et al.*, 2010) reported that adding 160 g/day of infused free ALA in the diet for lactating cows increased ALA content in milk by 41-fold, and also resulted in significant increases in milk EPA and DPA by two-fold and three-fold, respectively. However, supplementation with vegetable seed and oils only marginally increased milk EPA, DHA, and DPA in both bovines and ovines, with the percentages of these FA often lower than 0.1 g/100g FA (Table 2.10). These findings indicated that the endogenous biosynthesis pathway of these *n-3* LC-PUFA from dietary ALA in dairy animals is limited.

Table 2.10. Effect of supplementing ruminants with plant-derived dietary sources on n-3 PUFA concentration in milk and milk products (g/100g fatty acids)

Diet	Species	Product	ALA	EPA	DHA	DPA	References
Control	Bovine	Milk	0.61	0.09	-	0.07	Khas *et al.* (2010)
40 g/day infused LNA-rich FA[1]			6.49	0.18	-	0.12	
80 g/day infused LNA-rich FA			12.42	0.22	-	0.16	
120 g/day infused LNA-rich FA			18.75	0.21	-	0.29	
160 g/day infused LNA-rich FA			25.38	0.22	-	0.23	
Control	Bovine	Milk	0.75	0.003	0.001	-	Caroprese *et al.* (2010)
Whole flaxseed			0.81	0.022	0.001	-	
Control	Bovine	Milk	0.41	0.05	-	0.05	Dai *et al.* (2011)
Rapeseed oil			0.38	0.06	-	0.04	
Peanut oil			0.33	0.06	-	0.06	
Sunflower seed oil			0.32	0.06	-	0.05	
Control	Bovine	Milk	0.83	0.09	0.01	0.13	Otto *et al.* (2014)
25ml/kg DM[2] Canola oil			0.85	0.09	0.01	0.14	
35 ml/kg DM canola oil			0.95	0.08	0.01	0.12	
50 ml/kg DM canola oil			0.97	0.08	0.00	0.11	
Control	Bovine	Milk	0.28	0.02	-	-	Cattani *et al.* (2014)
500 g/day extruded flaxseed			0.50	0.02	-	-	
1000 g/day extruded flaxseed			0.59	0.02	-	-	
Linseed oil	Bovine	Milk	0.249	0.019	-	0.014	Li *et al.* (2015)
Safflower oil			0.180	0.013	-	0.007	
Control	Bovine	Milk	0.19	0.012	0.004	0.037	Welter *et al.* (2016)
3% Canola oil			0.36	0.011	0.003	0.034	
6% Canola oil			0.35	0.011	0.003	0.033	
Control	Bovine	Milk	0.19	-	0.019	-	Vanbergue *et al.* (2018)
Extruded linseed			0.51	-	0.008	-	
Palm oil	Ovine	Milk	0.52	0.04	0.02	0.08	Bodas *et al.* (2010)
Olive oil			0.36	0.03	0.02	0.06	
Soybean oil			0.53	0.03	0.02	0.07	
Linseed oil			1.07	0.05	0.04	0.11	
Control	Ovine	Milk	1.21	0.05	0.05	-	Mughetti *et al.* (2012)
100 g extruded linseed			1.65	0.06	0.09	-	
200 g extruded linseed			2.26	0.06	0.10	-	
Control	Ovine	Milk	0.57	0.07	0.05	0.08	Caroprese *et al.* (2016)
Seaweed			0.59	0.06	0.04	0.08	
Whole flaxseed			1.53	0.08	0.05	0.09	
Seaweed + Whole flaxseed			1.32	0.08	0.06	0.10	

Table 2.10. ***(Continued)*** Effect of supplementing ruminants with plant-derived dietary sources on *n-3* PUFA concentration in milk and milk products (g/100g fatty acids)

Diet	Species	Product	ALA	EPA	DHA	DPA	References
Control	Ovine	Milk	0.62	0.08	0.04	0.08	Nguyen *et al.* (2018b)
Canola oil			0.73	0.09	0.06	0.13	
Rice bran oil			0.51	0.07	0.04	0.10	
Flaxseed oil			1.74	0.11	0.06	0.15	
Safflower oil			0.67	0.07	0.06	0.10	
Control	Ovine	Milk	0.31	0.04	0.02	0.08	Parentet *et al.* (2018)
Canola oil			0.26	0.03	0.02	0.07	
Sunflower oil			0.24	0.03	0.02	0.07	
Castor oil			0.28	0.05	0.01	0.08	
Control	Bovine	Cheese	0.29	0.02	-	-	Cattani *et al.* (2014)
500 g/day extruded Flaxseed at			0.50	0.02	-	-	
1000 g/day extruded Flaxseed at			0.61	0.02	-	-	
Palm oil	Ovine	Cheese	0.54	0.04	0.02	0.07	Bodas *et al.* (2010)
Olive oil			0.36	0.03	0.03	0.06	
Soybean oil			0.51	0.03	0.02	0.06	
Linseed oil			1.04	0.03	0.03	0.09	
Control	Ovine	Cheese	1.18	0.02	0.03	-	Mughetti *et al.* (2012)
100 g extruded linseed			1.84	0.04	0.05	-	
200 g extruded linseed			2.02	0.04	0.06	-	
Control	Ovine	Cheese	0.71	0.11	0.06	0.12	Nguyen *et al.* (2019)
Canola oil			0.79	0.11	0.06	0.13	
Rice bran oil			0.63	0.10	0.06	0.12	
Flaxseed oil			1.30	0.11	0.06	0.13	
Safflower oil			0.71	0.11	0.08	0.13	
Control	Ovine	Yogurt	0.0	-	-	-	Bianchi *et al.* (2017)
2% Palm oil			0.0	-	-	-	
4% Palm oil			0.28	-	-	-	
6% Palm oil			0.31	-	-	-	

[1] FA: fatty acid
[2] DM: dry matter

Marine lipid sources

Feeding dairy animals with marine oil resulted in the highest *n-3* LC-PUFA concentration in milk and milk products (Table 2.11) among all types of lipid supplements examined. Previous studies also confirmed the efficiency of utilising rumen-protected forms of marine products

that were markedly higher than in the untreated controls; mainly as a result of the lesser extent of ruminal biohydrogenation with the rumen-protected diets (Kitessa *et al.*, 2001a). Kitessa *et al.* reported that the content of EPA and DHA, which are generally little in milk (Tables 2.9 & 2.10), could be increased by supplementing both dairy cattle (Kitessa *et al.*, 2004) and ewes (Kitessa *et al.*, 2003) with rumen-protected fish oil. The proportion of DHA, the most essential *n-3* LC-PUFA, observed in these studies, exceeded 1% of the total FA. Similarly, an effective incorporation rate of DHA from a marine algae supplement, an alternative to fish oil into milk, was also confirmed by a number of studies (Table 2.11). This transfer rate appears to be higher as observed in ovine (Papadopoulos *et al.*, 2002) than in bovine (Boeckaert *et al.*, 2008). Results presented in Table 2.11 also indicate that supplementing fish oil is more advantageous than marine algae in terms of improving milk EPA and DPA content.

Recent focus on achieving quantitatively significant amounts of *n-3* PUFA per standard serve of milk and milk products has occurred (Nguyen *et al.*, 2018b; Nguyen *et al.*, 2019). This absolute FA concentration data, may be more accurate than the proportion (expressed as %FA) itself, since fat percentage of milk from different species varies widely (Park *et al.*, 2007), and such quantitative data can potentially assist consumers in purchasing decisions. One serve of fresh milk produced from grazing ewes supplemented with rumen-protected EPA + DHA contains 62 mg of total *n-3* LC-PUFA, three-fold higher than the control group (Nguyen *et al.*, 2018b). This result is higher than the concentration of total EPA + DHA + DPA in one serve of cooked lamb meat (55 mg) reported by Flakemore *et al.* (2017). In achieving 60 mg/serving, this sheep milk can also be considered as achieving a "good source" level of *n-3* LC-PUFA adhering to Food Standards Australia and New Zealand (FSANZ) (2002). Although the inclusion of fish oil into ruminant diets might have a negative effect on meat quality such as possible rancidity and abnormal flavour in cooked or grilled lamb (Watkins *et al.*, 2013), no side effects on milk and milk products have been reported. Nguyen *et al.* (2019) observed no differences in sensory eating traits between ripened cheese processed from milk produced by dairy sheep supplemented with rumen-protected marine source and the unsupplemented group.

However, the higher cost of the marine oil source possibly limits its utilisation as a routine supplementation for dairy ruminants (Woods and Fearon, 2009).

Table 2.11. Effect of supplementing ruminants with dietary marine sources on n-3 PUFA concentration of milk and milk products (g/100g fatty acids)

Diet	Species	Product	ALA	EPA	DHA	DPA	References
Control	Bovine	Milk	0.54	-	0.00	-	Franklin *et al.* (1999)
Protected algae			0.49	-	0.76	-	
Unprotected algae			0.47	-	0.46	-	
Control	Bovine	Milk	0.86	0.0	0.0	-	Kitessa *et al.* (2004)
Rumen-protected tuna oil			1.28	0.61	1.09	-	
Control	Bovine	Milk	0.21	0.03	0.00	0.07	Shingfield *et al.* (2006)
Fish oil and sunflower oil			0.23	0.11	0.07	0.16	
Control	Bovine	Milk	0.50	-	0.09	-	Boeckaert *et al.* (2008)
Marine algae			0.42	-	1.01	-	
ABO/ABO[1]	Bovine	Milk	14.4	0.22	-	0.22	Kazama *et al.* (2010)
RUM/ABO[2]			4.78	0.14	-	0.22	
RUM/RUM[3]			2.33	0.09	-	0.12	
ABO/RUM[4]			11.6	0.16	-	0.18	
Control	Bovine	Milk	0.75	0.003	0.001	-	Caroprese *et al.* (2010)
Fish oil			0.84	0.060	0.117	-	
Control	Bovine	Milk	0.45	0.06	0.10	-	Vargas-Bello-Pérez *et*
Fish oil			0.62	0.10	0.21	-	*al.* (2015b)
Fish oil + palm oil			0.69	0.09	0.14	-	
Control	Bovine	Milk	0.41	0.06	0.03	0.09	Kairenius *et al.* (2015)
Ultrarefined fish oil at 75 g/day			0.38	0.06	0.03	0.08	
Ultrarefined fish oil at 150 g/day			0.39	0.07	0.05	0.10	
Ultrarefined fish oil at 300 g/day			0.48	0.17	0.10	0.18	
Control	Bovine	Milk	0.19	-	0.019	-	Vanbergue *et al.* (2018)
Microalgae DHA Gold®			0.25	-	0.444	-	
Extruded linseed + DHA Gold®			0.46	-	0.170	-	
Control	Ovine	Milk	0.33	ND	ND	ND	Papadopoulos *et al.*
Low algae (23.5 g)			0.31	0.04	0.43	0.21	(2002)
Medium algae (47 g)			0.33	0.12	0.69	0.28	
High algae (94 g)			0.25	0.21	1.24	0.31	

Table 2.11. ***(Continued)*** Effect of supplementing ruminants with dietary marine sources on *n-3* PUFA concentration of milk and milk products (g/100g fatty acids)

Diet	Species	Product	ALA	EPA	DHA	DPA	References
Control	Ovine	Milk	0.53	0.05	0.03	0.10	Toral *et al.* (2010)
Sunflower oil (SO)			0.41	0.04	0.02	0.07	
SO + 8 g/ kg DM of Marine Algae			0.37	0.05	0.17	0.10	
SO + 16 g/ kg DM of Marine Algae			0.36	0.09	0.46	0.13	
SO + 24 g/ kg DM of Marine Algae			0.34	0.10	0.57	0.15	
Sunflower oil	Ovine	Milk	0.49	0.04	0.05	0.10	Bichi *et al.* (2013)
Sunflower oil + Marine algae			0.48	0.06	0.38	0.12	
Control	Ovine	Milk	0.62	0.08	0.04	0.08	Nguyen *et al.* (2018b)
Rumen-protected EPA+DHA oil			0.74	0.17	0.19	0.23	
Control	Bovine	Cheese	0.01	0.05	0.09	-	Vargas-Bello-Pérez *et*
Fish oil			0.02	0.12	0.34	-	*al.* (2015b)
Fish oil + palm oil			0.01	0.09	0.18	-	
Control	Ovine	Cheese	0.71	0.11	0.06	0.12	Nguyen *et al.* (2019)
Rumen-protected EPA+DHA			1.02	0.16	0.15	0.19	

[1] ABO/ABO diet contains abomasal flax oil and hulls infusion.

[2] RUM/ABO diet contains flax oil placed in the rumen and hulls infused in the abomasum.

[3] RUM/RUM diet contains flax oil and hulls placed in the rumen and abomasal infusion of water.

[4] ABO/RUM diet contains flax hulls administered in the rumen and abomasal flax oil infusion

2.4.5.3. Genetic manipulation as a potential tool for the enrichment of dairy products with n-3 PUFA

Attempts at understanding and estimating genetic parameters influencing milk FA content that may be beneficial for human health had been made a decade ago (Soyeurt *et al.*, 2007; Stoop *et al.*, 2008). Up to the present time, low heritabilities (<0.1) for individual *n-3* PUFA (Table 2.12) were consistently reported in dairy cows (Stoop *et al.*, 2008; Bilal *et al.*, 2014; Pegolo *et al.*, 2016) and dairy sheep (Correddu *et al.*, 2018), indicating a low impact of genetics or breed on the concentration of *n-3* PUFA. This observation probably arises because the fatty acids longer than 18 carbon chains are not *de novo* synthesised in the mammary gland, but are circulated from the blood which contains lipids that originated from the diet (Chilliard *et al.*, 2000). Moderate heritabilities for the whole group of *n-3* PUFA were reported by Boichard *et*

al. (2014) and Maroteau *et al.* (2014), and could be explained by major contribution of the short-chained *n-3* PUFA. First identified in the human genome in 2000 (Marquardt *et al.*, 2000), fatty acid desaturase 1 and 2 (FADS1 and FADS2) are considered as the major candidate genes that regulate the endogenous synthesis of *n-3* LC-PUFA from ALA in mammals including ruminants (Malau-Aduli *et al.*, 2011; Malau-Aduli and Kashani, 2015; Malau-Aduli *et al.*, 2016; Malau-Aduli *et al.*, 2019). The first effort to define the association between these two encoding genes and *n-3* PUFA in the milk of Holstein cows (Ibeagha-Awemu *et al.*, 2014) found that three significant single nucleotide polymorphism (SNP) markers within FADS1 and FADS2 were associated with EPA. Apart from the two well-characterized FADS1 and FADS2, Ibeagha-Awemu *et al.* (Ibeagha-Awemu *et al.*, 2016) uncovered more potential candidate genes with several novel SNPs that were significantly associated with milk EPA and DPA. Consequently, by employing these potential genetic markers, future research can investigate the specific relationships between combining genetics and other environmental strategies such as nutritional supplementation for elevating *n-3* LC PUFA in milk.

Table 2.12. Heritability estimates of major individual and group of n-3 fatty acids

Breed	Species	Unit	ALA	EPA	DPA	*n-3*	Reference
Holstein-Friesians	Bovine	%FA	0.09	-	-	-	Stoop *et al.* (2008)
Holsteins	Bovine	% FA	0.06	0.04	0.01	-	Bilal *et al.* (2014)
Brown Swiss	Bovine	% FA	0.093	0.045	0.039	0.085	Pegolo *et al.* (2016)
Sarda	Ovine	% FA	0.02	-	-	-	Correddu *et al.* (2018)
Holstein	Bovine	% fat	-	-	-	0.26	Boichard *et al.* (2014)
Saanen	Caprine		-	-	-	0.23	
Lacaune	Ovine		-	-	-	0.18	
Alpine	Caprine	% fat	-	-	-	0.28	Maroteau *et al.* (2014)
Saanen	Caprine		-	-	-	0.25	

2.5. Nutritional aspect of sheep cheese and factors driving cheese eating quality

2.5.1. Nutritional aspects of sheep cheese

Similar to cow and goat cheese, the major nutritional components of sheep cheese include fat and protein. Fat and protein concentration in sheep cheese varies widely across different breeds depending largely on the composition of the raw milk and cheese processing method (Raynal-Ljutovac *et al.*, 2008) (Table 2.13).

Table 2.13. Major nutritional properties of sheep cheese (%) (Source: Raynal-Ljutovac et al. (2008)

Cheese	Breed	Age of cheese	Total solids	Fat	Protein
Ricotta	Sarda		30	18	
Canestrato Pugliese		1-56 days	39	31	25
Canestrato Pugliese		10-12 months	67	30	27
Fiore Sardo			70	29	28
Pecorino Romano			65	30	27
Manchego	Manchega	90 days	63		
Soft lactic		0-33 days	53	26	
Manchego		1-9 months	37	31	25
Feta		3-240 days	45	22	18
Serra da Estrela		1, 7, 21, 35 days			
Manchego			66		
Serena			58		
Halloumi		Fresh	65	32	23
Terrincho		0-60 days	46	25	21
Pecorino	Sarda	1 day/60 days	70	37/36	26
Manchego	Manchega	90 days	70	30/42/37	23
Los Pedroches	Merinos	2-100 days	35	31/33	26
Robiola delle Langhe		1, 11, 28 days	49	24	18
Roquefort			57	33	19
Ossau-Iraty			61	32	24

In general, ruminant dairy products containing a high proportion of SFA contribute to cardiovascular diseases (Mozaffarian *et al.*, 2010). Menotti *et al.* (1999) reported a positive correlation between milk and cheese consumption and coronary heart disease (R = 0.6 and R=0.407, respectively). However, this meta-analysis failed to mention the specific type of cheese, particularly the animal species these cheeses were produced from. Three weeks' consumption of sheep cheese in contrast, was demonstrated to significantly increase the plasma concentrations of α-linolenic, conjugated linoleic, vaccenic, and eicosapentaenoic acids at the expense of low-density lipoprotein (LDL) concentration reduction by 7% in adults diagnosed with mild hypercholesterolemia (Pintus *et al.*, 2013). According to Mihaylova *et al.* (2012), the reduction in LDL could inhibit the risk of major vascular diseases. Under the same production technology, sheep cheese contains a higher level of healthy fatty acids for human consumption including conjugated linoleic acid (CLA) and total n-3 PUFA (Prandini *et al.*, 2011; Aguilar *et al.*, 2014) than cow and goat cheeses. Beneficial human health effects were also indicated by the lower content of palmitic acid (16:0) in sheep cheese in comparison with other ruminant cheese (Aguilar *et al.*, 2014). This medium-chain FA intake is well-known to have a positive association with CHD risk in humans (Praagman *et al.*, 2016).

2.5.2. Factors driving cheese eating quality

Appearance, flavour (taste and aroma) and texture are very important features that determine cheese quality (Fox *et al.*, 2000) and affect consumer choice (Awad *et al.*, 2007). Cheese flavour defined as organoleptic properties, is the most important quality attribute formed by a large complex of sapid and aromatic compounds (McSweeney and Sousa, 2000). These elements are developed through biochemical and microbiological activities during cheese manufacture (McSweeney, 2004). Therefore, factors that influence different stages of cheese making including milk supply, rennet (coagulant), starter, non-starter lactic acid bacteria, cheese composition, ripening temperature and their complex interactions all contribute to

cheese quality (Fox *et al.*, 2000) (Figure 2.7). This complexity explains some of the reasons behind unsuccessful efforts to produce consistent premium-quality cheese (Fox *et al.*, 2000).

The foundation of cheese flavour is based on the quality of raw milk contributed mainly by total somatic cell count (SCC) and chemical composition (Amenu and Deeth, 2007; Murphy *et al.*, 2016) that are indirectly influenced by animals' characteristics and nutrient inputs (Coulon *et al.*, 2004). SCC has a negative correlation with cheese quality (Andreatta *et al.*, 2007; Mazal *et al.*, 2007; Murphy *et al.*, 2016). Milk utilised for cheese making is recommended to contain less than 300,000/ml of SCC (Fox *et al.*, 2000). Milk content with emphasis on casein, fat, and calcium contents as discussed in Section 2.2, has a major impact on both cheese yield and composition and subsequently affects cheese texture and quality. Therefore, all genetic and non-genetic parameters, discussed in Section 2.2, as features that impact on the composition of milk, may indirectly influence cheese quality. Bonanno *et al.* (2013) observed smoother texture, sweeter, and more acidic taste in cheese produced from Cinisara than Brown cow milk, those are the local breeds in Sicily, Italy. In contrast, Brown cow milk produces cheese with more bitterness and saltiness.

The review by Kilcawley *et al.* (2018) suggested that pasture-based diets for dairy cows increase yellow density of ripened cheese resulting in the enhancement of cheese eating quality. Different levels of concentrate supplementation for grazing dairy cows also modifies cheese sensory properties (Bovolenta *et al.*, 2009). The relationships between lipid supplementation of dairy animals and fatty acid changes and cheese eating quality have been examined, but still remain unclear. Najera *et al.* (2017) and Vargas-Bello-Perez *et al.* (2015a,b) reported a neutral effect of rapeseed oilcake and different vegetable oil supplements on cheese sensory attributes in sheep and cows, respectively. On the other hand, Sympoura *et al.* (2009) demonstrated the ability of altering odour compounds in cheese produced from cow milk fed extruded linseed. The influence of milk fatty acid composition on cheese sensory attributes probably because volatile flavour compounds that form the cheese flavour are derived from raw milk fatty acids (McSweeney, 2004).

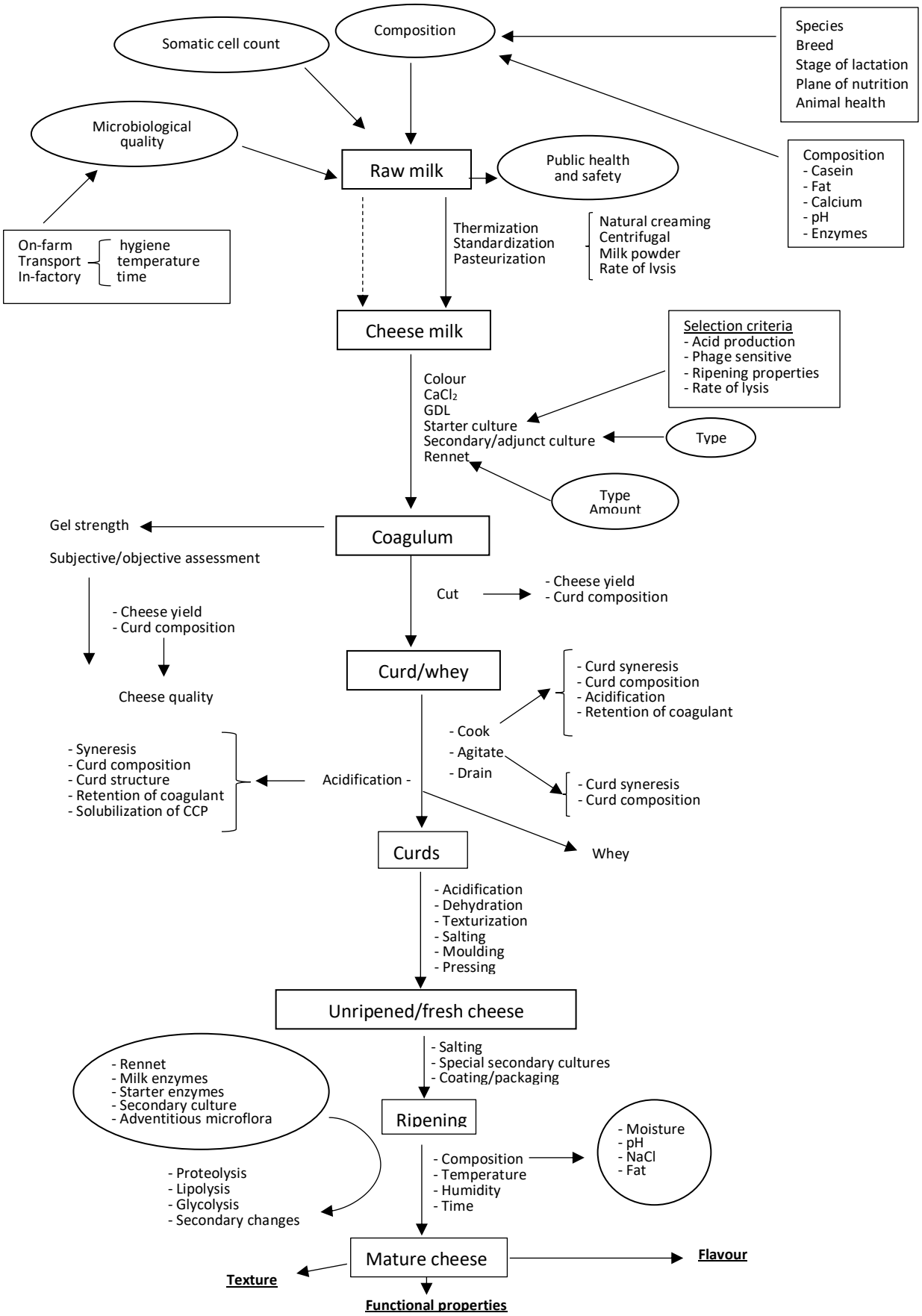

Figure 2.7. Factors affect cheese quality, adapted from (Fox *et al.*, 2000)

2.6. Justification and Research Objectives of the study

Feeding programs in animal production should take into account animal requirements for optimal growth, productive performance and product quality. Other important production parameters include feed availability, costs of nutrients, animal health and wellbeing. Furthermore, feed supplementation at a commercial level should not have negative impacts on consumer ethics, eating quality and acceptability.

Evidence from published literature suggests that the nutritional content of feeds has an impact on milk production, milk composition, and fatty acid profile of sheep milk, with subsequent implications on the value of cheese product. It is also evident that there are considerable existing knowledge gaps in the use of plant oil supplementation on dairy sheep and its products, especially under Australian pasture-based production conditions. Therefore, the aim of this study is to compare the utilisation of different oils of plant origin as supplementary feed sources and evaluating their incorporation into dairy sheep milk and cheese for commercial production.

Therefore, the research objectives needed to address the identified knowledge gaps are:

- To compare and evaluate the impact of different oils of plant origin and rumen-protected EPA + DHA as dietary supplements on grazing dairy sheep performance, including: body conformation, feed intake, milk yield, milk composition and milk and cheese fatty acid profiles.
- To examine cheese eating quality and consumer acceptability of products from dairy sheep supplemented with different types of oils.

Chapter 3: Supplementing dairy ewes grazing low quality pastures with plant-derived and rumen-protected oils containing EPA + DHA pellets increases body condition score and milk, fat, and protein yields

3.1. Abstract

The Australian dairy sheep industry is small and mostly based on a natural grass grazing system, which can limit productivity. The current study tested different plant oil-infused and rumen protected polyunsaturated fats and their interactions with sire breeds to improve lactation traits and body condition scores (BCS) of ewes grazing low quality pastures. It was hypothesised that supplementing lactating ewe's diets with plant-derived polyunsaturated oils would improve milk production and composition without compromising BCS. Sixty ewes (n = 10/treatment) in mid-lactation, balanced by sire breed, parity, milk yield, body condition score, and liveweight, were supplemented with: (1) control: wheat-based pellets without oil inclusion; wheat-based pellets including; (2) canola oil (CO); (3) rice bran oil (RBO); (4) flaxseed oil (FSO); (5); safflower oil (SFO); and (6) rumen protected marine oil containing eicosapentaenoic acid and docosahexaenoic acid (RPO). Except for the control group, all supplementary diets included the same level of 50 mL/kg DM of oil and all diets were isocaloric and isonitrogenous. Experimental animals were grazed in the same paddock with *ad libitum* access to pasture, hay, and water during the 10-week study. RPO was the most effective diet that enhanced milk, fat, and protein yields by approximately 30%, 13%, and 31%, respectively ($p < 0.0001$). A significant increase in milk production was also observed with CO, RBO, and SFO treatments ($p < 0.0001$) by 8.5%, 8.9%, and 16.1%, respectively. Breed significantly influenced animal performance with higher milk yields recorded for crossbred Awassi x East Friesian (AW x EF) (578 g/day) vs. purebred Awassi (452 g/day) ($p < 0.0001$). This study

provides empirical evidence for the use of rumen-protected and plant-derived oil-infused pellets as supplements under low quality pasture grazing conditions to improve the production performance of purebred Awassi and crossbred AW x EF ewes.

3.2. Introduction

Although previously published studies have demonstrated that sheep milk has more nutritional value compared to cow milk (Park *et al.*, 2007; Silanikove *et al.*, 2015), the contribution of milk derived from sheep to national milk production in Australia is relatively low. As of 2013, there were 13 commercial farms producing 550,000 litres of milk annually (AgriFutures Australia, 2013) compared to 9 billion litres of milk produced by dairy cows nationwide (Dairy Australia, 2018). Milk yield and composition are influenced by various factors, including diet, breed, age, management practices, health, and the environment (Caja and Bocquier, 2000; Abd Allah *et al.*, 2011; Ayadi *et al.*, 2014). Dietary supplementation with fat is considered as an effective tool to improve milk yield and alter milk composition (Hristov *et al.*, 2004; Kennelly *et al.*, 2005). Plant derived oils are a potential source of dietary fat and have been used in ruminant feeds to increase the energy density of diets and modify the milk fatty acid profile (Caja and Bocquier, 2000; Chilliard *et al.*, 2003; Pulina *et al.*, 2006), with the aim of increasing n-3 long–chain (≥C20) polyunsaturated fatty acids (n-3 LC-PUFA) in dairy products. This is because high consumption of n-3 LC-PUFA in humans has been demonstrated to inhibit adipogenic, diabetogenic, atherogenic (McGuire and McGuire, 2000), inflammatory (Calder, 2012; Calder, 2013), and carcinogenic (Belury, 2002) diseases and lower the risk of developing Alzheimer's disease (Calon and Cole, 2007). A number of authors have demonstrated that while dietary fat supplements can enhance milk yield (Castro *et al.*, 2009; Bernal-Santos *et al.*, 2010; Otto *et al.*, 2015; Pirondini *et al.*, 2015), it is generally accompanied by a decrease in milk fat and protein compositions because of the negative correlation between milk solids concentration and milk yield in dairy sheep (Caja and Bocquier, 2000; Pulina *et al.*, 2005). This

could reduce the income of the producers as milk is generally traded based on total milk solids. For this reason, the use of fats as dietary sources to improve the milk yield of sheep used for commercial milk harvesting within Australia is not widely undertaken and is mostly applied as a supplement only during the dry seasons when pasture quality and quantity are low, in order to increase the energy intake of lactating animals (Akbaridoust *et al.*, 2014).

To our current knowledge, studies on the effect of dietary supplementation with rice bran, canola, and safflower oils on milk yield and composition have only been conducted with dairy cows (Bell *et al.*, 2006; Lunsin *et al.*, 2012a,b; Otto *et al.*, 2015) and goats (Park *et al.*, 2013), but not dairy ewes. The effects of supplementation with flaxseed on animal performance and milk fatty acid profiles have been studied with dairy ewes, however, these investigations supplemented flaxseed either as whole or extruded grain (Caroprese *et al.*, 2011; Mughetti *et al.*, 2012; Caroprese *et al.*, 2016). In addition, there has been a paucity of studies that have examined the effects of different dietary sources of supplementation on lactation and liveweight traits in grazing dairy ewes of different genetic backgrounds under the same management and feeding regime.

The major objective of the current work was to fill these knowledge gaps by comparing the lactation performance, milk composition, and body condition score of dairy ewes in mid lactation grazing low quality pastures and supplemented with canola, rice bran, flaxseed, safflower, and rumen protected oil-infused pellets. It was hypothesised that supplementing grazing dairy ewes with oils of different plant-derived and marine origins will have different effects on milk yield, milk composition, and body condition score.

3.3. Materials and Methods

3.3.1. Animal Management and Experimental Design

The use of animals and procedures performed in this study were all approved by the University of Tasmania Animal Ethics Committee (Permit No A0015657).

Sixty lactating Awassi and crossbred Awassi _ East Friesian ewes in mid-lactation, located in the South East of Tasmania (Grandvewe Cheeses Farm, Birchs Bay, Woodbridge, Tasmania, Australia), were included in a 10-week feeding trial where the ewes were kept in the same paddock and had *ad libitum* access to local natural velvet tussock grass, hay, and water. The experimental animals were allocated to six dietary treatments with each group balanced for liveweight (59±5.9 kg), breed, parity (2.8±0.5), body condition score (BCS), and milk yield (657±100 g/day). Treatments consisted of (1) commercial wheat-based pellets without oil inclusion (control); wheat-based pellets infused with 50 mL/kg DM of (2) canola (CO); (3) rice bran (RBO); (4) flaxseed (FSO); (5) safflower (SFO), and (6) rumen protected EPA + DHA (RPO) oils, as represented in Table 3.1. The RPO treatment was based on a modification of the microencapsulation of oil droplets in a protein-aldehyde matrix procedure (Scott *et al.*, 1971). All treatments were isocaloric and isonitrogenous (Table 3.2). Each ewe was fed 1 kg/day of the supplemented pellet individually in the milking parlour during milking time over a 10-week period with an initial two-week adjustment period, followed by an 8-week experimental period. In the first two weeks of the adjustment period, commercial pellets (control) for each treatment group were increasingly substituted at 100 g/day by the experimental diets, CO, RBO, FSO, SFO, and RPO, until the attainment of 1 kg/day on day 10 was achieved. Ewes were milked in the mornings at 0600 h and individual milk yield was electronically recorded by the La Laval platform using De Laval's Alpro Herd Management System software version 6.54 (De Laval, Tumba, Sweden).

Table 3.1. Ingredient composition of the experimental pellets[a.]

Items	Control	CO	RBO	FSO	SFO	RPO
Ingredient, g/kg						
Wheat	585	545	535	465	535	530
Paddy rice	210	210	220	280	210	215
Lupins	148	148	148	148	148	148
Canola oil, ml/kg	-	50	-	-	-	-
Flaxseed oil, ml/kg	-	-	-	50	-	-
Safflower oil, ml/kg	-	-	-	-	50	-
Rice bran oil, ml/kg	-	-	50	-	-	-
EPA+DHA, ml/kg	-	-	-	-	-	50
Ammonium sulphate	12.6	12.6	12.6	12.6	12.6	12.6
Salt	10	10	10	10	10	10
Limestone	20.9	20.9	20.9	20.9	20.9	20.9
Sheep premix	1	1	1	1	1	1
Acid buffer	6.25	6.25	6.25	6.25	6.25	6.25
Sodium bicarbonate	6.25	6.25	6.25	6.25	6.25	6.25

[a] Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO). Sheep premix contains Calcium, Phosphorous, Ammonium Chloride, Magnesium, Sulphur, Manganese, Iron, Zinc, Copper, Selenium, Cobalt, Iodine, Sodium Chloride, Vitamins, A, D E. Vitamin B1, Molasses.

3.3.2. Feed intake and body condition score

The amount of offered pellets and residuals were weighed daily to calculate supplement intake. Weekly feed samples were collected and stored at -20 °C for subsequent chemical analysis. Body condition score (BCS) was subjectively evaluated at weekly intervals on a scale of 1-5 (Kenyon *et al.*, 2014) by the same evaluator to ensure consistency and repeatability.

Table 3.2. Nutrient compositions[a] of basal and experimental diets[b]

Component (% DM)	Pasture	Hay	Control	CO	RBO	FSO	SFO	RPO
DM	96.5	95.5	91.5	93.0	91.6	90.0	91.7	91.6
OM	90.5	97.3	92.2	93.3	92.7	91.0	91.8	92.0
Ash	9.5	2.7	7.8	6.7	7.3	9.0	8.2	8.0
ADF	45.5	37.6	10.6	7.1	8.1	9.7	9.0	8.5
NDF	69.9	68.3	30.0	21.8	19.4	23.3	23.9	22.0
EE	1.4	1.2	3.3	5.7	5.2	5.4	5.0	5.1
CP	4.7	4.3	14.6	14.0	14.7	14.6	14.5	15.6
TDN	48.5	54.1	73.4	75.9	75.2	74.1	74.5	74.9
ME, MJ/kg DM	7.1	8.1	11.7	12.2	12.0	11.8	11.9	12.0

[a] Dry matter (DM), organic matter (OM), acid detergent fibre (ADF), neutral detergent fibre (NDF), ether extract (EE), crude protein (CP), (TDN) and metabolisable energy (ME).

[b] Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO).

3.3.3. Milk sample analyses

Weekly milk samples from each animal were bulked from daily milkings at 0600 h and stored in labelled plastic vials containing bronopol blue preservative at 4 °C before sending the samples off to Hadspen for compositional analysis at the officially contracted herd recording laboratory - TasHerd Pty Ltd, Hadspen, Tasmania. The Fourier Transformed Infrared spectrometry technology (Bentley Fourier Transform Spectrometer) was used to quantify milk composition. This system uses Bentley Flow Cytometry to measure the somatic cell count, while the Bentley Fourier Transform Spectrometer measures somatic cell count, milk fat, protein and lactose based on official laboratory analysis method (AOAC, 1990). The equation from Mavrogenis and Papachristoforou (1988) was used to calculate Fat-corrected milk (FCM):

6% FCM=M (0.453+0.091F), where "F" is the percentage of fat and "M" is milk yield (kg).

3.3.4. Chemical analysis of experimental and basal diets

Before analysing dry matter (DM), ash and chemical composition, samples of the basal and experimental diets were dried in a fan-forced oven at a constant temperature of 65 °C and subsequently ground through a 1 mm sieve using a Thomas Model 4 Laboratory Mill (Thomas Scientific). DM content was determined by placing the ground samples at 150 ºC in an oven for 24 h to remove moisture. The samples were combusted in a furnace set at 600 °C for 8 h to determine ash content. Neutral detergent fibre (NDF) and acid detergent fibre ADF were quantified using an ANKOM220 fibre analyser, while an ANKOM^{XT15} fat/oil extractor (ANKOM Technology Corp., Macedon, NY, USA) was used to measure ether extract. The crude protein percentage was calculated based on the value of nitrogen that was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser (Thermo Fisher Scientific, MA, USA). Metabolisable energy (ME) and total digestible nutrients (TDN) were calculated as per Weiss (1992). Table 2 shows the nutritional composition of the experimental diets.

3.3.5. Data and statistical analysis

All data were analysed using 'Statistical Analysis System' software (SAS, 2009). Initial descriptive summary statistics were computed with means, standard errors, and minimum and maximum values scrutinised for data entry errors and outliers. The data were then subjected to General Linear Model (PROC GLM) analysis, with different oil supplementation, sire breed, week of supplementation and their interactions fitted as fixed effects and feed intake, milk yield, milk composition, and body condition score as dependent variables. Level of significance threshold was $P < 0.05$ and differences between means were established using Duncan's multiple range and Turkey's probability pairwise comparison tests. The final statistical model used for the analysis was:

$$Yijk = \mu + SB_i + D_j + W_k + (SBD)_{ij} + (SBW)_{ik} + (DW)_{jk} + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, SB, D and W are the fixed effects of sire breed, diet and week of supplementation, respectively, brackets represent second-order interactions and e_{ijk} is the error term.

3.4. Results

The results of this study suggest that dietary treatments significantly influenced supplement intake of grazing dairy ewes (P<0.0001; Table 3.3), with DM intakes being greatest in control group, followed by the RBO, SFO, CO, RPO, and FSO groups respectively. Estimated intake of OM, ADF, NDF and CP followed a similar pattern to DMI with the greatest intakes observed in the control group except the intake of EE which was greatest in the RBO group (41 g/day). Breed and its interaction with supplementation had no significant effect on intake among treatments (Table 3.3).

Significant differences in dairy performance traits, milk composition, and body condition score were observed among all treatments (Table 3.4). Ewes receiving RPO produced the greatest milk yield at 628 g/day, followed by SFO, RBO, CO, FSO, and the control (P<0.0001). Inconsistent with milk yield, fat concentration was highest in milk from control (P=0.015), whereas RBO yielded the greatest content of protein (5.9 g/100g) (P<0.0001) resulting in the highest concentration of solids-non-fat (11.7 g/100g) in this group. Although milk from ewes fed RPO had the least proportion of fat at 6.6 (g/100g), this group produced the greatest fat yield (FY) (41 g/day; P=0.0008). In addition, RPO followed by SFO produced the most protein yield (P=0.0004). There were no significant differences among treatments in the percentage of milk lactose. The type of oil included in the dietary supplement affected BCS (P=0.0008), although the mean BCS of experimental ewes only varied from 2.1-2.3 (Table 3.4). Since the cell counts for healthy sheep range from 10 to 200×1000 cells/ml, cell counts of all treatments

ranged from 60 to 109×1000 cells/ml (Table 4) indicating that all experimental animals were free from intramammary infections during the feeding trial.

Table 3.3. Least square means and standard errors (LSM ±SEM) of experimental feed intake[a] (g/head/day).

Items	Feed intake	DMI	OM	ADF	NDF	EE	CP
Treatment[b] (T)							
Control	885.5[a]	810.3[a]	741.4[a]	85.9[a]	243.1[a]	26.7[e]	118.3[a]
CO	751.3[c]	698.7[c]	651.9[b]	49.6[e]	152.3[d]	39.8[b]	97.8[e]
RBO	860.4[b]	788.0[b]	730.5[a]	63.8[c]	152.9[d]	40.9[a]	115.8[b]
FSO	754.3[c]	678.9[d]	617.8[d]	65.9[b]	158.2[c]	36.7[c]	99.1[e]
SFO	767.1[c]	703.4[c]	645.8[bc]	63.3[c]	168.1[b]	35.2[d]	102.0[d]
RPO	753.9[c]	690.5[cd]	635.3[c]	58.7[d]	151.9[d]	35.2[d]	107.7[c]
Breed[c]							
AW	793.5	726.5	678.8	64.3	170.6	35.7	106.5
AW x EF	797.1	729.9	671.9	64.7	171.5	35.8	107.0
SEM	4.1	3.8	3.5	0.6	1.7	0.3	0.6
P-values							
Treatment	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Breed	0.4483	0.4384	0.4423	0.3670	0.3492	0.5652	0.4358
T x Breed	0.7877	0.7982	0.7993	0.7557	0.6935	0.8934	0.8082

[a] Dry matter intake (DMI), organic matter (OM), acid detergent fibre (ADF), neutral detergent fibre (NDF), ether extract (EE), crude protein (CP).

[b] Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO).

[c] Awassi (AW), East Friesian (EF), Awassi x East Friesian (AW x EF) crossbred.

Values with different superscripts within columns are significantly different (P<0.05).

It was evidenced that breed also had major impacts on milk production rather than milk composition (Table 3.4), with significantly higher milk (P<0.0001), fat (P<0.0001) and protein (P<0.0001) yields observed in crossbred AW x EF than purebred AW. There were minor

variations in terms of mean fat, protein and lactose contents between AW x EF and AW, despite the statistical difference in lactose percentage.

Table 3.4. Effect of supplementation with diverse plant-derived oils on body condition score and lactation performance traits[a].

Item	MY	FCM	Fat	FY	Protein	PY	Lactose	SNF	SCC	BCS
Treatment[b] (T)										
Control	484^{d}	542^{bc}	7.4^{a}	36^{bc}	5.4^{c}	26^{c}	4.9	10.9^{bc}	109^{a}	2.1^{c}
CO	525^{c}	573^{b}	7.2^{ab}	38^{b}	5.5^{bc}	29^{b}	4.9	11.1^{bc}	98^{ab}	2.3^{a}
RBO	527^{c}	578^{b}	7.2^{ab}	38^{b}	5.9^{a}	31^{b}	4.9	11.7^{a}	73^{c}	2.2^{bc}
FSO	489^{d}	523^{c}	6.9^{bc}	34^{c}	5.4^{c}	26^{c}	4.8	10.8^{c}	60^{c}	2.3^{a}
SFO	562^{b}	587^{b}	6.6^{c}	37^{b}	5.6^{b}	31^{ab}	4.8	11.2^{b}	105^{ab}	2.2^{bc}
RPO	628^{a}	649^{a}	6.6^{c}	41^{a}	5.4^{c}	34^{a}	4.8	11.0^{bc}	81^{bc}	2.2^{bc}
Breed[c] (B)										
AW	496^{b}	535^{b}	7.1	35^{b}	5.5	27^{b}	4.8^{b}	11.1	97^{a}	2.2^{b}
AW x EF	578^{a}	617^{a}	6.9	40^{a}	5.5	32^{a}	4.9^{a}	11.2	78^{b}	2.3^{a}
SEM	3.4	7.8	0.07	3.6	0.04	2.9	0.02	0.05	3.6	0.0
P-values										
Treatment	0.0001	0.0001	0.0001	0.0021	0.0001	0.0001	0.1689	0.0001	0.0002	0.0018
Breed (B)	0.0001	0.0001	0.1765	0.0001	0.7444	0.0001	0.0006	0.1351	0.115	0.0030
Week (W)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0257	0.0012	0.0001
T x B	0.0001	0.0001	0.0001	0.0002	0.0003	0.0001	0.0001	0.0257	0.0795	0.0002
T x W	1.0000	1.0000	0.9766	0.9999	0.8717	1.0000	0.8348	0.8039	0.3630	0.9999
B x W	0.9061	0.8724	0.9494	0.8517	0.9971	0.9380	0.6808	0.9910	0.9974	0.8640

[a] Milk yield (MY, g/day), fat-corrected milk (FCM, g/day), fat (g/100g milk), fat yield (FY, g/day), protein (g/100g milk), protein yield (PY, g/day), lactose (g/100g milk), solids-non-fat (SNF, g/100g milk), somatic cell count (SCC, ×1000 cells/ml), body condition score (BCS).
[b] Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO).
[c] Awassi (AW), East Friesian (EF), Awassi x East Friesian (AW x EF) crossbred.
Values with different superscripts within columns are significantly different ($P<0.05$).

Weekly trends for BCS and lactation traits are presented in Figures 3.1 and 3.2. As observed in all treatment groups, BCS, fat percentage and protein percentage (Figure 3.1a and 3.2a,b) increased, while milk yield decreased over the duration of the experimental period (Figure

3.1b). The best weekly milk yield trend was recorded in RPO group, where its decrease was smaller (4.9 at the start to 3.9 kg/week) than the other groups at the end of the trial.

Figure 3.3 presents significant interactions between oil supplementation and breed in milk yield ($P<0.0001$), fat percentage ($P<0.0001$), and protein percentage ($P=0.0003$). Regarding milk production, crossbred AW x EF ewes had greater responses to oil supplements than AW with the highest milk yield at 751 g/day observed in RPO group (Figure 3.3a). Breed and diet interactions, however, varied across treatments in which AW ewes fed with RBO produced the highest percentages of fat and protein (7.8, and 6.1 g/100g, respectively).

3.5. Discussion

3.5.1. Effect of dietary supplements on dry matter intake and body condition score

The decrease in DMI was inconsistent with previous studies that examined the effect of adding 2% plant oil in the diets of dairy ewes (Hervas *et al.*, 2008), but was similar to recent reports in dairy cows that found a negative impact of a high level supplemented oil on DMI (Shingfield *et al.*, 2006; Mapato *et al.*, 2010; Lunsin *et al.*, 2012b; Ammah *et al.*, 2018). According to Illius *et al.* (Illius and Jessop, 1996) voluntary ruminant feed intake is affected by nutrient and energy flows related to ruminal fermentation. Adding high levels of oil in diets that was the case of the current study, may reduce diet acceptability (Petit *et al.*, 2005) which is caused by ruminal function reduction. Other studies have shown that oil addition to diets reduces fibre digestibility, DMI and feed palatability in ruminants, suggesting negative effects of plant oils on animals' appetite (Gonthier *et al.*, 2004). This occurs due to selection against microorganisms with cellulolytic capability leading to a decrease in ruminal fibre digestion (Gonthier *et al.*, 2004). Moreover, DMI differences among oil supplement groups (with the highest observed in RBO), indicates the effect of oil type on nutrient digestibility (Doreau and Chilliard, 1997).

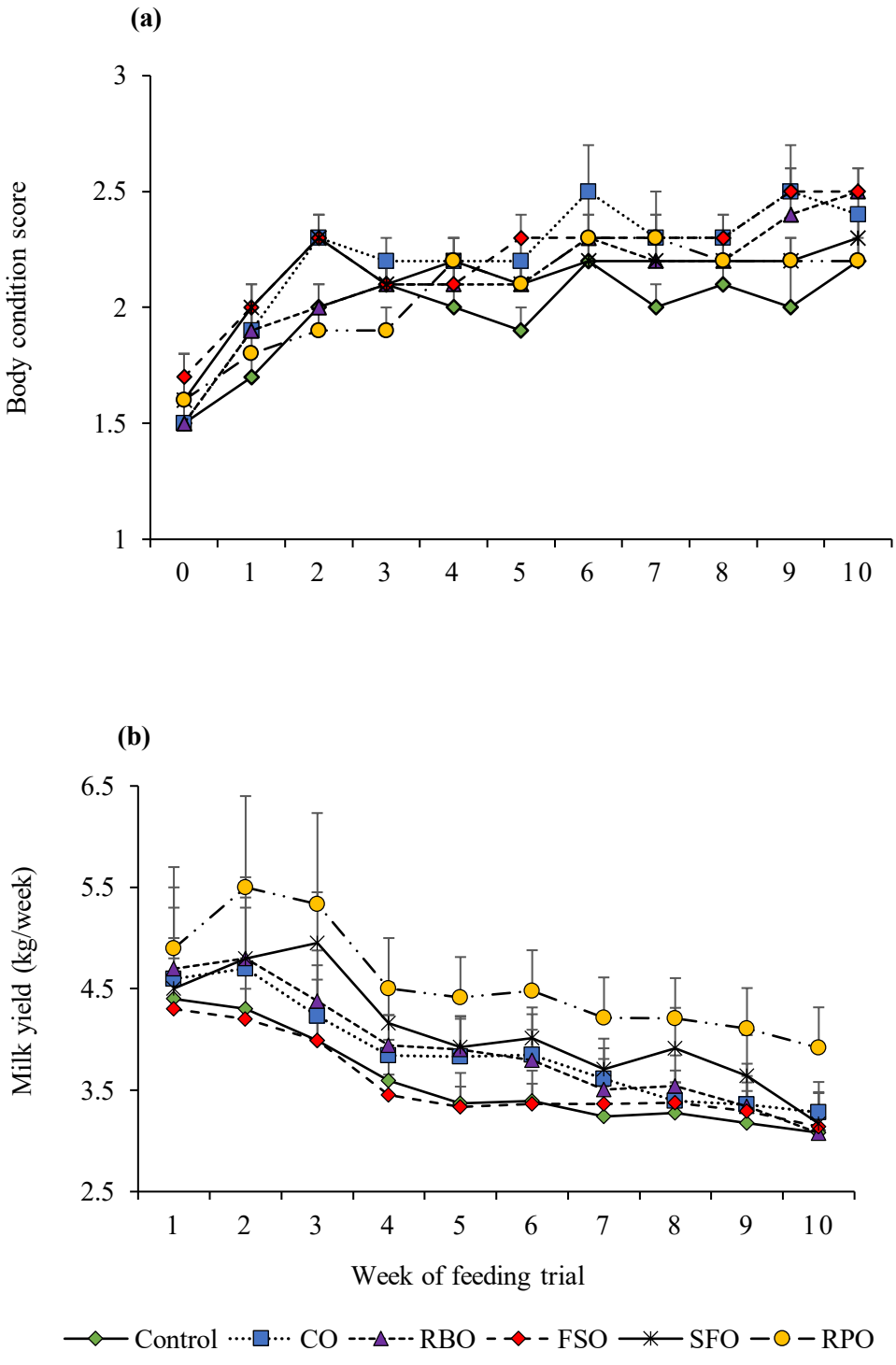

Figure 3.1. Weekly trends in body condition score (a) and milk yield (b).

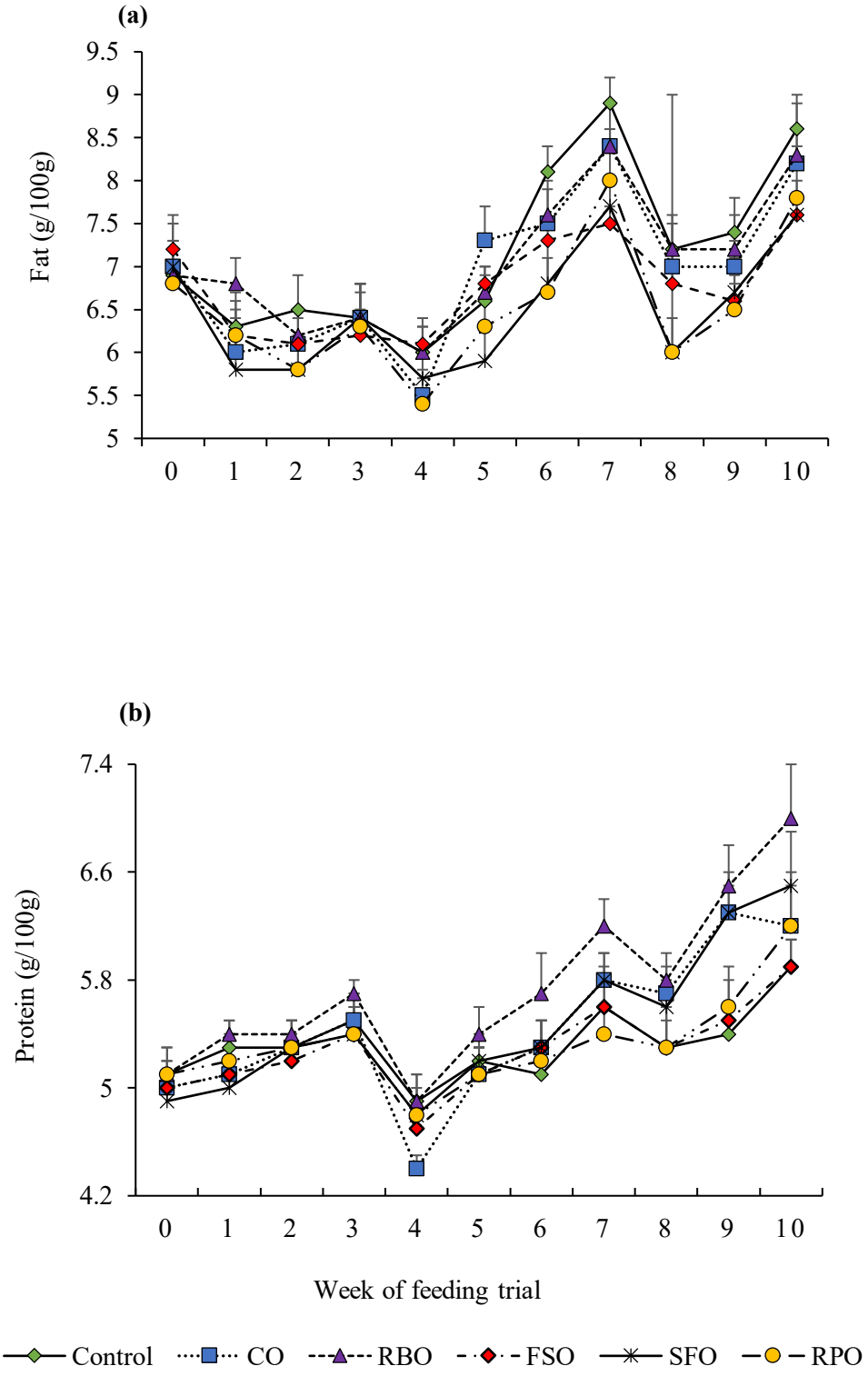

Figure 3.2. Weekly trends in milk fat (a) and milk protein (b) concentration.

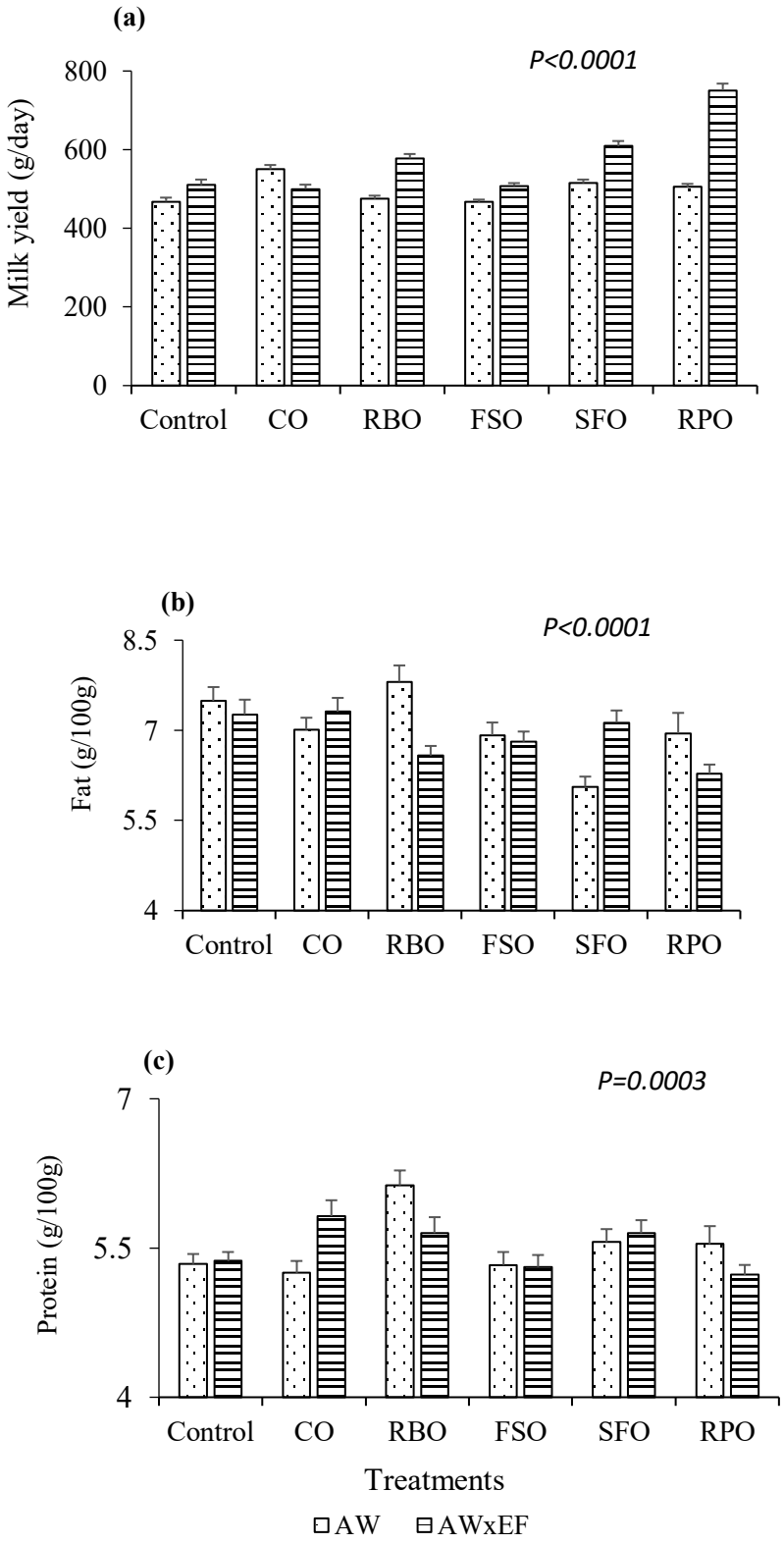

Figure 3.3. Supplementary diet and breed interactions on (a) milk yield, (b) milk fat, and (c) milk protein. Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO)

Known as an important indicator of cow heath status in dairy management, body condition score (BCS) is also regularly used to estimate fatness in the form of energy reserves as well as animal welfare status (Malau-Aduli and Anlade, 2002; Morgan-Davies *et al.*, 2008; Roche *et al.*, 2009; Phythian *et al.*, 2011). A meta-analysis by Kenyon et al. (Kenyon *et al.*, 2014) demonstrated a positive association between BCS at breeding and ewe reproductive traits (pregnancy rate and number of lambs born). Generally, these parameters increase as BCS increases from 2.0 to 3.0 (Kenyon *et al.*, 2004; Abdel-Mageed, 2009 ; Yilmaz *et al.*, 2011). At the commencement of the feeding trial, the average BCS of the experimental animals was 1.5; a reflection of the low quality pastures the ewes were grazing and a pointer to fat mobilisation from body reserves for sustaining milk synthesis (Komaragiri *et al.*, 1998). At the end of the feeding trial, average BCS values of ewes fed CO, RBO and FSO rose to 2.55, 2.60, and 2.55, respectively. These BCS were within the target of 2.5-3.0 (Kenyon *et al.*, 2014), which suggests that the use of such supplements could have a positive effect on not only milk yield, but also reproductive performance and the general welfare of dairy ewes.

3.5.2. Effect of dietary supplements on milk yield, and milk composition

Despite the wide accessibility and availability of canola and rice bran in Australia (Seymour *et al.*, 2012; Ricegrowers' Association of Australia, 2013), the extent of use of these plant lipid sources as dietary supplements in the Australian dairy industry is unknown. Supplementing diets with canola and rice bran oils in the current study increased milk yield without exerting negative effects on milk fat and protein compositions. Lunsin *et al.* (2012b) supplemented dairy cow diets with 2, 4, 6% rice bran oil in a confined system and did not observe any statistical variation in milk production. This was inconsistent with a reduction in the milk yield of dairy goats fed total mixed rations that included 5, 10 and 20% rice bran (Park *et al.*, 2013). In contrast, an increase in milk yield of RBO group observed in the current study suggests the advanced effect of rice bran oil inclusion in a pasture-based system compared to a confined

system. Regarding milk fat and protein concentrations, supplementation of grazing dairy ewes with rice bran oil in the current study, had no influence on milk fat. However, it significantly enhanced milk protein even though the potential to alter milk protein concentration by changing the dietary composition is considered less compared with the potential to alter milk fat composition (Kennelly *et al.*, 2005). This increment of change in protein composition in milk agrees with the findings of Park *et al.* (2013) in goat milk, but disagrees with a decrease observed in cows when the percentage of dietary RBO increased (Lunsin *et al.*, 2012b). On the other hand, supplementation of ewes in this study and cows (Otto *et al.*, 2015) in similar pasture-based dairy systems with 5% of CO demonstrated an increase in milk yield. However, while inclusion of CO had no statistically significant effect on all milk components of lactating ewes, Otto et al. (2015) reported marginal decreases in fat and protein percentages of cow milk. These contrasting results in response to rice bran and canola oil supplementation suggest that there could be physiological differences between species in lipid metabolisms that might need further investigation.

Variations in results assessing the effect of mostly whole or extruded flaxseed (Nudda *et al.*, 2014) and flaxseed oil (Antonacci *et al.*, 2018) on milk production and composition of dairy ewes have been reported. Akin to the current results, no statistical difference in milk production was observed when ewes were supplemented with extruded linseed at 128 g/day (Gomez-Cortes *et al.*, 2014) and 220 g/day (Nudda *et al.*, 2015) or linseed oil at 6% of estimated total DM intake (Antonacci *et al.*, 2018). These findings were in contrast with other authors who distinguished either an increase (Caroprese *et al.*, 2016) or a decrease (Mughetti *et al.*, 2012) in milk yield of dairy ewes fed 250 g/day of whole flaxseed or 200 g/day of extrude flaxseed respectively. Milk fat depression in response to supplementation with FSO in this study was supported by other studies in sheep (Gomez-Cortes *et al.*, 2014) and cows (Li *et al.*, 2015; Ammah *et al.*, 2018; Brossillon *et al.*, 2018), but disagrees with others that showed no changes

in sheep (Nudda *et al.*, 2015; Caroprese *et al.*, 2016; Antonacci *et al.*, 2018) or a minor increase in sheep (Mughetti *et al.*, 2012; Caroprese *et al.*, 2016), and goats (Nudda *et al.*, 2013). These variations might be due to the multi nutritional effects including energy balance, NDF concentration, feed particle size, when these factors were demonstrated to have strong correlations with milk yield and milk fat concentration (Pulina *et al.*, 2006).

Safflower, which is grown in over 60 countries (Glibert and Porter, 2008), has been used widely as a supplement in ruminant diets (Alizadeh *et al.*, 2012). Despite studies investigating the effects of using various types of safflower on bovine and caprine performance (Shingfield *et al.*, 2013), there is relatively little information on its effectiveness as a supplement for influencing milk yield and composition in lactating ewes. In this study, supplementation of grazing dairy ewes with SFO increased milk production by 16%. This supports the findings of Ahmadpour *et al.* (2017) who supplemented dairy cows with rolled safflower seed at 3 and 6% and reported increases in milk yield by 2 and 9% respectively. Other studies have, however, reported no significant effects on milk yield when the diets of lactating cows (Bell *et al.*, 2006; Dschaak *et al.*, 2011; Alizadeh *et al.*, 2012; Oguz *et al.*, 2014) and goats (Shi *et al.*, 2015) were supplemented with safflower oil or seed. Similarly, variable responses and changes in milk components had been observed when the diets of lactating goats or cows were supplemented with safflower. Regarding fat content, some results portrayed negative effects (Bell *et al.*, 2006; Shi *et al.*, 2015; Ammah *et al.*, 2018) which align with our results, while others did not observe any significant effects (Dschaak *et al.*, 2011; Alizadeh *et al.*, 2012; Oguz *et al.*, 2014; Ahmadpour *et al.*, 2017). The wide range of inclusion rates and variation in dietary components in these studies might have led to the variable responses reported.

A significant enhancement of milk yield by approximately 30% compared to the control animals, was observed in ewes supplemented with RPO. Increases in fat yield (13%) and protein yield (31%) were also observed. These improvements in milk yield and total solids

production play an important role in positively enhancing the economic benefits for dairy sheep producers as most sheep milk is used for cheese making (Balthazar *et al.*, 2017). The quantity of cheese that can be produced from sheep milk is limited by the concentrations of fat and especially protein, in raw milk (Pulina *et al.*, 2006). Reviews on bypass fat supplementation studies suggest a consistent increase in the milk production of lactating cows by 5.5-24% (Naik, 2013), while variable responses were presented in lactating ewes (Pulina *et al.*, 2006). According to Pulina *et al.* (2006), positive effects of supplementing rumen-protected fat on dairy sheep production performance generally occur with feeding trials longer than 4 weeks. This was confirmed in the current work, while short-term studies had a minor reduction or no change (Kitessa *et al.*, 2003; Appeddu *et al.*, 2004; Garcia *et al.*, 2005). In this study, we recorded a reduction in the concentration of milk fat in the RPO group. This agrees with the findings of Rotunno *et al.* (1998) who fed ewes with 4 and 8% rumen-protected fat, whereas this disagreed with consistent increase in milk fat concentration reported by Pulina *et al.* (2006). Differences in dietary components, type and dosage of protected fat, feeding regimes, or stage of lactation might have accounted for this contrasting set of outcomes.

3.5.3. Effect of breed on animal performance

The East Friesian (EF) breed of sheep was developed in northern Germany and the Netherlands, and has become one of the world's most productive dairy sheep. The EF has earned the reputation as the most productive dairy sheep breed in terms of milk yield (Haenlein, 2007). However, it has a low ability to adapt under unfavourable environmental conditions, especially excessive heat and humidity (Gootwine and Goot, 1996). Thus, this breed has been used widely in crossbreeding systems to improve milk production of local breeds in various temperate environments (Gootwine and Goot, 1996; Thomas *et al.*, 1998; Konečná *et al.*, 2013). Together with Awassi (AW), the predominant breed in The Eastern Mediterranean countries (Galal *et al.*, 2008), EF was introduced to Australia in the 1990s, and since, has been used more widely

in the dairy sheep industry as reported by the Australian Rural Industries Research and Development Corporation (Stubbs *et al.*, 2009). The improvement in milk yield without any negative effects on relative content of milk composition in crossbred ewes AW x EF was akin to Clement *et al.* (Clement *et al.*, 2006), whereas it was inconsistent with Gootwine and Goot (1996) who demonstrated similar milk volumes between AW and AW x EF. Local heat stress that leads to a depression of feed intake, milk production and reproduction (Silanikove, 2000; West, 2003), might be the principal factor for this performance variation by crossbreds in some studies. Moreover, statistically significant variation in the interaction between treatments and sire breed regarding milk production and composition, but not supplement intake, in the current research, suggests that gene regulation may be involved in experimental oil metabolism. Therefore, identification of regulated genes for milk yield and composition in response to plant and rumen-protected oil supplements needs to be investigated.

3.6. Conclusion

The current study demonstrated that canola, rice bran, safflower and rumen-protected EPA+DHA could improve lactation traits without any negative impact on BCS of dairy ewes grazing low quality pasture. Under the same nutrition and management conditions, crossbred AW x EF significantly showed greater lactation performance than AW. Utilising these oil supplements combined with crossbreeding the AW and EF sheep breeds, is therefore, recommended for Australian sheep milk producers in pasture-based systems. In addition, the novel potential of supplementing dairy sheep with rice bran and canola oils explored in this study, may need further research to better elucidate their metabolic mechanisms.

Chapter 4: Supplementing grazing dairy ewes with plant-derived oil and rumen-protected EPA+DHA enhances health – beneficial n-3 long-chain polyunsaturated fatty acids in sheep milk

4.1. Abstract

This study investigated the impact of supplementing dairy ewes in mid lactation with rumen-protected (RPO) pellets containing eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) or pellets infused with 50 ml/kg DM of either canola (CO), rice bran (RBO), flaxseed (FSO) or safflower (SFO) oils on enhancing the concentration of n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA) in milk. It is hypothesized that including these oils in the diet of grazing dairy ewes will improve milk fatty acid (FA) composition by increasing levels of n-3 LC-PUFA. Sixty grazing dairy ewes balanced by sire breed and parity were randomly allocated to one of 6 treatments: 1) Control: commercial pellets without oil inclusion; 2) pellets containing 50 ml/kg DM of CO; 3) RBO; 4) FSO; 5) SFO; and 6) RPO at the rate of 1 kg/day for each ewe for 8 weeks. Weekly bulked daily milk FA analysis showed that RPO was the most effective diet at elevating n-3 LC-PUFA content by twofold, threefold and fivefold greater concentrations of EPA, DPA and DHA respectively, than the control (0.17 vs 0.08%, 0.23 vs 0.08%, 0.19 vs 0.04%) ($P<0.0001$). FSO improved levels of EPA (0.11%) and DPA (0.15%), while CO increased DPA (0.13%) ($P<0.0001$). FSO and RPO reached the 'source' and good source' of n-3 LC-PUFA (ΣEPA+DHA+DPA) contents of 35.1 and 61.7 mg/250 ml, respectively. These findings recommend that rumen protected pellets containing EPA + DHA, flaxseed and potentially canola oil supplements, can be used to improve the content of n-3 LC-PUFA in dairy ewe milk.

4.2. Introduction

More attention is being paid to human diets rich in n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA) because of the potent ability of these natural ingredients to inhibit adipogenic, diabetogenic, atherogenic (McGuire and McGuire, 2000), inflammatory (Calder, 2012; Calder, 2013) and carcinogenic (Belury, 2002; Calder, 2004) diseases and their effects. Furthermore, high consumption of n-3 LC-PUFA is typically associated with a lower incidence of depression, a decreased prevalence of age-related memory loss and a lower risk of developing Alzheimer's disease (Laugharne, 1996; Kalmijn *et al.*, 1997; Calon and Cole, 2007). This has led to a large number of studies aimed at increasing LC-PUFA content in dairy products for human consumption (Bargo *et al.*, 2006; Luna *et al.*, 2008; Shingfield *et al.*, 2012; Park *et al.*, 2013; Aprianita *et al.*, 2014; Otto *et al.*, 2014).

Although the fatty acid (FA) profile in ruminant tissues and milk is difficult to modify because of ruminal fermentation by microorganisms (Demeyer and Doreau, 1999), various studies have shown that manipulating the diet or feeding regime could enhance the ruminal escape rate of unsaturated fatty acids (UFA) from feeds to tissues (Chilliard *et al.*, 2007; Otto *et al.*, 2014; Manso *et al.*, 2016). FA profiles in milk are derived from 4 different sources including: *de novo* synthesis in mammary gland, diet, ruminal biohydrogenation and body reserves in which dietary FA directly or indirectly contribute half of the C_{16} and all of the long-chain FA (Chilliard *et al.*, 2007). Therefore, dietary supplementation with UFA is one of the ways to alter milk fat profile (Glasser *et al.*, 2008a; Hristov *et al.*, 2011) and increase the PUFA proportion of milk fat (Leiber *et al.*, 2011; Sterk *et al.*, 2011; Buccioni *et al.*, 2015). However, previous studies have mainly focused on cow milk despite the fact that consumption of sheep milk has more nutritional advantages such as the higher levels of protein and fat, and smaller size of fat globules (Park *et al.*, 2007; Silanikove *et al.*, 2015), compared to cow milk.

A comprehensive review of recent developments in altering the FA composition of ruminant-derived foods (Shingfield *et al.*, 2013) stated that the "potential to increase 20:5n-3 and 22:6n-3 in milk is extremely limited" and the proportion of eicosapentaenoic acid (EPA, 20:5n-3) typically is less than 0.1 % of total FA in ruminant milk. This present study, however, explores the novelty of utilising rumen-protected EPA+DHA and a variety of oil pellets at 50ml/kg DM, to elevate the levels of n-3 LC-PUFA (∑EPA+DHA+DPA) to 'source' and 'good source' levels of 30 and 60 mg / standard serve as determnined by FSANZ (2012), respectively, in sheep milk. Furthermore, under Australian on-farm production conditions, a combination of limited research, scarcity of published studies investigating the effect of different oils of plant origin and the current absence of the use of rumen-protected oil in the diets of grazing dairy ewes for enhancing milk n-3 LC–PUFA content represent a major knowledge gap that this study intends to fill. Therefore, it was hypothesized that supplementing grazing dairy ewes with different sources of dietary n-3 oils will affect the concentration of n-3 LC-PUFA in milk. The main objective of this study was to evaluate the effects of adding CO, RBO, FSO, SFO and RPO to the diets of grazing dairy ewes on the proportions and concentrations of beneficial LC-PUFA, particularly n-3 and also n-6 PUFA in sheep milk.

4.3. Materials and methods

This experiment was carried out at Grandvewe Cheeses Farm, Birchs Bay, Woodbridge, Tasmania, Australia, in accordance with the University of Tasmania Animal Ethics Committee guidelines, 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Animal Ethics Permit Number A0015657).

4.3.1. Animal management and experimental design

A completely randomised experimental design comprising sixty lactating Awassi and Awassi x East Friesian crossbred ewes in mid-lactation at Grandvewe Cheeses Farm was utilised in allocating the animals into one of the following 6 treatments: (1) Control: existing on-farm commercial wheat-based pellets without oil inclusion; wheat-based pellets infused with (2) canola (CO); (3) rice bran (RBO); (4) flaxseed (FSO); (5) safflower (SFO) and (6) rumen protected EPA+DHA (RPO) oils. The RPO treatment was based on a modification of the microencapsulation of oil droplets in a protein-aldehyde matrix procedure (Scott *et al.*, 1971). The same level of 50 ml/kg DM of oil was included in all supplementary diets except for the control treatment and all treatments were isocaloric and isonitrogenous. The nutritional composition of the experimental diets is shown in Table 4.1. Experimental animals were balanced by breed, parity (2.8±0.5), liveweight (59±5.9 kg), and milk yield (657±100 g/day) in each treatment and kept in the same paddock with *ad libitum* access to pasture, hay and fresh water. Each ewe was fed 1 kg/day of the supplemental pellets individually during milking time for a total of 10 weeks including an initial two-week adjustment period, followed by an 8-week experimental period. During the initial two-week adjustment period, the proportions of experimental diets CO, RBO, FSO, SFO, and RPO were gradually increased by 100 g/day the attainment of 1 kg/day on day 10. Ewes were milked in the mornings at 0600 h in batches of ten according to their treatment groups and individual milk yield automatically recorded by the Alfa Laval platform. The daily milk yield per ewe was sampled and the weekly samples bulked for milk composition analysis and processing into cheese.

4.3.2. Chemical analysis of experimental feeds

The chemical composition of the basal and experimental diets was determined by the standard AOAC procedure previously reported in detail (AOAC, 1990). Briefly, samples were dried in a fan-forced oven at a constant temperature of 65ºC before being ground through a 1 mm sieve

using a Thomas Model 4 Laboratory Mill (Thomas Scientific) and then used for analysing dry matter (DM), ash content and chemical composition. Ground samples were placed at 150°C in an oven for 24 h in order to remove moisture and determine DM content. The samples were combusted in a furnace set at 600°C for 8 h to determine ash content. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were quantified using an ANKOM220 fibre analyser and ether extract was measured using an ANKOM^{XT15} fat/oil extractor (ANKOM Technology, Macedo, NY, USA). The crude protein percentage was calculated based on the value of nitrogen that was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser. Metabolisable energy (ME) and total digestible nutrients (TDN) were calculated as per Weiss (1992)

Table 4.1. Nutrient composition[a] of experimental diets[b].

Component (% DM)	Pasture	Hay	Control	CO	RBO	FSO	SFO	RPO
DM	96.5	95.5	91.5	93.0	91.6	90.0	91.7	91.6
OM	90.5	97.3	92.2	93.3	92.7	91.0	91.8	92.0
Ash	9.5	2.7	7.8	6.7	7.3	9.0	8.2	8.0
ADF	45.5	37.6	10.6	7.1	8.1	9.7	9.0	8.5
NDF	69.9	68.3	30.0	21.8	19.4	23.3	23.9	22.0
EE	1.4	1.2	3.3	5.7	5.2	5.4	5.0	5.1
CP	4.7	4.3	14.6	14.0	14.7	14.6	14.5	15.6
TDN	48.5	54.1	73.4	75.9	75.2	74.1	74.5	74.9
ME, MJ/kg DM	7.1	8.1	11.7	12.2	12.0	11.8	11.9	12.0

[a] Dry matter (DM), organic matter (OM), acid detergent fibre (ADF), neutral detergent fibre (NDF), ether extract (EE), crude protein (CP), total digestible nutrients (TDN) and metabolisable energy (ME).
[b] Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO).

4.3.3. Milk sample collection and fatty acid analysis

Daily fresh milk samples from each dairy sheep (100 ml/sample) were collected and bulked each week into two sets of plastic vials containing bronopol blue milk preservative. One set of of milk samples was stored at 4ºC and later sent to TasHerd Pty Ltd, Hadspen, Tasmania, for milk composition analyses. The other part was flushed with nitrogen and stored at -20ºC before weekly analysis right through to the end of the experiment. Representative aliquots from individual milk samples collected over the 8 weeks of the experimental period were used to analyse milk FA profile.

Milk samples were analysed for fatty acid composition using a gas liquid chromatography (GC) method (Otto *et al.*, 2014). Approximately 5 mg of diet samples and 0.5 g of milk samples were accurately weighed into methylating tubes and freeze-dried to remove moisture. The dried materials were methylated in a solution of methanol/HCl/dichloromethane (10/1/1; 3 ml; 80ºC 2hr) to produce fatty acid methy esters (FAME), which were extracted (hexane/dichloromethane; 4/1, 2 ml, 3x) and transferred to glass GC vials. FAME were diluted with dichloromethane containing C19:0 FAME as the internal injection standard before analyses were performed using an Agilent Technologies 7890B gas chromatograph equipped with an equity™-1 fused silica capillary column (15 m x 0.1 mm internal diameter and 0.1 µm film thickness), a flame ionisation detector, a split/splitless injector and an Agilent Technologies 7683B Series autosampler. The temperature profile of the oven was 120ºC for 1 minute which was then increased by 10ºC/min to 270ºC, and then by 5ºC/min to 310ºC. ChemStation software (Agilent Technologies, Palo Alto, CA, USA) was used to quantify peak areas. FAME identities were confirmed by GC-mass spectrometry (GC-MS) analysis using a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and Thermoquest Xcalibur software (Austin, Texas USA).

4.3.4. Statistical analysis

Statistical analysis of all collected data was performed in SAS version 9.2 (SAS Institute, Cary, NC, USA). Since the control pellets were the existing commercial pellets without any oil on the farm that all the ewes were already used to before the feeding trial begun, individual variability between and within animals were minimised to the barest minimum with enough replications of animals in each treatment group. Initially means, standard deviations, standard errors, minimum and maximum values of data were computed using PROC MEANS and these were scrutinized for any data entry errors. Selected fatty acids were then evaluated using General Linear Model (PROC GLM) analysis, with different oil supplementation, sire breed, parity, and week of experiment fitted as fixed effects and their second-order interactions. The changes between the initial and final liveweight measurements at the end of the adaptation period were fitted as covariates in the analytical model. Level of significance threshold was $P < 0.05$ and differences between means were established using Duncan's multiple range and Tukey's probability pairwise comparison tests.

4.4. Results

Sire breed, parity, week of experiment and their interactions with diet had no significant ($p > 0.05$) influence on FA profile during experimental period, hence these interactions were eliminated from all tables.

The values of the main fatty acids (expressed as % of total fatty acids) in the supplements are depicted in Table 4.2, which shows that the FSO group had the highest proportion of total PUFA followed by the SFO group. In terms of individual PUFA, SFO had the greatest percentage (56.5%) of linoleic acid (LA), FSO had the highest alpha linolenic acid (ALA) level (20.5%) while the highest n-3 LC-PUFA level (4.7%, EPA+DHA+DPA) was observed in RPO. These differences in FA profile of the supplements resulted in significant variations in milk yield, composition and absolute content of FA per standard serve of milk (mg/250 ml) within the dietary treatments (Tables 4.3 and 4.4).

Table 4.2. Fatty acid[a] compositions (% of total fatty acids) of basal (pasture, hay) and experimental diets

Fatty acid	Pasture	Hay	Control	CO	RBO	FSO	SFO	RPO
12:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
14:0	1.60	2.85	0.05	0.14	0.14	0.14	0.22	2.35
15:0	0.47	0.89	0.13	0.06	0.09	0.12	0.10	0.36
16:0	18.8	35.8	21.8	12.4	17.4	11.0	14.0	19.5
17:0	0.38	0.92	0.19	0.08	0.08	0.09	0.08	0.24
18:0	4.48	7.57	1.05	1.12	2.28	3.38	2.26	2.86
20:0	3.55	5.81	0.40	0.57	0.59	0.37	0.47	0.45
22:0	4.28	5.60	0.34	0.31	0.49	0.29	0.19	0.43
24:0	4.43	5.06	0.39	0.36	0.63	0.22	0.33	0.37
16:1n-9c	0.20	0.00	0.01	0.00	0.01	0.00	0.02	0.00
16:1n-7c	0.65	0.55	0.13	0.27	0.20	0.25	0.22	4.03
16:1n-7t	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
18:1n-9c	10.2	0.0	20.5	41.8	31.8	18.8	20.3	20.9
18:1n-7c	1.31	1.15	1.22	2.54	1.11	1.83	1.48	1.90
C18:1n-7t	0.08	0.37	0.00	0.00	0.00	0.00	0.00	0.00
18:2CLA$_a$	0.01	0.02	0.15	0.02	0.08	0.00	0.04	0.05
18:2CLA$_b$	0.00	0.41	0.00	0.00	0.00	0.00	0.00	0.00
18:2n-6 LA	18.7	8.6	47.0	33.8	41.1	40.9	56.5	35.7
18:3n-3 ALA	24.1	12.2	3.0	4.8	2.3	20.5	1.7	2.7
20:4n-6 ARA	0.05	0.24	0.00	0.00	0.00	0.00	0.00	0.23
20:5n-3 EPA	0.00	0.00	0.00	0.13	0.10	0.12	0.07	2.50
22:6n-3 DHA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.63
22:5n-3 DPA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.54
ΣSFA	41.2	69.1	25.3	15.3	22.0	16.0	18.0	27.1
ΣMUFA	15.0	7.1	23.4	45.7	34.0	21.7	22.9	28.3
ΣPUFA	43.8	23.8	51.3	39.0	44.0	62.3	59.1	44.6
Σn-6 PUFA	19.5	10.7	47.8	34.0	41.4	41.6	57.0	37.1
Σn-3 PUFA	24.2	12.3	3.1	4.9	2.4	20.6	1.7	7.4

[a] Linoleic acid (LA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), total saturated fatty acids (ΣSFA), total monounsaturated fatty acids (ΣMUFA), and total polyunsaturated fatty acids (ΣPUFA).

ΣSFA is the sum of 12:0, 13:0, i14:0,14:0, i15:0, a15:0,15:0, i16:0, 16:0, i17:0, 17:0, i18:0, 18:0, 18:0FALD, 19:0, 20:0, 21:0, 22:0, 23:0, 24:0.

ΣMUFA is the sum of 14:1, 16:1n-9c, 16:1n-7c, 16:1n-7t, 16:1n-5c, 16:1n-13t,17:1n-8(+a17:0), 18:1n-9c, 18:1n-7c, 18:1n-7t, 18:1a, 18:1b , 18:1c, 18:1FALD ,19:1a, 19:1b, 20:1n-11c, 20:1n-9c, 20:1n-7c, 20:1n-5c, 22:1n-11c, 22:1n-9c, 24:1n-9c.

ΣPUFA is the sum of 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, 22:6n-3, 22:5n-3, 18:3n-6, 18:4n-3, 18:2CLAa, 18:2CLAb, 18:2CLAc, 20:3, 20:3n-6, 20:4n-3, 20:2n-6, 22:5n-6, 22:4n-6.

Σn-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

Σn-3 PUFA is the sum of 18:3n-3, 20:5n-3, 22:6n-3, 22:5n-3, 18:4n-3.

ΣEPA+DHA+DPA is the sum of 20:5n-3; 22:6n-3 and 22:5n-3.

Supplementation had a significant effect on milk yield and composition (Table 4.3). Ewes receiving RPO produced the greatest milk yield at 628 g/day, followed by SFO (562 g/day), RBO (527 g/day), CO (525 g/day), FSO (489 g/day), and control (484 g/day) ($P<0.0001$). Inconsistent with milk yield, fat percentage was highest in milk from control (7.4 %) ($P=0.015$), whereas RBO yielded the most protein (5.9 %) ($P<0.0001$). Although milk from ewes fed RPO had the least proportion of fat at 6.6 (g/100g), this group produced the greatest fat yield (FY) (41 g/day; $P=0.0008$). In addition, RPO followed by SFO, produced the most protein yield ($P=0.0004$). There were no significant differences among treatments in the percentage of lactose.

Table 4.4 shows that total PUFA in milk of FSO and SFO groups increased significantly ($p<0.0001$) compared to the control group; ewes receiving SFO produced the greatest proportion of total PUFA (7.2±1.1%, expressed as percentage of total FA), followed by FSO (6.6±0.3%), RPO (5.5±0.2%), RBO (4.8±0.5%), control (4.8+0.2%), and CO (4.7±0.2%)

respectively. This finding was alligned with the difference ($p<0.0001$) in content of Σn-6 that was also highest in milk from SFO ewes (5.8±0.3%). Supplemented diets were also significant sources of variation in the proportion of Σn-3, as well as n-3 LC-PUFA in the milk ($p<0.0001$). The relative proportion of Σn-3 PUFA was highest in milk from FSO (2.07±0.08), whereas RPO yielded the highest percentage of EPA (0.17±0.01%), DHA (0.19±0.01%), and also DPA (0.23±0.02%). There were no significant differences among control, CO and RBO in the percentages of Σn-6 PUFA and Σn-3PUFA.

Interaction between diets and week of supplementation on the concentration of n-3, and n-6 PUFA, as well as n-3 LC-PUFA are presented in Figure 4.1. Changes started in the first week of adaptation period when the control diet was increasingly being replaced by experimental diets with a rapid increase in milk LA (Figure 4.1a) for SFO, ALA (Figure 4.1b)for FSO, EPA (Figure 4.1c), DHA (Figure 4.1d), and DPA (Figure 4.1e) for RPO up to week 3. Concentrations of these beneficial FA then remained stable with minor changes from weeks 3 to 10 in all treatments.

The health beneficial fatty acids of milk were also presented in quantitative terms per standard serve (mg/250 ml of milk) to further investigate the potential nutritional benefits to consumers. The absolute content of beneficial shorter chain (SC, $\leq C_{18}$) and long-chain n-3 and n-6 PUFA per standard serve (mg/250 ml of milk) are portrayed in Table 4.5. Consistent with the percentage FA composition results, ewes fed FSO produced the greatest amount of ALA (185.2±5.8 mg/250 ml), and Σn-3 PUFA (221±7.6 mg/250 ml), while SFO and RPO fed ewes had the highest Σn-6 PUFA (639±30.6 mg/250 ml) and ΣEPA+DHA+DPA (61.7±5.0 mg/250 ml) respectively. The extent of these increases were from 1.5 to 3 times the values observed in the control group (ALA, Σn-3 PUFA, Σn-6 PUFA, and ΣEPA+DHA+DPA contents were 74.1±7.1; 99.6±7.4; 441±36.5; and 24.1±2.1 mg/250 ml respectively).

4.5. Discussion

To our current knowledge, there is a paucity of peer-reviewed published literature assessing the incorporation of health beneficial n-3 long-chain polyunsaturated fatty acids from canola and rice bran oils into sheep milk under on-farm grazing management. Among treatments in this study, CO and RBO had only minor effects on altering milk FA composition. Adding rice bran oil to the diet of grazing dairy ewes in this study had no major effect on the concentration of PUFA, but induced a significant increase in milk saturated fatty acids (SFA). Inconsistent with (Lunsin *et al.*, 2012b) who reported that the content of 14:0 and 16:0 in milk was lower while the content of 18:0 was higher in response to supplementing dairy cows with rice bran oil, this current study observed the reverse trend for these short and medium-chain SFA. However, it was demonstrated (Park *et al.*, 2013) that there were no statistical differences in the proportions of 14:0 and 16:0 in milk when assessing the effect of supplementing rice bran to the diets of dairy goats. Disparities between cow, sheep and goat milk in the concentrations of medium chain SFA in diets supplemented with rice bran and rice bran oil could be the result of species differences in mammary lipid metabolism (Chilliard *et al.*, 2014). PUFA incorporation observed in CO group was largely similar to the control, except for the minor but statistically significant elevation of the proportions of 18:1n-7c and 22:5n-3 (DPA). Increases in the concentrations of 18:1 isomers have been reported in lactating cows supplemented with canola oil (Otto *et al.*, 2014) and canola seed (Chichlowski *et al.*, 2005). Desaturation of 18:0 fatty acids in the mammary gland could be the reason behind the elevation of 18:1cis9 fatty acid concentration found in milk (Enjalbert *et al.*, 1998). Increase in ewe milk DPA on the other hand, was contradictory to the finding of Otto *et al*. (2014) who reported a decrease in n-3 LC-PUFA in cow milk; thus suggesting physiological differences between ovine and bovine lipid metabolisms in response to canola oil supplementation. Further research is therefore needed in order to better elucidate the use of canola and rice bran oils as dietary supplements for dairy ewes.

Table 4.3. Ewe milk yield and composition (Means ± SE)

Item	Control	CO	RBO	FSO	SFO	RPO	P-value
MY	484±8.0^{d}	525±7.8^{c}	527±7.1^{c}	489±4.7^{d}	562±8.2^{b}	628±10.8^{a}	0.0001
Milk composition							
Fat (%)	7.4±0.2^{a}	7.2±0.1^{ab}	7.2±0.2^{ab}	6.9±0.1^{bc}	6.6±0.1^{c}	6.6±0.2^{c}	0.015
FY	36±7.2^{bc}	38±8.8^{b}	38±8.1^{b}	34±5.7^{c}	37±9.3^{b}	41±10.5^{a}	0.0008
Protein (%)	5.4±0.1^{c}	5.5±0.1^{bc}	5.9±0.1^{a}	5.4±0.1^{c}	5.6±0.1^{b}	5.4±0.2^{c}	0.0001
PY	26±7.2^{c}	29±7.4^{b}	31±6.2^{b}	26±3.5^{c}	31±6.4^{b}	34±8.9^{a}	0.0004
Lactose (%)	4.9±0.0	4.9±0.0	4.9±0.0	4.8±0.0	4.8±0.0	4.8±0.1	0.524
SNF (%)	10.9±0.1^{bc}	11.1±0.1^{bc}	11.7±0.1^{a}	10.8±0.1^{c}	11.2±0.1^{b}	11.0±0.1^{bc}	0.0001

Milk yield (MY, g/day), fat yield (FY, g/day), protein yield (PY, g/day), solids non-fat (SNF). Abreviations are as defined in Table 4.1.

In stark contrast, milk from ewes receiving FSO pellets showed a marked improvement in PUFA composition, recording the highest Σn-3 PUFA due to its high ALA proportion compared to all the other diets. These increases were in accordance with other studies that supplemented dairy sheep and cows with whole or extruded flaxseed (Caroprese *et al.*, 2010; Mughetti *et al.*, 2012; Caroprese *et al.*, 2016). The relatively high level of PUFA in the FSO diet also resulted in increased biohydrogenation of 18:2 and 18:3 in the rumen into 18:0, which is an inhibitor of *de novo* fatty acid synthesis of short-chain FA including 16:0 (Chilliard *et al.*, 2003; Buccioni *et al.*, 2012a). Therefore, the percentage of 16:0 in the FSO treatment in the current study decreased significantly, also consistent with a better outcome in terms of the overall FA profile from a human health perspective; this feature is because a high percentage of 16:0 is associated with cardiovascular problems (Noakes *et al.*, 1996). In agreement with Caroprese *et al.* (2010), the proportions of EPA and DPA in milk from ewes supplemented with FSO in our study were also higher compared to the control group (0.11 vs 0.08%, and 0.15 vs 0.08%; respectively). This finding confirms that the concentration ALA was the essential precursor for the synthesis of EPA (Leonard *et al.*, 2004), while EPA was the precursor for DPA synthesis (Gregory *et al.*, 2013) through enzymatic elongation and desaturation in adipose tissue of mammals (Leonard *et al.*, 2004).

Table 4.4. Fatty acid profiles of ewe milk (as % of total fatty acids ± SE)

Fatty acid	Control	CO	RBO	FSO	SFO	RPO	P-value
12:0	2.82±0.28	3.33±0.53	4.37±0.59	2.56±0.26	3.15±0.30	3.43±0.45	0.0667
13:0	0.05±0.02	0.02±0.00	0.07±0.02	0.02±0.01	0.05±0.02	0.03±0.01	0.1408
14:0	11.2±0.56^{b}	12.1±1.07^{b}	14.7±0.84^{a}	10.8±0.61^{b}	12.4±0.60^{b}	12.7±0.71^{ab}	0.0138
15:0	1.12±0.03	1.04±0.03	1.04±0.05	1.02±0.03	1.05±0.04	1.09±0.03	0.4118
16:0	30.6±0.8^{bc}	29.8±1.7^{c}	35.4±1.5^{a}	26.7±0.7^{d}	30.3±1.3^{bc}	33.1±0.9^{ab}	0.0001
17:0	0.75±0.03^{a}	0.59±0.03^{cd}	0.53±0.03^{d}	0.65±0.02^{bc}	0.58±0.02^{cd}	0.72±0.03^{ab}	0.0001
18:0	11.8±0.7^{a}	10.6±1.05^{ab}	7.9±1.1^{b}	12.0±0.8^{a}	9.1±0.8^{b}	8.7±0.7^{b}	0.0255
20:0	0.43±0.02^{b}	0.42±0.05^{b}	0.31±0.04^{b}	0.38±0.02^{b}	0.33±0.03^{b}	0.58±0.06^{a}	0.0001
22:0	0.12±0.01^{bc}	0.13±0.02^{bc}	0.08±0.02^{c}	0.15±0.02^{ab}	0.11±0.01^{bc}	0.18±0.02^{a}	0.0043
24:0	0.10±0.00	0.07±0.02	0.05±0.02	0.09±0.01	0.06±0.02	0.08±0.01	0.0949
16:1n-9c	0.34±0.02^{a}	0.36±0.02^{a}	0.34±0.02^{a}	0.28±0.01^{b}	0.33±0.02^{ab}	0.22±0.01^{c}	0.0001
16:1n-7c	1.40±0.09^{bc}	1.45±0.14^{bc}	1.81±1.20^{a}	1.15±0.11^{c}	1.66±0.12^{ab}	1.63±0.11^{ab}	0.05
16:1n-7t	0.35±0.02^{b}	0.26±0.03^{c}	0.22±0.02^{c}	0.50±0.04^{a}	0.39±0.03^{b}	0.53±0.04^{a}	0.0001
18:1n-9c	23.0±0.8^{ab}	23.5±1.8^{a}	18.9±1.2^{cd}	21.5±0.9^{ab}	19.9±0.9^{bc}	16.3±0.9^{d}	0.0047
18:1n-7c	1.17±0.16^{c}	2.19±0.12^{a}	1.63±0.04^{bc}	1.90±0.10^{ab}	1.63±0.28^{bc}	2.14±0.09^{a}	0.0017
C18:1n-7t	3.46±0.17^{b}	3.49±0.32^{b}	2.74±0.16^{b}	6.47±0.40^{a}	5.53±0.57^{a}	6.06±0.47^{a}	0.0001
18:2CLA$_{a}$	0.20±0.00^{b}	0.18±0.01^{b}	0.19±0.06^{b}	0.31±0.02^{a}	0.22±0.01^{b}	0.24±0.03^{ab}	0.0363
18:2CLA$_{b}$	0.04±0.01^{b}	0.04±0.02^{b}	0.05±0.02^{ab}	0.09±0.01^{a}	0.09±0.01^{a}	0.07±0.02^{ab}	0.0333
18:2n-6 LA	3.27±0.16^{bc}	2.94±0.13^{c}	3.52 ±0.35^{bc}	3.76±0.19^{b}	5.29±0.30^{a}	3.10±0.13^{bc}	0.0001
18:3n-3 ALA	0.62±0.05^{bc}	0.73±0.03^{b}	0.51±0.06^{c}	1.74±0.08^{a}	0.67±0.04^{bc}	0.74±0.04^{b}	0.0001
20:4n-6 ARA	0.23±0.02^{bc}	0.23±0.02^{bc}	0.28±0.02^{ab}	0.21±0.01^{c}	0.32±0.02^{a}	0.22±0.01^{c}	0.0004
20:5n-3 EPA	0.08±0.00^{c}	0.09±0.00^{c}	0.07±0.01^{c}	0.11±0.00^{b}	0.07±0.01^{c}	0.17±0.01^{a}	0.0001
22:6n-3 DHA	0.04±0.01^{b}	0.06±0.01^{b}	0.04±0.02^{b}	0.06±0.01^{b}	0.06±0.01^{b}	0.19±0.01^{a}	0.0001
22:5n-3 DPA	0.08±0.02^{d}	0.13±0.01^{bc}	0.10±0.02^{cd}	0.15±0.01^{b}	0.10±0.02^{cd}	0.23±0.01^{a}	0.0001
ΣSFA	62.5±0.8^{b}	61.1±2.0^{bc}	66.9±1.5^{a}	57.8±1.4^{c}	60.3±1.2^{bc}	63.9±1.1^{ab}	0.0007
ΣMUFA	32.7±1.9^{ab}	34.2±0.7^{ab}	28.3±1.3^{c}	35.5±1.2^{a}	32.5±1.1^{ab}	30.6±1.0^{bc}	0.0036
ΣPUFA	4.8±0.2^{b}	4.7±0.2^{b}	4.8±0.5^{b}	6.7±0.3^{a}	7.2±1.1^{a}	5.5±0.2^{b}	0.0001
Σn-6 PUFA	3.7±0.2^{b}	3.4±0.1^{b}	3.7±0.4^{b}	4.1±0.2^{b}	5.9±0.3^{a}	3.8±0.2^{b}	0.0001
Σn-3 PUFA	0.84±0.05^{cd}	1.01±0.04^{c}	0.72±0.07^{d}	2.07±0.08^{a}	0.90±0.06^{cd}	1.36±0.05^{b}	0.0001
PUFA/SFA	0.08±0.00^{b}	0.08±0.00^{b}	0.07±0.01^{b}	0.12±0.01^{a}	0.12±0.02^{a}	0.09±0.01^{b}	0.0001
Σn-6/ Σn-3	4.5±0.4^{c}	3.4±0.2^{d}	5.5±0.5^{b}	2.0±0.0^{e}	6.7±0.3^{a}	2.8±0.1^{de}	0.0001
AI	2.05±0.11^{bc}	2.21±0.38^{abc}	2.72±0.19^{a}	1.71±0.12^{c}	2.07±0.16^{bc}	2.39±0.17^{ab}	0.0237
IT	0.32±0.01^{abc}	0.32±02^{abc}	0.39±0.02^{a}	0.27±0.02^{c}	0.31±0.02^{bc}	0.35±0.02^{ab}	0.0172

Values with different superscript are significantly different
IA = Atherogenic index [12:0 + (4*14:0) +16:0] / [(ΣPUFA) + (ΣMUFA)], and IT = Thrombogenic index [14:0 + 16:0 + 18:0]/[(0.5*ΣMUFA) + (0.5*Σ n-6) + (3*Σn-3)+(n-3/ n-6)] calculated as per Ulbricht and Southgate (Ulbricht and Southgate, 1991)
All abreviations are as defined in Tables 4.1 and 4.2.

Safflower has been known as the richest source of linoleic acid among oilseeds, although its use as a supplement for dairy sheep has not been previously reported. The total relative level of PUFA in milk from the SFO group in our study increased significantly by 149% (7.2 vs 4.8%) and mainly due to an increase in the level of linoleic acid (5.3 vs 3.3%). These findings were in agreement with outcomes from recent studies in lactating bovines that evaluated the effect of safflower oil (Bell *et al.*, 2006; Li *et al.*, 2015) as well as safflower seed (Alizadeh *et al.*, 2012) on milk fat composition. Post-ruminal infusion increased the escape rate of LA from biohydrogenation in the rumen for eventual absorption and subsequent transfer into milk fat in the mammary gland (Shingfield *et al.*, 2013). As a consequence, the proportion of 18:0 in SFO also reduced significantly. In contrast, the result from Bell *et al.* (2006) showed significant increase in 18:0. In terms of n-3 PUFA, concentration differences between the control and SFO in our study align with the outcomes reported by Li *et al.* (2015). Therefore, the inclusion of safflower oil in diets would not improve the concentration n-3 LC-PUFA in milk for both dairy cows and dairy sheep.

The highest level of n-3 LC-PUFA in the diet resulting in significant increases in these FA in milk was for ewes receiving RPO, a result that is in agreement with previous studies that examined milk fat n-3 LC-PUFA in reponse to fish oil supplements in cow (Kitessa *et al.*, 2004), sheep (Kitessa *et al.*, 2003; Toral *et al.*, 2010; Toral *et al.*, 2015) and goat (Kitessa *et al.*, 2001b). These increases suggest that the use of n-3 LC-PUFA in protected oil was able to facilitate an escape from ruminal biohydrogenation that reduces the double bonds of LC-PUFA due to the activity of rumen microbial communities. It has been reported that sheep fed protected tuna oil pellets increased significantly the proportion of DHA that bypassed the rumen than those fed unprotected tuna oil pellets (Kitessa *et al.*, 2001a). According to (Palmquist, 2009), the potential lengthening of 20:5n-3 to 22:5n-3 and shortening of 22:6n-3 to 22:5n-3 in body tissues induced higher rates of transfer from the supplementary diet into

milk for 22:5n-3 in comparision with 20:5n-3 and 22:6n-3. In agreement with (Kitessa *et al.*, 2001a; Toral *et al.*, 2015) the concentrations of EPA, DHA, and DPA increased in inverse proportions to that of 18:0 in milk from RPO group. This further difference is probably because of the lower levels of 18:0 in the RPO, and therefore reduced availability for its direct incorporation in the mammary gland.

Precise interactions between the stage of lactation and dietary effects on FA composition were observed. This is because experimental animals were fed the same control diet before the start of experiment that minimized the effects of random variabilty on this relationship. Changes in LA in SFO (Figure 4.1a) and ALA in FSO during adaptation period in this study (Figure 4.1b) were akin to previous findings (Roy *et al.*, 2006) that demonstrated significant increases in these FA on day 6 after feeding dairy cows with high-concentrate diets suplemented with sunflower oil and hay diet supplemented with linseed oil, respectively. Our results demonstrating rapid increases in EPA, DHA and DPA in RPO treatment (Figure 4.1 c, d, e) align with those of (Kitessa *et al.*, 2001a).

Examination of the nutritional value per standard serve size of milk (250 ml) identified by the National Health and Medical Research Council of Australia (NHMRC, 2013) (expressed as mg/250 ml of milk) showed that the content of total n-3 PUFA in milk from ewes fed FSO was the greatest at 221 mg/250 ml and provided 34% of the recommended 650 mg daily consumption for humans (Simopoulos *et al.*, 2000). In parallel with total n-3 PUFA, the FSO dietary treatment also showed the highest content of ALA at 185.2 mg per serve that accounted for approximately 14.2% and 23.2% of the adequate intake level for men and women (1.3-0.8 g/day), respectively, recommended by NHMRC (2006). This value was higher than the contribution of cooked lamb meat to ALA needs at 3.7% and 6.1% (Flakemore *et al.*, 2017). In terms of linoleic acid, milk from ewes supplemented with SFO showed the highest content among treatment groups with each serve containing 575 mg/250 ml that covered 4.4%-7.2% of the proposed daily allowance (NHMRC, 2006).

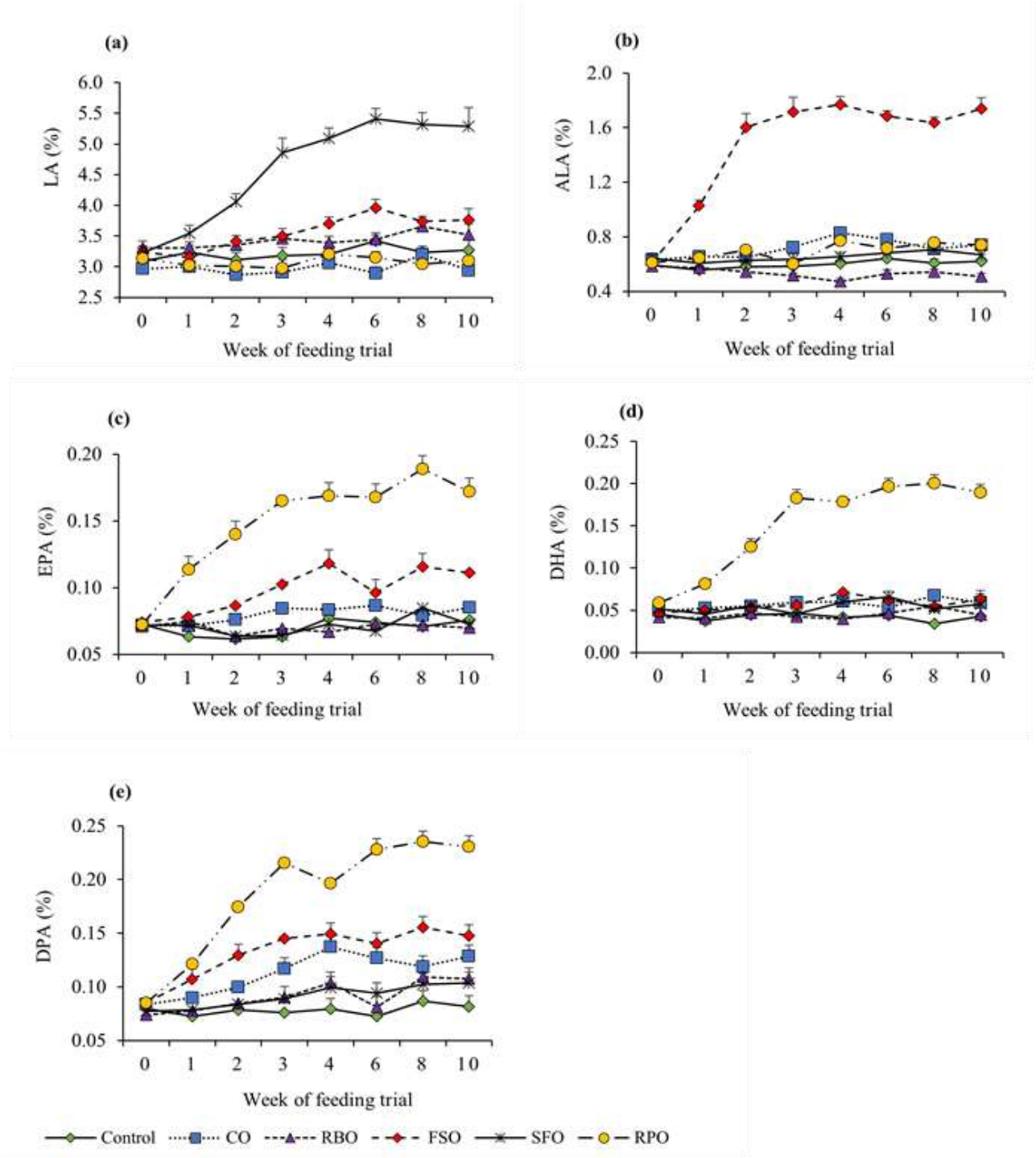

Figure 4.1. Interactions between supplemented diets and week of supplementation on the concentrations of LA (a), ALA (b), EPA (c), DHA (d), and DPA (e) in milk

Consumption of DPA also has beneficial effects on human health, such as lowering the risk of heart diseases (Rissanen *et al.*, 2000; Phang *et al.*, 2009; Mozaffarian *et al.*, 2013), and inhibition of inflammation (Chen *et al.*, 2012); however, previous studies on n-3 LC-PUFA

have often not considered or included DPA, but rather focused mainly on EPA and DHA. This shortcoming may be in part, due to pure DPA not being available as a commercial product, and therefore its effects for both animal models and ultimately human consumers, remain very much understudied. Its structure is similar to EPA and DHA (Byelashov *et al.*, 2015). In our study, DPA contributed more than 40% of total n-3 LC-PUFA in all treatments (Table 4.4), thus the intake of n-3 LC-PUFA should include DPA as suggested by NHMRC (2006). The content of total n-3 LC-PUFA (ΣEPA+DHA+DPA) in a cup of milk from RPO supplemented ewes supplied 38.6% of the requirement for men and 68.6% for women compared with current recommendations (NHMRC, 2006). Furthermore, one serve of milk produced from FSO and RPO supplemented diets contains more than 30 mg, and 60 mg repectively , hence they can be considered as 'source' and 'good source' of n-3 LC-PUFA respectively (FSANZ, 2012). These findings suggest that for consumers who do not habitually eat fish or fish products, sheep milk can be considered as an alternative and/or complimentary source of n-3 LC-PUFA.

Table 4.5. Mean concentrations (± SE) (mg/250 ml of milk) of n-3 and n-6 PUFA and LC-PUFA

FA (mg/250 ml)	Control	CO	RBO	FSO	SFO	RPO	P-value
18:2n-6 LA	392±32.0^{b}	326±18.2^{b}	431±20.3^{b}	403±17.7^{b}	575±27.9^{a}	320±18.5^{b}	0.0001
18:3n-3 ALA	74.1±7.1^{b}	80.6±4.8^{b}	67.7±3.4^{b}	185.2±5.8^{a}	72.9±4.2^{b}	74.5±3.7^{b}	0.0001
20:5n-3 EPA	9.1±0.6^{c}	9.6±0.6^{c}	8.6±0.5^{c}	12.0±0.7^{b}	8.0±0.7^{c}	17.8±1.2^{a}	0.0001
22:6n-3 DHA	5.4±0.89^{b}	7.2±0.9^{b}	5.7±0.7^{b}	7.0±0.8^{b}	6.2±0.5^{b}	19.8±1.8^{a}	0.0001
22:5n-3 DPA	9.7±2.0^{c}	15.2±1.7^{bc}	13.1±2.0^{bc}	16.1±1.6^{b}	11.5±2.1^{bc}	24.1±2.1^{a}	0.0001
ΣSFA	7426±348	6987±456	7988±385	6295±393	6646±342	6667±483	0.2167
ΣMUFA	3917±235	3915±341	3574±270	3838±196	3553±149	3146±188	0.2433
ΣPUFA	578±42.5^{b}	526±28.0^{b}	630±36.4^{b}	712±28.0^{a}	781±34.9^{a}	565±31.0^{b}	0.0001
Σn-6 PUFA	441±36.5^{b}	380±21.2^{b}	486±27.5^{b}	440±19.0^{b}	639±30.6^{a}	386±24.7^{b}	0.0001
Σn-3 PUFA	99.6±7.4^{c}	113±7.6^{c}	95.8±4.4^{c}	221±7.6^{a}	98.5±6.4^{c}	140±6.8^{b}	0.0001
ΣEPA+DHA + DPA	24.1±2.1^{c}	31.9±3.1^{bc}	27.5±3.2^{bc}	35.1±3.0^{b}	25.6±2.8^{bc}	61.7±5.0^{a}	0.0001

Values in rows with different superscripts are significantly different
All abreviations are as defined in Tables 4.1 and 4.2.

4.6. Conclusion

This study evaluated the ability of different plant oils and rumen bypass protected EPA+DHA to alter milk fatty acid composition (as percent total FA) and concentration (as mg/250 ml) toward enhancing n-3 LC-PUFA of grazing dairy ewes. Results demonstrated that rumen bypass protected EPA+DHA was the most effective supplementary diet at elevating milk n-3 LC-PUFA levels of EPA, DHA and DPA. The addition of flaxseed and canola oils in the diets also increased the levels of these milk n-3 LC-PUFA, but to a lesser extent. The inclusion of flaxseed oilwas the most effective at increasing the amount of α-linoleic acid. Therefore, in terms of nutritional value to consumers, our research clearly validates the health beneficial impact of adding flaxseed and rumen protected EPA + DHA oils to grazing dairy ewe diets to produce high quality milk with elevated levels of n-3 LC-PUFA. Although diets including rice bran and canola oil demonstrated minor effects on milk fat composition of grazing dairy sheep in this study, further research is necessary to fill the knowledge gap regarding the optimum level of oil inclusion in the diet of lactating ewes.

Chapter 5: Enhancement of dairy sheep cheese eating quality with increased n-3 long-chain polyunsaturated fatty acids

5.1. Abstract

This study investigated the effect of different plant oil-infused and rumen-protected wheat-based pellets containing eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) on n-3 long-chain (≥C20) polyunsaturated fatty acids (n-3 LC-PUFA) content, fatty acid recovery and sensory attributes of ripened cheese from dairy sheep. During a ten-week supplementary feeding trial, sixty dairy ewes balanced by liveweight, milk yield, parity, and sire breed were randomly divided into six groups that were (1) supplemented with on-farm existing commercial wheat-based pellets without oil inclusion (Control) or supplemented with wheat-based pellets infused with 50 ml/kg DM of oils from (2) canola, (3) rice bran, (4) flaxseed, (5) safflower (SFO), and (6) rumen protected EPA+DHA. Milk samples from each treatment were collected separately by sire breed during the experimental period for cheese processing at the end of the experiment. Twelve batches of cheese (2 batches per treatment) were processed and ripened for 120 d. Three cheese samples were collected and analysed for each cheese making session (total of 36 cheese samples) at day 120 of ripening. Processed cheese of RPO had the highest total n-3 LC-PUFA [total EPA + DHA + docosapentaenoic acid (DPA, 22:5n-3] content compared with the control (0.49 vs 0.28%). Flaxseed elicited the greatest enhancement of α-linolenic acid (ALA, 18:3n3), whereas safflower was the most effective diet in enhancing the level of linoleic acid (18:2n6) in cheese (1.29 vs 0.71%; 4.8 vs 3.3%; respectively). Parallel recoveries of n-3 and n-6 LC-PUFA were observed across all treatments except for α-linolenic acid (P=0.0090), and EPA. Cheese eating sensory traits were also highly affected by oil supplementation with the highest score of 7.5 in cheese from the

rice bran and flaxseed treatments. These results provide new insights into the biological mechanisms and processes that determine dairy ewe milk productivity by underpinning the vital biological role of n-3 LC-PUFA in not only enhancing the healthy composition of cheese from ewes, but also translating it into consumer acceptability.

5.2. Introduction

Over the last three decades, numerous studies have examined the health benefits of n-3 long-chain ($\geq C_{20}$) PUFA (n-3 LC-PUFA) and consistently demonstrated their vital role in inhibiting chronic diseases. According to the global status report on non-communicable diseases of the World Health Organization (Mendis and Chestnov, 2014), 38 million people died of chronic diseases and unhealthy dietary habits, with the deficiency of n-3 LC-PUFA intake as one of the main causes of death. In response to this health concern, a large number of studies aimed at increasing n-3 LC-PUFA content in human foods have emerged. Cheese is the most popular long shelf-life dairy product and its global production has been predicted to increase by 19% between 2008 and 2020 (OECD/FAO, 2011). Among the numerous cheese varieties, sheep cheese has been reported to have higher levels of beneficial PUFA than cow and goat cheeses under the same processing conditions (Prandini *et al.*, 2011). Therefore, the enhancement of health beneficial fatty acids (FA) in sheep cheese have been of immense research interest (Zhang *et al.*, 2006a,b; Bodas *et al.*, 2010; Buccioni *et al.*, 2012b).

The FA profile of cheese, especially beneficial FA that have positive effects on human health, primarily depends on the FA composition of raw milk rather than cheese processing technology (Collomb *et al.*, 2006; Bisig *et al.*, 2007; Prandini *et al.*, 2011). Because dietary FA contributes all of the long-chain FA in milk fat (Chilliard *et al.*, 2007), dietary supplementation of ruminants with unsaturated fatty acids (UFA) remains one of the most popular ways to alter milk fat profile (Reynolds *et al.*, 2006; Zhang *et al.*, 2006a,b; Castro *et al.*, 2009; Gomez-Cortes *et al.*, 2011) and increase the proportion of PUFA in sheep milk and cheese. However, recent research has only focused on limited sources of unsaturated plant lipids, mostly linseed, soybeans, safflower and

sunflower (Nudda *et al.*, 2014). This suggests the need for further investigations into the effect of other available plant oil sources including canola and rice bran on FA composition of dairy sheep products. Furthermore, about 30% of milk processed in Australia is used for the production of cheese (Dairy Australia, 2016). However, to the best of our knowledge, available published literature about the concentration of beneficial FA including n-3 LC-PUFA in cheese under Australian on-farm production conditions is at best scanty or non-existent, thus representing a major knowledge gap that this study intends to fill. Therefore, the objective of this study was to determine the effects of supplementing grazing Australian dairy ewes with oil-infused canola, rice bran, flaxseed, safflower and rumen-protected eicosapentaenoic acid and docosahexaenoic acid pellets on the concentrations and recovery of LC-PUFA as well as the eating quality of sheep cheese. It was hypothesized that supplementing grazing dairy ewes with different sources of dietary oils, including those containing n-3 and n-6 PUFA, will affect the concentration and recovery of n-3 LC-PUFA, and alter the appearance, texture, taste, flavour and aroma of ripened cheese.

5.3. Materials and methods

5.3.1. Animals and treatments

This study was carried out at Grandvewe Cheeses Farm, Birchs Bay, Woodbridge, Tasmania, Australia, with all experimental protocols approved by the University of Tasmania Animal Ethics Committee in accordance with the 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Animal Ethics Permit Number A0015657).

Sixty lactating Awassi and Awassi x East Friesian crossbred ewes in mid-lactation were randomly assigned to six groups balanced by breed, parity (2.8±0.5), liveweight (59±5.9 kg), and milk yield (657±100 g/day). The six groups were (1) supplemented with on-farm existing commercial wheat-based pellets without oil inclusion (control) or supplemented with wheat-

based pellets infused with 50 mL/kg DM of (2) canola (CO); (3) rice bran (RBO); (4) flaxseed (FSO); (5) safflower (SFO) and (6) rumen-protected eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA; treatment referred to as RPO). The RPO treatment was based on a modification of the microencapsulation of oil droplets in a protein-aldehyde matrix procedure (Scott *et al.*, 1971). All supplementary diets were isocaloric and isonitrogenous. The nutritional composition of the experimental diets is shown in Table 5.1. Ewes were grazed as a single mob in the same paddock with free access to local natural velvet tussock grass, hay and fresh water. Each ewe received 1 kg of the pellet supplements daily during milking time at 0600 h for 10 weeks including an initial two-week adjustment period, followed by an 8-week experimental period. The proportions of experimental diets CO, RBO, FSO, SFO, and RPO were gradually increased by 100 g/day during the transition period until the attainment of 1 kg/day per head on day 10 of the adjustment period. Milk in each group were collected separately by sire breed during experimental period using the De Laval Sheep Milking Platform and store at the farm in sanitised plastic containers at -20 ºC for cheese processing at the end of the experiment.

5.3.2. Cheese making

Twelve batches were processed following Grandvewe Cheeses farm standard protocols without pasteurization. Briefly, raw milk was heated to 38 ºC in a cheese vat and rennet was added to form curd. The curds were then cut, stirred into very small pieces, and left to compact at the bottom of the vat. Then, the curd was removed from the vat and the whey was drained and put into small, 1-kg plastic mould blocks. After brine-salting for 24 h, the cheeses were transferred to a ripening room adjusted to remain at constant temperature (11-12 °C) and 75-80% relative humidity for 120 d. Three cheese samples of each batch were randomly collected and stored in air-tight bags at -20 ºC until analysed at d 120 (36 cheese samples in total), when they were ripened and ready for trading.

5.3.3. Chemical analysis of experimental feeds

Samples of supplementary and basal diets were collected weekly and stored at -20 ºC during the feeding trial, then dried at a constant temperature of 65 ºC in a fan-forced oven before being ground to pass through a 1-mm screen using a Thomas Model 4 Laboratory Mill (Thomas Scientific, Swedesboro, NJ), and then used for analysing DM and ash contents according to AOAC (1990). An ANKOM220 fibre analyser was used to analyse NDF and ADF, and ether extract was measured using an ANKOMXT15 fat/oil extractor (ANKOM Technology, Macedon, NY, USA). The CP percentage was calculated based on the value of nitrogen that was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser (Thermo Scientific, Waltham, MA, USA). Metabolisable energy (ME) and total digestible nutrients (TDN) were calculated as per Weiss (1992). The chemical compositions of the control, treatments, pasture and hay are presented in Table 5.1.

Table 5.1. Proximate analysis[a] of experimental diets[b]

Component (% DM)	Pasture	Hay	Control	CO	RBO	FSO	SFO	RPO
DM	96.5	95.5	91.5	93.0	91.6	90.0	91.7	91.6
OM	90.5	97.3	92.2	93.3	92.7	91.0	91.8	92.0
Ash	9.5	2.7	7.8	6.7	7.3	9.0	8.2	8.0
ADF	45.5	37.6	10.6	7.1	8.1	9.7	9.0	8.5
NDF	69.9	68.3	30.0	21.8	19.4	23.3	23.9	22.0
EE	1.4	1.2	3.3	5.7	5.2	5.4	5.0	5.1
CP	4.7	4.3	14.6	14.0	14.7	14.6	14.5	15.6
TDN	48.5	54.1	73.4	75.9	75.2	74.1	74.5	74.9
ME, MJ/kg DM	7.1	8.1	11.7	12.2	12.0	11.8	11.9	12.0

[a] Dry matter (DM), organic matter (OM), acid detergent fibre (ADF), neutral detergent fibre (NDF), ether extract (EE), crude protein (CP), total digestible nutrients (TDN) and metabolisable energy (ME).

[b] Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO).

5.3.4. Fatty acid analysis

Approximately 5 mg of diet samples and 0.5 g of raw milk samples were accurately weighed into methylation tubes and freeze-dried to remove moisture according to Otto *et al.* (2014), and 0.1 g of unground cheese sample was extracted for total lipids according to the procedures reported by Malau-Aduli *et al.* (2016). The dried feed samples and aliquots of the extracted lipids from cheese samples were then methylated in a solution of methanol/HCl/dichloromethane (10/1/1; 3 mL; 80°C for 2 h) to produce fatty acid methyl esters (FAME), which were extracted (hexane/dichloromethane, 4/1; 2 mL, 3×) and transferred to glass GC vials. The FAME were diluted with dichloromethane containing C19:0 FAME as the internal injection standard before analyses were performed using an Agilent Technologies (Santa Clara, CA, USA) 7890B gas chromatograph equipped with an Equity-1 fused silica capillary column (15 m × 0.1-mm i.d. and 0.1- µ m film thickness), a flame ionization detector, a split/splitless injector, and an Agilent Technologies 7683B Series autosampler. Oven temperature profile was set initially at 120°C for 1 min and was raised to 270°C at 10°C/min, then to 310°C at 5°C/min. ChemStation software (Agilent Technologies) was used to quantify peak areas. We performed GC-MS analyses on selected samples to confirm FA identities using a Thermo Scientific 1310 GC coupled with a TSQ triple quadrupole. Samples were injected using a Tripleplus RSH autosampler with a nonpolar HP-5 Ultra 2 bonded-phase column (50 m × 0.32-mm i.d. × 0.17-µm film thickness; Agilent Technologies). The HP-5 column was of similar polarity to the column used for GC analyses. The initial oven temperature of 45°C was held for 1 min, followed by temperature programming at 30°C/min to 140°C, then at 3°C/min to 310°C, where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were as follows: electron impact energy = 70 eV; emission current = 250

μA; transfer line = 310°C; source temperature = 240°C; scan rate = 0.8 scan/s; mass range = 40 to 650 Da. Mass spectra were acquired and processed with Thermo Scientific Xcalibur software.

5.3.5. Calculation of cheese FA recovery

Recovery of cheese for selected individual FA [$REC_{(FA)}$)] was calculated using the formula described by (Cattani *et al.*, 2014):

$$REC_{(FA)} = \frac{\text{Cheese fatty acid (g)}}{\text{Milk fatty acid (g)}} \times \frac{\text{Cheese fat (g)}}{\text{Milk fat (g)}}$$

5.3.6. Consumer sensory evaluation

Following the procedure of Fuentes *et al.* (2015), a sensory evaluation test was conducted at the University of Tasmania by trained consumer panelists comprising 25 male and female volunteer staff and students (aged between 20 and 55 yr) who habitually consume cheese in their diets. The assessors had a training session before the official testing so that they were familiar with the whole process of hedonic evaluation of sensory characteristics. As demonstrated by Lim (2011), the 9-point hedonic scale is easy, simple, reliable, and highly effective for quantifying sensory differences among foods and for predicting consumer acceptability; hence, it was chosen in this study. In both sessions, cheese was refrigerated until served as 1.5-cm3 cubes in a completely randomized block design in a tasting room at 20°C. Panelists were given the opportunity to taste each sample 5 times, including 2 of the cheeses for pretesting sessions (Mughetti *et al.*, 2012). Unsalted crackers and water were used to remove any lingering effects of the previous samples. A 9-point hedonic scale was used to rate the degree of liking, appearance, aroma, flavor, texture, and overall acceptability, where 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither

like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = like extremely.

5.3.7. Statistical analysis

Statistical analysis of all collected data was performed in SAS version 9.2 (SAS Institute, Cary, NC). Initially means, standard deviations, standard errors, minimum and maximum values of data were computed using PROC MEANS, and these were scrutinized for any data entry errors. Selected cheese FA were subjected to a General Linear Model (PROC GLM), mixed model analysis with treatment, breed, and their second-order interactions fitted as fixed effects, while milk yield and ewe age were fitted as random effects. All non-significant interactions were removed from the final analytical model. Level of significance threshold was set at $P < 0.05$ and differences between means were established using Tukey's probability pairwise comparison test.

5.4. Results

5.4.1. FA composition and recovery of the main n-6 and n-3 LC-PUFA in ripened cheese

As depicted on Table 5.2, there were differences in FA percentages between the diets. These differences resulted in significant variations in selected raw milk FA (Table 5.3), and cheese FA (Table 5.4). The largest improvement in the proportion of total n-3 PUFA was recorded in cheese produced from milk from ewes fed FSO and RPO ($P<0.001$; 161% and 158% respectively), compared to the control (unsupplemented) group. The enrichment of total n-3 PUFA in cheese produced from animals in the FSO group was mainly due to the significant increase in the proportion of α−linolenic acid (ALA, 18:3n-3, 1.30%) compared with the control (0.71%). observed in cheese produced from animals in the RPO treatment compared with the control group was attributable to increases in the proportions of ALA (1.02 vs 0.71%) as well as total n-3 LC-PUFA [EPA + DHA + docosapentaenoic acid (DPA); 0.50 vs. 0.29%; $P < 0.001$; Table 5.4]. In terms of linoleic acid (LA), the greatest percentage was observed in the

cheese produced from the SFO group, which increased by 147% compared with the control group (4.78 vs 3.26%) and followed by FSO (3.79%), RPO (3.52%), RBO (3.45%), and CO (3.06%; $P < 0.001$).

Table 5.2. Selected fatty acid[a] compositions of pasture, hay and experimental diets[b] (% of total fatty acids)

Fatty acid	Pasture	Hay	Control	CO	RBO	FSO	SFO	RPO
14:0	1.60	2.85	0.05	0.14	0.14	0.14	0.22	2.35
16:0	18.8	35.8	21.8	12.4	17.4	11.0	14.0	19.5
18:0	4.48	7.57	1.05	1.12	2.28	3.38	2.26	2.86
16:1n7c	0.65	0.55	0.13	0.27	0.20	0.25	0.22	4.03
18:1n9c	10.2	0.0	20.5	41.8	31.8	18.8	20.3	20.9
18:1n7c	1.31	1.15	1.22	2.54	1.11	1.83	1.48	1.90
18:2n6 LA	18.7	8.6	47.0	33.8	41.1	40.9	56.5	35.7
18:3n3 ALA	24.1	12.2	3.0	4.8	2.3	20.5	1.7	2.7
20:5n3 EPA	0.00	0.00	0.00	0.13	0.10	0.12	0.07	2.50
22:6n3 DHA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.63
22:5n3 DPA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.54
ΣSFA	41.2	69.1	25.3	15.3	22.0	16.0	18.0	27.1
ΣMUFA	15.0	7.1	23.4	45.7	34.0	21.7	22.9	28.3
ΣPUFA	43.8	23.8	51.3	39.0	44.0	62.3	59.1	44.6
Σn6 PUFA	19.5	10.7	47.8	34.0	41.4	41.6	57.0	37.1
Σn3 PUFA	24.2	12.3	3.1	4.9	2.4	20.6	1.7	7.4
ΣEPA+DHA+DHA	0.00	0.00	0.00	0.13	0.10	0.12	0.07	4.67

[a] Linoleic acid (LA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), total saturated fatty acids (ΣSFA), total monounsaturated fatty acids (ΣMUFA), and total polyunsaturated fatty acids (ΣPUFA).

[b] Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO).

Table 5.3. Selected fatty acid composition of raw milk (as % of total fatty acids)

Fatty acid	Control	CO	RBO	FSO	SFO	RPO	SEM	P-value
12:0	2.68	3.11	3.89	2.62	3.42	3.37	0.20	0.5019
14:0	11.13^{b}	11.75^{b}	15.22^{a}	11.17^{b}	13.15^{ab}	12.42^{ab}	0.45	0.0484
15:0	1.09	1.06	1.01	0.97	1.09	1.08	0.02	0.3425
16:0	31.38^{ab}	29.61^{b}	36.95^{a}	27.11^{b}	31.58^{ab}	32.22^{ab}	0.83	0.0294
17:0	0.71^{a}	0.59^{b}	0.48^{c}	0.62^{ab}	0.57^{bc}	0.71^{a}	0.02	0.0002
18:0	11.42^{a}	10.99^{ab}	7.48^{b}	11.57^{a}	8.19^{ab}	8.63^{ab}	0.50	0.0500
20:0	0.42^{bc}	0.45^{b}	0.29^{c}	0.39^{bc}	0.31^{bc}	0.60^{a}	0.02	0.0009
22:0	0.13^{b}	0.13^{b}	0.08^{c}	0.15^{ab}	0.09^{bc}	0.19^{a}	0.01	0.0022
24:0	0.07^{abc}	0.07^{abc}	0.04^{c}	0.08^{ab}	0.05^{bc}	0.09^{a}	0.00	0.0335
16:1n-9c	0.35^{a}	0.35^{a}	0.31^{a}	0.26^{b}	0.32^{a}	0.21^{b}	0.01	0.0001
16:1n-7c	1.49	1.47	1.82	1.33	1.75	1.72	0.07	0.3007
16:1n-7t	0.36^{c}	0.28^{cd}	0.23^{d}	0.48^{b}	0.38^{ab}	0.59^{a}	0.02	0.0001
18:1n-9c	23.22^{ab}	24.03^{a}	18.10^{bc}	21.86^{ab}	18.57^{bc}	16.29^{c}	0.77	0.0194
18:1n-7c	1.49^{c}	2.15^{ab}	1.58^{bc}	1.84^{abc}	1.69^{abc}	2.23^{a}	0.08	0.0466
18:1n-7t	3.18^{b}	3.42^{b}	2.54^{b}	6.15^{a}	5.34^{a}	6.77^{a}	0.33	0.0001
18:2 CLA	0.24	0.22	0.32	0.36	0.29	0.35	0.03	0.5052
18:2n-6 LA	3.15^{b}	2.86^{b}	3.54^{b}	3.62^{b}	5.30^{a}	3.08^{b}	0.16	0.0001
18:3n-3 ALA	0.56^{bc}	0.69^{bc}	0.53^{c}	1.69^{a}	0.65^{bc}	0.71^{b}	0.07	0.0001
20:4n-6 ARA	0.24^{bc}	0.23^{bc}	0.26^{b}	0.18^{c}	0.34^{a}	0.19^{c}	0.01	0.0001
20:5n-3 EPA	0.07^{cd}	0.08^{c}	0.06^{d}	0.11^{b}	0.07^{cd}	0.17^{a}	0.01	0.0001
22:6n-3 DHA	0.04^{b}	0.06^{b}	0.05^{b}	0.06^{b}	0.06^{b}	0.19^{a}	0.01	0.0001
22:5n-3 DPA	0.09^{b}	0.13^{b}	0.11^{b}	0.13^{b}	0.13^{b}	0.23^{a}	0.01	0.0001
ΣSFA	62.44^{ab}	60.82^{b}	67.73^{a}	58.02^{b}	61.68^{ab}	62.72^{ab}	0.89	0.0001
ΣMUFA	32.89^{ab}	34.57^{a}	27.18^{b}	35.61^{a}	31.08^{ab}	31.66^{ab}	0.84	0.0038
ΣPUFA	4.66^{d}	4.61^{d}	5.09^{cd}	6.37^{ab}	7.25^{a}	5.62^{bc}	0.19	0.0001
Σn-6 PUFA	3.55^{b}	3.37^{b}	3.95^{b}	3.95^{b}	5.93^{a}	3.88^{b}	0.17	0.0001
Σn-3 PUFA	0.78^{cd}	0.96^{c}	0.75^{d}	1.99^{a}	0.91^{cd}	1.33^{b}	0.08	0.0001
PUFA/SFA	0.08^{b}	0.08^{b}	0.08^{b}	0.11^{a}	0.12^{a}	0.09^{b}	0.00	0.0006
Σn-6/ Σn-3	4.74^{b}	3.57^{c}	5.32^{b}	1.99^{d}	6.58^{a}	2.92^{cd}	0.30	0.0001

Row means bearing different superscripts within a fixed factor significantly differ.

Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO), linoleic acid (LA), α-linolenic acid (ALA), Arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), total saturated fatty acids (ΣSFA), total monounsaturated fatty acids (ΣMUFA), and total polyunsaturated fatty acids (ΣPUFA).

Table 5.4. Selected fatty acid composition of ripened cheese from ewe milk (as % of total fatty acids)

Fatty acid	Control	CO	RBO	FSO	SFO	RPO	SEM	P-value
12:0	0.79	1.12	1.36	1.02	1.19	1.37	0.13	0.854
14:0	9.48^{b}	10.59^{ab}	11.89^{a}	10.17^{b}	10.64^{ab}	10.53^{b}	0.20	0.0222
15:0	1.15^{a}	1.04^{c}	1.01^{d}	1.02^{d}	1.06^{c}	1.09^{b}	0.01	0.0001
16:0	30.68^{d}	30.69^{d}	34.25^{a}	30.72^{d}	32.11^{c}	33.09^{b}	0.25	0.0001
17:0	0.83^{a}	0.65^{c}	0.58^{d}	0.65^{c}	0.65^{c}	0.73^{b}	0.68	0.0001
18:0	12.22^{a}	11.06^{c}	9.72^{d}	11.41^{b}	9.79^{d}	9.87^{d}	0.17	0.0001
20:0	0.51^{a}	0.43^{b}	0.39^{c}	0.39^{c}	0.42^{bc}	0.54^{a}	0.01	0.0001
22:0	0.17^{a}	0.12^{b}	0.11^{b}	0.13^{b}	0.14^{b}	0.17^{a}	0.01	0.0005
24:0	0.08	0.05	0.05	0.05	0.05	0.07	0.00	0.1125
16:1n-9c	0.34^{c}	0.41^{a}	0.35^{b}	0.29^{e}	0.32^{d}	0.25^{f}	0.01	0.0001
16:1n-7c	1.44^{c}	1.45^{c}	1.72^{a}	1.41^{c}	1.73^{a}	1.62^{b}	0.02	0.0001
16:1n-7t	0.34^{c}	0.29^{d}	0.25^{e}	0.42^{b}	0.43^{b}	0.52^{a}	0.02	0.0001
18:1n-9c	24.54^{a}	24.61^{a}	21.87^{b}	21.24^{b}	20.23^{c}	18.39^{d}	0.39	0.0001
18:1n-7c	1.58^{f}	2.36^{a}	1.99^{d}	1.92^{e}	2.08^{c}	2.21^{b}	0.04	0.0001
18:1n-7t	3.84^{c}	3.79^{c}	3.37^{d}	5.67^{b}	5.62^{b}	6.41^{a}	0.20	0.0001
18:2 CLA	0.98^{b}	0.84^{c}	0.84^{c}	1.23^{a}	1.09^{b}	1.07^{b}	0.03	0.0001
18:2n-6 LA	3.26^{d}	3.06^{e}	3.45^{c}	3.79^{b}	4.78^{a}	3.52^{c}	0.10	0.0001
18:3n-3 ALA	0.71^{d}	0.79^{c}	0.63^{e}	1.30^{a}	0.71^{d}	1.02^{b}	0.04	0.0001
20:4n-6 ARA	0.22^{cd}	0.23bc	0.26^{ab}	0.21^{cd}	0.27^{a}	0.19^{d}	0.01	0.0001
20:5n-3 EPA	0.11^{b}	0.11^{b}	0.10^{b}	0.11^{b}	0.11^{b}	0.16^{a}	0.01	0.0001
22:6n-3 DHA	0.06^{b}	0.06^{b}	0.06^{b}	0.06^{b}	0.08^{b}	0.15^{a}	0.01	0.0001
22:5n-3 DPA	0.12^{b}	0.13^{b}	0.12^{b}	0.13^{b}	0.13^{b}	0.19^{a}	0.01	0.0008
ΣSFA	59.02^{bc}	58.45^{c}	61.73^{a}	58.36^{c}	58.76^{bc}	60.16^{b}	0.26	0.0001
ΣMUFA	35.12^{ab}	35.96^{a}	32.42^{e}	34.47^{bc}	33.66^{cd}	33.20^{de}	0.24	0.0001
ΣPUFA	5.86^{d}	5.59d	5.85^{d}	7.17^{b}	7.58^{a}	6.64^{c}	0.13	0.0001
Σn-6 PUFA	3.73^{c}	3.51^{d}	3.94^{c}	4.25^{b}	5.33^{a}	3.90^{c}	0.10	0.0001
Σn-3 PUFA	1.00^{bc}	1.09^{b}	0.91^{c}	1.61^{a}	1.04^{b}	1.58^{a}	0.05	0.0001
PUFA/SFA	0.09^{c}	0.09^{c}	0.09^{c}	0.12^{a}	0.13^{a}	0.11^{b}	0.00	0.0001
Σn-6/ Σn-3	3.72^{c}	3.21^{d}	4.34^{b}	2.65^{e}	5.17^{a}	2.47^{e}	0.16	0.0001

Row means bearing different superscripts within a fixed factor significantly differ.

Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO), linoleic acid (LA), α-linolenic acid (ALA), Arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), total saturated fatty acids (ΣSFA), total monounsaturated fatty acids (ΣMUFA), and total polyunsaturated fatty acids (ΣPUFA).

The effect of oil supplements on the recovery of main n-6 and n-3 LC-PUFA is depicted in Table 5.5. In terms of individual FA, there were significant differences in due to dietary supplementation, with the highest transferred rates of ALA and EPA observed in RPO (1.09) and SFO (1.03). The recoveries of group FA had similar patterns across treatments except for n-3 PUFA ($P < 0.0514$), which ranged from a minimum of 0.56 in FSO to a maximum of 0.86 in RPO.

Table 5.5. Recovery of n-3 and n-6 PUFA in ripened cheese

Fatty acid	Control	CO	RBO	FSO	SFO	RPO	SEM	P-value
18:2n-6 LA	0.66	0.67	0.64	0.73	0.63	0.83	0.02	0.1045
18:3n-3 ALA	0.84^{ab}	0.73^{bc}	0.78^{bc}	0.54^{c}	0.75^{bc}	1.09^{a}	0.04	0.0090
20:5n-3 EPA	0.97^{ab}	0.83^{abc}	1.02^{a}	0.76^{bc}	1.03^{a}	0.68^{c}	0.03	0.0098
22:6n-3 DHA	0.89	0.64	0.87	0.74	0.95	0.52	0.05	0.1347
22:5n-3 DPA	0.77	0.70	0.73	0.70	0.68	0.59	0.03	0.8512
ΣEPA+DHA+DHA	0.87	0.72	0.84	0.72	0.83	0.59	0.03	0.1745
ΣSFA	0.59	0.61	0.59	0.69	0.64	0.68	0.01	0.2070
ΣMUFA	0.68	0.69	0.79	0.67	0.74	0.75	0.02	0.5989
ΣPUFA	0.79	0.77	0.75	0.78	0.72	0.86	0.02	0.5743
Σn-6 PUFA	0.67	0.66	0.66	0.75	0.63	0.74	0.02	0.5503
Σn-3 PUFA	0.83^{a}	0.73^{ab}	0.79^{a}	0.56^{b}	0.78^{ab}	0.86^{a}	0.03	0.0514

Row means bearing different superscripts within a fixed factor significantly differ.
Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO), linoleic acid (LA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), total saturated fatty acids (ΣSFA), total monounsaturated fatty acids (ΣMUFA), and total polyunsaturated fatty acids (ΣPUFA).

5.4.2. Concentration of the main n-6 and n-3 LC-PUFA per standard serve of cheese and cheese sensory test

To investigate the potential nutritional benefits to consumers, LA and the main n-3 LC-PUFA of cheese were also analysed in quantitative terms per serving (mg/40 g; Table 5.6). In relation to the proportion of FA (expressed as % of total FA), cheese in the FSO treatment had the greatest amount of ALA (113.8 mg/40 g), and RPO and SFO had the highest total n-3 LC-PUFA (44.3 mg/40 g) and LA (414.3 mg/40 g), respectively.

Table 5.6. Mean values of main LC-PUFA in one standard serve of cheese (mg/40 g)

Fatty Acid	Control	CO	RBO	FSO	SFO	RPO	SEM	P-value
18:2n6 LA	302.7^{b}	235.4^{c}	297.8^{b}	329.7^{b}	414.3^{a}	320.8^{b}	11.3	0.0001
18:3n3 ALA	66.3^{c}	61.1^{cd}	54.3^{d}	113.8^{a}	61.9^{cd}	92.0^{b}	4.0	0.0001
20:5n3 EPA	10.6^{b}	8.2^{c}	9.2^{bc}	10.2^{bc}	9.1^{bc}	14.3^{a}	0.5	0.0001
22:6n3 DHA	4.9^{b}	4.4^{b}	4.7^{b}	5.2^{b}	6.1^{b}	12.8^{a}	0.6	0.0001
22:5n3 DPA	10.7^{b}	10.0^{b}	10.3^{b}	11.8^{b}	10.5^{b}	17.1^{a}	0.6	0.0001
ΣEPA + DHA	15.5^{b}	12.6^{b}	13.9^{b}	15.4^{b}	15.1^{b}	27.2^{a}	1.0	0.0001
ΣEPA + DHA +DPA	22.5^{b}	22.5^{b}	24.2^{b}	27.3^{b}	25.7^{b}	44.3^{a}	1.5	0.0001

Row means bearing different superscripts within a fixed factor significantly differ.
Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO), linoleic acid (LA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA).

Despite the application of the same cheese making process to all treatments, the varied preferences of cheese tasting panelists across treatments reflect the effect of dietary supplements on eating quality of the final product. Cheeses made from RBO and FSO milk yielded the highest scores for all sensory traits, with an overall acceptability or overall liking of 7.5, implying that the consumers' preferences for these cheeses were between "like

moderately" and "like very much". In contrast, the CO cheese had a slightly negative effect on sensory attributes, with the lowest overall liking rating from the panelists at 4.4 (slightly dislike) compared with 5.7 (neither like nor dislike) in the control (Table 5.7). Inclusion of RPO and SFO in the diets elicited similar levels of all tested sensory attributes except for the improvement in appearance at 6.1 and 6.2, respectively. As shown in Table 5.8, there were strong positive correlations between all sensory traits, in which flavour was the key assessed attribute contributing to the consumer enjoyment of sheep cheese ($r = 0.91$; $P < 0.001$). Partial sums of SFA and PUFA were correlated positively with eating quality traits, ranging from weak to moderate, whereas correlations were strongly negative ($r = -0.58$ to 0.76) for the sum of MUFA. However, correlations between selected individual and partial sums of FA were not statically significant (Table 5.8).

5.5. Discussion

5.5.1. Effect of oil supplementations on the proportion of beneficial PUFA in ripened cheese

Cheese produced from FSO milk showed a marked improvement in total n-3 PUFA due to its high ALA proportion compared with all other treatments. Our findings agree with previous results from other studies that also examined the effect of adding flaxseed to dairy sheep (Zhang *et al.*, 2006b; Bodas *et al.*, 2010; Mughetti *et al.*, 2012) and dairy cow (Santillo *et al.*, 2016) diets on the FA profile of cheese. The high level of ALA in the diet supplemented to the ewes accounted for this enrichment. Although ruminal biohydrogenation of ALA for most diets is very high and varies from 85 to 100% (Glasser *et al.,* 2008b; Shingfield *et al.*, 2010), the postruminal infusion, increased the passage rate of ALA through the rumen for eventual absorption in the small intestine and subsequent transfer and conversion into milk fat in the mammary gland (Glasser *et al.*, 2008a). Typically, supplementation with oilseeds rich in ALA, such as flaxseed, also has the potential to enhance the concentration of 20:5n-3 (EPA) in milk and the final product through the elongation and desaturation of 18:3n-3 in mammalian tissues

(Leonard *et al.*, 2004). Kazama *et al.* (2010) and Khas *et al.* (2010) reported a minor but significant increase in EPA content in dairy cows. However, outcomes observed in the current study are in agreement with the results of Cattani *et al.* (2014) and Bodas *et al.* (2010), who did not find any significant correlation between ALA in the diet and EPA content in the milk product. Species differences in mammary lipid metabolism could be the principal reason for these disparities (Chilliard *et al.*, 2014).

The greatest improvement of n-3 LC-PUFA was observed in RPO cheese. This outcome was in agreement with several studies that targeted an increase in EPA and DHA contents in ovine milk by supplementing lactating ewes with diets containing high levels of these ingredients (Kitessa *et al.*, 2003; Reynolds *et al.*, 2006; Toral *et al.*, 2010a,b; Bichi *et al.*, 2013) Polyunsaturated FA, EPA, and DHA are also biohydrogenated in the rumen, resulting in the production of 18:0 (Palmquist *et al.*, 2005), but this occurs to a lesser extent than for the biohydrogenation of LA and ALA (Chilliard *et al.*, 2000). Given the relatively low absorption rate from the small intestine into the mammary gland, ranging from 14.3 to 33.0% for EPA and 13.3 to 25.0% for DHA (Shingfield *et al.*, 2013), it is not surprising that the proportion of these 2 n-3 LC-PUFA in dairy products is generally very low. However, the significant improvement in total n-3 PUFA content at the expense of a significant decrease of 18:0 in the RPO treatment (Table 5.3) indicates that using rumen-protected EPA + DHA in supplementary diets is an effective physiological and biochemical by-pass of rumen biohydrogenation and a smart escape from the negative effect of typical fat metabolism in dairy sheep. This substitution of long-chain SFA with PUFA in human diets has been reported to reduce the risk of coronary heart disease (Hu *et al.*, 1999). In addition, because of the resultant elevated total n-3 PUFA, the inclusion of flaxseed oil and rumen- protected EPA + DHA significantly reduced the ratio of n-6 to n-3 FA (2.65/1 and 2.47/1, respectively). These ratio values are close to the ratio of 2.5/1 that had been reported to have a positive effect in colorectal cancer patients by reducing rectal

cell proliferation and between the ratio of 2 to 3/1 that inhibited inflammation in rheumatoid arthritis (Simopoulos, 2002).

Although safflower has been known as one of the richest sources of LA, studies on the influence of supplementing dairy sheep diets with safflower oil on FA compositions have not been found by the authors. The largest improvement of LA, observed in the SFO group, was due to the high proportion of this FA in the diet. This present finding is in line with previously reported results by Bell *et al.* (2006) and Li *et al.* (2015), who evaluated the effect of safflower oil on dairy cows' FA. These outcomes also supported the conclusion of Shingfield *et al.* (2013) that similar to ALA, the amount of LA during mammary gland secretion strongly depends on the amount of FA in the abomasum. The highest ratio of n-6 to n-3 (5.17 vs. 3.72) occurred in SFO cheese over the control, and as a result of LA enhancement, this ratio is still within the optimal n-6/n-3 ratio of <6:1 (Zymon *et al.*, 2014).

Rice bran is an agricultural by-product known to be an effective energy and FA dietary feed source for livestock due to its high content of oleic, linoleic, and palmitic acids (Warren and Farrell, 1990; Cicero and Derosa, 2005). Canola, on the other hand, has an ideal ratio of n-6 to n-3 PUFA (Sakhno, 2010) and is by far the largest oilseed crop in Australia (Seymour *et al.*, 2012). Despite its availability and accessibility, peer-reviewed published information on the use of these plant lipid sources in the Australian dairy sheep industry is limited. The limited outcome of supplementing sheep diets with rice bran and canola oils to enhance the availability of desired PUFA in cheese in this study aligned with previous studies in cow milk (Lunsin *et al.*, 2012b; Otto *et al.,* 2014) and goat milk (Mir *et al.*, 1999; Park *et al.*, 2013). Adding rice bran oil in the diet for experimental animals increased the concentration of medium-chain SFA 14:0 and 16:0 in cheese, and this agrees with the results of Park *et al. (*2013), who observed increased proportions of SFA 14:0 and 16:0 in goat milk due to the high proportions of these FA in rice bran oil. The increased proportions of these 2 SFA show the potentially negative

effect of the RBO diet on cheese FA profile in lactating ewes in terms of human health concerns. This is because, a high consumption level of 14:0 and 16:0 in humans may increase low-density lipoprotein cholesterol, which is associated with atherosclerotic cardiovascular disease (Siri-Tarino *et al.*, 2010).

5.5.2. Effect of oil supplementations on the recovery of LC-PUFA of ripened cheese

To date, there is a paucity of studies assessing FA retention in ripened cheese produced from sheep milk supplemented with oil pellets. Cattani *et al.* (2014) demonstrated no statistical differences in the recovery of group FA, including SFA, MUFA, PUFA, n-6 and n-3 PUFA and all individual FA, including LA, ALA, and EPA in ripened cheese processed from milk produced by cows supplemented with 500 or 1,000 g of extruded flaxseed. Our investigation, however, observed significant differences in the transfer rates for ALA, EPA, and total n-3 PUFA from raw milk to ripened cheese in grazing dairy sheep. This disparity between the 2 studies could be the result of differences in dietary supplements, species, or cheese making processes. Further research is therefore needed to better elucidate the effect of adding oils in dietary supplements for dairy ewes on the recovery of FA in ripened cheese.

5.5.3. Effect of oil supplementations on the concentration of LC-PUFA in absolute terms (mg/40 g) and eating quality of ripened cheese

In 2016, 260,000 t of cheese was traded in Australia, where the annual cheese consumption was estimated to be 13.5 kg per person (Dairy Australia, 2016). These values make cheese the second major dairy product after milk for the Australian domestic market. To assist in the promotion of the health and nutritional benefits of dairy products as one of the key focuses of the Australian dairy industry, our research examined the nutritional value of cheese from ewe milk in terms of selected beneficial LC-PUFA per standard serving (mg/40 g; NHMRC, 2013). These absolute data can potentially assist consumers in purchasing decisions. The LA at 414.3 mg/serving in the SFO treatment corresponds to a 137% increase compared with the control.

Adhering to Australian nutritional values, the content of this essential n-6 FA contributes only a small proportion of approximately 3.2 and 6.2% of the 13 and 8 g/d required for adequate intake in men and women, respectively (NHMRC, 2006). The increase of ALA in FSO cheese was almost double the value observed in the control, accounting for approximately 8.8 and 14.2% of the adequate intake level requirements for men and women (1.3 and 0.8 g/d), respectively (NHMRC, 2006). In comparison, the ALA content in 1 serving of cheese produced from the FSO treatment was markedly higher than that in a 100-g serving of cooked lamb meat at 48.5 mg/100 g, as reported by Flakemore *et al.* (2017).

Table 5.7. Effect of different diets on sensory eating quality of sheep cheese

	Treatment	Appearance	Aroma	Flavour	Texture	Overall liking
	Control	5.0^{c}	5.9^{ab}	5.8^{b}	5.6^{b}	5.7^{b}
	CO	5.0^{c}	5.6^{b}	4.2^{c}	5.0^{b}	4.4^{c}
	FSO	7.0^{a}	6.7^{a}	7.3^{a}	7.0^{a}	7.5^{a}
	RBO	7.3^{a}	6.6^{a}	7.5^{a}	7.0^{a}	7.5^{a}
	RPO	6.1^{b}	6.1^{ab}	5.5^{b}	5.7^{b}	5.7^{b}
	SFO	6.2^{b}	6.5^{a}	5.7^{b}	5.8^{b}	6.0^{b}
SEM		0.13	0.10	0.15	0.14	0.15
P value		0.0001	0.0097	0.0001	0.0001	0.0001

Row means bearing different superscripts within a fixed factor significantly differ.
Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO).

Previous studies on n-3 LC-PUFA often have generally focused on EPA and DHA rather than also including DPA, although DPA consumption also contributes to reduced risk of heart disease (Rissanen *et al.*, 2000; Phang *et al.*, 2009; Chen *et al.*, 2012) and the inhibition of inflammation (Chen *et al.*, 2012). This shortcoming may in part be due to pure DPA not being produced at commercial levels; consequently, its effects in both animal models and ultimately human consumers remain very much understudied. In line with suggested dietary targets by

NHMRC (2006), this study has also included EPA, DHA, and DPA in the total n-3 LCPUFA. In 1 serving of RPO cheese, the content of total n-3 LC-PUFA at 44.3 mg accounts for 28 and 49% of the daily dietary target of 90 and 160 mg/d required for men and women, respectively. The minimum recommended daily intake of milk and alternative products of 2.5 servings per day (NHMRC, 2013) corresponds to 100 g of cheese/d in adults; at this serving size, RPO cheese can provide up to 69% of the total n-3 LC-PUFA requirement for men and exceeds the requirement for women, as depicted in Figure 5.1. Moreover, at an excess of 30 mg/serving, RPO cheese can be considered as meeting the "source" (30 mg/serving) level of n-3 LCPUFA based on FSANZ (2012) guidelines.

Table 5.8. Pearson's correlation coefficients between sensory eating quality traits and selected fatty acids of sheep cheese

Item	Appearance	Aroma	Flavour	Texture	Overall liking
Appearance					
Aroma	0.52***				
Flavour	0.61***	0.56***			
Texture	0.57***	0.53***	0.72***		
Overall liking	0.65***	0.62***	0.91***	0.76***	
18:2 CLA	0.46	0.40	0.39	0.32	0.37
18:2n-6 LA	0.40	0.59	0.22	0.20	0.28
18:3n-3 ALA	0.45	0.16	0.31	0.31	0.28
20:5n-3 EPA	-0.09	-0.24	-0.29	-0.29	-0.27
22:6n-3 DHA	0.01	-0.08	-0.23	-0.22	-0.19
22:5n-3 DPA	0.02	-0.16	-0.25	-0.23	-0.22
ΣSFA	0.36	0.41	0.38	0.43	0.43
ΣMUFA	-0.68	-0.76	-0.58	-0.61	-0.64
ΣPUFA	0.52	0.57	0.31	0.28	0.35
Σn-6 PUFA	0.39	0.59	0.22	0.21	0.28
Σn-3 PUFA	0.37	0.09	0.18	0.18	0.16

Degree of correlation: 0.00-0.19 (very weak), 0.20-0.39 (weak), 0.40-0.59 (moderate), 0.60-0.79 (strong), 0.80-1.0 (very strong).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Conjugated linoleic acid (CLA), linoleic acid (LA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), total saturated fatty acids (ΣSFA), total monounsaturated fatty acids (ΣMUFA), and total polyunsaturated fatty acids (ΣPUFA).

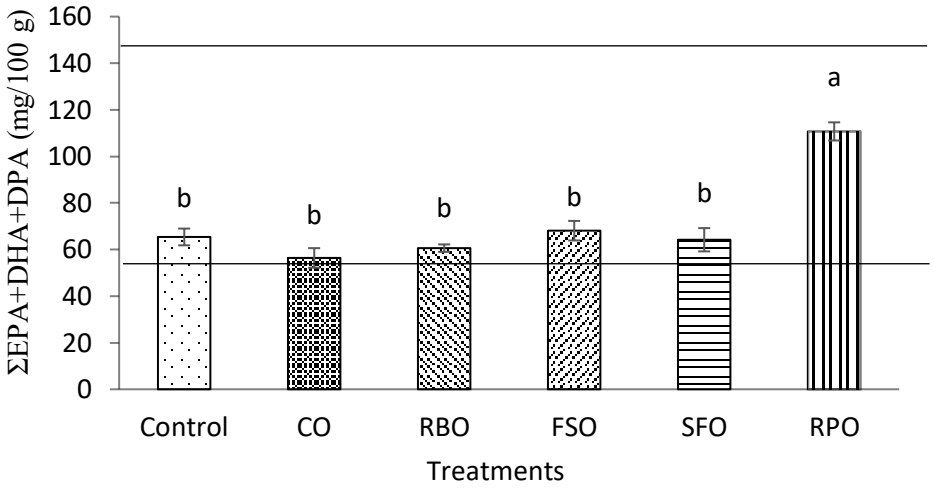

Figure 5.1. Mean values of ΣEPA+DHA+DPA (mg/100 g) in cheese. Different letters indicate significant differences between treatments ($p<0.05$). Lines indicate daily required intake of n-3 LC-PUFA for women (90 mg/day) and men (160 mg/day), respectively.
Canola oil (CO), rice bran oil (RBO), flaxseed oil (FSO), safflower oil (SFO), rumen-protected oil (RPO), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA).

Besides nutritional value, appearance, aroma, flavour, and texture are very important features of commercial cheese marketing that affect consumer choice (Awad *et al.*, 2007). The enhancement of all sensory eating traits observed in the RBO and FSO groups were inconsistent with previous studies that showed a minor effect on cheese sensory attributes made with milk from sheep (Najera *et al.*, 2017) or cows (Ryhanen *et al.*, 2005; Vargas-Bello-Perez *et al.*, 2015a) supplemented different plant oils. In the current study, no statistical correlation between FA content and sensory attributes was observed although levels of MUFA showed a strongly negative effect on cheese eating quality. In addition, cheese flavour is due to contributions from complex agents including mainly milk constituents like casein, milk fat,

lactose and citrate (Singh *et al.*, 2003) combined with the activity of microorganisms during the ripening period (Pastorino *et al.*, 2003; Sheehan and Guinee, 2004; Awad *et al.*, 2007). This current study primarily focused on improving the FA profile of ripened cheese. Further research, therefore, is recommended to investigate the effect of utilising milk from ewes supplemented with flaxseed and rice bran oils on cheese manufacture focusing on microbiological, biochemical, and chemical aspects of cheese ripening.

5.6. Conclusion

Outcomes of this novel on-farm study demonstrated a valuable process of increasing the healthy food functionality and consumer acceptability of sheep cheese fortified with dietary n-3 LC-PUFA. Feeding rumen bypass-protected EPA + DHA to grazing dairy sheep offers the best strategy to increase desired n-3 LCPUFA in cheese without any negative effect on eating quality. Inclusion of flaxseed oil elevates not only the concentration of ALA but also all sensory attributes significantly. The diet containing safflower oil was the most effective in relation to LA enhancement, with a minor improvement in customer sensory evaluation results. Diets including rice bran and canola oil demonstrated minor effects on cheese PUFA, and rice bran oil had a positive effect on cheese eating quality in this study. Due to the current paucity of data and knowledge in the published literature, further research should be performed regarding the optimum level of rice bran and canola oil supplementation in grazing dairy sheep to further elevate the proportion of valuable cheese FA. In addition, precise interactions between cheese processing and oil supplementation also need to be examined to maximize transferred rate of healthy FA from sheep milk into ripened cheese.

Chapter 6: General Discussion and Conclusion

The series of studies identified variations in animal performance, production, composition, FA profiles, and quality of milk and cheese of dairy ewes in an on-farm, pasture-based system. These variations attributable to supplementation with different n-3 PUFA oil sources from canola, rice bran, flaxseed, safflower and rumen-protected oil pellets in combination with sire breed, had impacts on:

1. Feed intake and body condition score;
2. Milk yield and composition;
3. Fatty acid compositions of milk and cheese;
4. Recovery of n-3 PUFA in cheese; and
5. Cheese eating quality;

In **Chapter 3**, the hypothesis that a lower dry matter intake due to the high energy density of oil infused pellets will be compensated by higher milk production, body condition score and overall performance of dairy sheep in an on-farm pasture-based system was tested**.** A significant decrease in DMI of ewes in all groups supplemented with oil pellets was evident. It confirms the negative association between high level of oil inclusion and feed intake of dairy ruminants reported by Mapato *et al.*, (2010) and Ammah *et al.*, (2018). The reverse impact of high level of oil inclusion on feed intake can be explained by the reduction in ruminal function (Petit *et al.*, 2005) primarily in response to higher energy density of the diets requiring lesser volume of feed intake (Illius and Jessop, 1996). Besides, another driving factor influencing feed intake in ruminants is palatability (Baumont *et al.*, 2000; Kawamoto *et al.*, 2001). Rice bran has a comparatively higher palatability which might have contributed to the highest DMI found in RBO among oil supplemented groups. The pelleted experimental diets were different

in physical characteristics (hardness, aroma and integrity). These quality features, particularly hardness, have been demonstrated to significantly influence animal preferences for pellets (Thomas and vander Poel, 1996). However, the focus of this current research was not on the relationship between the attributes of pellet quality from different oil origins and voluntary intake of dairy ewes.

Regarding body condition score, previous studies consistently confirmed a greater BCS loss in early lactation (Ruegg and Milton, 1995; Roche *et al.*, 2007) due to fat mobilisation from body reserves for sustaining milk synthesis due to the imbalance of dietary energy (Komaragiri *et al.*, 1998; Berry *et al.*, 2007). Akin to that, very low BCS of experimental animals was observed at the commencement of the feeding trial (**Chapter 3**) which indicated that the pasture quality was insufficient. Supplementing these grazing dairy ewes with high energy sources was necessary to maintain animal welfare and productivity. This was demonstrated by the increase in BCS values during the feeding trial with more benefits improved BCS to ewes supplemented with CO, RBO, and FSO pellets than other diets. At the end of the feeding trial, BCS of ewes in these groups increased significantly within the optimum target of 2.5–3.0 for dairy sheep (Kenyon *et al.*, 2014).

More importantly, milk production (**Chapter 3**) responded positively to supplementation with oil-infused or rumen-protected pellets. Except for the flaxseed oil group, all supplementary diets significantly increased milk production compared with the control. Taken with DMI data, rumen-protected oil pellets was the most effective diet that enhanced the volume of milk, fat and protein by approximately 30, 13 and 31%, respectively. Moreover, the weekly trend in milk production over the whole experimental period for RPO seemed to be more stable than in other groups. This finding agrees with previous studies on the positive effect of bypass fat on the milk production of cows (Naik, 2013) and lactating ewes supplemented for longer than 4

weeks (Pulina *et al.*, 2006). Interestingly, the inclusion of rice bran oil in this study marginally, but statistically, enhanced milk protein composition in contrast to Depeters and Cant (1992) who were of the view that changing milk protein composition is less likely to occur compared with the potential to alter milk fat composition (Depeters and Cant, 1992). However, Park *et al.* (2013) reported an increase in protein composition in the milk of dairy goats, while a reduction was observed in cows (Lunsin *et al.*, 2012b). These variations could be explained by the disparity of species and feeding systems among studies. Not surprisingly, breed also had a major impact on the yields of milk, fat and protein. Milk production from crossbred AW x EF was significantly higher than purebred AW by 16.5%. This is because EF is the most productive dairy sheep breed in terms of milk yield worldwide, and generally used in crossbreeding systems to improve milk production of local breeds (Ugarte *et al.*, 2001). Sire breed also had a significant interaction with diet with greater responses in crossbred AW x EF ewes to oil supplements with respect to milk yield, particularly within the RPO group. In general, the results in Chapter 3 confirmed acceptance of the tested hypothesis and illustrated the positive effect of utilising oil supplements combined with a crossbreeding strategy between the AW and EF sheep breeds to improve animal performance, body condition score, milk production and composition in pasture-based dairy ewe production systems.

The study in **Chapter 4** was carried out to assess the effects of including rumen-protected pellets containing EPA+DHA or pellets infused with either canola, rice bran, flaxseed or safflower oils in the diet of grazing dairy ewes on milk n-3 PUFA content. The hypothesis tested was that supplementing grazing dairy ewes with different sources of dietary oils, including those containing n-3 and n-6 PUFA, would affect the concentration of n-3 LC-PUFA in milk without any negative effect on milk production. Results demonstrated that rumen bypass protected EPA + DHA was the most effective supplementary diet at elevating levels of n-3 LC-PUFA. The outstanding enhancement of EPA, DHA and DHA content by twofold,

threefold, and fivefold, respectively than the control in the current study, agrees with outcomes reported by Kitessa *et al.*, (2001a; 2004). The form of rumen-protected oil utilised in these works increased the escape rate of dietary n-3 LC-PUFA from ruminal biohydrogenation for eventual absorption in the abomasum and transfer into the mammary glands (Shingfield *et al.*, 2013).

The addition of flaxseed oil in the diets also significantly increased EPA and DPA content, but to a lesser extent compared to RPO. The increment of these FA in FSO group were positively associated with an increase in ALA, confirming the conversion of ALA to n-3 LC-PUFA in mammalian tissues (Leonard *et al.*, 2004). However, the proportions of DHA observed in the control, CO, RBO, FSO, SFO were not different despite the variation in milk ALA, suggesting that the conversion rate of ALA to DHA is limited. Therefore, supplementing a rich source of ALA such as flaxseed and canola oil at levels of 5% could improve EPA and DPA constituent but not DHA, in sheep milk.

In line with previous findings in dairy cows (Roy *et al.*, 2006) and sheep (Kitessa *et al.*, 2003), this study observed rapid changes in ALA, LA, and n-3 LC-PUFA contents in FSO, SFO, and RPO, respectively, during the feeding trial. These results indicated potential utilisation of oil supplements to produce desirable FA composition during ewe lactation for dairy sheep producers. No sire breed effect on n-3 PUFA concentration was found in this study, which demonstrated the low heritabilities of individual n-3 PUFA that were consistently reported by previous studies in dairy animals (Stoop *et al.*, 2008; Bilal *et al.*, 2014; Pegolo *et al.*, 2016; Correddu *et al.*, 2018). This would probably be as a result of half of the C16 and all of the long-chain fatty acids in milk being derived from dietary and body reserves (Chilliard *et al.*, 2007).

By elevating the content of n-3 PUFA, the inclusion of canola, flaxseed and rumen-protected oil in the diets of grazing dairy ewes, noticeably decreased the n-6:n-3 ratio. This would benefit

consumers in terms of reducing the risk of inflammation (Di Nicolantonio and O'Keefe, 2018). This reduction in n6:n3 ratio is caused by competition between mediators such as the E-series and D-series resolvins transformed from n-3 LC-PUFA and inflammation mediators from arachidonic acid (20:4n-6, ARA) (Calder, 2006; Kunnumakkara *et al.*, 2018). To potentially assist consumers in purchasing decisions, quantitative data regarding FA concentration was investigated in this chapter. It was concluded that fresh milk produced from ewes supplemented with flaxseed and rumen-protected oil achieved "source" and "good source" of n-3 LC-PUFA respectively, in line with Food Standards Australia and New Zealand (FSANZ, 2002).

The objectives described in **Chapter 5** were to investigate the influence of supplementing different plant oil-infused and rumen-protected wheat-based pellets for grazing dairy ewes on n-3 LC-PUFA concentration, fatty acid recovery and sensory attribute of ripened cheese. It was hypothesized that supplementing grazing dairy ewes with different sources of dietary n-3 oils would affect the concentration and recovery of n-3 LC-PUFA and alter the eating sensory traits of ripened cheese. Akin to the finding of Cattani *et al.*, (2014), there were obvious parallels between FA composition of milk and ripened cheese observed in this study. Diets containing flaxseed oil, safflower oil and rumen bypass-protected EPA + DHA effectively increased the concentration of ALA, LA, and n-3 LC-PUFA, respectively. This is because FA composition of ripened cheese primarily depends on the FA profile of milk that was demonstrated by a number of studies (Collomb *et al.*, 2006; Bisig *et al.*, 2007; Prandini *et al.*, 2011). Therefore, similar to the findings in **Chapter 4**, a significant increase in n-3 LC-PUFA was found in RPO cheese, while FSO and SFO cheeses had the highest levels of ALA and LA, respectively. According to NHMRC (2006), a standard serve of 40 mg cheese produced from RPO contributes approximately 28 and 49% of the 90 and 160 mg/day required for adequate intake in men and women, respectively. The content of total n-3 LC-PUFA at 44.3 mg also exceeded the "source" (30 mg/serving) level of n-3 LC-PUFA (FSANZ, 2002). Regarding the effect of

dietary supplements on major PUFA recovery, this investigation found similar conversion rate of n-3 and n-6 LC-PUFA except for α-linolenic acid and EPA. The specific mechanism explaining the association between FA retention in cheese and FA content of raw milk is unknown due to paucity of studies on this theme.

Besides nutritional value, sensory quality attributes critically affect the acceptability of commercial foods in general (Morales and Tsimidou, 2000) and consumer choice of cheese in particular (Awad *et al.*, 2007). Thus, **Chapter 5** also investigated the effects of oil supplements on cheese eating quality and the association between sensory features and fatty acid composition. In contrast with findings in sheep (Najera *et al.*, 2017) and cows (Vargas-Bello-Perez *et al.*, 2015a) where it was observed that supplementation with different plant oils had minor effect on cheese quality, the inclusion of rice bran and flaxseed oil in this study significantly enhanced all sensory traits. Disparities in forage sources, feeding system and type of oil supplement could be the reasons for this variation. However, this study could not find any statistically significant correlation between sensory attributes and FA content. Cheese quality is affected by a complexity of factors and their interactions during cheese processing. These factors include milk supply, rennet (coagulant), starter, non-starter lactic acid bacteria, cheese composition and ripening temperature (Fox *et al.*, 2000). Thus further research is needed to clarify which factors could interact with oil supplementation, particularly rice bran and flaxseed oil regarding changes cheese eating quality.

In summary, this on-farm study utilising different plant oil derived and rumen-protected oil supplements for grazing dairy sheep demonstrate an improvement in overall animal performance, lactation traits, and milk and cheese quality. The inclusion of rumen bypass-protected EPA + DHA pellets offers the best strategy to increase milk productivity and desirable n-3 LC-PUFA content in both fresh milk and cheese without any adverse effect on

sensory attributes. The usage of flaxseed oil supplement considerably elevates the content of ALA and cheese eating quality, but has no incremental effect on milk production. The best approach to enhance n-6 PUFA is through supplementing ewes with safflower oil that also significantly improves milk yield, although to a lesser extent than rumen-protected oil. At present, the practical use of canola and rice bran supplements for dairy sheep in grazing systems is limited. This first effort shows that there is a promising potential of including rice bran oil in supplementary diets aimed at improving milk production, whilst at the same time, significantly increasing consumer acceptability of the processed cheese product. On the other hand, canola oil supplementation has only minor effects on dairy sheep productivity and product quality. Therefore, together with potential easy access to oil supplements, the use of these supplementation strategies depends on different on-farm goals focussing on enhancing animal performance, milk production, milk composition, milk and cheese fatty acid composition, or consumer acceptability of ripened cheese. It was also shown that sire breed independently interacts with oil supplementation to influence milk production, but not milk and cheese quality. The use of crossbred AW x EF ewes for improving milk, fat and protein yields is recommended for producers in pasture-based systems to increase the farm gate value of dairy sheep.

Further research investigations are needed to better elucidate the:

1) Effect of canola and rice bran oil on animal performance, milk and cheese FA concentration of dairy sheep in different management systems;
2) Profitable margins of oil supplementation for sheep producers;
3) The biosynthesis pathway of n-3 LC-PUFA from α-linolenic acid and the biohydrogenation mechanism of individual n-3 LC-PUFA;
4) Activities of fat regulatory genes on the concentration of n-3 PUFA and their as associations with lipid supplementation;

5) Effect of oil supplementation for dairy ewes on biochemical and microbiological activities during cheese manufacture contributing to cheese quality.

Limitations of the study:

- This study was only conducted in Southern Tasmania where the climatic conditions and production systems may not be generally applicable to the whole country. Therefore, similar research in different production conditions in different regions of the country will comprehensively elucidate the findings revealed in the current study.
- Profitability margins were not computed due to lack of feed costs associated with each supplement. Future investigations on profitability margins of using oil based supplementation for dairy sheep production will fill this knowledge gap.

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Appendices

This section contains all the information on published chapters, supplementary data and declarations

Appendix 1

nutrients

MDPI

Review

Enhancing Omega-3 Long-Chain Polyunsaturated Fatty Acid Content of Dairy-Derived Foods for Human Consumption

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Abstract: Omega-3 polyunsaturated fatty acids (n-3 PUFA) are termed essential fatty acids because they cannot be synthesized de novo by humans due to the lack of delta-12 and delta-15 desaturase enzymes and must therefore be acquired from the diet. n-3 PUFA include α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic (EPA, 20:5n-3), docosahexaenoic (DHA, 22:6n-3), and the less recognized docosapentaenoic acid (DPA, 22:5n-3). The three long-chain ($\geq C_{20}$) n-3 PUFA (n-3 LC-PUFA), EPA, DHA, and DPA play an important role in human health by reducing the risk of chronic diseases. Up to the present time, seafood, and in particular, fish oil-derived products, have been the richest sources of n-3 LC-PUFA. The human diet generally contains insufficient amounts of these essential FA due largely to the low consumption of seafood. This issue provides opportunities to enrich the content of n-3 PUFA in other common food groups. Milk and milk products have traditionally been a major component of human diets, but are also among some of the poorest sources of n-3 PUFA. Consideration of the high consumption of milk and its processed products worldwide and the human health benefits has led to a large number of studies targeting the enhancement of n-3 PUFA content in dairy products. The main objective of this review was to evaluate the major strategies that have been employed to enhance n-3 PUFA content in dairy products and to unravel potential knowledge gaps for further research on this topic. Nutritional manipulation to date has been the main approach for altering milk fatty acids (FA) in ruminants. However, the main challenge is ruminal biohydrogenation in which dietary PUFA are hydrogenated into monounsaturated FA and/or ultimately, saturated FA, due to rumen microbial activities. The inclusion of oil seed and vegetable oil in dairy animal diets significantly elevates ALA content, while the addition of rumen-protected marine-derived supplements is the most effective way to increase the concentration of EPA, DHA, and DPA in dairy products. In our view, the mechanisms of n-3 LC-PUFA biosynthesis pathway from ALA and the biohydrogenation of individual n-3 LC-PUFA in ruminants need to be better elucidated. Identified knowledge gaps regarding the activities of candidate genes regulating the concentrations of n-3 PUFA and the responses of ruminants to specific lipid supplementation regimes are also critical to a greater understanding of nutrition-genetics interactions driving lipid metabolism.

Appendix 2

animals

MDPI

Article

Supplementing Dairy Ewes Grazing Low Quality Pastures with Plant-Derived and Rumen-Protected Oils Containing Eicosapentaenoic Acid and Docosahexaenoic Acid Pellets Increases Body Condition Score and Milk, Fat, and Protein Yields

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Simple Summary: This study evaluated the lactation performance and body condition scores of purebred Awassi and Awassi × East Friesian crossbred dairy ewes grazing low quality pastures and supplemented with diverse plant-derived oil enriched pellets under on-farm management conditions. The origin and treatment of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to get the rumen protected EPA + DHA treatment was based on a modification of the microencapsulation of oil droplets in a protein-aldehyde matrix procedure. The results demonstrated that supplementation with rumen (EPA + DHA) and oil-infused pellets improved milk, fat, and protein yields by approximately 30%, 13%, and 31% respectively, and crossbred ewes produced more milk than purebreds. These results are very useful for dairy sheep producers in improving ewe lactation performance, milk quality, and body condition score under low quality pasture grazing conditions.

Abstract: The Australian dairy sheep industry is small and mostly based on a natural grass grazing system, which can limit productivity. The current study tested different plant oil-infused and rumen protected polyunsaturated fats and their interactions with sire breeds to improve lactation traits and body condition scores (BCS) of ewes grazing low quality pastures. It was hypothesised that supplementing lactating ewe's diets with plant-derived polyunsaturated oils would improve milk production and composition without compromising BCS. Sixty ewes (n = 10/treatment) in mid-lactation, balanced by sire breed, parity, milk yield, body condition score, and liveweight, were supplemented with: (1) control: wheat-based pellets without oil inclusion; wheat-based pellets including; (2) canola oil (CO); (3) rice bran oil (RBO); (4) flaxseed oil (FSO); (5); safflower oil (SFO); and (6) rumen protected marine oil containing eicosapentaenoic acid and docosahexaenoic acid (RPO). Except for the control group, all supplementary diets included the same level of 50 mL/kg DM of oil and all diets were isocaloric and isonitrogenous. Experimental animals were grazed in the same paddock with *ad libitum* access to pasture, hay, and water during the 10-week study. RPO was the most effective diet that enhanced milk, fat, and protein yields by approximately 30%, 13%, and 31%,

Appendix 3

RESEARCH ARTICLE

Sheep Milk

European Journal of Lipid Science and Technology

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Supplementing Grazing Dairy Ewes with Plant-Derived Oil and Rumen-Protected EPA+DHA Pellets Enhances Health-Beneficial n−3 Long-Chain Polyunsaturated Fatty Acids in Sheep Milk

*Quang V. Nguyen, Van H. Le, Don V. Nguyen, Bunmi S. Malau-Aduli, Peter D. Nichols, and Aduli E. O. Malau-Aduli**

This study investigates the impact of supplementing dairy ewes in mid lactation with rumen-protected (RPO) pellets containing eicosapentaenoic acid (EPA, 20:5n−3) and docosahexaenoic acid (DHA, 22:6n−3) or pellets infused with 50 mL kg^{-1} DM of either canola (CO), rice bran (RBO), flaxseed (FSO), or safflower (SFO) oils on enhancing the concentration of n−3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n−3 LC-PUFA) in milk. It is hypothesized that including these oils in the diet of grazing dairy ewes will improve milk fatty acid (FA) composition by increasing levels of n−3 LC-PUFA. Sixty grazing dairy ewes balanced by sire breed and parity are randomly allocated to one of 6 treatments: 1) Control: commercial pellets without oil inclusion; 2) pellets containing 50 mL kg^{-1} DM of CO; 3) RBO; 4) FSO; 5) SFO; and 6) RPO at the rate of 1 kg day^{-1} for each ewe for 8 weeks. Weekly bulked daily milk FA analysis shows RPO has the most efficiency at elevating n−3 LC-PUFA content by twofold, threefold, and fivefold greater concentrations of EPA, DPA, and DHA, respectively, than the control (0.17 vs 0.08%, 0.23 vs 0.08%, 0.19 vs 0.04%) ($P < 0.0001$). FSO improves levels of EPA (0.11%) and DPA (0.15%), while CO increases DPA (0.13%) ($P < 0.0001$). FSO and RPO reached the "source" and good "source" of n−3 LC-PUFA (ΣEPA + DHA + DPA) contents of 35.1 and 61.7 mg 250 mL^{-1}, respectively. These findings recommend that rumen protected pellets containing EPA + DHA, flaxseed, and potentially canola oil supplements, can be used to improve the content of n−3 LC-PUFA in dairy ewe milk.

Practical Applications: The results promote the potential for production of premium quality and health-beneficial fresh milk through dietary supplementation of grazing dairy ewes with plant-derived oil and rumen-protected EPA + DHA.

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Appendix 4

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Enhancement of dairy sheep cheese eating quality with increased n-3 long-chain polyunsaturated fatty acids

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ABSTRACT

This study investigated the effect of different plant oil-infused and rumen-protected wheat-based pellets containing eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) on n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (LC-PUFA) content, fatty acid recovery, and sensory attributes of ripened cheese from dairy sheep. During a 10-wk supplementary feeding trial, 60 dairy ewes balanced by live weight, milk yield, parity, and sire breed were randomly divided into 6 groups that were (1) supplemented with on-farm existing commercial wheat-based pellets without oil inclusion (control) or supplemented with wheat-based pellets infused with 50 mL/kg dry matter of oils from (2) canola, (3) rice bran, (4) flaxseed, (5) safflower, and (6) rumen-protected EPA + DHA. Milk samples from each treatment were collected separately by sire breed during the experimental period for cheese processing at the end of the experiment. Twelve batches of cheese (2 batches per treatment) were processed and ripened for 120 d. Three cheese samples were collected and analyzed for each cheese making session (total of 36 cheese samples) at d 120 of ripening. Processed cheese of rumen-protected EPA + DHA had the most efficiency at elevating total n-3 LC-PUFA [total EPA + DHA + docosapentaenoic acid (DPA, 22:5n-3] content compared with the control (0.49 vs. 0.28%). Flaxseed elicited the greatest enhancement of α-linolenic acid (ALA, 18:3n-3), whereas safflower was the most effective diet in enhancing the level of linoleic acid (18:2n-6) in cheese (1.29 vs. 0.71% and 4.8 vs. 3.3%, respectively). Parallel recoveries of n-3 and n-6 LC-PUFA were observed across all treatments except for α-linolenic acid and EPA. Cheese eating sensory traits were also highly affected by oil supplementation, with the highest score of 7.5 in cheese from the rice bran and flaxseed treatments. These results provide new insights into the biological mechanisms and processes that determine dairy ewe milk productivity by underpinning the vital biological role of n-3 LC-PUFA in not only enhancing the healthy composition of cheese from ewes but also translating it into consumer acceptability.

Key words: sheep cheese, n-3 long-chain polyunsaturated fatty acid, sensory eating quality, food functionality, oil

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