Harper Adams University

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The effect of feeding microalgae on rumen fermentation, milk and cheese fatty acid profile and fertility in dairy cows

Bethan Eluned Till

(B.Sc. Veterinary Bioscience, The University of Glasgow, 2014)

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Harper Adams University,

Edgmond, Shropshire, TF10 8NB

Declaration

I declare that this thesis is my original work and it has been written by myself and that it has not been accepted in any previous application for a degree. I have acknowledged all the sources of information which have been used in this thesis.

Bethan Eluned Till
2018

Abstract

The objectives of this thesis were to determine the effects of Algae (ALG), a supplement high in docosahexaenoic acid (DHA), on the biohydrogenation rate of DHA *in vitro*, and the effect feeding ALG to dairy cows had on milk FA profile and indicators of fertility. The first study was conducted *in vitro* to determine the effect of different inclusion levels of ALG or fish oil (FO) on the biohydrogenation of DHA. Results showed that DHA was extensively biohydrogenated at all time-points but was lower at the higher inclusion levels of ALG. The first cow study investigated the effect of rate of inclusion of ALG (0, 50, 100 or 150 g/ALG per cow/d) on milk and cheese FA profile, and cheese taste. The results demonstrated differences ($P < 0.05$) in milk fat yield, diet digestibility and milk and cheese FA profiles, but no difference ($P > 0.05$) in DM intake, milk yield, or cheese yield. A number of cheese taste attributes were affected. Milk and cheese DHA content were increased by 0.29 g/100 g. The second cow study determined the effect of long-term feeding of ALG on milk FA profile, and indicators of fertility. Cows were fed 100 g/ALG per day from 3 weeks post calving for 14 weeks. Results showed no differences ($P > 0.05$) in DM intake, milk yield or milk fat yield, but there were differences ($P < 0.05$) in the milk FA profiles and milk progesterone levels, with milk DHA content increasing in the ALG fed cows from week 2 of study onwards, and peaking at week 14 at 0.38 g/100 g. Plasma concentrations of the prostaglandin $F_2\alpha$ metabolite were not significantly ($P = 0.126$) lower in ALG fed cows who had improved overall conception rates (55.6 % v.48 %). It was concluded from these experiments that supplementation of 100 g/ALG per cow/d is the most appropriate inclusion level to increase milk DHA content, avoiding rumen adaptation and negative effects on animal performance.

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There has been a lot of sweat, blood and tears, but it has been the best four years of my life,

Diolch yn fawr iawn.

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B E Till, J A Huntington, J Taylor-Pickard and L A Sinclair, 2016. Dietary inclusion of marine algae and its impact on milk composition and performance in dairy cows. Book of Abstracts of the 67th Annual Meeting of the European Federation of Animal Science, Vol 22, p.272

B E Till, J A Huntington, J Taylor-Pickard and L A Sinclair, 2017. Rate of inclusion of microalgae and its effect on whole tract digestibility and milk fatty acid profile in Holstein-Friesian dairy cows. Advances in Animal Biosciences: The future of Animal Science, Vol 8 part 1, p.79

B E Till, J A Huntington, J Taylor-Pickard and L A Sinclair, 2017. Improving the long chain fatty acid profile of milk and cheese in dairy cows by supplementation with microalgae. American Dairy Science Association Annual Meeting 2017, p. 79

B E Till, J A Huntington, J Taylor-Pickard and L A Sinclair, 2018. Supplementation of dairy cow diets with docosahexaenoic acid enriched microalgae and its effect on milk fatty acid profile over time. Advances in Animal Biosciences, p. 155

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Abbreviations

˚C	Degree Celsius
AA	Arachidonic acid
AI	Artificial insemination
ALA	α- Linolenic acid
ALG	Microalgae
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BHB	β-hydroxy butyrate
CH_4	Methane
CLA	Conjugated linoleic acid
CLA	Corpus luteum
CO2	Carbon dioxide
CVD	Cardiovascular disease
DHA	Docosapentaenoic acid
DMI	Dry matter intake
DMI	Dry matter
E_2	Oestriadol -17β
EPA	Eicosapentaenoic acid
FA	Fatty acid
FO	Fish oil
FSH	Follicle-stimulating hormone
GC	Gas chromatography
GnRH	Gonadotrophin-releasing hormone
IFN†	Interferon†
IPUAC	International union of pure and Applied Chemistry
LA	Linoleic acid
LC	Long-chain
LH	Luteinising hormone
LT	Leukotrienes
LX	Lipoxins
MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
n-9	Omega-9
NDF	Neutral detergent fibre
NEFA	Non-esterified fatty acid
OM	Organic matter
P_4	Progesterone
PG	Prostaglandins
$PGF_2α$	13-14-dihydro-15keto prostaglandin $F_2α$
PGFM	Prostaglandin $F_2α$ metabolite
PGHS	Prostaglandin H synthase pathway
PUFA	Polyunsaturated fatty acid
SARA	Subacute rumen acidosis
SEM	Standard error of the mean

SFA	Saturated fatty acid
TMR	Total mixed ration
TNF-α	Tumor necrosis factor α
TX	Thromboxanes
UFA	Unsaturated fatty acid
VFA	Volatile fatty acid

CHAPTER 1: Literature Review

1.1 Introduction

There is a large body of evidence to support the beneficial effects of long chain omega-3 polyunsaturated fatty acids (LC *n*-3 PUFA) on human health, especially regarding cardiovascular diseases (CVD) (De Lorgelil and Salen, 2012; Gibbs *et al.,* 2010). Many studies have concluded that the majority of the population, especially those in Western countries are failing to meet the recommended daily intake (>0.2g a day) of LC *n*-3 PUFA, (Calder, 2018; Meyer, *et al*. 2003), emphasising the need for strategies to increase the availability and consumption of these essential fatty acids. There is currently a considerable amount of interest in altering the fatty acid composition of milk and cheese as an alternative dietary source of the two main LC *n*-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Vargas-Bello-Perez *et al*., 2015). Fish oils and microalgae (ALG) have been shown to have the potential to enhance the LC *n*-3 PUFA content of ruminant products when they are supplemented in the diet (Chow *et al.,* 2004).

An added benefit to feeding ALG is the positive effect fat supplementation has on the reproductive performance of dairy cows (Rodney *et al*., 2015). Fertility in dairy cows has declined over the past five decade (Rodney *et al*., 2015). There is considerable interest in finding ways to prevent the increase in in-fertility and culling of cows which are unable to conceive. Improvements in fertility are caused by the type of fatty acid (FA) supplemented and not just an increase in energy intake (Staples *et al*., 1998). Supplementing dairy cows with *n*-3 FA have shown to improve indicators of fertility (Ambrose *et al*., 2006, Petit and Twagiramungu, 2006). Microalgae is rich in the LC *n*-3 PUFA DHA, and may increase the uptake of DHA into membrane phospholipids, the 3 series of prostaglandins (PG) are involved in improving the environment for embryo implantation and survival by decreasing the secretion of PG metabolites (Dong Hyeon *et al*., 2016).

Relatively few studies have been conducted on ALG, mainly due to the difficulty in obtaining sufficient quantities at an economic price (Stamey *et al*., 2012). Developments in the production of ALG have however meant that a commercial source is now available (Stamey *et al*., 2012).

1.2 Lipids

1.2.1 Classifications

Lipids, along with carbohydrates, proteins and nucleic acids, are one of the four major classes of biologically organic molecules found in all living organisms (Whitney and Rolfes, 2013). Lipids are a group of substances which are insoluble in water but soluble in organic compounds and are found in plant and animal tissue (Starr *et al*., 2016). They

exist as components of biological membranes and act as electron carriers and substrate carriers in enzymatic reactions (McDonald *et al*., 2011). Lipids can be divided into two major sub-classes; neutral lipids which lack a free polar group and contain long hydrophobic hydrocarbon chains such as triacylglycerol, waxes and terpenes, and polar lipids; which include polar hydrophilic groups such as phospholipids and glycolipids (Lordan *et al*., 2017). Fats and oils are major form of energy storage in both plant and animals (Whitney and Rolfes, 2013).

1.2.2 Triglyceride

Lipids are esters of FA with the trihydric alcohol glycerol; when all three alcohol groups are esterified by FA, the compound is a triacylglycerol (or triglyceride; Figure 1) (Starr *et al*., 2016). Triglycerides are synthesised through a series of condensation reactions which combine a hydrogen atom from the glycerol and from a hydroxyl group of the FA, forming a molecule of water; leaving a bond between the molecules (Whitney and Rolfes, 2013). The positions occupied by the FA chain are not identical, and are designated as positions *sn*-1, *sn*-2 and *sn*-3 (McDonald *et al*., 2011). The different positions are recognisable by enzymes, leading to different reactions at different positions (McDonald *et al*., 2011). Triacylglycerols can exist as simple triacylglycerols when all three of the FA residues are the same, or as mixed triacglycerols when more than one FA is involved in the esterification (Starr *et al*., 2016). Under the influence of lipase, the process of fat breakdown may take place (McDonald *et al*., 2011). During lipolysis one or two FA residue may be removed from the glycerol producing FA mixtures of mono- and dicylglycerols with a free FA (McDonald *et al*., 2011). When lipolysis occurs in edible fat it may be rendered unacceptable to the consumer (Whitney and Rolfes, 2013). Lipolysis occurs before the hydrogenation of fats in the rumen and is discussed in section 1.6.2.

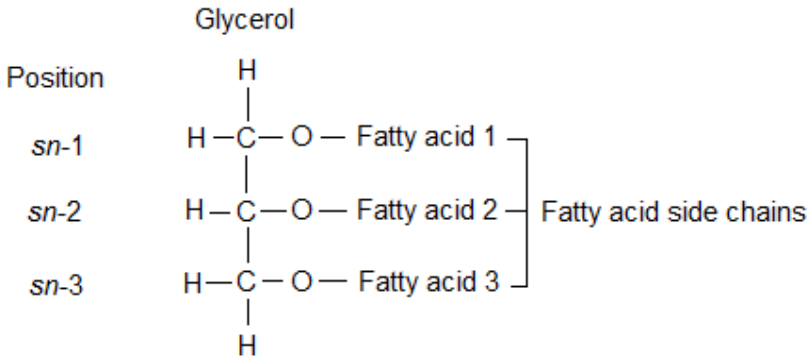


Figure 1.1. The structure of a triglyceride adapted from Starr *et al*., (2016)

1.2.3 Glycolipids

In glycolipids two of the alcohol groups of the glycerol are esterified by FA and the third is linked to a sugar residue, such as galactose (McDonald *et al*., 2011). They are involved in cellular recognition, which is important to the immune response system and help maintain the stability of cell membranes (Schnaar, 2004). The lipids of grasses and clovers are predominantly (60 %) galactolipids, which form the majority of the dietary fat of ruminants (McDonald *et al*., 2011). Monogalactosyl is the main type of galactolipids of grasses, but some digalactosyl compounds are also present (McDonald *et al*., 2011). There are five major FA in forage plant, (C16:0, C18:0, C18:1*n*-6, C18:2*n*-6 and 18:3*n*-3) (Van Soest, 1994). The FA associated with galactolipids of grasses and clovers contain high amounts of linoleic (C18:2*n*-6; LA) and α-linolenic acids (C18:3*n*-3; ALA) (Van Soest, 1994). As the plant matures the concentration of galactolipids declines as the proportion of leaves to stem changes (Van Soest, 1994). Galactolipids can be broken down by rumen microoragnisms to release galactose, FA and glycerol (McDonald *et al*., 2011).

1.2.4 Phospholipids

Phospholipids are a major component of cell membranes consisting of a hydrophilic head group and hydrophobic tail, and forms a lipid bilayer due to their amphiphilic characterisation (Lordan *et al*., 2017). The FA composition of phospholipids varies, but usually the *sn*-2 position contains an unsaturated fatty acid (UFA) such as C18:1 *cis*-9, LA, ALA, arachidonic acid (AA) or EPA, whereas *sn*-1 position contains a saturated fatty acid (SFA) (Lordan *et al*., 2017). The ratio of UFA to SFA of the phospholipid is very important, as the degree of saturation directly affects the fluidity of the cell membrane and cellular processes, such as the formation of lipid rafts (Lordan *et al*., 2017). The lipid rafts are involved in carrying SFA, which are involved in the processes of apoptosis and cellular proliferation, and UFA which act as precursors for the synthesis of eicosanoids.

1.3 Fatty acid structure and nomenclature

1.3.1 Fatty acid nomenclature

Fatty acids are hydrocarbon derivatives that are present in the form of fat and oils in all living organisms (Nelson and Cox, 2005).The FA exist as hydrocarbon chains of various lengths from 4 – 36 carbons that terminate with a carboxyl group at one end and a methyl group at the other, and are held together by simple bonds (Berg *et al.*, 2012; Fig 1.2).

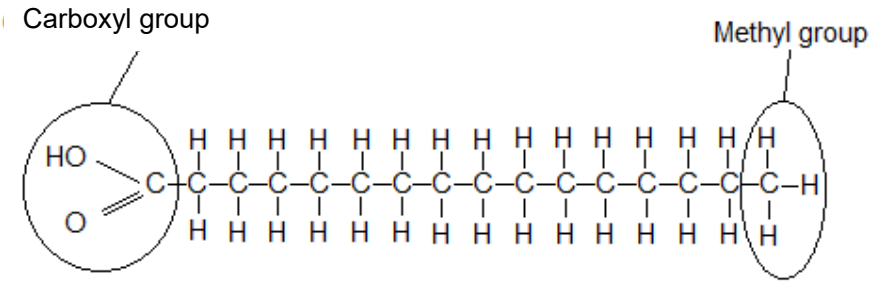


Figure 1.2. The structure of the saturated fatty acid C16:0 (Berg *et al.*, 2012).

Fatty acids differ from one another by the length of the hydrocarbon chain, degree of unsaturation and by the position and configuration of the double bonds in the chain which can affect the extent of rancidity and lipolysis (Robinson and Wilbey, 1998). Fully SFA are unbranched and contain no double bonds, whereas UFA contain one or more double bonds (Robinson and Wilbey, 1998). Those containing one double bond are known as monounsaturated FA (MUFA), and those containing two or more double bonds are termed polyunsaturated fatty acids (PUFA) (Ruxton *et al*., 2004). The position of the double bond in the chain may change location, but generally the first double bond will occur between carbons 9 and 10 counting from the methyl group in higher plants and animals (Prasad, 2013). The configuration of the bond will change the spatial arrangement of the molecules in the FA despite it having the same molecular formula, because a double bond cannot rotate (Berdanier and Berdanier, 2015). This restricted rotation forms geometric isomers called *cis* and *trans* (Nelson and Cox, 2005). A *cis* isomer will have the hydrogen atoms on the same side of the double bond, whereas the *trans* isomer will have the hydrogen atoms on opposite sides of the double bond (Gurr, 1987; Fig 1.3).

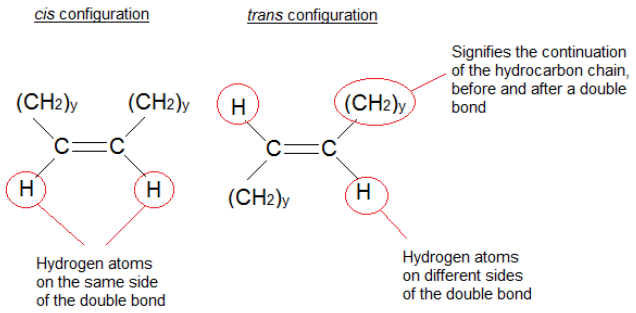


Figure 1.3. The difference between a *cis* and *trans* bond found in unsaturated fatty acids, adapted from Nelson and Cox (2005).

Many FA have common names which are usually derived from the plant or animal they were first isolated from. For example palmitic acid was discovered in palm oil (Berdanier and Berdanier, 2015), but as more FA were discovered other means were required. Simple methods of naming these FA has been developed based on the International union of Pure and Applied Chemistry (IUPAC) and nomenclature that are more technically clear and descriptive. The straight chain hydrocarbon name is modified, the final '*e*' in the hydrocarbon name exchanged for '*oic*'; for example hexadecan**e** becomes hexadecan**oic** (C16:0). If a FA has one double bond in the hydrocarbon chain an '*e*' is exchanged for the '*a*,' resulting in hexadec**e**noic, two double bonds leads to hexadeca**die**noic, and three double bonds will be hexadeca**tri**enoic (Berg *et al*., 2012). The configuration of the bond is also shown by adding *cis* or *trans* in front of a number which specifies at which carbon the unsaturated double bond occurs, which will be the first carbon of the pair involved in the bond counting from the methyl end (Berg *et al.*, 2012). The systematic names for FA may be abbreviated and given a shorthand designation which specify the chain length and number of double bonds separated by a colon (Nelson and Cox, 2005). For example oleic acid, an 18 carbon long FA with one double bond is C18:1. The exact position of the double bonds are then indicated by a superscript number following a Δ *delta* symbol if naming from the carboxyl end of the FA or by the *n* symbol if naming from the methyl end, therefore cis $\Delta^{9,12}$-18:2 and 18:2*n*-6 both represent LA (Berg *et al.*, 2012, Table 1.1).

Table 1.1. Some of the most common fatty acids and other essential fatty acids found in plant and animal organisms (Berg *et al*., 2012).

Carbon skeleton	Systemic name	Common name
C4:0	Butanoic acid	Butyric acid
C6:0	Hexanoic acid	Caproic acid
C8:0	Octanoic acid	Caprylic acid
C10:0	Decanoic acid	Capric acid
C12:0	Dodecanoic acid	Lauric acid
C14:0	Tetradecanoic acid	Myristic acid
C14:1	*cis*-9- tetradecanoic acid	Myristoleic acid
C16:0	Hexadecanoic acid	Palmitic acid
C16:1	*cis*-9- hexadecanoic acid	Palmitelaidic acid
C17:0	Heptadecanoic acid	Margaric acid
C18:0	Octadecanoic acid	Stearic acid
C18:1*n*-9	*trans*-9- octadecenoic acid	Elaidic acid
C18: 1*n*-10	*trans*-10- octadecenoic acid	Isooleic acid
C18:1*n*-11	*trans*-11- octadecenoic acid	Vaccenic acid
C18: 1*n*-12	*trans*-12- octadecanoic acid	-
C18:1*n*-9	*cis*-9- octadecenoic acid	Oleic acid
C18:2*n*-6	*cis*-9, 12- octadecadienoic acid	Linoleic acid (LA)
C18: 2*n*-9	*cis*-9, *trans*-10- octadecadienoic acid	*cis*-9, *trans*-10 CLA[1]
C18: 2*n*-10	*trans*-10, *cis*-12 octadecadienoic acid	*trans*-10, *cis*-12 CLA[1]
C20:0	Eicosanoic acid	Arachidic acid
C18:3*n*-3	*cis*-9,12,15 octadecenoic acid	α-Linolenic acid (ALA)
C20:4*n*-6	*cis*-7,10,13,16- docosatetraenoic acid	Arachidonic acid (AA)
C20:5*n*-3	*cis*-5,8,11,14,17- eicosapentaenoate	Eicosapentaenoic acid (EPA)
C22:0	Docosanoic acid	Behenic acid
C22:5*n*-3	*cis*-7, 10, 13, 16, 19- docosapentaenoic acid	Docosapentaenoic acid (DPA)
C22:6*n*-3	*cis*-4,7,10,13,16,19 - docosahexanoic acid	Docosahexaenoic acid (DHA)

[1]Conjugated linoleic acid

1.3.2 Elongation and desaturation of FA

The ability to synthesise different FA is important to all organisms, as lipids play an important role in many cellular activities (Nelson and Cox, 2005). Not only are they the principal store for energy but they are important in pigmentation, transportation, hormone development, anchors for membrane proteins and are involved in extracellular and intracellular messaging (Nelson and Cox, 2005).

Fatty acids are synthesized by numerous enzymes that together are called *fatty acid synthase* and require the involvement of malonyl-CoA an intermediate produced from acetyl-CoA (Wakil, 1960). Palmitate, a C16:0 FA is constructed by a repeated four step

sequence, involving condensation, reduction, dehydration and a final reduction step (Berg *et al.*, 2012). Once formed, free palmitate is released from further elongation making it the principal product of the *fatty acid synthase* system in animal cells (Berg *et al.*, 2012). In certain plants such as coconut and palm, the chain is terminated earlier, resulting in 90% of FA in the oils of these plants containing between 8 and 14 carbons long (Nelson and Cox, 2005).

Elongation is the process by which a two-carbon unit is added to the carboxyl end of the FA, which is donated by malonyl-CoA in a catalyzed reaction (Berg *et al.*, 2012). Palmitic acid may be lengthened to form C18:0 or even longer SFA through the fatty acid elongation system (Leonard *et al*., 2004). This system has been known since the 1960's and a review by Wakil (1960) described how it was favoured over the idea of total FA synthesis from acetyl CoA. Elongation of FA in the mammary gland by elongase is limited (Mida *et al*., 2012), mammary lipogenesis will be discussed later in section 1.6.6.

When SFA are oxidised a double bond is introduced to the carbon chain, a process called desaturation (Leonard *et al*., 2004, see Figure 1.4). Desaturation of SFA may occur in plants such as grass, and ALG, where more than one double bond is introduced to C16:0 and C18:0. First the MUFA C16:1 (palmitoleate) and 18:1 (oleate) are produced (Leonard *et al*., 2004). These FA are the two most common monounsaturated FA in animal tissues and have a single *cis* bond between C-9 and C-10 (Berg *et al.*, 2012). Further desaturation of C18:1 *cis*-9 will produce LA, and ALA (Monroig *et al*., 2013). Further elongation and desaturation of ALA results in the formation of EPA and DHA, which are important *n*-3 FA (Figure 1.5).

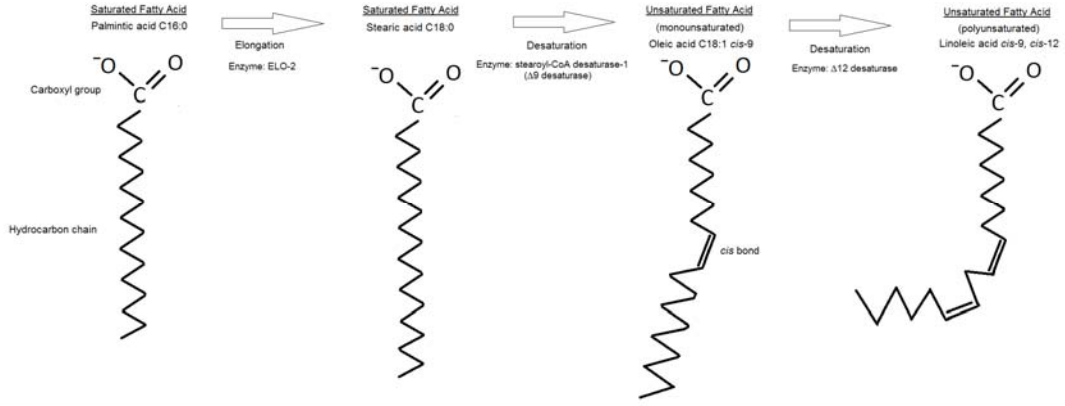


Figure 1.4. Palmitic acid, a 16 carbon long SFA is elongated to form C18:0, and desaturation of this SFA leads to the production of C18:1 *cis*-9, this can be further desaturated to form LA in terrestrial plants, marine phytoplankton and some protozoa and insects but not in mammals (Leonard *et al*., 2004).

Polyunsaturated fatty acids may be classified as either *n*-3, *n*-6 (omega-6) or *n*-9 (omega-9) based on the location of the last double bond. Unlike plants mammals lack the enzyme to introduce double bonds past carbon 9 in the hydrocarbon chain (Berg *et al.*, 2012) making LA and ALA essential fatty acids which must be obtained from the diet through the consumption of plant material. Arachidonic acid, EPA and DHA however can be synthesised by mammals from dietary intake of LA and ALA (Abedi and Sahari., 2014). The conversion efficiency to AA, EPA and DHA however is very low (Abedi and Sahari., 2014; Sinclair *et al*., 2007; Gerster, 1998). Plourde and Cunnane (2007) reported that *in vivo* studies in humans ≈5% of ALA is converted to EPA and <0.5% of ALA is converted to DHA. The direct uptake of these LC (long chain) -PUFA from the diet is therefore a more effective means to alter tissue or milk composition (Ruxton *et al*., 2004).

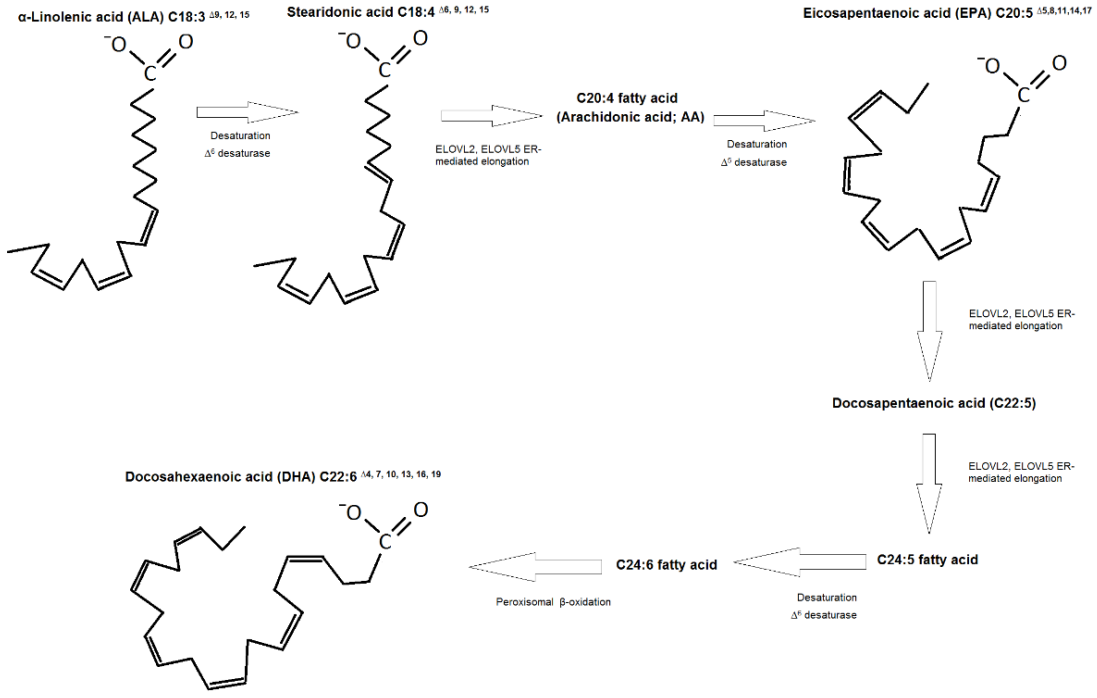


Figure 1.5. α-Linolenic acid, a 18 carbon long PUFA undergoes desaturation and elongation to form a C24:6 fatty acid which is then oxidised to produce DHA. The synthesis of DHA from ALA does occur in mammals, but the conversion efficiency is very low. Adapted from Rizzi *et al*., (2013)

1.3.3 Metabolism of fatty acids to eicosanoids of series 1, 2 and 3

Eicosanoids include PG, thromboxanes (TX), leukotrienes (LT) and lipoxins (LX) compounds which are derived from 20 carbon PUFA (Mayes and Botham., 2003). These

eicosanoids are considered to act as local hormones functioning through G-protein-linked receptors to stimulate their biochemical effects (Mayes and Botham., 2003). Prostaglandins and TX are products of the prostaglandin H synthase pathway (PGHS), which consists of two enzymes, cyclooxygenase and peroxidase (Figure.6). The substrate for their synthesis is AA which is released from the phospholipid through the action of phospholipase A_2 (Lordan *et al*., 2017). The activity of phospholipase A_2 can be stimulated by the binding of oxytocin to the oxytocin receptors (Mattos *et al*., 2000). Arachidonic acid can be acquired either from the diet or synthesised *de novo* from LA, and is stored in the phospholipids of the cellular plasma membrane (Mattos *et al*., 2000). The products of the PGHS pathway (PG_2 and TX_2) are called prostanoids and may be converted to PGD_2, PGE_2, $PGF_{2\alpha}$, TXA_2 and prostacyclin (PGI_2) (Mattos *et al*., 2000). The lipoxygenase pathway competes against the PGHS pathway for the same AA substrate. Synthesised from the later pathway are the LT_4 and LX_4 eicosanoids which have important roles in inflammation and the resolution of inflammation (Mattos *et al*., 2000).

Polyunsaturated FA have major roles in the endocrine system, with different series of PG deriving from different PUFA (Otto *et al*., 2014). The 1 and 2 series derive from *n*-6 FA and are involved in uterine involution and subsequent sequential ovulation post-partum (Otto *et al*., 2014). The 3- series PG are derived from *n*-3 FA and are involved in improving the environment for embryo implantation and survival by decreasing the secretion of prostaglandin metabolites, resulting in increased lifespan of the corpus luteum (Dong Hyeon *et al*., 2016).

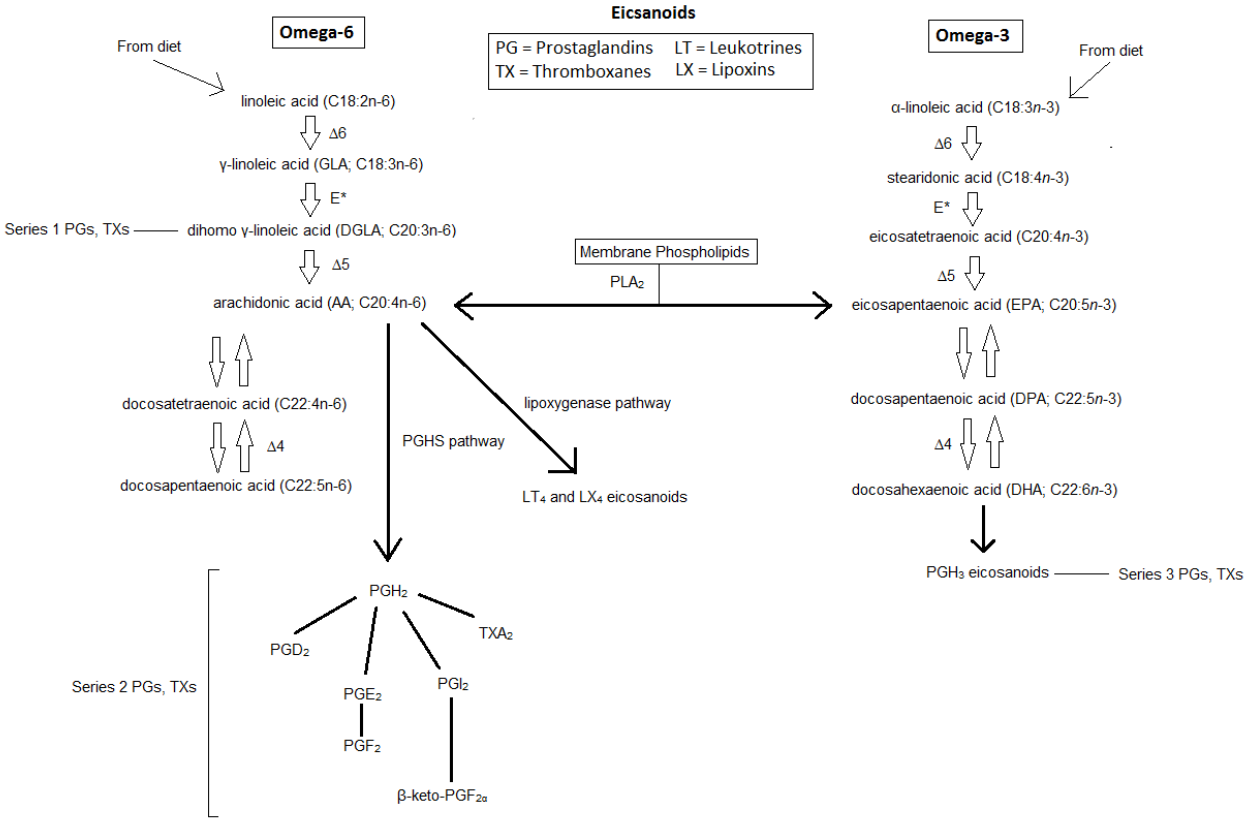


Figure 1.6. The metabolism of dietary *n*-6 and *n*-3 polyunsaturated fatty acids. The amount of each fatty acid incorporated depends on the amount of precursor present in the diet. Adapted from Mattos *et al*., (2000) and Mayes and Botham, (2003). Δ6 = activity of Δ6 desaturase; Δ5 = activity of Δ5 desaturase; Δ4 = activity of Δ4 desaturase; E* = elongation.

1.4 Fatty acids and human health

1.4.1 Uptake of EPA and DHA into cell membranes

Essential FA are nutrients of primary importance for human health, and decades of research has demonstrated the significance of an adequate intake of *n*-3, a sub class of essential PUFA in the prevention of several diseases, particularly CVD (Astorg *et al*., 2004). The most important *n*-3 FAs regarding human health are EPA and DHA. Both of these FA are termed very LC *n*-3 PUFA due to the length of their hydrocarbons, with EPA having 20 carbons and DHA having 22 carbons in its chain. They are found in high amounts in seafood, especially fatty fish and in supplements like FO and cod liver oil. They may be synthesised from ALA as described in fig 1.5 (section 1.3.2), but the capacity of this pathway is extremely limited (Williams and Burdge, 2006). The pathway involves enzymes which are shared with the conversion of LA to AA. In many Western

diets the intake of LA is much greater than ALA, favouring the conversion of LA to AA over the conversions of ALA to EPA and DHA (Calder, 2014).

All FAs, including EPA and DHA are transported in the bloodstream esterified into triacylglycerols, phospholipids and cholesterol esters as components of lipoproteins and non-covalently bound to albumin in the non-esterified form (Calder, 2014). They are found in cell membranes esterified into phospholipids and stored in adipose tissue esterified into triacylglycerols. The FA composition of cell membranes are important in determining the physical characteristics of that cell membrane, how they change to external stimuli and the functional activities of membrane bound proteins (Marventano *et al*., 2015). The proportion of EPA to DHA within any of the functional pools differ, but often DHA is present in a greater amount than EPA, especially in regions of the eye and brain (Calder, 2014). It was reported that DHA contributed to 18% of FA in adult human brain grey matter, and Makrides *et al*., (1994) reported an average DHA content of 8 and 12 % in infant cerebral cortex and retina respectively, whilst EPA concentrations were less than 0.05 and 0.1% respectively.

Increased intake of EPA and DHA are reflected in blood lipid, blood cell, and many tissue pools. Rafts (which are cell membrane domains) have specific lipid and FA composition and act as a platform for intracellular signalling pathways, EPA and DHA can modify raft formation, modifying intracellular signalling pathways and transcription factors (Alessandri *et al*., 2004). A second consequence of increased EPA and DHA in cell membrane phospholipids, and the decreased abundance of AA, is the effect on the biosynthesis of various PG, TX and LT, discussed in section 1.3.3. As both *n*-3 and *n*-6 PUFAs compete for the same metabolic enzymes, an imbalance in the *n*-3:*n*-6 ratio may result in altered equilibrium in cell membrane composition (Marventano *et al*., 2015).

1.4.2 Human health benefits of EPA and DHA

1.4.2.1 Cardiovascular disease

Cardiovascular diseases includes heart disease, cerebrovascular disease, and peripheral vascular disease. The major causes of death as a result of CVD are heart attacks and strokes (Calder, 2014). Mensink *et al*, (2003) reported that the replacement of SFA with PUFA reduces the risk of CVD, based on changes in plasma cholesterol. Williams and Burdge (2006) reported how human experimental studies, animal experiments and cell-culture studies, have shown the beneficial effects of consuming oily fish for the uptake of LC *n*-3 PUFA into the body, and the associated health benefits. Results from these studies and others indicate that consumption of EPA and DHA reduces the risk of CVD outcomes in Western populations (Alessandri *et al*., 2004; Marventano *et al*., 2015). Chronic inflammation is the cause of many CVD and the anti-inflammatory effect of long chain *n*-3 PUFA lowers inflammation, and can lower the

concentrations of plasma triacylglycerol and blood pressure (Swanson *et al*., 2012). The results of these modifications include improved blood flow and reduced build-up of fatty deposits within the blood vessel walls. Intakes of LC *n*-3 PUFA in people with CVD has also been investigated, with several studies reporting a lower death rate in patient receiving LC *n*-3 PUFA (Hibbeln *et al*., 2006). Doses of 500-900 mg of EPA + DHA a day for 2 years, 885 mg/d of EPA + DHA for 1 year, 3.5 years, 3.9 years and 5 years have been studied (Tavazzi *et al*., 2008; Yokoyama *et al*., 2007). Three mechanisms have been suggested for the therapeutic effects on LC *n*-3 PUFA regarding reduced likelihood of myocardial infarction (e.g heart attack) and mortality. These mechanisms are discussed by Calder (2014) and include altered cardiac electrophysiology seen as lower heart rate, increased heart rate variability, and fewer arrhythmias, making the heart more able to respond to stress. Secondly the anti-thrombotic action results in lowered likelihood of clot formation, or weaker clots that are unable to stop blood flow to affected organs. Thirdly is the anti-inflammatory effect of LC *n*-3 PUFA, which stabilises atherosclerotic plaques preventing their rupture. The European Food Safety Authority (2012) suggest that the intakes of *n*-3 PUFAs should be 2-4 g a day to reach clinically relevant effects on preventing CVD, and intakes of 250 mg a day are sufficient for the maintenance of general cardiovascular health in healthy adults and children (Marventano *et al*., 2015).

1.4.2.2 Cancer

There is inconsistency in findings from studies investigating the effects of LC *n*-3 PUFA consumption and risk of colorectal, prostate, and breast cancers, but many prospective and case studies suggest the risk is lowered (Calder., 2014). In addition to lowering the risk of developing cancer, Elia *et al.,* (2006) reported that supplementing patients with existing cancer with EPA and DHA improved their appetite, energy intake, body weight and quality of life. Other studies have also reported higher physical activity, and less fatigue in cancer patients supplemented with 2.9 and 1.8 g of EPA and DHA (van der Meij *et al*.,2012; Mocellin *et al*., 2007). The LC *n*-3 PUFA, EPA and DHA can directly influence cancer cells, they exert a range of biological activities that may influence tumour cell proliferation and sustainability, with DHA promoting cell apoptosis (Marventano *et al*., 2015; Zarate *et al.,* 2017). They also replace the *n*-6 FA AA in cell membranes resulting in reduced production of mediators that drive tumour cell proliferation and tumour growth. It has also been reported that response to chemotherapy is improved, although the mechanism behind this is unclear (Calder, 2014).

1.4.2.3 Brain development

The amount of DHA in the brain increases rapidly with growth, and an adequate supply of LC *n*-3 PUFA seems essential for optimal visual, neural and behaviour

development (Calder, 2014), therefore LC *n*-3 PUFA have important roles in brain function throughout life. Lower levels of EPA and DHA have been found in blood of children with attention deficit hyperactivity disorder and autistic disorders, suggesting that these disorders could be related to FA deficiencies (Richardson., 2004). It has also been reported by Hibbeln (1998) that there is a correlation between high annual fish consumption and lower prevalence of depression, again suggesting a protective effect of LC *n*-3 PUFA against mental health issues. Recent studies have also reported low blood levels of LC *n*-3 PUFA in patients suffering dementia, and post mortem studies have reported lower levels of DHA in the brains of Alzheimer's disease sufferers (Cunnane *et al*., 2012; Tully *et al*., 2003).

1.4.3 Consumption of EPA and DHA

Dietary habits of humans has changed drastically over the past 10 000 years, despite their genetic profile remaining pretty similar, leading to the development of diseases (Marventano *et al.,* 2015). This has led to the implementation of dietary guidelines in all aspect of human nutrition. Policies implemented in most developed countries recommend a decrease in the consumption of SFA and an increase in the consumption of LC *n*-3 PUFA from fish and plant sources (Simopoulos, 2016). Most studies have concluded that the majority of the population, especially those in Western countries are failing to meet the recommended daily intake of LC *n*-3 PUFA (>0.2g/d) (Meyer *et al*., 2003), emphasising the need for strategies to increase the availability and consumption of these essential FA. It is estimated that the mean current intake of LC *n*-3 PUFA for UK adults is 244 mg/d (Gibbs *et al.,* 2010), considerably below the recommended intake of 450 mg/person/d (SACN/COT, 2004). The ratio of *n*-6 to *n*-3 and their effect on health is becoming increasingly important in recent research due to their opposing physiological functions, signifying a balance in their consumption is important for homeostasis and development (Schmitz and Ecker, 2008). Kris-Etherton *et al.,* (2000) stated how this ratio has decreased in the diet over the years in the United States from 12.4:1 to 10.6:1, but it is still much higher than the recommended value of 2.3:1 (Kris-Etherton *et al.,* 2000). However Bernard *et al*., (2013) stated that this ratio has actually increased over the last decade in many Western counties suggesting that Western diets are deficient in *n*-3 PUFA, and have excessive amounts of *n*-6 PUFA (Simopoulos, 2008). To reach the desired ratio, at least a 4-fold increase in the consumption of *n*-3 PUFA in the form of DHA and EPA is needed. This may be accomplished by increasing fish consumption or supplementation, but the future sustainability of these sources are uncertain (Williams and Burdge, 2006). Kris-Etherton *et al*., (2000) suggest alternative strategies through the use of biotechnology or food enrichment.

Marine fish like mammals rely on the dietary acquisition of essential FA. Fish will acquire these FA through the consumption of phytoplankton that are the abundant natural producers of LC *n*-3 PUFA at the base of the food chain (Givens *et al*., 2000). Concerns regarding the sustainability of the continued use of FO supplements in fish stocks by aquaculture has recently been raised (Naylor *et al*., 2001) as natural resources that provide these oils are in danger of being exhausted (Napier and Sayanova 2005). Plant oils and animal fats have been used in fish feeds as an alternative way to supply their dietary energy requirement. Torstensen *et al*., (2005) conducted a study on feeding Atlantic salmon 75% or 100% vegetable oil as a FO replacement and reported that with a reduction in dietary FO there was also a reduction in the levels of LC *n*-3 PUFA content in the fish meat.

Lock *et al*., (2014) discussed how nutritional quality is increasingly an important consideration in food choice by consumers because of their growing awareness of the link between diet and health. Much research is investigating ways of manipulating animal feeds in an attempt to increase *n*-3 content of eggs, milk, and meat (Scollan *et al*., 2014). Some major obstacles which are faced are the added expense and “off” flavours in food products as well as the extensive biohydrogenation of LC *n*-3 PUFA that takes place in the rumen (Wood and Enser, 2017). Because of these complications eggs are the only animal product available on the market which are enriched with *n*-3 FA (Bruneel *et al*., 2013; Lewis *et al*., 2000).

Ruminant products have been criticized for their higher level of SFA and low levels of PUFA (Lock *et al*., 2014). This is mainly due to extensive rumen biohydrogenation of dietary PUFA (Lourenco *et al*., 2005), (see section 1.6). This however has led to a miss-perception of dairy products having negative health benefits, whereas in reality they include important high-quality protein, and many minerals and vitamins (Lock *et al*., 2014). There is currently considerable amount of interest in in altering the FA composition of milk and cheese as an alternative source for EPA and DHA. The amount of PUFA in dairy products is as low as 2%, whereas the percentage of SFA is as high as 70% (Elgersma *et al*., 2006). A diet low in SFA, and cholesterol is advised by nutritionist in many countries (Lichtenstein *et al*., 2006), but the intake of SFA currently exceeds the recommended levels by 10-11% of total energy intake (Kliem and Shingfield, 2016). Milk fat is a complex lipid that contains over 400 different FAs, with SFA containing 4-18 carbons, C18:1 *cis*-9, C18:1 *trans* and LA being the most abundant (Glasser *et al*., 2008). The milk fatty acids composition of mammals is linked to intrinsic (animal breed, genotype, lactation, and pregnancy stages) or extrinsic (environmental) factors (Chilliard and Ferlay, 2004) with milk fat containing between 65 and 75 g SFA/ 100g of total FA. Lactation stage is linked to lipid storage during early lactation, but otherwise has little effect on the FA profile of milk, whereas diets can have marked effects on milk FA composition. Altering milk fat

composition offers the opportunity to lower consumption of SFA without requiring a change in consumers eating habits. Milk fat can be altered by feeding cows with oilseeds, plant oils and marine lipids (Kliem and Shingfield, 2016). The altered FA profile will differ according to oil source fed, form of lipid and degree of processing and the basal diet of the cow.

There is insufficient evidence to conclude on whether altering milk fat composition would lower CVD risk, but the available data suggests a beneficial effect (Mensink *et al*, 2003). Changes to the cows diet therefore provides an easy way for farmers to rapidly modulate milk FA composition (Chilliard and Ferlay, 2004). Lucas *et al*., (2005) also reported how cheese making technology has a minimal effect on FA composition. Cheese was made from cow and goats milk using four different cheese making technologies, the cheese FA profile was similar to that of the milk used to produce it, the only difference was seen between samples made from different species or those fed different diets (Lucas *et al*., 2005), therefore changes in the nutrition of ruminants in order to modify cheese FA is also possible. In another recent study, Manuelian *et al.,* (2017) examined how specie, breed and the type of cheese produced may vary cheese FA content, and reported a that hard cheese (moisture content <35 %) had higher content f PUFA compared to the soft cheeses (moisture > 45%).

1.5 Fatty acids sources for ruminants

1.5.1 Forages

The type of forages consumed by dairy cows has a large effect on both nutritional and sensorial characteristics of milk and dairy products (Kalac and Samkova, 2010). Dairy cows derive FA for milk fat synthesis from the diet and rumen microorganisms (400-450 g/kg), from *de novo* biosynthesis in the mammary gland (500 g/kg) and from metabolism of adipose tissue (<100 g/kg) (Kalac and Samkova, 2010).

Forages, either fresh or preserved, make up the staple component of ruminant diets (Van Soest, 1994). The total FA content in forages ranges between 20-50 g/kg dry matter (DM; Kalac and Samkove, 2010). There are five major FA in forage plant, (C16:0, C18:0, C18:1*n*-6, LA and ALA) (Van Soest, 1994), making forages important sources of precursors of *n*-3 and *n*-6 series of FA, and the cheapest and safest sources of FA in ruminants diets. The concentration of each individual FA varies depending on the plant species, growth stage, temperature and light intensity (Table 1.2; Dewhurst *et al*., 2001). In two consecutive years (2003 and 2004) Wyss *et al*., (2006) investigated the FA composition of three grass/clover mixtures over three cuts in both years. Mixture A contained only grasses, mixture B grasses and red clover, and mixture C contained grasses, red clover and lucerne. The dominant FA was ALA in all three mixtures, varying between 8.6 and 12.8 g/kg DM. The highest values in 2004 were found in the 2nd cut and

the lowest in the 3rd cut, whereas no trend was found in 2003. The concentration of ALA was higher in 2003 compared to 2004, which was suggested to be because of the higher amount of cocksfoot grass, a finding in agreement with Dewhurst *et al*., (2001) who reported a higher concentration of ALA in ryegrass compared to cocksfoot. The concentrations of all other major FA was very similar between mixtures and years. Alves *et al*., (2011) examined the effect of ensiling and silage additives on FA composition of ryegrass and maize, and reported no difference in the FA content (g/100 g FA) of LA and ALA during ensiling. In contrast Kalac and Samkove (2010) reported a decrease in LA and ALA content of maize and grass silages when exposed to air for up to 24 hrs, which is what occurs to silages prior to feed out due to the opening of clamps.

Table 1.2. Fatty acid composition of different grass species cut in July

Species	Fatty acid content (g/kg DM)				
	C16:0	C18:0	C18:1*n*-1	LA	ALA
Dactylis glomerata	3.91	0.92	0.45	2.85	10.56
Festuca arundinacea	4.21	1.06	0.96	2.55	11.98
Festuca pratensis	4.09	0.99	1.04	2.74	10.95
L. x boucheanum	4.32	0.98	1.44	3.28	10.51
Lolium multiflorum	3.05	0.94	0.84	2.26	6.94
X Festulolium	3.96	0.87	1.22	3.04	10.39
Lolium perenne	4.30	1.01	1.24	2.90	11.42
Phleum pratense	4.05	1.05	1.05	3.18	10.43

Data from Dewhurst *et al.,* 2001

1.5.2 Oils

Dairy cows need a tremendous amount of energy in order to support the demands of maintenance, milk production and reproduction (Van Saun and Sniffen, 1996). Concentrates are higher in energy density than forages, but to maintain rumen function, adequate dietary effective fibre is required in order to reduce the risk of subacute rumen acidosis (SARA) (Zebeli *et al.,* 2012), therefore concentrate usage in the diet is limited. The use of supplemental fats and oils in dairy cow ration has developed over the past few decades, and is now considered standard practice (National Research Council, 2001). Fats are higher in energy density than carbohydrates and proteins (9 v 3.75 v 4 kcal per g) and are supplemented in order to increase the energy density of the diet leading to increased energy intake if dry matter intake (DMI) is not decreased (Harvatine and Allen, 2006). With an increased energy intake, the dairy cow will have an improved energy balance, milk production and reproduction (Zebeli *et al*., 2012). Fat supplements differ in

FA source, form and type, and will consequently result in different production effects (Harvatine and Allen, 2006). Feeding excessive amount of UFA can reduce methane (CH_4) emissions, but it can also lead to a reduction in feed intake, which must be avoided (Bayat *et al.,* 2018). Modifying the FA content of animal products such as milk and meat to improve product quality, by increasing the content of PUFA is of great interest, especially for the consumer due to the health benefits of PUFA (Marventano *et al*., 2015). Omega-3 FA also have a positive influence on the reproductive and immune systems of dairy cows (Santos *et al*., 2008). Calcium salts are high in palmitic acid (C16:0), and are made from palm oil or soyabean. Higher levels of C16:0 are delivered to the cow in order to increase milk fat yield, as C16:0 is found in high concentrations in milk (Lock *et al*., 2013; Vyas *et al*., 2012). A diet enriched in LC-FA can result in a higher production of propionate and a lower production of acetate and butyrate, decreasing milk fat (Weisbjerg *et al*., 2008). It has also been reported that diets rich in PUFA such as rapeseed oil may inhibit the formation of precursors for milk fat in the rumen, and also inhibit *de novo* synthesis, referred to as milk fat depression in dairy cows (see section 1.6.6; Bauman and Griinari, 2001). Increasing the dietary supplementation of a specific FA does not mean that this FA will be increased in the milk or meat. This is due to biohydrogenation in the rumen, which is discussed in section 1.6.4. Protected fats and oils have been developed that are less susceptible to rumen biohydrogenation, with the FA of these rumen protected fats passing through the rumen to be digested and absorbed post-ruminal, and possibly be incorporated into milk fat (Jenkins and Bridges, 2007). Table 1.3 shows the different FA composition of a number of oilseeds.

Table 1.3. Average FA content (g/100 g total FA) of various commonly used fat sources

Oil source	Fatty acid content (g/100 g FA)								
	C14:0	C16:0	C16:1	C18:0	C18:1	LA	ALA	EPA	DHA
Linseed (n=22)	0.1	6.1	0.1	3.4	18.8	16.3	54.4	-	-
Rapeseed (n=24)	0.2	4.8	0.3	2.1	60.5	20.8	9.2	-	-
Soybean (n=44)	0.1	11.4	0.1	4.1	22.3	53.5	7	-	-
Sunflower seed (n=13)	0.1	5.1	0.1	4.3	21.6	66.8	0.2	-	-

Data from Glasser *et al*, 2008.

1.5.3 Marine oils

Marine oil supplementation of ruminant diets is a strategy used to increase the intake of beneficial FA, such as EPA and DHA, which are then incorporated into products such as milk and meat (Chow *et al.,* 2004). Many studies have been conducted on supplementing ALA rich oils (such as linseed) to ruminants as they are able to be

elongated to EPA and DHA (Barcelo-coblijn and Murphy, 2009; Mach *et al*., 2013). The conversion of ALA to these LC PUFA is however extremely low and inefficient (Plourde and Cunnane, 2007). Fish oil contain a high natural source of *n*-3, in the form of EPA and DHA, Table 1.4 shows the various FA composition of FO used in a number of animal studies. Fish oil supplementation has been shown to increase the concentrations of desirable FA, conjugated linoleic acids (CLA), EPA and DHA in milk, but also causes a decrease in milk fat content (Kairenius *et al*., 2015). Therefore the quantity used must be minimised in order to reduce the negative impact of feeding PUFA (Kairenius *et al*., 2015). Another problem regarding FO is its availability. There is already competition for FO as human nutritional supplement, which is starting to threaten its supply for aquaculture feed (Shepherd and Bachis, 2014). The growth in the demand for FO and the insecure supply are causing price inflation of FO (Shepherd and Bachis, 2014). This has led to an increase in the use of vegetable oils as a substitute for FO in aquaculture feeds, reducing the *n*-3 content of fish (Shepherd and Bachis, 2014).

An alternative source of pre-formed EPA and DHA for ruminants is ALG (Stamey *et al*., 2012). Its use in animal feed can be traced back to the 1950's (Lum *et al*., 2013), but due to the lack of controlled growing environments, it's only recently that technology has enabled ALG to be grown heterotrophically with a defined growth medium, typically supplemented with glucose (Bumbak *et al*., 2011). The controlled growing environment offers a more consistent nutrient profile for the ALG compared to sources of phototropic marine algae (Bumbak *et al*., 2011). Biomass densities of up to 400 g/kg DM per litre of water can be produced heterotrophically, the only limiter to growth being oxygen (Givens *et al*., 2000). There is a considerable amount of interest in ALG production, as heterotrophic ALG offers significant opportunities either as feed supplements, biofuels or for the treatment of disease (Bumbak *et al*., 2011).Table 1.4 shows the FA composition of ALG used in a number of animal studies.

Table 1.4. Fatty acid composition (g/100 g total FA) of various FO and ALG used in ruminant feed studies

Oil source	Fatty acid content (g/100 g FA)									Reference
	C14:0	C16:0	C16:1	C18:0	C18:1	LA	ALA	EPA	DHA	
ALG	10.1	26.3		0.9	1.1	0.32	0.17	0.04	37.8	Boeckaert *et al*., (2007a)
ALG	9.1	24.9	-	0.5	-	-	0.30	1.6	42.4	Moate *et al*., (2013)
ALG	-	52.6	-	1.4	0.13	-	0.03	0.41	30	Sinedino *et al*., (2017)
ALG	8.4	23.5	0.1	0.5	0.2	-	-	1.4	39.5	Stamey *et al*., (2012)
ALG	5.3	25	-	32.5	0.06	-	-	1.6	24.2	Vahmani *et al*., (2013)
FO	8.3	16.9	10.9	3.2	5.9	1.5	2.1	13.2	12.5	Ballou *et al*., (2009)
FO	8.2	16.6	9.6	3.7	13	1.4	2.9	11.5	10.3	Fatahnia *et al*., (2008)
FO	2.2	15.5	2.5	53.3	12.7	3.2	3.3	2.5	1.9	Moallem *et al*., (2013)
FO	-	20.8	12.4	8.4	9.7	1.7	2.0	12.6	7.8	Moussavi *et al*., (2007)
FO	0.4	12.7	-	38.7	4.1	0.6	1.29	15.9	12.3	Vahmani *et al*., (2013)

1.6 Metabolic pathways

1.6.1 Microbial ecosystem of the rumen

Ruminants have four compartments to their stomachs; the rumen, reticulum, omasum and abomasum (Membrive, 2016). The rumen is the largest compartment of the digestive tract and has a volume of 100 L or more in an adult dairy cow, and a volume of around 10 L in sheep (Hobson and Stewart, 1997). The microbial ecosystem of the rumen contains a very complex population of bacteria, protozoa, archea and fungi, that live in a symbiotic manner with the cow (Doreau *et al*., 2015). The rumen of a cow contains approximately 10^{10} to 10^{11} bacteria and 10^{5} to 10^{6} protozoa per mL of rumen content (Lock and Bauman, 2004). The normal temperature of the rumen is between 38-39 °C, and it has a normal pH range of 6.0 – 6.7 (Buccioni *et al*., 2012). Any changes to these conditions can influence the microbial population and its fermentation products (Buccioni *et al*., 2012). Mammalian enzymes cannot break down cellulose or xylan, which are major components of forages that's are found in ruminant's diet (Lourenco *et al*., 2010). In order to digest the resistant polymers, the ruminant requires microbial enzymes and the microbes grow by fermenting sugars released through digestion into ATP (Lourenco *et al*., 2010). The main products produced through the pathway to generate ATP for microbial growth are volatile FA (VFA), mainly being acetic, propionic and butyric acids, and form the main energy substrate for ruminants (Figure 1.7) (Doreau *et al*, 2015). Gases (CO_2 and CH_4) are also produced as waste (Russell and Wallace, 1997). Nitrogen is also required for microbial growth, and is obtained from plant protein or non-protein nitrogenous sources (Laurenco *et al*., 2010). Proteins are hydrolysed to amino acids and peptides, the amino acids then undergo deamination where an amino group is removed to form ammonia (Figure 1.8; Husveth, 2011). The metabolism of dietary lipid is not essential to provide nutrients to rumen microbes, as they are capable of synthesising their own FA (Lourenco *et al*., 2010). Lipid metabolism is important to microbes as some FA are toxic and can prevent their growth (Maia *et al*., 2010). Fatty acid metabolism in the rumen has a major influence on the FA composition of ruminant products, such as meat and milk (Jenkins *et al*., 2014). Through lipolysis and biohydrogenation, two major processes which occur during rumen fermentation (described in section 1.6.4), the FA which reach the duodenum and absorbed in the small intestines are different to the FA composition of the diet (Buccioni *et al*., 2012). Feed particles can remain in the rumen for two days, with the actual time depending on the rate of degradation of particles, which is affected by particle size, as particles cannot leave the rumen until they have been reduced to millimetre size by the combination of rumination and microbial action (Membrive, 2016). Any liquid or particles small enough may leave the rumen within 8-10 h (Membrive, 2016). Increased forage particle size has been shown to improve fibre, OM and starch digestibility (Yang *et al*., 2002), as shorter particles pass through the rumen too quick to be digested properly.

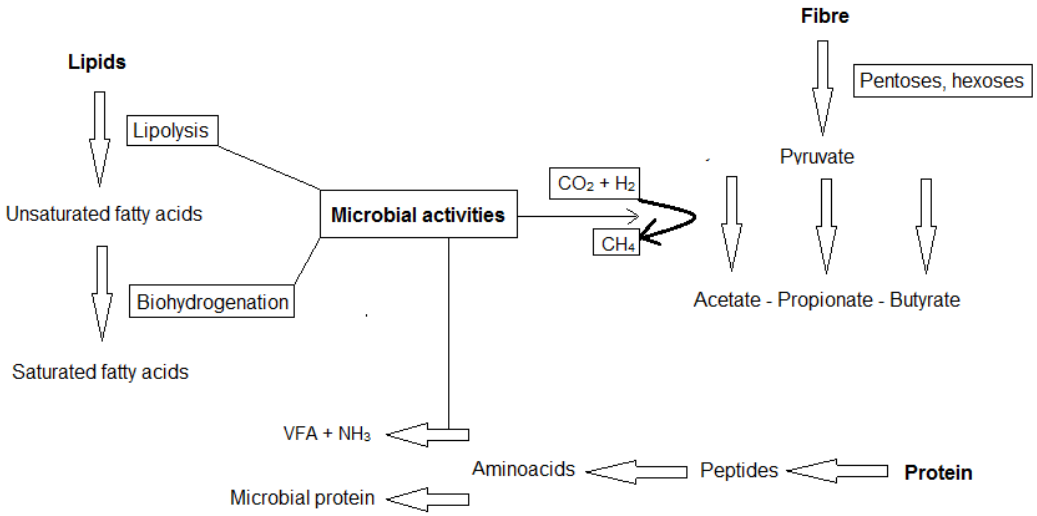


Figure 1.7. The main metabolic pathways for lipid, fibre and protein in the rumen and the role of rumen microbes. Adapted from Lourenco *et al.,* (2010).

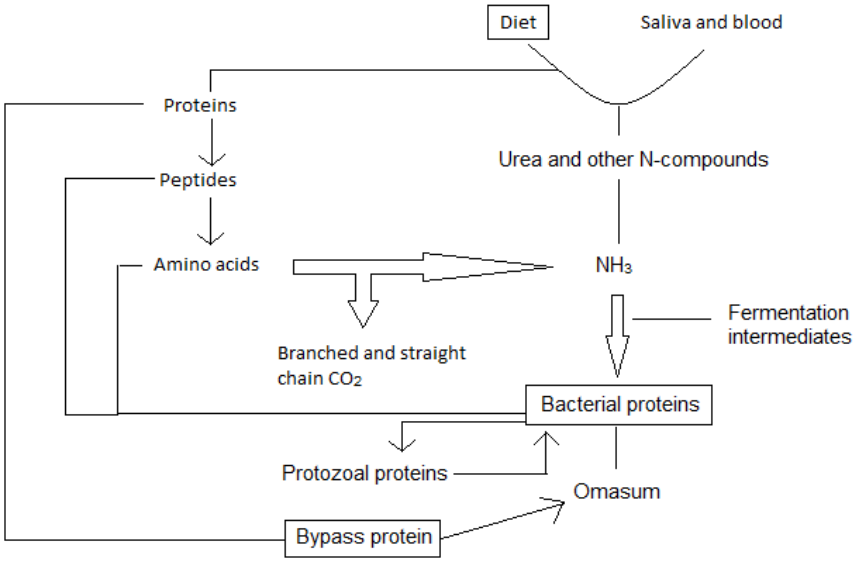


Figure 1.8. Degradation of protein in the rumen (Husveth, 2011)

Many of the bacteria population were isolated in the 1940s and 50s due to the development of strictly anaerobic techniques and medium that stimulated the bacterial habitat (Hungate 1947). Some of the more important bacterial species are listed in Table 1.5, along with the substrate they utilise and the products of the fermentation. Many of these strains are discussed by Krause and Russell (1996) and detail the most predominant rumen bacteria involved in lipolysis. With modern techniques based on 16 sRNA gene sequence analysis, it is believed that over 2000 different species of bacteria exists in the rumen (Firkins, 2010). One of the first isolate to be named was *Anaerovibrio*

lipolytica, by Hungate (1966), a gram – negative, anaerobic, curved rod bacterium which hydrolyses linseed oil triglycerides, and fermented glycerol. Its activity was found to be greatest at a pH of 7.4 and at 20-22°C, and was shown to be present in the rumen at around 10^7 ml/l (Hobson and Stewart, 1997). A diverse range of flagellated bacterium were isolated by Bryant and Small (1956), which were later grouped and classified as *Butyriuibrio fibrisolvens,* an important bacteria for the production of butyrate, the third most important VFA in the rumen.

Table 1.5. Important species of rumen bacteria involved in fibre, starch, and soluble sugar digestion in the rumen (from Dryden, 2008)

Species	Description[a]	Important function	Energy sources[b]	Fermentation products						
				Formic acid	Acetic acid	Propionic acid	n-Butyric acid	iso-Butyric acid	Lactic acid	Succinic acid
Butyrivibrio fibrisolvens	Gram +ve rods	Fibre digestion	Xylans, pectin (β glucans, starch)	+	+		+			
Fibrobacter succinogenes	Gram -ve rods	Fibre digestion	β glucans, glucose (pectin, starch)	+	+					+
Ruminococcus albus	Gram -ve single or paired cocci	Fibre digestion	β glucans, cellobiose, xylans	+	+					
Ruminococcus flavefaciens	Catalase -ve streptococci	Fibre digestion	β glucans (xylans)	+	+					+
Eubacterium ruminantium	Gram +ve rods	Starch and soluble sugar digestion	Glucose (xylan, pectin)	+			+		+	
Lactobacillus spp.	Gram +ve rods	Starch and soluble sugar digestion	Glucose (xylan, pectin)						+	
Megasphaera elsdenii	Gram -ve large cocci	Starch and soluble sugar digestion	Lactate, glucose (glycerol)		+	+	+			

Prevotella amylophilus		Starch and soluble sugar digestion	Starch, monosaccharides	+	+	+	+		+
Prevotella ruminicola	Gram -ve rods	Starch and soluble sugar digestion	Glucose and other monosaccharides (xylans, pectin, starch)	+	+		+		+
Ruminobacter amylophilus	Gram -ve rods	Starch and soluble sugar digestion	Starch (xylose)	+	+				+
Selenomonas lactilytica		Starch and soluble sugar digestion	Lactate, sugars		+	+			+
Selenomonas ruminantium	Gram -ve rods	Starch and soluble sugar digestion	Glucose, xylose and other monosaccharides (starch, sucrose)		+	+		+	
Streptococcus bovi	Gram +ve cocci	Starch and soluble sugar digestion	Starch, glucose (xylans, pectin)					+	
Succinomonas amylolytica	Gram -ve rods	Starch and soluble sugar digestion	Starch, maltose, fructose						+

[a] +ve = positive; -ve = negative
[b] Alternative substrates for some strains are shown in brackets.

Ruminal bacteria have been divided into two groups by Kemp and Lander (1984) based on the reactions and end products of biohydrogenation. Members of group A hydrogenate LA and ALA to C18:1 *trans*-11, with some strains also producing C18:2 *trans*-11 *cis*-15 from ALA (Harfoot and Hazlewood, 1997). This group of bacteria are incapable of hydrogenating C18:1 FA. Group B bacteria are able to hydrogenate a wide variety of C18:1 FA, including *cis*-9 and *trans*-11 FA, as well as hydrogenating LA to C18:0 (Harfoot and Hazlewood, 1997). Only three isolates of this group are known, two species of *Fusocillus* and an unnamed Gram-negative rod, R8/5 (Harfoot and Hazlewood, 1997).

Another microbial group that inhabits the rumen are archaea, they are strictly anaerobic methanogens and grow using H_2, reducing CO_2 to CH_4 (Janssen and Kirs, 2008). Efficient removal of H_2 leads to VFA formation and increased fermentation by eliminating the inhibitory effect of H_2 on microbial fermentation, making archaea very important in rumen function despite only being present as a small part of the rumen microbial biomass (Janssen and Kris, 2008). Over the past decade research on methanogens has become very attractive, because CH_4 emissions from ruminants contribute to global greenhouse gas emissions and represent a loss of feed energy (Patra *et al*., 2017). In a recent study by Henderson *et al*., (2015) who investigated the microbial community composition of 32 animal species, the rumen archaea population is less diverse than the bacterial community, with the 5 dominant methanogen groups compromising 89.2 % of the archaeal community. Feeding fats to ruminants has been shown to lower CH_4 emissions, with PUFA being more potent than UFA (Bayat *et al*., 2018; Grainger ad Beauchemin, 2011). The metabolic activities of rumen methanogens is lowered in the presence of PUFA, and when supplemented at high concentrations animal performance is reduced due to a reduction in feed digestion and fermentation with a change in archaeal composition (Patra *et al*., 2017).

1.6.2 Lipid hydrolysis

Lipolysis is when free FA are released from esters to allow biohydrogenation to occur, which is the reduction to the number of double bonds on the carbon chain of a FA (Buccioni *et al*., 2012). Firstly, when dietary lipids enter the rumen, hydrolases produced by rumen bacteria hydrolyses the ester linkages found in triglycerides, phospholipids and glycolipids, causing the release of constituent free FA, glycerol and small amounts of mono- and diglycerides (Lock and Bauman, 2004; Buccioni *et al*., 2012). The lipase enzyme involved in the hydrolysis is extracellular and is formed by the bacterium *Anaerovibrio lipolytica* (Jenkins, 1993). *Butyrivibrio fibrisolvens* is another esterase active bacterium that has been well studied with over 30 strains identified (Hespell and O'Bryan-Shah, 1988). It is involved in hydrolysing saponins, tributyrin and galactolipids, with only a

few strains able to hydrolyse LCFA (Hespell and O'Bryan-Shah, 1988). The extent of hydrolysis is generally high (>85%) (Fiorenti *et al*., 2015). Many factors affect the rate and extent of hydrolysis, and have been discussed by Harfoot and Hazlewood (1997) and Doreau and Chilliard (1997), and state that the extent of hydrolysis is higher in diets rich in fats and highest in diets rich in protein. Once liberated as free fatty acids, any FA can be isomerized and hydgrogenated by microbial isomerases and reductases (Doreau *et al*., 2015).

1.6.3 Microbial lipids and metabolism

D*e-novo* synthesis of microbial lipids also take place in the rumen however it is assumed that the microbial lipids released upon the death and lysis of microbes are immediately hydrolysed forming both saturated and unsaturated free FA (Harfoot and Hazlewood, 1997). As a results of microbial synthesis and biohydrogenation, the FA contained in rumen lipids and post ruminal digesta differ from those present in the diet (Buccinoni *et al*., 2012).

1.6.4 Biohydrogenation

1.6.4.1 Biohydrogeantion of LA and ALA

Microbial biohydrogenation is the process of converting UFA to more saturated end products by gut microbes, via isomerisation to *trans* FA intermediates (Li *et al*., 2012). Biohydrogenation is mainly a result of the activity of rumen bacteria, usually those attached to feed particles, rather than those in free liquid (Buccioni *et al*., 2012). The bacteria involved in biohydrogenation are mostly celluolytic, with *Butyrivibrio fibrisolvens* being the most important (Buccioni *et al*., 2012). *Butyrivibrio proteoclasticus* is another important bacterium that was re-classified by Moon *et al*. (2008), and is the only bacterium isolated from the rumen capable of converting PUFA to SFA. The biohydrogenation pathways of both LA and ALA have been well studied (Figure 1.9). The first step in the biohydrogenation pathway of LA is the isomerisation by *cis*-12, *trans*-11 isomerase which turns the *cis*-12 bond into a *trans*-11 bond forming *cis*-9, *trans*-11 CLA, before being hydrogenated to form a mixture of C18:1 *trans* isomers (Jenkins *et al.,* 2014*)*. In mixed ruminal bacteria, C18:1 *trans* is further hydrogenated to SFA C18:0 (Li *et al*., 2012). Differently to LA, ALA is isomerised to *cis*-9, *trans*-11, *cis*-15 C18:3 before being hydrogenated to a nonconjugated C18:2 as the main final product (Jenkins *et al*., 2014).

The rate of biohydrogenation is very extensive with 80 % of LA converted to C18:0 within 72 h (Maia *et al*., 2010).

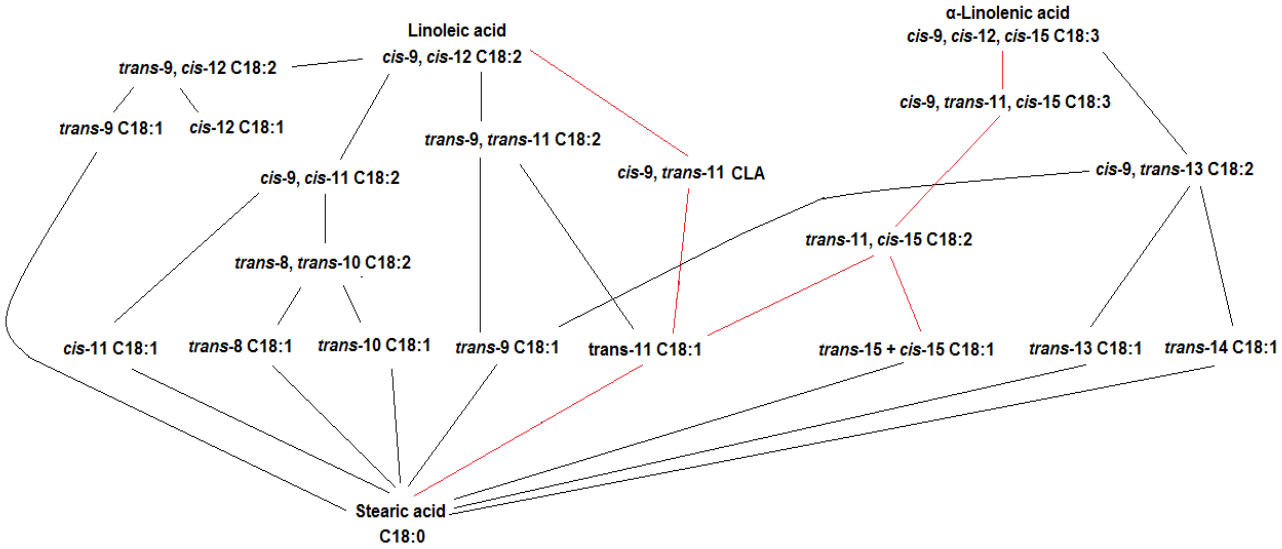


Figure 1.9. Biohydrogenation pathway of linoleic acid (LA) and α-linolenic acid (ALA), with the main pathway highlighted in red. Adapted from Lourenco *et al.* (2010) and Buccioni *et al*., (2012).

The extent of biohydrogenation may be reduced if the lipid included in the diet is protected, as Fiorenti *et al*., (2015) reported when 10 rumen and duodenal fistulated steers were fed diets containing no fat, palm oil, linseed oil, protected fat and whole soyabean and the biohydrogenation extent of C18:1 *cis*-9, LA and total UFA was decreased with the protected fat treatment.

1.6.4.2 Biohydrogenation of EPA and DHA

The biohydrogenation pathway of EPA and DHA is not as well-known as that of LA and ALA. Both the LC *n*-3 PUFA have a low transfer efficiency from the diet to ruminant derived products which suggests they undergo ruminal biohydrogenation (Jenkins *et al*., 2014). Dohme *et al*., (2003) studied the effects of two different FO types and soy oil on lipolysis and EPA and DHA biohydrogenation in a short term batch culture experiment. Each oil was incubated at six different levels of 12.5, 25, 50, 75, 100 and 125 mg for either 24 or 48 h. It was reported that both FO treatments had lower lipolysis rates compared to soy oil, and that lipolysis was further reduced when oil levels increased at 24 h of incubation, it was concluded that lipase activity was reduced due to the increasing presence of LC *n*-3 PUFA EPA and DHA. Dohme *et al*., (2003) also found that the biohydrogenation rates of EPA and DHA was affected by FO type (being lowest in the treatment containing more *n*-3 FA), and that the biohydrogenation differences between 24

and 48 h were less pronounced with increasing oil levels compared to 0 and 24 h. The reduction in biohydrogenation was suggested to be caused by PUFA adversely affecting the activity of specific rumen bacteria. In a similar study AbuGhazaleh and Jenkins (2004) examined the disappearance of pure DHA and EPA in ruminal cultures *in vitro* when incubated at different levels of 5, 10, 15 and 20 mg. It was reported that EPA and DHA are both extensively biohydrogenated and produced a large number of UFA with 1 to 5 double bonds. The disappearance of DHA was increased over time and was highest in the lower level treatment of 5 mg. The results for the disappearance of EPA was similar to that of DHA (AbuGhazaleh and Jenkins, 2004).

Kairenius *et al*., (2011) attempted to identify the biohydrogenation intermediates of LC FA in cows fed 250 g/d of FO. In the study a total of 27 novel 20-, 21- and 22-carbon FA containing at least one *trans* double bond and several unique all *cis* double bonds LC PUFA were detected in the omasal digesta of the cows fed FO. Many of the biohydrogeantion intermediates contained *trans* double bonds, which indicated that the biohydrogeantion of LC PUFA involves the isomerisation of *cis* double bond(s) (Kairenius *et al*., 2011). More recently Aldai *et al*., (2018) investigated the products formed during the initial stages of DHA biohydrogenation using mixed ruminal microorganisms from sheep, and reported that, products of DHA biohydrogenation started to appear after 1 h of incubation, with many peaks evident in the C22:0 region of GC-FID analysis. Aldai *et al*., (2018) also found that half the DHA was rapidly metabolised within the first 2.5 h, and up to 80 % after 6 h. It was also reported that there was no evidence of chain shortening of DHA to C18 FAs, which is in accordance with previous reports by AbuGhazaleh and Jenkins (2004) and Klein and Jenkins (2011). Aldai *et al*., (2018) identified two groups of transient metabolites, mono-*trans* methylene interrupted- DHA (group of 5 peaks on GS-MS) and mono-conjugate- DHA (group of two major and several minor peaks on GC-MS). As these metabolites only last for a short time the duration of the *in vitro* experiment was important, longer duration time points could have led to missing them completely, and shorter time points could possibly lead to identify further transient metabolites (Aldai *et al*., 2018). Figure 1.10 illustrates the proposed pathways of DHA metabolism. In the case of the mono-conjugate-DHA metabolites, it is still unknown which *cis* double bond(s) in DHA is/are isomerised for their production. *Cis*-12, *trans*-11 isomerase contained by *Butyrivibrio fibrosolvens* is known to cause the transition from a *cis*-12 to a *trans*-11 bond in the biohydrogenation of LA as mentioned earlier, but this enzyme would be inactive towards DHA because of the different positions of the *cis* double bonds, therefore other isomerases must be present within mixed rumen microorganisms (Aldai *et al*., 2018). Kairenius *et al*., (2011) proposed that the hydrogenation of EPA involves the reduction of the *cis* double bonds at position 5, 8 and 11 explaining the formation and accumulation of C20:2*n*-3 and C20:3*n*-3. It was also suggested that one of the main transformations of

DHA in the rumen involved the removal of the double bond between carbon atom 4 and 5 followed by the reduction of the double bond at position 7. Further studies are required to examine the fate of EPA and DHA as to date only Aldai *et al*., (2018) has managed to identify conjugated intermediates in the biohydrogenation of DHA.

Kim *at al* (2008) and Huws *et al*. (2010) have both used group-specific PCR to analyse bacterial communities in cows that were fed FO which altered the digesta FA composition, but only weak correlations were found between numbers of *B. proteoclasticus* and duodenal flow of C18:0. Another study by Boeckaert *et al*., (2008) performed a similar analysis on cows fed DHA rich ALG and found changes in a group of bacteria which there are no cultivated strains. Results from these studies indicate that there are other microbial species involved in biohydrogenation, especially in the biohydrogenation of LC PUFA, which have not yet been cultivated.

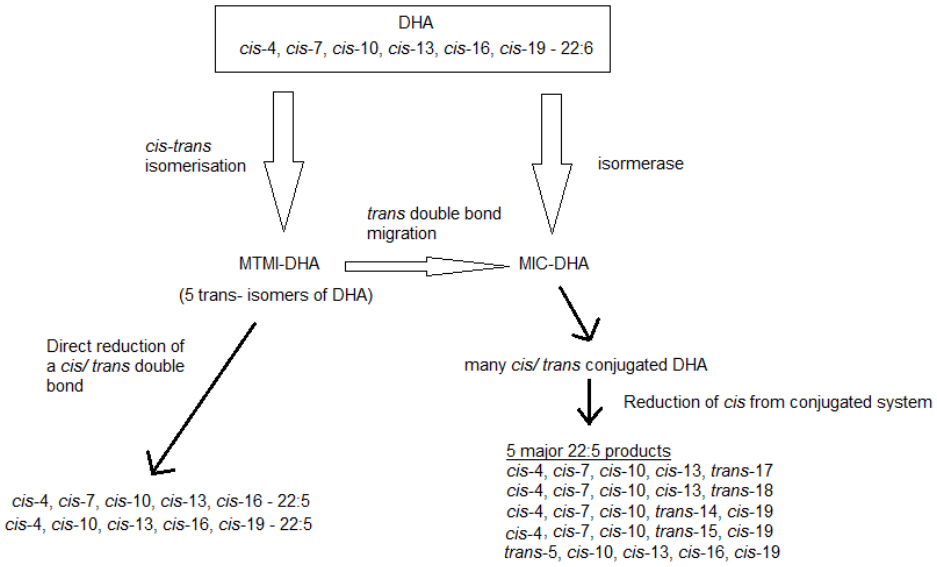


Figure 1.10. Biohydrogenation pathway of DHA. MTMI mono-*trans* methylene interrupted, MIC- mono-conjugated (Aldai *et al*., 2018)

1.6.4.3 Role of protozoa

Protozoa accounts for 50 % of the biomass of the rumen, but despite this their role in the rumen microbial ecosystem is unclear (Newbold *et al*., 2015). Studies have been conducted where ruminal protozoa has been removed by chemical and physical techniques, and results show that rumen protozoa are not essential for animal survival (Williams and Coleman, 1993). In a meta-analysis by Newbold *et al*., 2015, the main effects of protozoa removal in 23 *in vivo* studies were compared, it was confirmed that protozoal removal deceased rumen digestibility of OM and NDF, and was concluded to be caused by a loss of protozoal fibrolytic activity. Newbold *et al*., 2015, also reported greater

post-ruminal digestion, therefore the difference in whole tract digestibility between animals having no protozoa or those with protozoa in their rumen was less pronounced. Vargas *et al*., 2017, investigated the changes in the rumen microbiota when adding sunflower oil or marine oils to a ruminant diet, and reported a decrease in the number of protozoa but not total bacteria when marine oils were supplemented compared to the control and sunflower oil treatment. Similarily, Boeckaert *et al*., 2007a supplemented ALG to rumen-fistulated dairy cows to look at the effects on the microbial population. Supplementing ALG caused incomplete rumen biohydrogenation of PUFA, and quantitative PCR indicated that ALG supplementation was associated with a decrease in the number of ciliated protozoa. In contrast to this observation, protozoa appear to protect PUFA from biohydrogenation in the rumen, increasing the duodenal flow of PUFA and monounsaturated FA (MUFA) when steers were fed diets high in chloroplast (Huws *et al*., 2012). This is an agreement with the meta-analysis of Newbold *et al*., 2015, who observed an increase in SFA and decrease in MUFA and PUFA in the duodenal flow when protozoa were removed from the rumen.

1.6.5 Manipulating biohydrogenation

Rumen biohydrogenation is affected by many dietary factors. When the fibre content of the diet is lowered, and the concentrate content is increased there is a reduction in the number of cellulolytic bacteria (Loor *et al*., 2004), and other biohydrogenation pathways occur with an increase in *trans* isomers. The maturity stage of forages can also affect biohydrogenation, as well as particle size. Small feed particles pass through the rumen quicker, therefore rumen microbes has less time to hydrogenate the UFA. Added dietary fat also influences biohydrogenation, and induce the synthesis of intermediates such as CLAs and *trans* isomers. The effects of dietary lipids on manipulating biohydrogenation in order to modulate milk FA is discussed in section 1.7.3.

1.6.6 Mammary lipogenesis

Milk is a high nutritional value food, and contains 12 -14 % solid matter composed of proteins (2.5 – 4 %), fat (35 %) and lactose (~ 5 %) (Osorio *et al*., 2016). To improve the efficiency of milk production, milk fat, protein and lactose synthesis needs to be increased (Osorio *et al*., 2016). This can be achieved by combining genetic improvements and good management, especially nutrition. Milk fat is composed of more than 95 % triacylglycerol, (with three FAs esterified into the glycerol-3-phosphate backbone), and approximately 2 % of diacylglycerol (Ma, 2012). Milk fat contains over 400 FAs, making it a very complicated natural fat (Glasser *et al*., 2008). Milk fat synthesis requires the coordination of multiple biochemical processes and cellular events in the mammary epithelium, including FA activation, transport, desaturation, triacylglyceride synthesis, milk fat globule formation and secretion (Figure 1.11; Harvatine *et al*., 2009). There are two

sources of FA for milk fat synthesis, *de novo* FA synthesis in the mammary epithelial cells, which consist of short and medium chained FA (chain length < C16:0) and preformed FA uptake from blood circulation, which are derived from the diet, consisting of long chain FAs (chain length > C16:0) (Hussein *et al*., 2013). Due to biohydrogenation discussed in section 1.6.4 around 70 % of FA in milk are saturated, with C16:0 being the most abundant followed by C14:0 and C18:0 (Glasser *et al*., 2008).The most abundant MUFA is C18:1 *cis*-9, and the most abundant PUFA are LA and ALA, approximately 2.5 % of milk FA are *trans* FA, the most abundant being C18:1 *trans*-11 (Ma, 2012).

1.6.6.1 *De novo* FA synthesis

In ruminants the substrate for *de novo* FA synthesis in mammary epithelial cells is acetate produced from rumen fermentation, and β-hydroxybutyrate produced by the rumen epithelium from absorbed butyrate (Ma, 2012). Firstly acetate is converted into acetyl-CoA by acyl-CoA synthetase short-chain family (ASSC), the acetyl-CoA is then converted into a malonyl-CoA, catalysed by acetyl-CoA carboxylase, which is the rate limiting step of *de novo* FA synthesis (Hurley and Loor, 2011). Next a condensation step occurs when another acetyl-CoA and the malonyl-CoA covalently link together (releasing CoA and CO_2) producing acetoacetyl- acyl carrier protein (ACP), following a reduction step the acetoacetyl-ACP is converted to β-hydroxybutryl-ACP (using one nicotinamide adenine dinucleotide phosphate; NADPH2) (Hurley and Loor, 2011). This is followed by a dehydration step producing crotonyl-ACP (releasing a water molecule) and a reduction step converting the crotonyl-ACP to butryl-ACP (using a second NADPH2) (Hurley and Loor, 2011). Butryl-ACP then condenses with another molenyl-CoA starting another cycle. Despite Malonyl-CoA being a three carbon primer, one is lost in the condensation step and therefore only two carbons are added to the growing FA chain. Transacylase is involved in the termination of fatty acid synthesis up to 16 carbons (Ma, 2012).

1.6.6.2 Preformed FA uptake

The preformed FA which are taken up from the circulation are released from circulating lipoproteins by lipoprotein lipase (LPL) or are nonesterified FA bound to albumin that originate from body fat mobilisation (Clegg *et al.,* 2001). Usually less than 10 % of preformed FA in milk are from fat mobilisation, but it can increase when cows are in negative energy balance (Bauman and Griinari, 2003). The exact mechanism as to how the FA traverse the capillary epithelium and interstitial space to reach the alveolar cell is unknown, but is suggested to be a coordinate activity between the fatty acid translocase (CD36) and the fatty acid tranporter (SLC27A) (Osorio *et al*., 2016). Once the FA have entered the mammary epithelial cells they are activated by acetyl-CoA synthetase long-chain family (ACSL) enzyme and channelled towards triacylglyceride synthesis.

1.6.6.3 Triacylglyceride synthesis and milk secretion

The tricylglyceride synthesis is carried out in the smooth endoplasmic reticulum membrane (Osorio *et al*., 2016). The initial step with high concentration of long-chain FA (mostly saturated) is unsaturation generally by Δ9 stearoyl-CoA desaturase. The first step in tricylglyceride synthesis is the activation of long-chain FA through the addition of a phosphate group by a glycerol-3-phosphate acyltransferase (GPAT) forming a lysophosphatidic acid, this then becomes the substrate for 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT), forming phosphatidic acid (Osorio *et al*., 2016). A phosphate group is then removed by a phosphatidate phosphatase (LPIN). A 3rd long-chain FA is then added by a diacglycerol O-acyltransferase (DGATI) forming a tricylglyceride molecule.

Milk fat droplets are formed in the intermediate space of the endoplasmic reticulum bilayer membrane by the incorporation of newly synthesised triacglycerides, with an important role of adipophilin (PLIN2). The droplets are released into the cytosol as a result of the interaction between xanthine dehydrogenase (XDH), PLIN2 and butyrophilin (BTNIAI), coated with lipids and proteins from the cytoplasmic half of the endoplasmic reticulum (Osorio *et al*., 2016). In the cytoplasm, some milk lipid droplets merge into larger droplets before and during secretion (Ma, 2012). The milk fat droplets become enveloped in the plasma membrane and pinch off from the cell, forming fat globules surrounded by the apical membrane ready for secretion (Osorio *et al*., 2016).

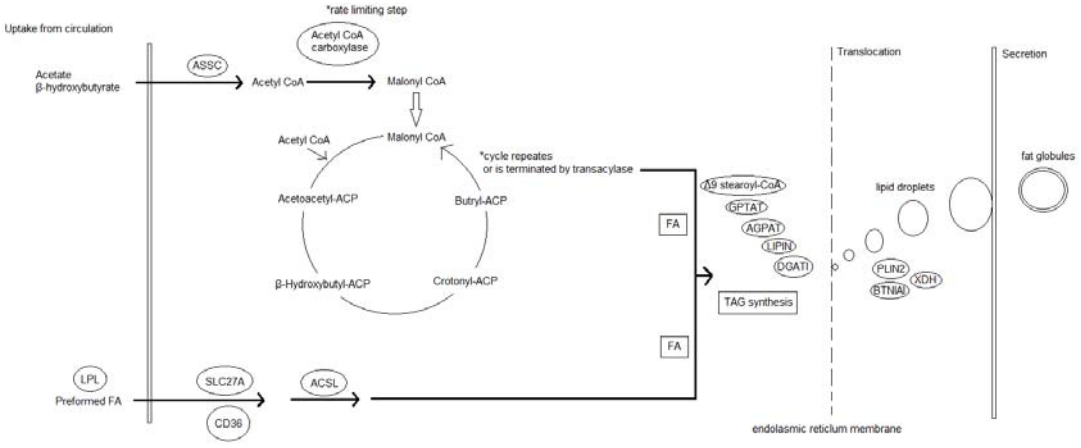


Figure 1.11. Activities and pathways during the synthesis and secretion of milk fat. Key proteins are shown in ovals:lipoprotein lipase (LPL); acyl-CoA synthetase short-chain family (ASSC); fatty acid translocase (CD36); fatty acid transporter (SLC27A); glycerol-3-phosphate acyltransferase (GPAT); 1-acylglycerol-3-phosphate O-acyltransferase

(AGPAT); phosphatidate phosphatase (LPIN); diacglycerol O-acyltransferase (DGATI); adipophilin (PLIN2); xanthine dehydrogenase (XDH) and butyrophilin (BTNIAI). FA= fatty acid; TAG = triacylglycerol. Adapted from Harvatine *et al*., (2009).

1.7 Effects of lipid supplementation on animal performance and milk fat content and quality

1.7.1 Performance

Feeding fats to dairy cows can increase the energy density of the diet, increasing energy intake if DMI is not reduced (Wullepit *et al*., 2012). Increasing the energy content of the diet can help limit the length and severity of negative energy balance, especially during early lactation (Meignan *et al*., 2017). Fat supplementation can also modify milk FA profile, increasing milk fat content of health promoting LC-PUFA and CLAs (Kliem and Shingfied, 2016). Increasing knowledge about the health benefits of *n*-3 FAs, especially EPA and DHA, have raised consumers demand for healthier dairy products (Meignan *et al*., 2017). The most common fat sources used to supplement dairy rations are vegetable oils, such as linseed, soyabean and sunflower oil, but more recently research has been undertaken on the use of FO and algae oil. As mentioned in section 1.5, vegetable and marine oils have a high content of PUFA in the form of ALA, LA, EPA and DHA, which undergo biohydrogenation in the rumen. The degree of unsaturation of these oils can affect specific rumen microorganisms (Pirondini *et al*., 2015), affecting animal performance. Studies on supplementing fats to dairy cows have resulted in varied results on DMI, milk yield and milk fat content and yield, which are summarised in Table 1.6. The results indicate differences between types of fat used, the amount added, and the composition of the basal diet.

Dry matter intake can decrease in cows when supplemented with high levels of PUFA, as reported by Franklin *et al*., (1999) who fed 910 g of ALG and reported a decrease of 4 kg/d, Mattos *et al*., (2004) who fed 200 g of FO and reported a decrease of 2-4 kg/d and Moate *et al*., (2013) who reported a linear decrease in DMI as the inclusion level of DHA increased in the diet from 0 to 75 g/DHA per cow/d. Studies that have reported a decrease in DMI often report a decrease in milk yield. On the other hand some studies have reported no effect on DMI and an increase in milk yield when supplementing cows with *n*-3 FA (Mach *et al*., 2013; Sinedino *et al*., 2017).

One of the largest challenges when supplementing fat is milk fat depression as. Milk fat depression is when milk fat content decreases whereas other milk components and yield remain the same (Bauman and Griinari, 2003). Milk fat is an important component of milk, contributing to the energy density of milk, and is important for many dairy products physical properties, manufacturing quality and organoleptic characteristics (Harvatine *et al*., 2009). Farmers are also often paid on milk quality, and increasing milk

fat is an opportunity to increase milk price, and therefore milk fat depression can have an economic effect on farmers (Lock, 2010). Milk fat content is very variable, and is affected by genetics, stage of lactation and nutritional factors (Harvatine *et al*., 2009). Diet induced milk fat depression is of great research interest and has been extensively investigated over the past 30 years. Diet induced milk fat depression involves an inter-relationship between ruminal fermentation and mammary tissue metabolism (Harvatine *et al*., 2009), and can be split into two groups; the first being caused by diets containing large amounts of fermentable carbohydrates or reduced amount of forage, and the second being diets supplemented with PUFAs (Ma, 2012). Many theories have been proposed to explain milk fat depression, including the glucogenic-insulin theory which is based on different tissue responses to insulin (Ma, 2012), and the biohydrogenation theory which is when the biohydrogenation pathway is altered under certain dietary conditions resulting in the formation of unique FA intermediates that inhibit milk fat synthesis (Bauman and Griinari, 2003). *Trans*-10, *cis*-12- CLA is formed during the metabolism of LA in the rumen under certain conditions of altered rumen metabolism, which occurs with feeding PUFA where *trans*-10, *cis*-12- CLA is produced instead of *cis*-9, *trans*-11 CLA (Figure 1.8; Wallace *et al*., 2007; Harvatine *et al*., 2009). *Trans*-10, *cis*-12- CLA is the only FA intermediate known to cause milk fat depression (Ventto *et al*., 2017). Earlier studies have shown that when supplemented to dairy cows at a rate of 10 g/d *trans*-10, *cis*-12 supplementation had no effect on any milk component except milk fat content and yield, that was reduced by 42 and 44 % respectively (Baumgard *et al*., 2000). Baumgard *et al*., (2001) also investigated abomasal infusion of *trans*-10, *cis*-12 CLA at doses of 0, 3.5, 7 and 14 g/d and both milk fat content and yield decreased with increasing concentration of *trans*-10, *cis*-12 supplementation by 25, 33 and 50 % and 24, 37 and 46 % respectively. Peterson *et al*., (2002), used an even lower concentration of *trans*-10, *cis*-12 CLA for abomasal infusion of dairy cows and reported a reduction in milk fat yield of 7 % when supplemented at a dose of 1.25 g/ d, and a reduction of 29 % when supplemented at a higher dose of 5 g/d. Some studies have reported that feeding FO (Bharatham *et al*., 2008) or ALG (Glover *et al*., 2012) to dairy cows increases milk content of *trans*-10, *cis*-12 CLA and results in milk fat depression. In contrast Loor *et al.,* (2005) reported no change in milk fat content of *trans*-10, *cis*-12 CLA when milk fat depression was induced, suggesting other biohydrogenation intermediates are involved in milk fat depression.

Table 1.6. Summary of studies evaluating the effects of oil supplementation on dairy cow performance

Study	Treatment[1]	Design	Effect on DMI	Effect on milk yield	Effect on milk fat
AbuGhazaleh *et al*., (2003)	1 % FO plus 2 % commercial fat high in C18:0, 1 % FO plus 2 % fat high in C18:1 *cis*-9 (sunflower seeds), 1 % FO plus 2 % fat high in LA (sunflower seeds), 1 % FO plus 2 % fat high in ALA (linseed)	4 ruminally fistulated cows in mid-lactation, 4x4 latin square design, 21 d adaption, 7 d sampling	No effect	No effect	The diets that included C18:1 *cis*-9 and LA reduced milk fat %
Boeckaert *et al*., (2008)	Experiment 1: Control (no added oil). ALG (2 % of fresh intake). Experiment 2: ALG (9.35 g of DHA/ kg of DM)	Experiment 1: Four cannulated cows, latin square design, 10 d adaption, 11 d sampling Experiment 2: Three rumen cannulated cows were fed ALG for 20 days, continuous design	Experiment 1: ALG diet decreased DMI (P = 0.004). Experiment 2: ALG diet decreased DMI from d 13 onwards (P = 0.003).	Experiment 1: Milk yield decreased in ALG fed cows (P = 0.015). Experiment 2: Milk yield decreased over time when supplementing ALG (P <0.001)	Experiment 1: Milk fat % was not affected, milk fat yield decreased when feeding ALG (P = 0.009). Experiment 2: Milk fat % and yield decreased over time (P <0.001).
Franklin *et al*., (1999)	Control (no added oil). Protected ALG (910 g). Unprotected ALG (910 g).	30 mid-lactation cow. 1 week adaption, 6 week sampling.	Decreased with addition of both protected and unprotected ALG in the diet (P < 0.05)	No effect	Milk fat % and yield decreased with the addition of both protected and unprotected ALG in the diet (P < 0.05)
Glover *et al.,* (2012)	Pasture or TMR basal ration supplemented with 100 g of ALG protected with an inert fat or an inert fat without the ALG	8 cows in early-mid lactation, 4x4 latin square design, 21 d adaptation 7 d sampling	No effect of ALG on DMI, only basal ration	No effect	Rumen protected ALG reduced milk fat % on both basal rations (P = 0.007)

Mach *et al*., (2013)	Control (no added oil). Linseed (13 % extruded linseed)	Fat supplement was fed from 3 weeks pre-partum until 6 weeks post-partum.	No effect	10 % increase in milk yield in cows fed linseed (P = 0.01)	Milk fat % was 8 % lower in linseed fed cows (P < 0.05). But there was no effect on milk fat yield
Mattos *et al*., (2004)	200 g/ d of FO or olive oil	Oils were fed from calving till 21 days post-partum	Decreased by 18.1 % when FO replaced OO	No effect	Milk fat % was unaffected, but ilk fat yield decreased when feeding FO (P = 0.03)
Moate *et al*., (2013)	Four treatments containing, 0, 25, 50 or 75 g/ DHA per cow/d	Day 0-14 adaptation, day 15-30 sampling period	Decreased linearly as the inclusion level of DHA increased in the diet (P = 0.02)	No effect	Linear decrease in milk fat yield as level of DHA increased (P = 0.01)
Moran *et al*., (2017)	Control (no added algae). ALG (supplemented at 6 g/kg DM).	12 week continuous design	No effect	For the total 12 week period, milk yield was 5.4 % higher in algae fed cows (P = 0.095)	Milk fat % was lower in ALG fed cows (P = 0.0001)
Shingfield *et al.,* (2006)	Control (no supplemented oil). FSO (45 g mixture of FO and SO)	Continuous design lasting 28 days	Decreased when feeding FSO (P < 0.01)	Decreased when feeding FSO (P = 0.06)	Milk fat % and yield decreased when feeding FSO (P < 0.01)
Sinedino *et al*., (2017)	Control (no added ALG). ALG (containing 10 % DHA).	Continuous design lasting 120 days	No effect	Feeding ALG increased milk yield by 0.9 kg/ d (P < 0.01)	Feeding ALG decreased milk fat % and yield (P < 0.02).
Stamey *et al*., (2012)	Control (no added oil). 29 g/d of DHA. 14.5 g/d DHA	4 x 4 latin square design, 14 d washout, 7 d of sampling	No effect	No effect	No effect
Vahmani *et al.,* (2013)	Pasture system or confined system, supplemented with no oil (control) rumen protected ALG or rumen protected FO	48 cows, fed treatment diets from 30 d pre-partum till 90 days post-partum	No effect of lipid supplement	No effect of lipid supplement	ALG supplement reduced fat % but not yield
Ventto *et al*., (2017)	L (low concentrate diet FC ratio of 65:35, no	Four Finish Ayrshire cows fitted with rumen	Intake was higher in high concentrate	No effect of treatment	Milk fat was lowest in HSO treatment, but higher in

	added lipid). LSO (low-concentrate diet FC ratio of 65:35 containing 50 g SO/ kg DM). H (high concentrate diet FC ratio of 35:65, no added lipid. HSO (high concentrate diet FC ratio of 35:65, containing 50 g SO /kg DM.	cannulae were used in a 4 x 4 latin square design, with 14-d adaption followed by 12-d of sampling	diets, with no effect of lipid supplement		LSO compared to H (P < 0.01)
Welter *et al.,* (2016)	Control (no added oil), 3 % inclusion of canola oil, 6 % inclusion of canola oil on a DM basis	18 mid-lactation cows, 3x3 latin square design, 14 d adaption, 7 d sampling	-	Decreased linearly with increasing inclusion of canola oil (P = 0.0001)	Milk fat % and yield decreased linearly with increasing inclusion of canola oil (P = 0.009 and 0.0002 respectively)

[1]FO = fish oil; SO = sunflower oil; TMR = total mixed ration; FC = forage: concentrate ratio; DM = dry matter

1.7.2 Diet digestibility

In recent years FA metabolism and digestibility has received a lot of interest in order to provide information for optimal FA supplementation (Boerman *et al*., 2015). There has also been considerable research conducted on ways to reduce CH_4 production in dairy cows by feeding PUFA (Martin *et al*., 2008; Chilliard *et al*., 2009). Dietary fat often decreases neutral detergent fibre (NDF) digestibility in sheep and steers (Weld and Armentano., 2017), but evidence of fat supplementation on NDF digestibility in dairy cows is lacking. It has been reported that UFA have a more negative effect on rumen fermentation than calcium salts or hydrogenated fats (NRC, 2001). This could be explained by the effects UFA have on the rumen microbial population, for example Maia *et al*., (2007) investigated *in-vitro* the toxicity of PUFA on rumen microbes, and reported that all cellulolytic bacteria and some butyrate-producing bacteria did not grow in the presence of PUFA at concentrations of 50 µg/ml, with toxicity of PUFA ranked as EPA > DHA > ALA > LA. Despite this, in a meta-analysis by Weld and Armentano (2017) that examined the effects of adding fat to diets of lactating dairy cows on NDF digestibility, rumen NDF digestibility was not affected by fat supplementation, and it was suggested that the microbes responsible for fibre digestion were unaffected. Pirondini *et al*., (2015) reported an increase in whole-tract NDF digestibility when FO was supplemented to dairy cows, although the reason for this effect was unclear. In contrast, Stokes *et al*., (2015) reported a linear decrease in DM, organic matter (OM) and NDF digestibility when different levels of ALG (0, 10, 20, 30 % DM basis) was fed to sheep, and Martin *et al*., (2008) also reported a decrease in DM, OM, and NDF digestibility in dairy cows fed crude linseed, extruded linseed or linseed oil on a 5 % DM basis. To date, no studies have examined the effect of ALG supplementation on DM, OM or NDF digestibility in dairy cows.

1.7.3 Milk FA profile

As discussed in section 1.4.3, there has been considerable interest in modifying the FA profile of ruminant products for the benefit of the consumer. Milk fat contains a high proportion of SFA and lower proportions of UFA compared with the dietary fat (Kliem and Shingfied, 2016). This is a consequence of rumen biohydrogenation (discussed in section 1.6.4) or the incorporation of PUFA into cholesterol esters and phospholipids component of lipoproteins (discussed in section 1.3.3). Studies involving abomasum or duodenum infusion of oils have reported an enrichment of specific FA in milk by avoiding rumen metabolism. Kazama *et al.,* (2010) infused dairy cows with linseed oil in the rumen and abomasum, and when administered through the abomasum compared to the rumen the milk fat content of ALA increased from 2.33 to 14.4 g/100 g FA. In another study by Loor *et al*., (2005), milk fat content of EPA and DHA was increased by duodenal infusion of FO compared to the control diet or when infused ruminally (EPA= 1.47 v 0.08 and 0.36; DHA

= 0.47 v 0.04 and 0.17). Diet is the main factor influencing milk fat composition, and a review from Kalac and Samkova (2010) comparing studies that have investigated the effects of season, different forage species and forage to concentrate ratio on milk FA profiles concluded that during the summer months, the proportion of ALA, C18:1 *trans*-11 and CLA in bovine milk was higher than in the winter months, this is due to the oxidation of forage PUFA during wilting and drying. Organic milk from cows fed grass-clover silages instead of maize and concentrates had higher CLA and total *n*-3 content (Kalac and Samkova, 2010). Milk fat composition can also be altered directly by feeding cows with oilseeds, plant oils and marine lipids (Kliem and Shingfield, 2016). Previous studies have shown that feeding linseed oil to dairy cows can increase the milk fat content of ALA (Suksombat *et al*., 2016; Mustafa *et al*., 2003), and that feeding soyabean or sunflower oil can increase milk fat content of LA (AlZahal *et al*., 2008; Halmemies-Beauchet *et al*., 2011). Despite the increase in total *n*-3 in milk fat by increasing milk fat content of ALA, increases in EPA or DHA is reported to be very low when feeding linseed oil (Suksombat *et al*., 2016), as it does not contain either FA.

The literature shows that in order to increase the content of EPA and DHA in dairy products marine oils must be fed to dairy cows, and Table 1.7 provides a summary of studies that have modified milk FA profiles by feeding marine oils to dairy cows. Comparing studies can be difficult as some do not include a control diet, and many have different sources and durations of supplementation. Studies that have supplemented for a longer period of time have often milk sampled on more than one occasion, but despite this only report one mean value (Franklin *et al*., 1999; Sinedino *et al*., 2017). Despite the differences between studies, it is still quite clear that marine oils have the potential to substantially modify milk FA profile. The milk fat content of C18:0 is generally higher in milk from cows fed a control diet, and lowest in those fed marine oils (Table 1.7). Milk fat content of C18:1 *trans* is also greater in cows fed marine oils compared to control diet (Table 1.7). In general, the total content of SFA is also decreased whereas the total amount of PUFA is increased with oil supplementation (Table 1.7), demonstrating that the biohydrogenation of UFA to their saturated form has been reduced by marine oil supplementation. Figure 1.12 illustrates the difference in milk fat content of both EPA and DHA when cows in 8 studies that supplemented no oil, less than 200 g of oil or 200 g or more of marine oil per day. As the dietary inclusion level of the marine oil increased so does the milk fat content of both EPA and DHA.

Rumen biohydrogenation leads to poor transfer efficiency of LC-PUFA from the diet to milk (Woods and Fearon, 2009). Wright *et al*., (1999) reported a linear decrease in the transfer efficiency of DHA with increasing inclusion levels of FO to dairy cows, with transfer efficiencies decreasing from 34.4 % to 10.9 % as the inclusion level of FO increased from 4.5 to 29.1 % of DMI. More recently Moran *et al*., (2017) reported an

increase in transfer efficiency of DHA when 148 g of ALG was fed to dairy cows as the days of supplementation increased. The transfer efficiency increased from 10.4 % at day 7 of supplementation to 21.6 % at day 84 of supplementation. Moran *et al*., (2018) conducted another study feeding 100 g of ALG per cow/d, and reported a lower transfer efficiency of 2.96 % at day 7 of supplementation that only increased to 7.08 % by day 84 of supplementation. Therefore both the amount of oil supplemented and the duration of supplementation can affect transfer efficiency of DHA from the diet into milk.

Table 1.7. Summary of studies examining the effect of marine oil supplementation to dairy cows on milk FA profile

Study	Source[a]	Intake[b]	Duration[c]	Milk fatty acid composition (g/ 100 g FA)								
				C18:0	C18:1 *trans*	LA	CLA	ALA	EPA	DHA	ΣSFA	ΣPUFA
AbuGhazaleh *et al*., (2009)	FO	150 g/d	21 d	9.20	11.80	1.53	3.41	0.35	0.04	0.03	-	-
	FO + ALG	100 g/d FO 50 g/d ALG	21 d	8.75	12.83	1.50	3.69	0.31	0.03	0.04	-	-
	FO + ALG	50 g/d FO 100 g/d ALG	21 d	7.32	13.87	1.62	4.47	0.35	0.04	0.06	-	-
	ALG	150 g/d	21 d	6.85	13.53	1.77	4.21	0.29	0.04	0.05	-	-
Boeckaert *et al*., (2008)	Control	0	21 d	10.2	2.04	1.89	0.51	0.50	-	0.09	65.5	3.06
	ALG	2 % of fresh	21 d	3.59	11.62	1.37	1.18	0.42	-	1.10	53.9	4.70
Franklin *et al*., (1999)	Control	0	6 week	12.20	-	2.83	0.37	0.54	-	0.00	65.7	4.03
	Protected ALG	910 g/d	6 week	4.96	-	2.54	2.31	0.49	-	0.76	61.6	6.47
	Unprotected ALG	910 g/d	6 week	4.26	-	2.73	2.62	0.47	-	0.46	62.7	6.53
Glover *et al*., (2012)	Control (Pasture)	0	28 d	-	-	2.35	3.52	0.68	0.06	0.06	60.2	5.20
	Pasture + ALG	200 g/d	28 d	-	-	2.56	4.18	0.83	0.08	0.26	61.3	6.54
	Control (TMR)	0	28 d	-	-	2.26	3.12	0.38	0.06	0.10	66.9	4.58
	TMR + ALG	200 g/d	28 d	-	-	2.43	3.59	0.35	0.05	0.22	61.9	5.45
Moate *et al*., (2013)	Control	0	30 d	6.65	-	1.39	0.57	0.77	0.08	0.04	75.4	5.62
	ALG	125 g/d	30 d	4.67	-	1.73	1.37	0.80	0.07	0.36	70.8	8.14
	ALG	250 g/d	30 d	2.01	-	1.75	1.99	0.69	0.11	0.60	70.6	9.82
	ALG	375 g/d	30 d	1.39	-	1.57	2.04	0.58	0.17	0.91	71.9	10.4
Moran *et al*., (2017)	Control	0	84 d	10.92	1.99	2.35	0.30	0.34	0.03	0.00	71.9	3.41
	ALG	148 g/d	84 d	9.74	7.03	2.42	0.86	0.86	0.05	0.37	67.8	4.32

Moran *et al*., (2018)	Control	0	84 d	8.28	1.74	1.76	0.41	0.24	0.02	0.00	74.2	2.75
	ALG	100 g/d	84 d	8.57	2.92	1.83	0.60	0.25	0.03	0.10	72.6	3.04
Rego *et al*., (2005)	Control	0	28 d	11.97	5.92	2.51	2.25	0.99	0.07	0.06	54.8	-
	FO	160 g/d	28 d	10.35	8.53	1.99	3.23	1.06	0.18	0.17	51.1	-
	FO	320 g/d	28 d	7.68	11.96	0.65	3.64	1.03	0.33	0.43	46.7	-
Sinedino *et al*., (2017)	Control	0	78 d	11.04		2.45	0.40	0.27	0.02	0.002	65.9	3.18
	ALG	100 g/d	78 d	11.60		2.72	0.58	0.32	0.03	0.24	62.1	3.63

[a]ALG = microalgae; FO = fish oil; TMR = total mixed ration
[b]Intake of supplemented fat
[c]Duration the oil was supplemented for in days or weeks

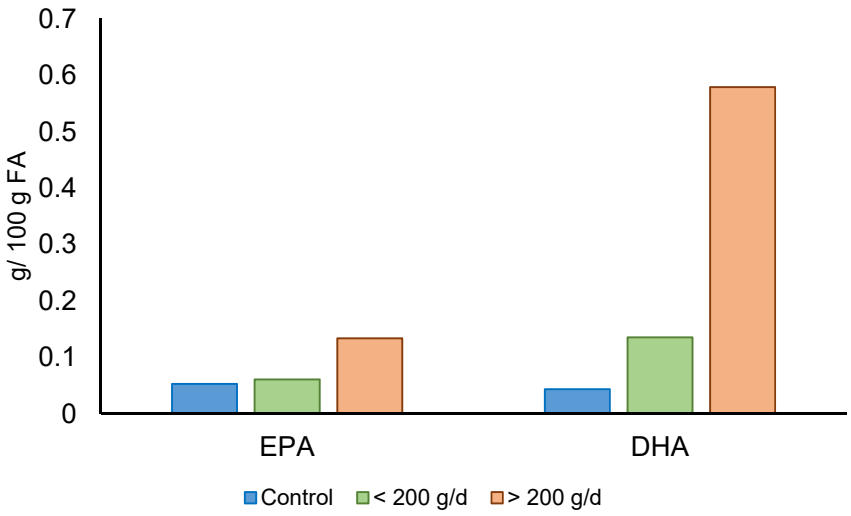


Figure 1.12 Increase in milk FA content of EPA and DHA in dairy cows fed 0, less than 200 g, or 200 g or more of supplemented marine oil (AbuGhazaleh *et al*., 2009; Franklin *et al*., 1999; Glover *et al*., 2012; Moate *et al*., 2013; Moran *et al*., 2017; Moran *et al*., 2018; Rego *et al*., 2005; Sinedino *et al*., 2017)

1.7.4 Effect of long term supplementation of PUFA on milk FA profiles

To date there has been limited research on the effect of long term feeding of *n*-3 PUFA to dairy cows. Many studies that have been conducted have been latin square designs, which despite including adaptation periods may still include some carry over effects of feeding PUFA as reported by Smith *et al*., (1993). Many studies that have fed *n*-3 PUFA for longer periods than 6 weeks often focused mainly on the effect of *n*-3 FA supplementation on fertility parameters (discussed in Section 1.9). Further limitations exist when looking at the effect of long-term feeding of PUFA on individual milk FA, as most studies have not reported the change in milk FA profile over time. A study by Bichi *et al.,* (2013) reported the change in numerous FA profiles over a 54 d period when supplementing ewes with 8 g of ALG added to 25 g of sunflower oil or a control diet containing 25 g of sunflower oil. An increase in C18:1 *trans*-11 and *cis*-9, *trans*-11 CLA was initially reported at day 6, followed by a decline over the remainder of the study. In contrast C18:1 *trans*-10 increased from day 6 of the study. Similarly, *trans*-10, *cis*-12 CLA increased over the period of the study. This change in FA biohydrogenation intermediates indicate a shift in biohydrogenation, with the rumen microbes adapting to ALG supplementation (Bichi *et al*., 2013). The milk fat content of DHA was reported to reach at maximum at week 3 of supplementation, and then remained constant (Bichi *et al*., 2013). Similarly, Moran *et al*., (2017) reported an increase in milk DHA content of cows fed ALG that reached a maximum at 3 weeks of supplementation before remaining constant. In contrast, in a more recent study, Moran *et al*., (2018) fed a lower level of ALG (100 v 148

g/cow/d), and reported an increase in milk DHA content that reached a maximum at day 54 of supplementation, which then decreased towards the end of the study (day 84). In contrast to all of the above, Shingfield *et al*., (2006) reported that milk DHA content of dairy cows reached a maximum at day 5 of FO and sunflower oil supplementation before levels decreased rapidly towards day 13 of the study. Existing research is therefore not consistent, with many studies using cows in late lactation, and no studies have supplemented cows in early lactation. Further investigation is therefore required in order to determine if rumen microbes adapt to PUFA with time with a decrease in milk DHA content.

1.7.5 Quality of PUFA enriched dairy products

Lipid oxidation is the process by which molecular oxygen reacts with UFA to form lipid peroxides (Walsh and Kerry, 2012). It's one of the main factors affecting shelf life, as it can lead to significant changes in sensory properties including odour, texture, flavour and colour (Jacobsen, 2010). Fish and seafood products which contain higher levels of PUFA are more susceptible to lipid oxidation (Walsh and Kerry, 2012). The effect of feeding supplements high in PUFA to dairy cows have on the sensory characteristics of dairy products can vary depending on the degree of unsaturation of the FA (Chen *et al*., 2004). Early studies have reported that dairy products high in PUFA have an oily texture, and give an oxidative, metallic, stale flavour (Wong *et al.,* 1973; Kieseker and Eustace, 1975). More recently Gonzalez *et al*., (2003) fed diets high in C18:1 *cis*-9 or high in LA to dairy cows, and reported that both treatments led to higher contents of UFA in butter and ice cream compared to the control diet. A reduction in the firmness of both butter and ice cream was observed with an increase in the UFA content of the dairy products but no difference was detected in the flavour evaluation (Gonzalez *et al*., 2003). In agreement to the findings of Gonzalez *et al*., (2003), Mallia *et al*., (2008) also identified no difference in the aromas characteristics of butter high in PUFA assessed by trained panellist. Further research is required on the oxidative stability of highly unsaturated dairy products.

1.8 The oestrus cycle of the dairy cow

1.8.1 Endocrine regulation

Cattle are polyoestrus animals, having more than one period of oestrus per year (Hafez and Hafez., 2000). The length of the bovine oestrous cycle can range from 18-24 days, averaging 21 (Forde *et al*., 2011). The cycle consists of two discrete phases: the luteal phase (lasting 14-18 days) and the follicular phase (lasting 4-6 days). The oestrus cycle involves the integration of multiple regulatory signals to stimulate follicle growth and maturation, ovulation of the preovulatory follicle, and synthesis of gonadal steroid and

peptide hormones (Cargile and Tracy, 2014). Figure 1.13 illustrates the oestrus cycle and follicular waves in the bovine.

Day 0 of the oestrus cycle is when the female displays oestrus by standing to be mounted or is restless and vocal, which can last from 2-24 hours. An elevation in circulating concentrations of oestriadol-17β (E_2) from the ovaries that is associated with enhanced follicular steroidogenesis during follicle development induces oestrus (Hansel and Convey., 1983). Increased concentrations of E_2 reaches a threshold 12-18 hours before the onset of oestrus, and along with a decrease in circulating concentration of progesterone (P_4) released from the ovaries, triggers a pre ovulatory surge in gonadotrophin-releasing hormone (GnRH) from the hypothalamus (Rahe *et al.,* 1980). A surge in GnRH induces a coincidental surge in follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary (Rahe *et al*., 1980). Approximately 30 hours after the onset of oestrus, ovulation is induced followed by an immediate decline in circulating concentrations of E_2 (Hafez and Hafez, 2000). The dominant follicle will only ovulate when serum P_4 concentrations are basal and LH pulses occur every 40-70 min for 2-3 days (Hafez and Hafez, 2000).

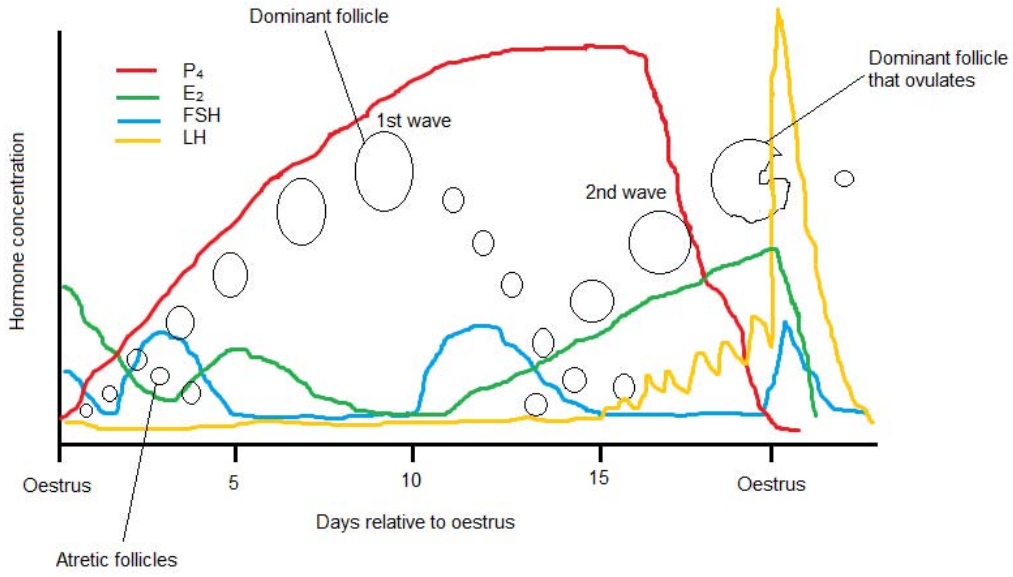


Figure 1.13. The oestrus cycle and follicular waves in the bovine. P_4 – progesterone, E_2 - oestriadol-17β, FSH – follicle stimulating hormone, LH - luteinising hormone (Forde *et al*., 2011).

Ovulation is followed by the luteal phase of the oestrus cycle (Forde *et al*., 2011). The first 3-4 days of the luteal phase is also known as met-oestrus, and is characterised by the formation of the CL from the collapsed ovulated follicle (Forde *et al*., 2011). The

follicular cells undergo functional transformation into luteal cells, which synthesise and release P_4 in readiness for the establishment of pregnancy and /or resumption of the oestrus cycle (Alila and Hansel, 1984). Following met-oestrus, the later stages of the luteal phase is known as di-oestrus (Hafez and Hafez, 2000). During di-oestrus P_4 levels reach their peak approximately 8 days after ovulation and remain elevated (Cargile and Tracy, 2014). During this phase concentrations of FSH and E_2 fluctuate in association with waves of follicular growth, however these dominant follicles that grow during the luteal phase of the oestrus cycle do not ovulate (Cargile and Tracy, 2014). Due to P_4 during the luteal phase of the oestrus cycle, negative feedback result in concentrations of circulating LH pulses that are too infrequent and inadequate for the ovulation of the dominant follicle (Rahe *et al*., 1980). At approximately day 17 of the oestrus cycle in the cow, luteolytic pulses of 13-14-dihydro-15keto prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are released by the endometrium causing luteal regression and a decrease in the concentrations of circulating P_4 (Hansel and Convey., 1983). With reduced P_4 concentration, the restraint on the release of GnRH and LH ceases, and the frequency of GnRH and LH release increases, bringing an end to the luteal phase and starts the follicular phase, known as pro-oestrus (Forde *et al*., 2011). With increased gonadotropin concentrations, follicle growth and maturation is promoted as well as elevations in E_2 concentrations and ultimately oestrus behaviour (Cargile and Tracy, 2014). The cyclic changes in gonadotropin and ovarian hormone release, and the resulting follicular activity are usually recurrent, except when pregnancy is established or the cow is suffering from a pathological condition (Forde *et al*., 2011). The majority of postpartum dairy cows will resume normal ovarian activity and ovulation within 15-45 days postpartum (Forde *et al*., 2011).

1.8.2 Function of the corpus luteum

The growth and demise of the CL during the oestrus cycle represents one of the most rapid dynamic processes in the body (Alila and Hansel, 1984). At ovulation, antral contents are evacuated and the wall of the ovulatory follicle collapses (Forde *et al*., 2011). The theca and granulosa cells of the pre-ovulatory follicle undergo vascularisation and luteinisation to form luteal cells, which is stimulated by LH (Alila and Hansel, 1984). These cells expand beyond the volume of the former follicular antrum, forming a functional CL (Alila and Hansel, 1984). Plasma P_4 levels are highly correlated with CL weight, volume, histomorphology, and ultrasound morphology, and is crucial for determining the duration of the oestrus cycle (Siqueira *et al*., 2009 ; Skarzynski *et al*., 2003). The function of the CL is to produce sufficient concentrations of P_4 throughout the luteal phase of the oestrus cycle to maintain pregnancy and during pregnancy to decrease gonadotrophin secretion and prevent behavioural oestrus occurring (Forde *et al*., 2011). Additionally the sustained

increase in P_4 concentration during the luteal phase of the oestrus cycle is involved in altering the expression pattern of genes in the uterus (Forde *et al*., 2011). These altered genes are important in order to initiate uterine receptivity, whether the cow is pregnant or not (Forde *et al*., 2011). If maternal recognition of pregnancy has not occurred by day 16 of the oestrus cycle, luteolysis of the CL occurs (Forde *et al*., 2011). Oxytocin receptors in the uterus binds oxytocin in the blood which proliferates the pulsatile secretion of $PGF_{2\alpha}$ (the major luteolytic hormone in ruminants) from the uterus, and induces the regression of the CL (Siqueira *et al*., 2009). Since the early 1970's when $PGF_{2\alpha}$ was recognised as the natural luteolytic hormone in cattle, it has been commonly used as a treatment for the induction and synchronization of oestrus in cattle (Odde, 1990). Studies have shown that the maturity of the CL at the time of $PGF_{2\alpha}$ treatment influences the luteolytic response, as $PGF_{2\alpha}$ does not induce luteolysis effectively during the first 5-6 days following oestrus (Momont and Seguin., 1984). This lack of responsiveness is still unclear, but it has been suggested that the mature CL may possess a positive feedback loop involving luteal oxytocin and tumor necrosis factor α (TNFα) that causes the release of endometrial $PGF_{2\alpha}$, sustaining luteolysis (Skarzynski *et al*., 2003). If this prediction is true multiple injections of $PGF_{2\alpha}$ will be required to complete luteolysis in an immature CL (Skarzynski *et al*., 2003).

1.8.3 Maternal recognition of pregnancy

Maternal recognition of pregnancy involves the process of generating signals that prevent luteal regression, allowing the CL to be preserved and continue to secrete P_4 (Cargile and Tracy, 2014). In cattle, secretion of interferon tau (IFNT) by the bovine conceptus along with P_4 stimulates luteotropic (a signal that stimulates luteal secretion of progesterone) and antiluteolytic (signals that block luteolysis by inhibiting endogenous luteolytic signals) signals, and it's secretion has been found to be highest at days 15-17, but has been observed up to day 28 of pregnancy (Arosh *et al*., 2004). This maternal recognition of pregnancy signal occurs in advance to the implantation of the conceptus, which begins during the third week of pregnancy (Cargile and Tracy, 2014). Elevated concentrations of $IFN_{\uparrow}$ from the conceptus will selectively increase the ratio of PGE_2 to $PGF_{2\alpha}$ (Asselin *et al*., 1997). This leads to an antiluteolytic effect on $IFN_{\uparrow}$ and a luteotropic effect on PGE_2 which eventually leads to prolonged CL lifespan and proper establishment of pregnancy (Arosh *et al*., 2004). In contrast an elevated ratio of $PGF_{2\alpha}$ to PGE_2 will stimulate uterine contractility and transport of $PGF_{2\alpha}$ towards the CL, initiating luteal regression, which can lead to pregnancy failure and a return to a new oestrus cycle (Lemley *et al*., 2015). Therefore prevention of CL regression and continual secretion of adequate P_4 is vital for successful maintenance of pregnancy to term. Progesterone is also vital in influencing the early uterine environment, increasing glandular uterine

secretions of nutrients and growth factors which are essential for early conceptus development (Lemley *et al*., 2015).

1.9 Nutrition and fertility

1.9.1 Factors affecting reproduction

Over the past five decades fertility in the dairy cow has declined as milk production per cow has increased (Walsh, 2011), although in recent years a slight improvement has been seen due to improved genetic selection (Berry *et al*., 2014). Reduced fertility includes delayed resumption of oestrous cycle post-partum, greater incidence of abnormal oestrous cycles and poorer conception rates to first and subsequent inseminations (Pryce *et al*., 2004). The lengthening of calving intervals in dairy cows has already been observed in the USA, UK and Portugal (Otto *et al*., 2014). Fertility is a multi-factorial trait and its deterioration has been caused by a combination of genetic, environmental and managerial factors and their interactions, making it difficult to determine the exact reason for its decline (Walsh *et al*., 2011). All commercial dairies desire the establishment and maintenance of a subsequent pregnancy as soon as possible following parturition, which would lead to another parturition and lactation cycle (Cargile and Tracy, 2014).

Successful reproductive efficiency in the dairy cow requires a low level of disease during the transition period, high submission rates to artificial insemination (AI), and high pregnancy rates per service. Other than infectious disease, the largest contributor to poor reproductive efficiency is the nutritional status of the cow (Lanyasunya *et al*., 2005). The nutritional status of the dairy cow involves the interaction between macronutrients and micronutrients and herd level management (Cargile and Tracy, 2014). In high-yielding dairy cows nutrition can have a significant effect on resumption of the ovarian cycle post-partum and on subsequent conception rates (Fouladi-Nashta *et al*., 2009). Dairy cows often experience negative energy balance at the beginning of lactation because the energy requirement for both metabolic processes and milk synthesis outweigh the amount being replenished through the diet (Otto *et al*., 2014). Poor nutrition during the transition period (3 weeks before parturition and 3 weeks post parturition) can result in reduced levels of plasma glucose, insulin, and low LH pulse frequency with increased β-hydroxy butyrate (BHB), non-esterified fatty acids (NEFA) and triacylglycerol plasma levels (Roche, 2006). In these conditions cows must mobilize lipids and protein reserves, increasing the incidents of metabolic diseases such as hypocalcaemia, acidosis, ketosis, and fatty liver which can then result in secondary metabolic diseases such as displaced abomasum (Roche, 2006). These metabolic disorders can reduce the reproductive efficiency of the cow.

1.9.2 Embryonic losses

Most embryonic losses in cattle occur before day 16 of pregnancy (Diskin and Morris, 2008.) At this stage of development some embryos may not have reached an appropriate size to inhibit the synthesis of $PGF_{2\alpha}$ during the critical stages of maternal recognition of pregnancy (Childs *et al*., 2008). Inhibition of the synthesis of $PGF_{2\alpha}$ could therefore increase the rates of embryo survival and pregnancy. Omega-3 PUFA such as EPA may inhibit uterine secretions of $PGF_{2\alpha}$ by competing with AA by means of COX, and DHA may compete with AA for PLA_2 enzymes (Mattos *et al*., 2000). This statement has been supported in studies carried out *in vitro*, which have demonstrated that EPA and DHA FAs can reduce the biosynthesis of the PG of the series 2 in cells and tissues (Mattos *et al*., 2003). *In vivo* studies have also reported a decrease in endometrial PG secretion along with a reduction in the secretion of AA and an increase in the concentrations of EPA in the endometrial phospholipid (Herrera-Camacho *et al.,* 2011). In a recent study by Sinedino *et al*., (2017), ALG supplementation improved conception rates to 1^{st} and 2^{nd} AI. In this study a reduction in the concentration of AA and gamma-linoleic acid in the plasma phospholipids was reported along with an increase in plasma DHA, limiting the amount of precursors available for the production of series 2 PG in the uterus (Sinedino *et al*., 2017).

1.9.3 Effect of dietary fats on reproduction in dairy cows

The diet of dairy cows are often supplemented with fat primarily to increase the energy density of the feed improving the energy status of the cow and enhancing animal performance by improving production, growth and reproduction (Santos *et al*., 2008). Several studies have reported an improved reproductive performance in lactating dairy cows fed supplemental fats (Mattos *et al*., 2000).

It has been suggested that the improvement in fertility by fat supplementation is not primarily a result of improved energy balance of the cow but is due to the effects of the dietary FA, especially the *n*-3 and *n*-6 FA families on reproductive responses in the pituitary, ovaries and uterus (Santos *et al.,* 2008). Dietary fat favours reproductive function by supplying energy and by actions on the reproductive process (Sinedino *et al*., 2017). Supplementation of dairy cows with fat was shown by Park *et al*., (1983) to increase intestinal synthesis of lipoprotein-cholesterol. This was supported by Bao *et al*., (1995) that diet changes in fat metabolism in cattle may modulate ovarian physiological processes by changing the availability of lipoproteins to the ovaries. These lipoproteins provide substrates for the steroid hormones (Bao *et al*., 1995). Cholesterol is reported to be the precursor for the synthesis of steroid hormones, the most important of these being P_4 and E_2 (Ball and Peters, 2004). Cholesterol is synthesised from acetate inside the cell, or is alternatively taken up from the blood (Ball and Peters, 2004). Another benefit to fat

supplementation is increased availability of FA precursors which increases steroid and eicosanoid secretion (described in section 1.3.3), which can alter ovarian and uterine function and affect pregnancy rates (Mattos *et al*., 2000). Reproduction in cattle therefore may be influenced more by the type of fat fed than how much is fed (Santos *et al*., 2008), and this concept is both important and challenging because as described earlier in section 1.6.4, ruminants extensively hydrogenate PUFA, limiting their supply for absorption in the small intestine. This makes fat supplementation of PUFA in the ruminant's diet and an improvement in the extent of delivery of these PUFA for absorption very important.

1.9.4 Dietary FA and their effect on reproduction

Previous studies of feeding different FA to cattle have shown a number of effects on reproductive function (Table 1.8), although responses have not been consistent. Most studies on the effect of supplemental fats on reproduction have compared *n*-3 rich and *n*-6 rich diets, with the *n*-3 diets usually consisting of ALA in the form of linseed oil, with a few evaluating the use of FO, which is rich in both EPA and DHA. Studies evaluating the effects of supplemental ALG, which is a rich source of DHA on reproductive performance in ruminants is limited, therefore further work is required in this field.

Petit *et al,* (2002) reported that feeding formaldehyde treated linseed (rich in ALA) increased the CL diameter and tended to decrease the concentration of PGFM in plasma in response to an oxytocin challenge. As discussed earlier, the size of CL corresponds to the amount of P_4 it secretes, and therefore a larger CL would secrete a greater amount of P_4 leading to improved IFNτ signalling and consequently a stronger recognition of pregnancy (Binelli and Thatcher, 1999). This could explain the improvement in conception rate found when cows were fed an increased level of ALA in another study by Petit *et al* in (2001). Similar to Petit *et al*., (2002), Childs *et al*., (2008) reported an increase in the size of the CL at day 7 of the oestrus cycle as the inclusion level of FO increased in the diet of the cows. Both FO and linseed oil are rich in *n*-3 FA, but the type of *n*-3 is different as FO are rich in LC *n*-3 PUFA compared to the shorter chain ALA found in linseed. This difference in type of *n*-3 may explain why Elis *et al*., (2016) reported an increase in the number of follicles and their size when FO was fed to dairy cows compared to soybeans but Fouladi-Nashta *et al*., (2009) reported no differences in follicle numbers or sizes when comparing Megalac, soybean and linseed diets.

Milk and plasma P_4 levels have also been reported to increase with dietary *n*-3 supplementation; Childs *et al*., (2008) reported that P_4 levels were higher as the inclusion level of FO increased in the diet, and Petit *et al*., (2001) reported an increase in P_4 when linseed was fed in comparison to Megalac. Both these results coincided with an increase in CL diameter. Another measurable fertility parameter is plasma concentrations of PGFM, the metabolite for $PGF_{2\alpha}$ (Wischral *et al*., 2001). The pulsatile secretion of $PGF_{2\alpha}$ during

the oestrus cycle mediates the regression of the CL, and feeding LC *n*-3 PUFA has been reported to reduce uterine $PGF_{2\alpha}$ secretion, and consequently a reduction in PGFM (Gulliver, 2012). A reduction in $PGF_{2\alpha}$ production would prevent the luteolysis of the CL and may prevent early embryo loss by helping with maintaining pregnancy (Otto *et al*., 2014). Mattos *et al*., (2004), Petit and Twagiramungu, (2006) and Robinson *et al*., (2002), all reported a decrease in plasma concentration of PGFM when feeding FO or linseed oil compared to olive oil, Megalac, soyabean or a control diet to dairy cows. However some findings are inconsistent, with Moussavi *et al*., (2007) reporting no difference in PGFM levels following feeding fish meal compared to a control diet. The inconsistency in results could be due to the duration of treatments which differ between studies from feeding the dietary treatments prepartum to not feeding them until 3 weeks postpartum. The treatment diets also differed in the amount of oil fed, which range from 65 to 700 g/cow per day. The quality of the forage in the basal diet may also have an effect, Bellows *et al*., (2001) was unable to improve pregnancy rate when a ration containing 6.5% fat was supplemented compared to a control diet, despite reporting an improvement in pregnancy rate in the first study when fat supplement were fed at a lower rate of 5.1% to beef heifers (Bellows *et al*., 2001). It was concluded that the better quality forage could have provided additional nutrients and in the study of Bellows *et al*., (2001) the fat effects of the supplement were masked. Many studies also don't include a control diet, making it harder to conclude whether the reduction in PGFM is due to the LC *n*-3 PUFA diet, or because the *n*-6 rich diet caused an increase in PGFM secretion.

Table 1.8 Summary of studies examining the effects of diets containing either *n*-3 or *n*-6 PUFA on reproduction responses in cattle

Study	Total no. of cows	Treatment diets[a]	Duration of treatment	Main findings[b]
Childs *et al*., (2008)	40 cross bred heifers	1) Control 2) 65 g FO 3) 140 g FO 4) 275 g FO	45 days	Plasma P_4 was higher in the high FO inclusion level compared to the low inclusion level. The CL on day 7 of oestrus increased in size as the inclusion level of FO increased from the control to the medium diet. PGFM concentration was highest in the higher FO inclusion treatment.
Elis *et al*., (2016)	25 Holstein dairy cows	1) FO 2) Soyabeans	Over two consecutive years in a switchback design	Oestrous duration was shorter in FO fed cows. There was a trend towards increased number of follicles and reduced early embryo mortality with the FO diet
Fouladi-Nashta *et al*., (2009)	12 Holstein dairy cows	1) Megalac 2) Soyabean (*n*-6) 3) LIN (*n*-3)	3x3 latin square design of 3 periods of 25 d duration, with 2 d adaption, allocated from 40-46 d postpartum	Number of follicles and their size did not differ between dietary treatments.
Moussavi *et al*., (2007)	25 Holstein dairy cows	1) Control 2) 1.25% Menhaden FM 3) 2.5% Menhaden FM 4) 5% Menhaden FM 5) 2.3% Ca salts of fish oil FA	From day 5-50 postpartum	Size of the dominant follicle increased as the inclusion level of fish meal increased. PGFM response not significantly different.
Mattos *et al*., (2004)	17 Holstein cows, 9 heifers	1) 200g FO 2) 200g Olive oil	From 21 d pre partum till 21 d postpartum	Cows fed FO had reduced blood plasma concentration of PGFM compared to olive oil during the first 3d postpartum
Moallem *et al*., (2013)	42 Holstein dairy cows	1) Encapsulated SFA, 240 and 560 g/d per cow prepartum and postpartum respectively	From day 256 of pregnancy until 100 d postpartum	Number of follicles was greater in LIN and FO treatments but size of dominant follicle did not differ. Folliculogenesis in the ovaries was increased in both LIN and FO treatments.

		2) LIN, 300 and 700 g/d per cow prepartum and postpartum respectively 3) FO at 300 and 700 g/d per cow prepartum and postpartum respectively		
Petit and Twagiramungu, (2006)	138 Holstein dairy cows	1) Whole LIN (*n*-3) 2) Megalac 3) Micronised Soyabeans (*n*-6)	From calving until pregnant/ 120 d postpartum	Conception rate was similar between treatments. Embryo mortality was higher when megalac and micronized soyabeans was fed. Mean value of plasma PGFM was lower in LIN fed cows, which also had a lower PGFM peak.
Petit *et al*., (2001)	35 Holstein dairy cows	1) Megalac 2) Extracted LIN	Week 9-19 postpartum	Length of oestrus cycle was shorter when fed megalac, conception rate to 1st AI was higher when fed LIN (87.5%). Mean P_4 value and peak was higher when fed LIN, with a peak of 35.2 μg L^{-1}
Robinson *et al*., (2002)	22 Fresian – Holstein cows	1) Control 2) LIN (*n*-3) 3) Soyabean meal (*n*-6)	For three oestrus cycles	Number of follicles on d5 was greater in cows fed soyabean meal but by d15 the number of follicles was greater in cows fed soyabean meal or LIN in comparison to the control. PGFM concentrations was higher in the soyabean meal fed cows compared to the control and LIN treatments.
Sinedino *et al*., (2017)	1800 Holstein dairy cows	1) Control 2) DHA enriched ALG	From 3 weeks postpartum for 120 d	Pregnancy rate to 1st and 2nd AI was significantly higher in ALG fed cows, ALG increased the proportion of primiparous cows that resumed oestrous cyclicity.

[a]Dietary treatments; FM= fish meal, LIN = linseed oil, FO = fish oil, ALG = algae, Ca = Calcium, FA = fatty acids
[b]Main findings; PGFM response = prostaglandin $F_{2\alpha}$ metabolite (13-14-dihydro-15keto prostaglandin $F_{2\alpha}$) response to an oxytocin injection, P_4 = progesterone

1.10 Summary of literature review

For the long-term benefit of human health, there is considerable interest in altering the FA profile of dairy products, particularly in increasing the content of health promoting LC-PUFAs, such as EPA and DHA. Milk FA are easily altered by dietary manipulation, with animals reared on pasture having been shown to have an improved milk FA composition, rich in ALA. From previous studies, marine oil supplementation has shown great potential to increase milk DHA content, although information on level of inclusion and adaptation over time is lacking. Over the past few decades fertility in the dairy cow has declined as milk production has increased. Previous studies have revealed that supplementation of LC *n*-3 PUFA has the potential to improve fertility. Additional studies to define the effect of feeding ALG on animal performance, diet digestibility, changes in milk FA profiles, and fertility are therefore required.

Hypothesis

That dietary manipulation of dairy cows with ALG rich in DHA will improve the milk FA profile, with an increase in health promoting DHA that will be maintained over time, and an improvement in indicators of dairy cow fertility.

Objective and aims

The objectives of these studies were to determine the effect of different inclusion levels of ALG on the biohydrogenation of FA *in vitro* and on milk FA profile *in vivo*. A secondary aim was to investigate the effect of altered milk FA profile on cheese yield and sensory characteristics. A third objective was to determine the long term effect of supplementation of ALG on milk FA profile, and the effect on indicators of fertility, including plasma PGFM concentrations, milk P_4 levels and conception rate.

The studies conducted will aim to:

- Understand the biohydrogenation pathways of LC-PUFA
- Investigate further the correlation between CH_4 output and LC-PUFA toxicity towards rumen microbes by measuring biohydrogenation
- Give further information on the mechanism behind milk fat depression by looking at the correlation (if any) between milk fat depression and certain milk FAs
- Determine the effect milk high in PUFA will have on the sensory characteristics of cheese
- Investigate the effect PUFA has on fertility, concentrating on the effect *n*-3 FA has on plasma PGFM concentration
- Determine if ALG supplementation can improve conception rates
- Investigate the effect ALG supplementation has on milk P4 levels on certain days post AI
- Understand the effect of long-term feeding of PUFA on individual milk FA to help determine if a shift in biohydrogenation pathways occurs

CHAPTER 2: Materials and Methods

2.1 Proximate analysis of samples

2.1.1 Dry matter determination

Dry matter content of the basal diets, total mixed ration (TMR) and faecal samples was determined according to the Association of Official Analytical Chemists (AOAC, 2012; 934.01). A subsample of the basal diet or bulked TMR were accurately weighed and oven dried at 105 °C for 48 hrs, until constant weight. Bulked faecal samples were oven dried at 70 °C (Philip Harris Ltd, England) for 48 hr, until constant weigh was reached. Samples were cooled in a desiccator and re weighed. Dry matter was calculated as:

$$\text{DM (g/kg)} = \frac{Weight\ of\ dry\ sample\ (g)}{Initial\ sample\ weight\ (g)} \times 1000 \qquad \text{Equation 1}$$

2.1.2 Ash and organic matter determination

Ash content of dried feed and faecal samples was determined according to the AOAC (2012; 924.05). Approximately 4 g of previously oven dried samples was accurately weighed into labelled pre weighed porcelain crucibles and heated overnight at 550 °C in a muffle furnace (Carbolite AAF 1100, Hope Valley, England). Samples were then cooled in a desiccator and re weighed. Ash content was calculated as:

$$\text{Ash (g/kg DM)} = \frac{Weight\ of\ ash\ (g)}{Initial\ sample\ weight\ (g)} \times 1000 \qquad \text{Equation 2}$$

Organic matter (OM) was calculated as:

$$\text{OM (g/kg DM)} = 1000 - \text{ash weight (g)} \qquad \text{Equation 3}$$

2.1.3 Crude protein determination

Crude protein (CP) content of dried feed was determined according to AOAC (2012; 988.05). Approximately 0.15 g of dried sample was accurately weighed into aluminium foil trays to determine N concentration using a C/N analyser (type FP-528, LECO Instruments, St. Joseph, MI, USA), operating the Dumas method (AOAC, 2000).

$$\text{CP (g/kg DM)} = \text{total nitrogen (g/kg DM)} \times 6.25 \qquad \text{Equation 4}$$

2.1.4 Neutral detergent fibre determination

Neutral detergent fibre content of dried feed and faecal samples was determined according to Van Soest *et al*., (1991) using fibretec apparatus (1020, FOSS, Warrington, UK). Approximately 0.5 g of sample was accurately weighed into a pre- weighed glass

crucible (porosity 1, Soham Scientific, Ely, UK) and placed into the apparatus. Neutral detergent reagent was made up by firstly dissolving 93 g of disodium ethylene diamine tetra-acetae dehydrate and 34 g of sodium borate in 3 L of deionised water. To this solution 150 g of sodium lauryl sulphate and 50 ml of tri-ethylene glycol was added. In another beaker 22.8 g of anhydrous disodium hydrogen phosphate was dissolved in approximately 500 ml of deionised water. Both solutions were then mixed and made up to 5 L using deionised water. The pH was adjusted to between pH 6.9 and 7.1 using either 0.1 M NaOH or 0.1 M HCl. Exactly 25 ml of the neutral detergent reagent and 0.5 ml of octan-1-ol (FOSS, Warrington, UK) was added to each sample. Samples were boiled for 30 min, then an additional 25 ml of cold neutral detergent reagent and 2 ml of α-amylase from *Bacillus subtilis* (MP biomedicals, LLC, UK) was added. Samples were boiled for a further 30 min, drained and washed 3 times with 30 ml of hot water under vacuum. A further 25 ml of hot water and 2 ml of α-amylase was added to each sample and after 15 min the samples were drained and washed under vacuum. The crucibles were removed from the apparatus and put in the oven set at 105 °C overnight. After drying, the samples were cooled in a desiccator, weighed and then placed in a muffle furnace at 550 °C overnight. Crucibles were cooled in a desiccator and re weighed.

$$\text{NDF (g/kg DM)} = \frac{residue\ weight\ (g) - ash\ weight\ (g)}{sample\ weight\ (g)} \times 1000 \qquad \text{Equation 5}$$

2.2 TMR and rumen fluid analysis

2.2.1 Determination of the FA content of TMR and rumen fluid samples

The FA content of dried TMR and freeze dried samples was determined according to Jenkins, (2010). The dried TMR samples were milled (Delongh KG 79, UK) to pass through a 2 mm screen, and the freeze dried rumen fluid were milled using a mortar and pestle, and 500mg accurately weighed into 50 ml glass Pyrex tubes and 2 ml of sodium methoxide (0.5 M in methanol) and 1 ml of FA internal standard (nonadecanoic acid) C19:0 (2 mg/50 ml methanol) added. The tubes were then vortexed for 5 seconds and incubated at 50 °C in a water bath (Grant Instrument Ltd, Cambridge) for 10 min, then cooled for 5 min, and 3 ml of 0.5 M HCl in methanol (GC derivatization; Sigma) added before incubation at 80 °C in a water bath for 10 min. After cooling for 7 min, 3 ml of hexane (>99.0 % GC; Sigma) was added followed by 10 ml of 6 % potassium carbonate (K_2CO_3), and the sample vortexed for 5 min. Anhydrous sodium sulphate (1 g) was then added along with 0.5 g of activated charcoal before centrifuging at 500 xg for 5 min, at 4 °C, (Refrigerated Centrifuges SIGMA 3-16PK). The solvent layer was extracted using a glass pipette into gas chromatography (GC) vials, and filtered using a 13 mm syringe filter

with a 0.22 um cellulose acetate membrane (Restek, Bellefonte, USA). The vials were stored at -20 °C prior to subsequent GC analysis.

Fatty acids were identified using a GC (model HP6890, Germany) fitted with an automatic sampler, flame ionization detector and 100 m column (CPSil88, Agilent Technologies, UK) as described by Lock *et al*., (2006). The oven temperature started at 70°C, was held for 2 min, followed by an increase of 8 °C/min until it reached 110 °C, held for 4 min, then increased 5°C/min to reach 170°C, held for 10min, and finally increased at 4 °C/min to 225 °C and held for 15 min. Each sample had a run time of 61.75 min and a post run time of 1 min at 70 °C. Peaks were identified by comparison of retention time with individual FAME standards (Sigma-Aldrich, UK) and corrected for recovery factors (Kaylegian *et al*., 2009).

2.2.2 Calculating FA content of TMR and rumen fluid samples

The individual FA content per vessel containing rumen fluid or TMR samples was calculated following the removal of the internal standard (IS), with C21:0 used as the internal standard for rumen fluid and C19:0 for TMR samples.

Corrected individual FA area (g/100g) = Equation 6

$$\left(\frac{\text{Individual FA area (g/100g)}}{100 \ - \ \text{IS area (g/100g)}}\right) \text{x}100$$

The corrected mg of total FA/g DM was then calculated as

Total FA/g DM = Equation 7

$$\left(\left(\frac{\text{IS (mg)}}{\text{Area IS / total FA area}}\right) - \text{IS (mg) added}\right) \text{x} \ \frac{1}{\text{weight of residue extracted (mg)}}$$

The mg of individual FA/g DM was calculated as:

Individual FA content (mg/g DM) = Equation 8

$$\left(\frac{\text{Total FA content (mg))}}{100}\right) \text{x corrected individual FA area (g/100g)}$$

2.3 Determination of whole tract digestibility by acid insoluble ash

Acid insoluble ash (AIA) was used as a marker to estimate feed digestibility (Van Keulen and Young, 1977) and was determined by weighing duplicate samples of 4 g of previously dried and ground faecal samples into ceramic crucibles and ashed in a muffle furnace (Carbolite AAF 1100, Hope Valley, England) for 4 h at 550°C, cooled and re-weighed. The ash residue was transferred into kjeldahl digestion tubes (Foss Tecator Digestor Unit, Hilleroed, Denmark) and 100 ml of 2M Hydrochloric acid added. Samples were boiled at 15 0°C for 10 min on the digester unit. After cooling the hydrolysate was filtered (Whatman No 41 filter paper, Fisher Scientific, UK) and washed with hot distilled water. The filer paper with ash residue was transferred back into the crucible and ashed for 12 h at 470 °C, cooled and re-weighed.

$$\text{AIA} = \frac{Weight\ of\ crucible\ (g) + ash - weight\ of\ crucible\ (g)}{Weight\ of\ dry\ sample\ (g)} \text{ x100} \qquad \text{Equation 9}$$

2.4 Milk sample analysis

2.4.1 Milk compositional analysis

For Experiment 2 milk compositional analysis (protein, fat and lactose content) was determined using a Milkoscan Minor 78110 auto analyser (Foss Electric, Denmark) that had been calibrated using standard samples (Eurofins ®, Wolverhampton, UK). Milk samples were collected from am and pm milkings and preserved using broad spectrum microtabs II (Advanced Instruments, inc, Massachusetts, USA) and stored at 4 °C. Samples were shaken and warmed to 40 °C for 15 min in a water bath (Clifton ®, Nickel Electro Ltd.UK) prior to analysis.

2.4.2 Fat extraction for FA determination (method 1)

Fat extraction in Experiment 2 followed the procedure of Hara and Radin (1987). Milk samples from individual cows were corrected for am and pm yield to produce 35 ml and placed into a 50 ml conical plastic tube. The bulked milk samples were centrifuged (Beckman, Avanti 30 Centrifuge, Harbor Boulevard, California) at 17,800 xg for 30 min at 8 °C. After centrifugation, approximately 300 mg of fat cake was transferred to 16 x150 extraction tubes (pre-rinsed with hexane) and 18 ml of hexane:isopropanol (3 parts hexane: 2 parts isopropanol, containing 50 mg butylated hydroxytoluene to prevent milk FA oxidation; HIP) was added per 1 g of fat cake and vortexed for 30 sec (5.4 ml for 300 mg of fat cake). Sodium sulfate (1 g/15 ml H_2O) was then added (12 ml per 1 g of fat cake/ 3.6 ml per 300 mg fat cake), and vortexed for 30 sec, let-to stand and vortexed again for a further 30 sec. The top layer was then transferred into 16 x 150 extraction tubes containing 1 g of sodium sulfate and pre-rinsed with hexane and were let to stand for 30

min. The top layer containing hexane and milk fat were then transferred to clean extraction tubes pre-rinsed with hexane and placed in a pre-heated water bath (40 °C), and the hexane evaporated under N_2. The lipid layer was then transferred into Eppendorf tubes and stored at -20 °C, prior to methylation.

2.4.3 Fat extraction for FA determination (method 2)

For experiment 3, fat extraction followed the method described by Feng *et al*., (2004). Milk was collected and corrected for am and pm milkings, and 20 ml was placed into a 50 ml conical plastic tube and the bulked milk samples were centrifuged (Beckman, Avanti 30 Centrifuge, Harbor Boulevard, California) at 17,800 xg for 30 min at 8 °C. An aliquot (1 g) of the fat-cake layer was then transferred to a 1.5-mL microtube and left at room temperature (~20 °C) for approximately 40 min until the fat cake melted. The microtube was then centrifuged at 19,300 xg for 20 min at room temperature using a microcentrifuge (MSE Micro Centaur; Sanyo Gallenkamp, Loughborough, UK). After centrifugation, the fat had separated into 3 layers: the top layer of lipid; the middle layer of protein, fat, and other water-insoluble solids; and the bottom layer of water. The top layer of lipid was then transferred into Eppendorf tubes and stored at -20 °C, prior to methylation.

2.4.4 Methylation of milk fat and FA determination

Methylation of the lipids was conducted according to the procedure described by Christie (1982) with modifications according to Chouinard *et al*., (1999). Approximately 50 mg of previously extracted lipid was weighed into extraction tubes pre-rinsed with hexane, and 2 ml of hexane and 40 µl of methyl acetate added and the tubes vortexed for 30 sec. Methylation reagent was prepared by mixing 1.75 ml methanol with 0.4 ml NaOMe, and 40µl added to each tube and vortexed for 2 min. Termination reagent was prepared up by weighing 1 g oxalic acid and placing in an oven (105 °C) for 30 min and then 30 ml of diethyl ether was added. After the samples had stood for 8 min after being vortexed for 2 min, 60 µl of the termination reagent was added to each sample tube and vortexed for 30 sec. A scoop (~200 mg) of calcium chloride was then added and stand for 1 h. The tubes were then centrifuged at 2600 xg for 5 min at 5 °C, before the top layer was transfer into GC vials for subsequent analysis on the GC.

Fatty acids were identified using a GC (model HP6890, Germany) fitted with an automatic sampler, flame ionization detector and 100 m column (CPSil88, Agilent Technologies, UK) as described by Lock *et al*., (2006). The oven temperature started at 70 °C, was held for 2 min, followed by an increase of 8 °C/min until it reached 110 °C, held for 4 min, then increased 5 °C/min to reach 170 °C, held for 10 min, and finally

increased at 4 °C/min to 225 °C and held for 15 min. Each sample had a run time of 61.75 min and a post run time of 1 min at 70 °C. Peaks were identified by comparison of retention time with individual FAME standards (Sigma-Aldrich, UK) and corrected for recovery factors (Kaylegian *et al*., 2009).

2.4.5 Milk progesterone

Milk P_4 levels were determined by enzyme immunoassay procedure (Ridgeway-M kit). The standards, plates and milk samples were brought to room temperature and the milk were shaken (Lab shaker, Kuhner, Switzerland) for 30 mins. The foil was stripped from the wells, which were then emptied into a sink and tapped dry onto a paper towel, before pipetting 10 µl of standard or sample to each well (leaving wells A1 and B1 empty). All standards were pipetted in duplicate and 200 µl of progesterone-enzyme label 1 was pipetted to each well (leaving A1 and B1 empty) and the plates left to incubate for 1 hr at room temperature. The incubated plates were then emptied and washed 3 times with cold water after tapping dry each time. The substrate solution was made up by dissolving the substrate with 25 ml of substrate buffer 2 and shaken for 10 mins, 200 µl of the dissolved substrate solution was then pipetted to each well and left for 30 mins for colour development to occur. The colour development of the samples was then compared with the standards. The plate optical density was read at 570 nm (FLUOstar OPTIMA, BMG Labtech, UK) after zeroing the machine using blank wells and the concentrations of the standards were computed from a standard curve. A strong pink colour indicated low P_4 and a weak pink colour indicated high P_4 levels. The progesterone concentrations of the milk samples were calculated by subtracting the blank wells from the absorbance reading of the rest of the plate. The standard curve was then plotted and a 4-parameter logistic fit was performed. The concentration of each sample was computed by reading the corresponding values on the x-axis of the standard curve.

2.5 Blood plasma analysis

Blood samples were collected into sodium heparinized vacutainers tubes (BD, MidMeds Limited, Herefordshire, UK) for albumin, BHB, total protein and urea determination, and into vacutainers containing potassium oxalate for glucose and NEFA determination. Samples were kept on ice immediately after being collected and were then centrifuged at 1000 xg for 15 min, and the plasma separated and stored at -20 °C prior to subsequent analysis. Plasma samples were analysed for albumin, BHB, total protein, urea, glucose and NEFA, using kit catalogue no's AB362; RB1008; TP245; UR221; GU611 and FA115, respectively (Randox Laboratories, County Antrium, UK) and a Cobas Mira Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK).

2.6 13,14-dihydro-15-keto-$PGF_{2\alpha}$ determination

Plasma PGFM was determined as Seals *et al*., (2002), blood samples were collected into sodium heparinised vacutainers (BD, MidMeds Limited, Herefordshire, UK) and centrifuged at 1000 xg for 15 min, and the plasma separated and stored at -20 °C prior to subsequent analysis. Plasma samples were analysed using a 13,14-dihydro-15-keto-$PGF_{2\alpha}$ ELISA kit (item 516671, Cayman Chemicals, Ann Arbor, USA). The first step was to pipette 100 µl of ELISA buffer to the non-specific binding (NSB) well and 50 µl to B_0 wells. In total there was 8 standards, and 50 µl of each was pipetted in duplicate. To each well was added, 50 µl in duplicate, of each milk sample and 50 µl of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ AChE tracer added except for the TA and Blank wells. Then 50 µl of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ AChE antiserum was added to each well except the TA, NSB and blank wells. The plate was then covered with plastic film and incubated for 18 hrs at 4 °C. For the development of the plate, the wells were first emptied and washed five times with wash buffer. Ellman's reagent was then reconstituted with ultrapure water and 200 µl immediately pipetted to each well, and 5 µl of tracer added to the TA well. The plate was then covered in plastic film, and the samples shaken (Environmental shaker-incubator ES-20, Grant bio, UK) in the dark to develop for 90 min. The plate was read at a wavelength of 420 nm. The absorbance was checked periodically until the B_0 wells reached a minimum of 0.3 AU (blank subtracted). To determine the PGFM concentrations the absorbance reading of the blank wells was first subtracted from the absorbance readings of the rest of the plate. The non-specific binding (NSB) wells and maximum binding (B_0) wells were averaged, and the NSB average subtracted from the B_0 average to give the corrected B_0.

$$\%B/\ B_0\ (\%\ \text{Bound/ Maximum bound}) = \left(\frac{Standard\ or\ Sample - NSB}{Corrected\ \text{Bo}}\right) \times 100 \qquad \text{Equation 10}$$

The $\%B/B_0$ for standards S1-S8 were plotted versus their PGFM concentrations using linear (y) and log(x) axes and a 4-parameter logistic fit was performed. The concentration of each sample was identified by the $\%B/B_0$ values on the standard curve.

2.7 Cheese analysis

2.7.1 Cheese compositional analysis

Cheese moisture content was determined using a moisture analyser (HB43-S, Mettler Toledo, Laboratory and Weighing Technologies, Leicester, UK; method ID 15550.05). The cheese samples were grated and 3 g was placed on the foil tray of the moisture analyser, the temperature of the analyser would be held at 130 °C and would run

between 9-10 min. Cheese yield was calculated according to Wedholm *et al*., (2009) and was expressed as gram cheese per 100 g of milk. Cheese fat content was determined using the Mojonnier method (AOAC, 989.05) using a Sotex system (HT 1043 extraction apparatus, FOSS, Warrington, UK). Approximately 1 g of fresh grated cheese samples was accurately weighed into cellulose extraction thimble (Whatman, Maidstone, UK), and cotton wool placed on top of the thimble. Samples were boiled at 40-50 °C in 25 ml petroleum ether (Analar, VWR, Lutterworth, UK) for half an hour. The thimbles were then rinsed for 30 min, and the petroleum ether evaporated. The ether extract (EE) was measured as:

$$\text{EE g/kg DM} = \frac{Fat\ weight\ (g)}{sample\ weight\ (g)} \times 100 \qquad \text{Equation 11}$$

2.7.2 Cheese FA determination

For cheese FA analysis, fat extraction followed the method of Coakley *et al*., (2007). A portion of each cheese sample was grated and 15 g of the grated cheese was mixed with 25 g ammonium thiocynate solution (30 % wt/vol) that was freshly prepared in 50 ml tubes. The tubes were then incubated for 1 hr in a 60 °C water bath, and shaken gently every 10 min. The samples were then centrifuged at 538 xg for 20 min, and the top cream layer transferred into culture tubes and frozen overnight. Methylation of the extracted cheese fat followed that of the milk samples (section 2.4.4)

CHAPTER 3: Experiment 1 - Effects of rate of inclusion of ALG and FO on the biohydrogenation of EPA and DHA *in vitro*

3.1 Introduction

In vitro techniques have been used extensively in feed evaluation and in studies of ruminal fermentation since the late 1970's (Pashaei *et al*., 2010). *In vitro* studies provide a cheap and quick method to determine rumen fermentation characteristics and to measure CH_4 output of various feeds, compared to the more expensive and time consuming *in vivo* studies as reviewed by Storm *et al*., (2012). Influence of PUFA on *in vitro* fermentation characteristics have been investigated by Sinclair *et al*., (2005) who investigated the effect of different oil types on vessel pH, gas production and biohydrogenation rates of PUFA, it was reported that vessel pH decreased over time, but was higher in the Control compared to the treatment vessels. Biohydrogenation of PUFA was extensive, but was lowest in the treatment containing ALG, although biohydrogenation did increase with time (Sinclair *et al*., 2005). In another *in vitro* study, Hassim *et al*., (2010), showed that increasing inclusion levels of oil palm fronds decreased the production of short chain FA, and linearly increased the production of acetate and decreased the proportion of propionate. Previous studies have shown that *in vitro* gas production and CH_4 output can also be altered by the addition of oils to a basal diet. Fievez *et al*., (2003) investigated the effect of two different FOs and soyabean oil on CH_4 production at 24 and 48 h of *in vitro* batch culture incubation and reported that CH_4 output was reduced by all three oil types, and was accompanied by increased propionate and reduced acetate production. Whereas Meale *et al*., (2012) compared the effects of different grasses, legumes and shrubs on gas and CH_4 production *in vitro*, and reported that both *Gliricidia sepium* and *Brachiaria ruziziens* produced less CH_4 despite having the highest cumulative gas production.

Fish oils and ALG have the potential to enhance the LC *n*-3 PUFA content of ruminant products when they are supplemented in the diet (Chow *et al.,* 2004). Marine oils containing LC *n*-3 PUFA are considered toxic to the rumen microbial population, and can inhibit the growth and activity of some biohydrogenating bacteria modifying the rumen microbial population as reported by Vargas *et al*., (2017) who observed a decrease in in the numbers of *S.bovis* and *Butyrivibrio* species with the addition of sunflower oil, FO and ALG oil to a control diet. With a change in the bacterial population lipid metabolism in the rumen is affected, leading to changes in the concentrations of certain FA that leave the rumen to be absorbed in the intestines (Vargas *et al*., 2017). A reduction in the concentration of C18:0 and an increase in content of biohydrogenation intermediates such as C18:1 *trans*-11 (which can be converted to *cis*-9, *trans*-11 CLA in the mammary gland

by the Δ-9 desaturase enzyme) have been reported in previous *in vitro* studies when different oils have been added to rumen fluid (Sinclair *et al.,* 2005). The strategy of including LC *n*-3 PUFA in the diet of dairy cows to reduce the extent of rumen biohydrogenation may lead to an increase supply of PUFA leaving the rumen to be absorbed from the small intestines, and consequently increasing the content of these PUFA in ruminant products.

An added benefit to feeding ALG to cattle is a reduction in the amount of CH_4 gas produced during rumen fermentation (Beauchemin, *et al*., 2009). Enteric CH_4 emissions from livestock account for approximately 38.9% of total anthropogenic CH_4 emissions and 5.7% of global anthropogenic emissions, contributing towards the problem of global warming (Johnson and Johnson, 1995; Beauchemin, *et al*., 2009). Today there is growing interest in developing practical strategies that will lead to a reduction in these emissions. Many *in vitro* studies have been conducted to determine the effect of different oil supplementation on FA biohydrogenation and CH_4 output, but relatively few have used ALG as a source and compared the effect of different inclusion levels of ALG on FA biohydrogenation and CH_4 output *in vitro,* therefore further work is required in this field.

Hypothesis

The LC *n*-3 PUFA in ALG and FO will resist biohydrogenation *in vitro*, increasing the potential for their uptake into milk, and will reduce the production of CH_4 gas, through their inhibitory effect on methanogens.

Objective and aims

The objective of the current study was to investigate, *in vitro*, the metabolism of LC *n*-3 PUFA and CH_4 production in the rumen by supplementing a basal diet with ALG and FO at varying levels of inclusion.

3.2 Material and methods

The study was conducted in accordance with the requirement of the Animals (Scientific Procedures) Act 1986 (amended 2013) and received approval by the Harper Adams University Ethical Committee.

3.2.1 Experimental design

The study was based on an *in vitro* batch culture technique as described by Sinclair *et al.,* (2005). The experimental design was a 3x4 factorial design plus control, with two oil sources; FO and ALG, which were supplemented to a basal diet of ground grass nuts at different inclusion rates of 20, 40, 60 and 80 mg of oil/g fresh weight.

3.2.2 Animals and sampling

Four wether sheep fitted with permanent rumen cannula were housed together with *ad-libitum* access to fresh water and grass hay. The sheep were fed ram master coarse mix (Wynnstay Group PLC, Powys, UK; Table 3.1) at a rate of 1 kg/sheep/ per d in one meal at 09 00 h. Sampling of rumen fluid was conducted after an adaption period of 14 days to the diet.

Table 3.1. Ingredient composition (g/kg) of the concentrate diet fed to sheep.

Ingredient	Amount (g/kg)
Wynnstay balancer PE	40
Cooked flaked barley	16
Flaked maize micronized	14
Flaked peas/ beans micronized	10
Molasses	6
Crushed oats	5
Flaked soya micronized	5
Lin-pro	4

[1]Contained (50% rape meal, 25% sunflower meal, 20% palm kernel, 5% molasses)

3.2.3 Diets and oil sources

The basal forage added to each *in-vitro* vessel was dried grass nuts that were ground through a 1.5 mm screen. This was supplemented with either FO or ALG at 5 rates of inclusion; 0, 20, 40, 60 and 80 mg of oil/g fresh weight (Table 3.2). The ALG was supplied by Alltech Inc (Kentucky, USA), and contained 580g oil/100g, The product was a pure heterotrophic ALG strain grown in a closed system of stainless steel vessels. The fish oil was supplied by UFAC (Newmarket, UK).

Table 3.2 Quantity of supplement and substrate added to 250ml duran bottles for the *in vitro* determination of the biohydrogenation of *n*-3 fatty acids.

Treatment	Rate of inclusion (mg oil/g fresh weight)	Supplement added (mg)	Ground grass nuts (mg)	Total added per vessel (mg)
FO	0	0	3000	3000
	20	60	3000	3060
	40	120	3000	3120
	60	180	3000	3180
	80	240	3000	3240
ALG	0	0	3000	3000
	20	103	2957	3060
	40	207	2913	3120
	60	310	2870	3180
	80	414	2826	3240

3.2.4 Experimental routine

Four 250 ml duran bottles modified to include a butyl rubber bung pushed through the schott (GL 45) cap and stainless steel washer, with an extra flat neoprene ring (R.H Nuttall LTD ,Great Brook Street, Birmingham, UK) placed below the washer were used for each treatment giving a total of 40 bottles. The control bottles included the basal diet of ground grass nuts, whilst blanks did not contain any additional oil or grass nuts. One bottle from each treatment was terminated by freezing at 6, 12, 24 and 48 h of incubation. The experiment was replicated to provide four values per time point treatment.

The rumen fluid (approximately 1 litre per animal) was collected from each animal at 11 00 h using a manual vacuum pump into a pre-warmed (39 °C) collection flask. The rumen fluid was then strained through four layers of muslin into a pre-warmed conical flask under a constant stream of CO_2, and kept in a water bath at 39 °C prior to being added to the fermentation vessels. Mixing of the rumen fluid (1.6 L) and buffer solution (6.4 L) (Tables 3.3 and 3.4) was conducted according to the procedure of Theodorou *et al*., (1994).

The fermentation vessels were pre-warmed at 39 °C in an incubator. To each vessel 200 ml of the buffer/ rumen mixture (80:20, v/v; pH 6.7) was added. The buffer was prepared 24 h before the experiment and autoclaved at 121 °C for 15 min to remove dissolved gases and then saturated with carbon dioxide by bubbling CO_2 gas through the mixture for 30 mins. All chemicals used in constituting the buffer solution were purchased from Sigma Aldrich®, UK.

Table 3.3. Solution composition of the buffer (to make 1 L)

Ingredient	Amount (ml)
Micromineral	0.1
Buffer solution	200
Macrominerals	200
Reducing solution	40
Indicator	1
Deionised water	559

Table 3.4. Chemical composition of the individual solutions that made the buffer solution

	Amount
Micromineral solution (g/100ml)	
Calcium chloride ($CaCl_2 \cdot H_2O$)	13.2
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	10
Cobalt chloride ($FeCl_3 \cdot 6H_2O$)	1
Iron chloride ($FeCl_3 \cdot 6H_2O$)	8
Buffer solution (g/1000ml)	
Ammonium hydrogen carbonate (NH_4CO_3)	4
Sodium hydrogen carbonate ($NaOHCO_3$)	35
Macromineral solution (g/1000ml)	
Di-sodium hydrogen orthophosphate ($Na_2HPO_4 \cdot 12H_2O$)	9.45
Potassium di-hydrogen ortho-phosphate (KH_2PO_4)	6.2
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	0.6
Reducing solution (g/100ml)	
Cystine HCl	0.625
Anaerobic indicator	
1 resazurin tablet dissolved in 50ml deionised water	

The accumulated head space gas pressure was measured manually at 0, 3, 6, 9, 12, 18, 24, 30, 39, 48 and 72 h after the addition of the substrates using a pressure transducer connected to a digital readout voltmeter (Tracker 220, Bailey and Mackey Ltd, UK). The gas was collected at each time point into labelled 50 ml syringes for subsequent CH_4 analysis.

At time points 6, 12, 24, and 48 h of incubation, fermentation was terminated by freezing the fluid for 1 h by placing in a freezer at -20 °C. The vessel contents were then mixed, their pH recorded and then transferred into plastic containers and stored at -20°C. The samples were then freeze dried prior to analysis for 7 days with the temperature of the condenser chamber maintained at -60 °C (Girovac GCD6/13, Norfolk, UK).

The freeze dried samples were prepared for FA determination and identification as described in section 2.2.1, and the FA content of each vessel was calculated as described in section 2.2.2. Ruminal biohydrogenation was calculated from the changes in the quantity of the individual FA in the residue of each vessel, compared with the amount added.

Biohydrogenation g/kg Equation 12

$$1000 - 1000\left(\frac{\text{Individual unsaturated FA in residue (mg / vessel)}}{\text{Individual unsaturated FA added (mg/vessel)}}\right)$$

3.2.7 *In vitro* gas production

Gas production (Gp; ml) was predicted by converting the pressure transducer readings (Pt; psi) using Boyle's Gas Law.

Equation 13

$$\mathrm{Gp} = \frac{\mathrm{Vh}}{\mathrm{Pa}} \mathrm{x\ Pt}$$

Where Vh represents headspace volume (ml) calculated by filling 20 x 250 ml duran bottles to the brim with water, and subtracting 200 (i.e volume of the media) from the volume measured, which provided a mean value of 107.33 ml; Pa signifies average standard atmospheric pressure (14.7 psi; Metrological Office, Bracknell, UK). Cumulative gas production was expressed per g DM after correcting for the blanks.

3.2.8 Methane analysis

Prior to analysis the GC (model 7890A, Aglient technologies, Buckinghamshire, UK) was calibrated using standard gas which contained 99% pure CH_4 gas (Puris®, Sigma Aldrich, UK). A calibration slope was created by manually injecting the GC with 10 ml of the standard CH_4 gas diluted with air to provide 25%, 50%, 75% and 100% CH_4 concentration. Peak area units were recorded for each methane. A straight line regression equation was derived from the standard gas samples against concentration and was used to determine the concentration of CH_4 gas in the test samples. Gas samples from the treatment were analysed manually by injecting 10 ml of each sample into the GC to clear through and fill a fixed loop which was then injected onto the packed column. The GC was equipped with an 80/100 mesh Porapak N column 1.8 m long (Sulpeco, Bellafonte, USA) and flame ionisation detector. The temperatures of the column, injector and detector were 170°C, 200°C and 300°C respectively, and the carrier gas (N_2) flow, H_2 flow and air flow were 34 ml/min, 30 ml/min and 400 ml/min. Each sample ran for 4 min with a post run of 2 min.

3.2.9 Statistical analysis

All data were checked for a normal distribution and were analysed as repeated measures analysis of variance using a 3 x 4 factorial design plus a control using Genstat 17 (VSN Int. Ltd., Oxford, UK) with the main effects of control, treatment, inclusion level, time and interactions. Within the FAs and pH analysis there was 4 inclusion levels per treatment and 4 time points. Within the gas production and CH_4 output analysis there was 4 inclusion levels per treatment and 10 time points.

3.3 Results

3.3.1 Fatty acid composition of the supplements

The FA composition of the supplements is provided in Table 3.5.The content of DHA was highest in the ALG, which was four times higher than the FO. In contrast EPA was considerably higher in FO. The basal diet (grass nuts) was highest in ALA, which was undetectable in the ALG. All three supplements had a low level of C18:0. The concentration of LA was similar between the grass nuts and FO but was undetectable in the ALG.

Table 3.5. The FA composition (mg/g) of the supplements

Fatty acids	Supplements		
	Grass nuts	ALG	FO
C14:0	137	55.1	34.6
C16:0	232	568	106
C16:1	-	1.18	40.1
C18:0	-	12.8	23.4
C18:1 *trans*-9	-	0.56	0.95
C18:1 *cis*-9	34.0	0.48	292
LA	132	-	106
ALA	318	-	37.1
C20:3*n*-6	-	8.47	0.30
C22:1*n*-9	-	0.77	35.3
EPA	-	3.13	46.1
C22:5*n*-5	-	57.2	6.75
DHA	-	246	61.1

3.3.2 Gas production and vessel pH

Vessel pH decreased across all treatments with time (P <0.001; Figure 3.1), with the highest value measured at 6 h of incubation (mean value of pH 6.29), and lowest at 48 h (mean value of pH 5.91). Vessel fluid pH was similar between the FO and ALG treatments and with level of inclusion. After 48 h of *in vitro* incubation however, pH was highest in the higher inclusion level of ALG which was very similar to the Control pH, and was lowest in the FO treatment.

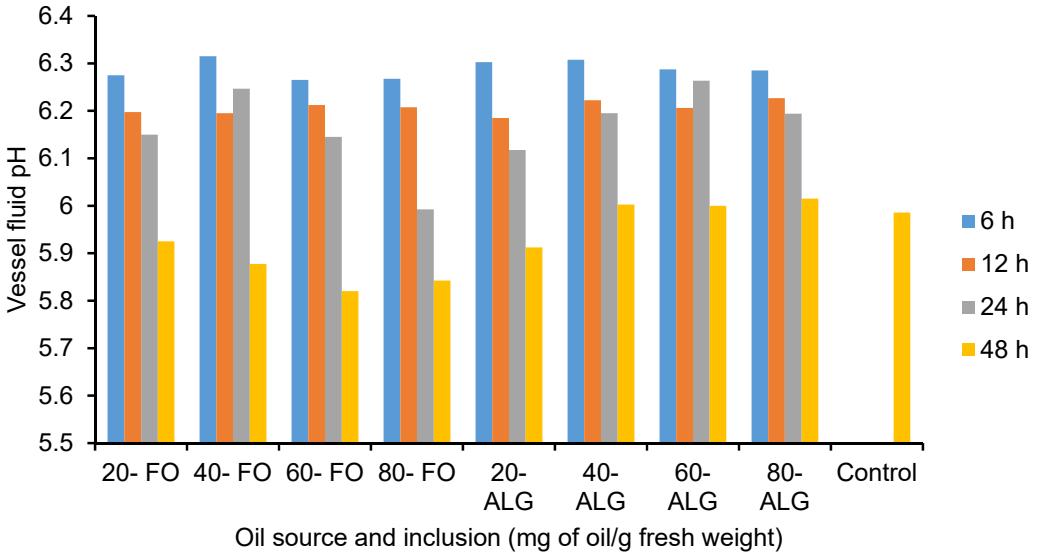


Figure 3.1. Fluid pH in vessels containing FO and ALG at different rates of inclusions during 6h, 12h, 24h and 48h of *in vitro* incubation. Treatment: *P*=0.192; Inclusion: *P* = 0.855; Time: *P* <0.001; Time x Treatment: *P* = 0.379; Time x Inclusion: *P* = 0.974; Time x Treatment x Inclusion: *P* = 0.781; s.e.d 0.11.

Gas production increased with time (*P* < 0.001; Figure 3.2) and reached asymptote at 72h of incubation for all treatments. There was an effect of treatment (*P* = 0.003) on gas production, with the addition of ALG resulting in the lowest amount of gas. An interaction was also seen between time, treatment and inclusion level (*P* < 0.001), with the FO inclusion level of 80 mg of oil/g having the greatest amount of gas production at all time-points and the 80 mg of oil/g ALG having the lowest gas production.

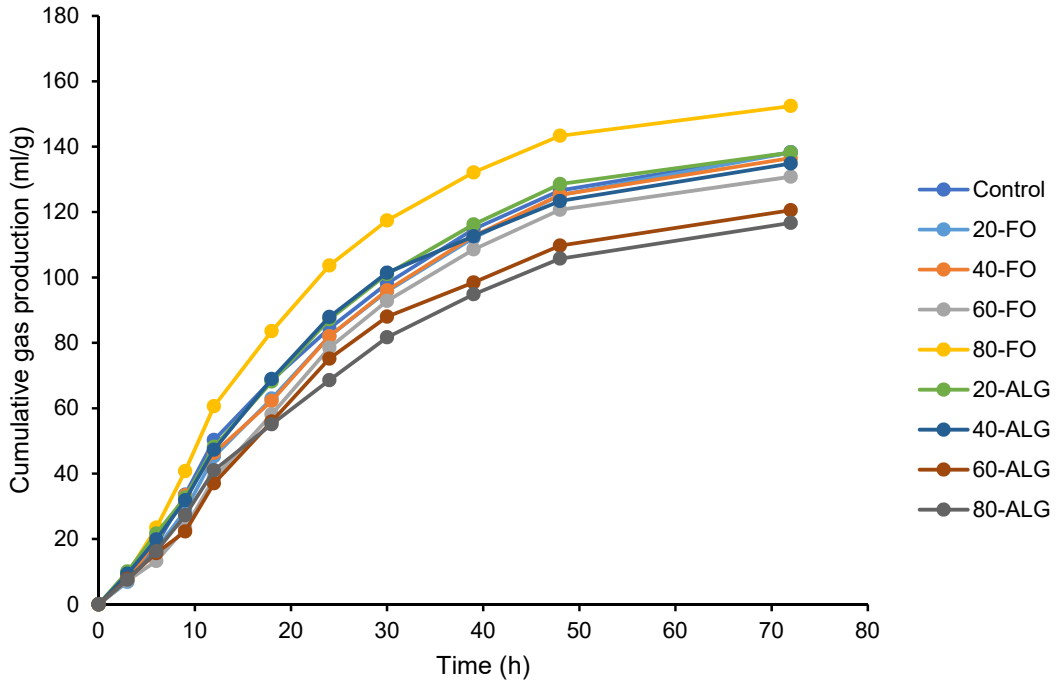


Figure 3.2. Cumulative gas production (ml/g) of FO and ALG treatments at different rates of inclusion (0, 20, 40, 60 and 80 mg of oil/g fresh weight) during 3, 6, 9, 12, 18, 24, 30, 39, 48 and 72 h of *in vitro* incubation at 39 °C; s.e.d: 27.97.

3.3.3 Methane Production

Methane output increased with time ($P < 0.001$; Fig.3.3) with average mean values increasing from 4.79 to 189 (ml/vessel) from 3 to 72 h of incubation, reaching asymptote at 72 h. The FO and ALG treatments reduced CH_4 output compared to the control from 24 h of *in vitro* incubation ($P = 0.03$). There was an effect of oil inclusion rate ($P = 0.007$), with the higher inclusion of 80 mg/g of both FO and ALG reducing CH_4 production to a greater extent than any of the other treatments from 18 h of *in vitro* incubation, by 72 h methane output was reduced by 19.2 and 13.8 % respectively compared to the Control. There was an interaction between oil inclusion level and time ($P = 0.018$), and by 24 h the Control treatment had a greater amount of CH_4 production and by 30 h the lower inclusion levels of oil also produced more CH_4 than the higher inclusions levels for both the FO and ALG treatments.

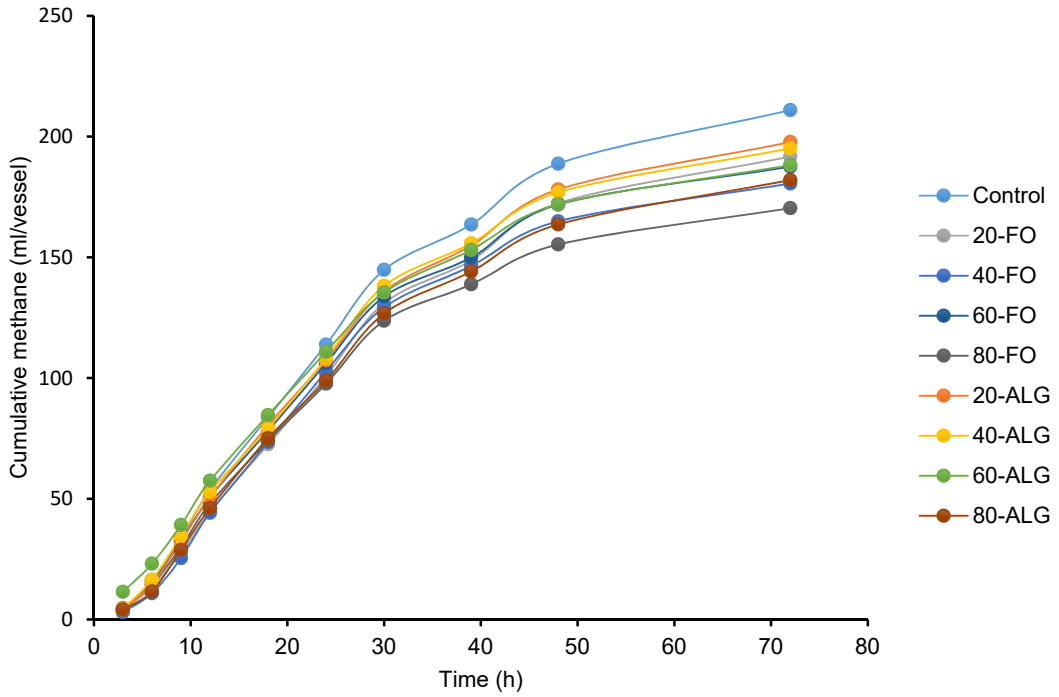


Figure 3.3. Cumulative CH_4 production (ml) of FO and ALG treatments at different rates of inclusions (0, 20, 40, 60 and 80 mg of oil/g fresh weight) during 3, 6, 9, 12, 18, 24, 30, 39, 48 and 72 h of *in vitro* incubation at 39 °C; s.e.d: 1.68.

3.3.4 Vessel FA content

The vessel content of C18:0 increased rapidly in the first 6 h of *in vitro* incubation and continued to increase with time (P<0.05; Tables 3.6 and 3.7). There was an effect of treatment (P <0.001), with FO containing a greater amount of C18:0 at all time-points compared to the ALG treatments at the same inclusion levels. The C18:1 *trans* FA content increased with time (P <0.001), with the greatest amount of 18:1 *trans* (10 + 11) and 18:1 *trans* -12 observed at 48 h of incubation at the higher inclusion of 80 mg of FO/g (mean value of 36.0 and 8.08 mg/vessel respectively). Vessel content of C18:1 *cis*-9 was greatest at 0 h, and declined with time (P < 0.05), with the largest decrease seen in the first 6 h. There was an interaction between time and treatment (P < 0.05) on C18:1*n*-9 concentration, the inclusion level of 20 mg of ALG/g had the lowest content of C18:1*n*-9, whilst vessels containing 80 mg of FO/g had the highest (mean value of 8.5 and 111 mg/vessel respectively).

The vessel content of LA decreased with time (P < 0.001), with the higher inclusion level of FO having the highest content at all times, and the ALG the lowest. There was an effect (P <0.001) of time on vessel content of ALA which decreased rapidly over time in all treatments, being lower in the ALG treatments with a 98% reduction at the 20 mg of ALG/g compared with a 89 % reduction with 80 mg of FO/g (P < 0.001). Neither of the

CLAs were detected at 0h, and there was no effect (P >0.05) of treatment, inclusion or time on *trans*-10, *cis*-12 CLA, but there was an effect of treatment and time on *cis*-9, *trans*-11 CLA (P < 0.05, P < 0.05); the vessel content of *cis*-9, *trans*-11 CLA decreased consistently with time after 6 h of incubation across all treatments, with 80 mg of ALG/g having the greatest amount at 48 h. A higher amount of EPA was detected at all time points in the FO treatments compared with the ALG (P < 0.05). In contrast, the vessel content of DHA was higher at all time points for the ALG treatment and there was an effect of inclusion level (P < 0.001), with the highest mean value after 48 h of *in vitro* incubation at an inclusion level of 80 mg of ALG/g (mean value of 16.1 mg/vessel).

Table 3.6. Vessel content (mg) of selected FA of FO and ALG treatments at different rates of inclusions during 6, 12, 24 and 48 h of *in vitro* incubation at 39°C.

Fatty acid		Treatment (oil and inclusion level mg/g)								s.e.d
		ALG	ALG	ALG	ALG	FO	FO	FO	FO	
	Time	20	40	60	80	20	40	60	80	
C18:0	0	1.32	2.65	3.97	5.30	1.40	2.81	4.21	5.62	
	6	42.8	48.4	54.5	51.1	72.2	55.1	52.2	54.9	
	12	48.5	47.9	49.5	43.0	51.8	54.6	49.2	56.7	10.20
	24	71.3	54.0	54.2	58.6	67.4	67.9	56.4	66.6	
	48	56.4	52.4	58.2	47.8	65.6	92.7	72.0	69.3	
C18:1 *trans*-9	0	0.06	0.12	0.17	0.23	0.06	0.11	0.17	0.23	
	6	5.51	8.89	12.3	11.4	11.6	10.4	9.55	11.1	
	12	8.01	7.79	9.43	8.37	9.74	10.8	10.2	16.9	3.68
	24	8.59	10.8	10.0	12.2	11.9	18.1	18.9	22.9	
	48	7.81	9.29	11.5	11.2	12.3	22.5	29.7	34.5	
C18:1 *trans*-10,11	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	6	17.1	19.1	18.2	13.3	19.3	21.5	16.9	23.9	
	12	23.8	22.3	21.9	17.1	26.3	31.1	22.2	37.1	5.75
	24	23.2	25.8	22.2	23.9	27.9	34.8	34.0	46.8	
	48	20.3	19.6	26.7	17.7	27.7	37.6	32.8	36.0	
C18:1 *trans*-12	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	6	1.90	2.22	2.23	1.85	3.25	3.26	3.08	4.04	
	12	2.46	2.49	3.18	1.96	3.42	3.94	3.45	5.41	0.89
	24	2.90	3.30	2.72	3.14	3.74	5.94	6.04	6.23	
	48	2.99	2.71	3.63	2.85	3.56	6.86	7.96	8.08	
C18:1 *cis*-9	0	100	100	100	100	119	137	154	172	
	6	8.50	10.7	12.1	11.1	77.7	66.0	81.4	111	
	12	11.4	10.2	10.7	8.70	39.3	57.6	62.8	101	12.5
	24	10.7	11.4	10.6	11.7	30.5	56.7	66.7	64.9	
	48	8.80	9.2	10.4	8.50	17.5	47.2	65.0	72.3	

Number of replicates per treatment per time point = 4

Table 3.6. Vessel content (mg) of selected FA of FO and ALG treatments at different rates of inclusions during 6, 12, 24 and 48 h of *in vitro* incubation at 39°C (contd).

Fatty acid		Treatment (oil and inclusion level mg/g)								s.e.d
	Time	ALG 20	ALG 40	ALG 60	ALG 80	FO 20	FO 40	FO 60	FO 80	
LA	0	391	385	379	374	403	409	416	422	
	6	6.56	6.75	7.61	10.1	15.8	18.4	20.4	26.3	
	12	5.39	4.99	4.86	4.66	8.79	11.2	11.0	14.9	2.68
	24	3.77	3.94	4.98	5.12	4.22	6.18	6.10	6.72	
	48	2.45	2.40	2.61	2.35	2.13	6.34	6.01	7.58	
ALA	0	939	925	911	897	955	957	959	961	
	6	5.82	6.31	7.00	10.4	11.8	9.34	9.70	12.1	
	12	4.64	5.18	4.77	5.18	6.20	6.12	6.75	7.72	1.79
	24	3.68	4.02	5.22	5.38	3.57	6.44	7.08	9.98	
	48	2.23	2.44	2.81	1.98	3.94	5.02	7.60	11.0	
C18:2 *cis*-9, *trans*-11 CLA	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	6	1.71	0.49	0.70	0.64	2.19	2.67	2.39	0.92	
	12	0.31	0.28	0.21	0.14	2.68	1.98	2.14	0.65	1.00
	24	0.33	0.28	0.40	0.30	1.78	1.83	0.65	0.67	
	48	0.14	0.27	0.35	0.38	0.37	0.43	1.62	0.79	
C18:2 *trans*-10, *cis*-12 CLA	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	6	0.60	0.73	0.56	0.62	0.81	0.41	0.39	0.37	
	12	0.41	0.51	0.60	0.31	0.46	0.28	1.78	0.36	0.57
	24	0.33	0.38	0.35	0.34	0.27	0.42	1.76	0.58	
	48	0.29	0.31	0.17	0.61	0.29	0.49	0.50	0.27	
EPA	0	3.22	6.48	9.71	13.0	31.1	62.1	93.2	124	
	6	0.80	1.06	1.05	1.18	1.76	0.91	1.29	1.86	
	12	0.94	0.67	1.31	0.51	0.69	0.98	1.46	1.73	0.49
	24	0.54	0.83	1.03	0.79	0.72	1.13	1.19	2.59	
	48	1.01	0.81	0.72	0.81	0.50	0.75	0.71	1.92	
DHA	0	25.4	51.0	76.4	101	3.66	7.32	11.0	14.7	
	6	2.84	6.46	11.12	25.9	1.69	0.97	1.16	1.43	
	12	2.54	10.6	9.12	19.3	0.43	0.90	1.33	1.84	2.43
	24	2.80	6.94	11.2	14.9	0.89	1.12	1.48	2.44	
	48	2.47	5.69	7.98	16.1	0.67	0.68	0.95	2.44	

Number of replicates per treatment per time point = 4

Table 3.7. *P* values of main effects and interactions of FO and ALG treatments at different rates of inclusions during 6, 12, 24 and 48 h of *in vitro* incubation at 39 °C

Fatty acid	Significance			Interaction			
	Treatment	Inclusion	Time	Treatment.Inclusion	Time.Treatment	Time.Inclusion	Time.Treatment.Inclusion
C18:0	<.001	0.613	0.001	0.322	0.121	0.604	0.378
C18:1 *trans*-9	<.001	<.001	<.001	0.023	<.001	<.001	0.313
C18:1 t*rans*-10,11	<.001	0.381	<.001	0.032	0.071	0.588	0.894
C18:1 *trans*- 12	<.001	0.003	<.001	0.003	0.013	0.364	0.265
C18:1 *cis*-9	<.001	<.001	0.006	<.001	0.009	0.574	0.451
LA	<.001	0.051	<.001	0.231	<.001	0.548	0.960
ALA	<.001	<.001	<.001	0.160	0.167	0.696	0.250
C18:2 cis- 9, trans- 11 CLA	0.012	0.663	0.013	0.801	0.193	0.512	0.507
C18:2 trans-10, cis-12 CLA	0.448	0.524	0.430	0.431	0.358	0.399	0.625
EPA	0.007	0.023	0.056	0.047	0.721	0.298	0.642
DHA	<.001	<.001	0.265	<.001	0.265	0.503	0.367

Number of replicates per treatment per time point = 4

3.3. 5 Biohydrogenation

The extent of biohydrogenation of EPA was higher in FO treatments (mean value over 900 g/kg at all time points) compared with ALG, (P < 0.001; Figure 3.4). The lowest inclusion level of ALG resulted in a lower extent of biohydrogenation than all other treatments at all time points (P < 0.001). The biohydrogenation of EPA also increased with time in all treatments (P = 0.032). Similar to EPA, DHA was extensively biohydrogenated over time (P = 0.03; Figure 3.5). At 48 h the extent of biohydrogenation was lowest at the highest inclusion level 80 mg of FO/g. There was no effect of treatment, but level of inclusion had an effect (P = 0.014), with the higher inclusion level of 80 mg of ALG/g reducing the extent of biohydrogenation at all time points compared to the lower inclusion levels for ALG treatments.

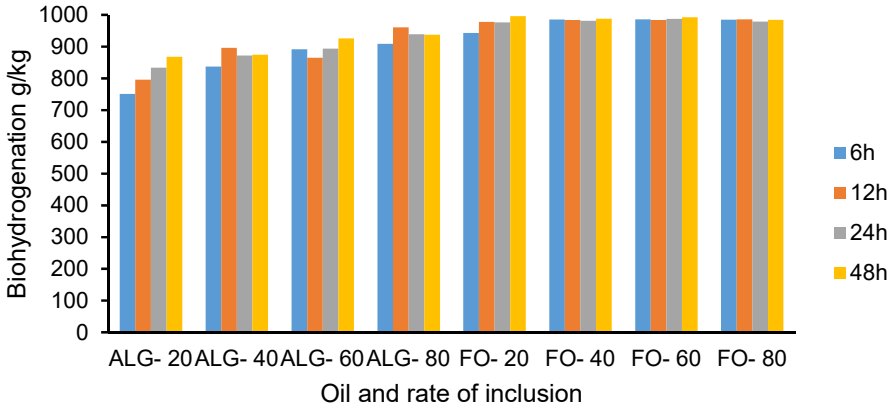


Figure 3.4. Biohydrogenation of EPA in vessels containing FO and ALG at different rates of inclusion during 6, 12, 24 and 48 h of *in vitro* incubation. Treatment: P < 0.001; Inclusion: P < 0.001; Time: P = 0.03; Treatment x Inclusion: P < 0.001; Time x Treatment: P = 0.302; Time x Inclusion: P =0.266; Time x Treatment x Inclusion: P = 0.826; s.e.d 29.10.

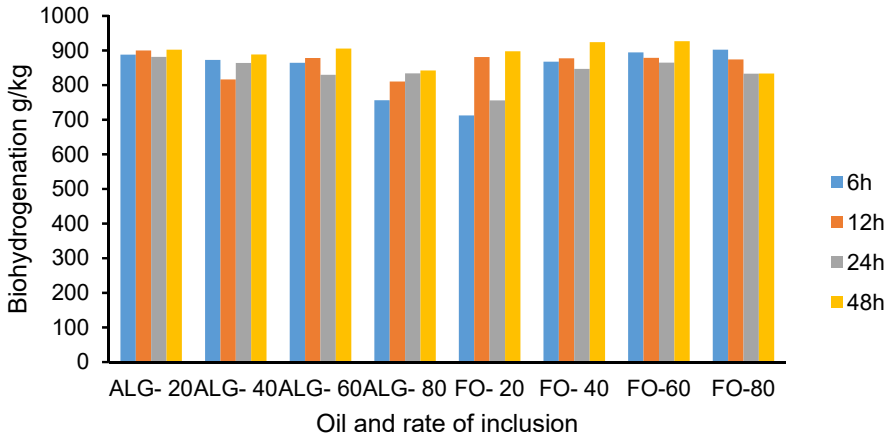


Figure 3.5. Biohydrogenation of DHA in vessels containing FO and ALG at different rates of inclusion during 6, 12, 24 and 48 h of *in vitro* incubation. Treatment: $P = 0.801$; Inclusion: $P = 0.014$; Time: $P = 0.03$; Treatment x Inclusion: $P < 0.001$; Time x Treatment: $P = 0.478$; Time x Inclusion: $P = 0.447$; Time x Treatment x inclusion: $P = 0.174$; s.e.d 23.65

3.4 Discussion

3.4.1 Gas production and vessel pH

Vessel pH reduced with time in all treatments (mean value of 6.29 at 6 h and 5.91 at 48 h), a finding in accordance with Troegeler-Meynadlet *et al*., (2006), who reported a decrease in vessel pH over a 24 h *in vitro* incubation. Both the FO and ALG treatments had a similar vessel pH throughout the *in vitro* incubation period, although after 48 h, the ALG and Control had a slightly higher pH compared to the FO treatment. Similar pH values were obtained by Sinclair *et al*., (2005) when treatments rich in ALA and LC *n*-3 PUFA were incubated *in vitro* for 48 h. Lower pH values have been associated with a reduction in the biohydrogenation of LA and ALA, and an accumulation in the production of C18:1 *trans*-11 (Fuentes *et al*., 2009; Ribeiro *et al*., 2007). In the current study the highest inclusion level of FO had the greater vessel content of both LA and ALA after 48 h of incubation associated with a lower vessel pH. Low ruminal pH (<6.0) can cause a shift in the rumen bacterial population as it has negative effects on fibrolytic bacteria and can cause a decrease in the population of amylolytic bacteria (Chen *et al*., 2011). In accordance to Chen *et al*., (2011), Troegeler-Meynadlet *et al.,* (2006) reported how the rate and efficiency of isomerisation in the first step of LA biohydrogenation decreased as the vessel pH reduced to below 6.0 over time.

An agreement was observed between a reduction in vessel pH and rate of gas production that was reduced over time, this inhibitory effect could be attributed to a reduction in rumen bacteria growth and activity as the vessel pH fell below 6 (Kessel and

Russell, 1996). The effect of low ruminal pH on bacterial growth has been well documented (Brock, 1969; Russell and Drombowski, 1980). Cumulative gas production profiles increased over time in all treatments, with high inclusions of ALG reducing gas production and FO increasing gas production compared to the Control. These finding conflict with Sinclair *et al*., (2005), who reported a reduction in gas production with a FO supplement. In another study by Yadeghari *et al*., (2015), gas production was increased at higher inclusion levels of essential oil after 24 h of incubation, which may be due to the adaption of rumen microbes to high inclusion levels of the oil. This is difficult to conclude in the current study as a reduction in total gas production was observed at the higher inclusion levels of ALG, these findings corresponds to that observed by Machado *et al.,* (2014) who reported the effects of marine and freshwater ALG on the reduction of total gas production *in vitro.* Low gas production has been associated with poor ruminal fermentation, as the main end products of microbial fermentation are volatile FAs, CO_2, CH_4 and ammonia (Harfoot and Hazlewood, 1997). The limitations of the *in vitro* study is the use of fermentation bottles with added buffer. Despite keeping the bottles in an incubator and shaking them, there was no movement of fluid in and out as a real rumen, and different studies may use different buffering techniques. A real rumen may also see a fluctuation in pH, correlating with feeding times.

3.4.2 Methane production

In the current study, CH_4 production was measured at ten different time points; 3, 6, 9, 12, 18, 24, 30, 39, 48 and 72 h. There was an increase in CH_4 production over time, which plateaued at 72 h in all treatments. Both the FO and ALG treatments reduced CH_4 production compared to the Control. Similar findings were reported by Machado *et al.,* (2014) when different ALG species were reported to reduce CH_4 production *in vitro*. This finding is also in agreement with a study by Fievez *et al.,* (2003) who reported an inhibition of CH_4 production both *in vitro* and *in vivo* when FO was added to the diet. A reduction in CH_4 production was also accompanied by an increased propionate and decreased acetate production (Fievez *et al*., 2003), which in accordance with Wachira *et al*., (2000) who reported an increase in the concentration of propionate when FO was supplemented to rumen cannulated sheep. Propionate production involves the process of H_2 utilisation, whilst acetate production involves H_2 production, therefore propionate production and methanogenesis are competing as alternative pathways (Moss *et al*., 2000). It has also been reported that methanogens lose the ability to use H_2 at low pH levels (Van Kessel and Russel, 1969), this would explain the reduction in CH_4 production over time as pH levels dropped to below 6.0. In the current study the different inclusion levels also affected CH_4 production, with higher inclusion levels of both the FO and ALG inhibiting CH_4 production more than the lower inclusion levels. These results are comparable to other

studies who have reported a greater reduction in CH_4 production when LC-PUFA are supplemented at higher levels (Patra and Yu, 2012; Fievez *et al.,* 2007a).

3.4.3 Fatty acid biohydrogenation *in vitro*

Polyunsaturated FA are known to be biohydrogenated in the rumen especially when supplemented at higher inclusion levels to ruminant diets (Beam *et al.,* 2000). The ruminal metabolism of FA in oil sources high in PUFA has been investigated both *in vitro* and in *vivo* (Fievez *et al*., 2007b, Sinclair *et al.,* 2005). In the current study the approach was to examine the change in various FAs over a 48 h *in vitro* incubation period, with two different oil sources rich in long chain *n*-3 PUFA and at four inclusion rates. The shift in the FA profile indicates that the batch culture technique used was reliable and the biohydrogenation of the FA was comparable to that reported by others (e.g. Sinclair *et al*., 2005). Over time there was an increase in the accumulation of C18:0 in both the FO and ALG treatments, which may have been a result of the biohydrogenation of ALA which was present in the grass nuts at the highest concentration, and is firstly hydrogenated to form C18:2 *cis*-9, *trans*-11 CLA, and then C18:1 *trans*-11 before the saturated C18:0 FA is formed (Boeckaert *et al.,* 2007a). At all time points the higher concentrations of ALG had a lower content of C18:0 which is in accordance with Lourenco *et al.,* (2007) who reported less C18:0 in treatments high in PUFA compared to the Control. In agreement with reports in the literature from *in vitro* studies, the supplementation of both DHA enriched ALG and FO was shown to inhibit the complete biohydrogenation of LA and ALA, leading to an accumulation of C18:1 *trans* 10+ 11 and C18:2 *cis*-9, *trans*-11 CLA (Boeckaert *et al.,* 2007b; Chow *et al.,* 2004). This is also in agreement with an *in vivo* study by Wachira *et al.,* (2000) that reported lower amounts of the biohydrogenation end product C18:0, and an increase in C18:1 *isomers* and C18:2 *cis*-9, *trans*-11 CLA at the duodenum in sheep when FO was added to the diet. The effect of ALG on the FA profile in the current study was also similar to an *in vivo* study by Boeckaert *at el.,* (2007a) who observed an accumulation of C18:1 *trans*-11 in the rumen fluid of dairy cows when their diet was supplemented with ALG. In the current study the vessel content of LA and ALA was comparable, similar to the findings reported by Sinclair *et al.,* (2005). An accumulation of C18:2 *trans*-10, *cis*-12 CLA an intermediary in the biohydrogenation of LA, was also observed in the ALG treatments, with a greater amount found in the higher inclusion levels. In a study by Kim *et al*., (2008) the addition of FO to Hereford x Friesian steers reduced the duodenal flow of C18:0, and it was suggested that FO might inhibit the enzyme that catalyses the final biohydrogenation step in the rumen, or prevents the proliferation of bacteria able to convert ALA and LA to C18:0.

The biohydrogenation of LC *n*-3 PUFA are not very well understood, and the metabolism of DHA in the rumen is less well characterised compared to ALA (Vlaeminick

et al., 2014). Previous studies have shown that the biohydrogenation of DHA is inhibited when inclusion levels are high (Klein and Jenkins, 2011). This was also observed in the current study where the ALG treatments had higher amounts of DHA at 0 h compared to FO, and by 48 h of *in vitro* incubation the amount of DHA remaining was higher in the ALG treatments, especially at the higher inclusion levels. The DHA present in the lowest inclusion level of FO was extensively biohydrogenated over time, which is in accordance with an *in vitro* batch culture study by Vlaeminck *et al.,* (2014). A correlation between DHA and C18:1 *isomers*, especially C18:1 *trans*-11 has been reported (Vlaeminick *et al*., 2008), with an accumulation of C18:1 *trans*-11 observed *in vitro* when freeze dried grass was supplemented with DHA. AbuGhazaleh and Jenkins (2004) reported that both DHA and EPA were lost during *in vitro* culture studies due to their transformation into other FA such as C22:6 and C20:5 isomers, and that the disappearance of EPA was greater than that of DHA, a finding similar to the current study. When using an *in vitro* batch culture Klein and Jenkins (2011) labelled DHA with ^{13}C in order to determine if DHA had a direct contribution to the accumulation of C18:1 *trans*-11, and reported that DHA was not directly involved in the increase of this FA. This suggests that DHA might alter the microorganisms present or the reaction pathway (Klein and Jenkins, 2011), and that further work is required for a better understanding of the metabolism of LC *n*-3 PUFA in the rumen.

3.5 Conclusion

Vessel pH decreased with time at all inclusion levels of ALG and FO as gas production increased. Methane output was reduced with the inclusion of ALG and FO compared to the Control, this may be attributed to a shift in the rumen bacteria population or due to a decrease in fermentable energy. By 72 h of incubation pH levels were low (<6.0) in all dietary treatment and methane production had plateaued. A higher inclusion level of ALG had a greater influence on inhibiting CH_4 production than FO, therefore ALG could be more toxic towards the rumen bacteria than FO. The metabolism of LA and ALA led to an accumulation of C18:1 *trans*-11, with a lower increase in vessel content of C18:0 with FO at the highest inclusion level. Vessel content of DHA after 48 h of incubation was higher in the ALG treatments, primarily due to its higher dietary inclusion level.

CHAPTER 4: Experiment 2 - Improving the DHA content of milk and cheese by supplementing dairy cows with ALG and the effect on cow performance

4.1 Introduction

Over the past century there has been a considerably body of research on the benefits of LC *n*-3 FA on human health (Calder, 2014; Kliem and Shingfield, 2016). Two important LC *n*-3 PUFA are EPA and DHA which, when provided in small quantities, can significantly decrease the likelihood of developing coronary heart disease via their role in modulating prostaglandin metabolism and decreasing blood triglycerides (Marventano *et al*., 2015). At high doses these LC *n*-3 PUFA can lower blood cholesterol and have antithrombotic and anti-inflammatory properties (Marventano *et al*., 2015; Swanson *et al*., 2012). These LC *n*-3 PUFA are also important for growth, development, immunity and insulin activity (Calder, 2014). In addition to the direct health benefits of PUFA, intermediates in the biohydrogenation of unsaturated FA in the rumen such as CLA have been shown to have health benefits including anti-carcinogenic properties in both animal models and human cancer cells (Lee *et al*., 2005; Gebauer *et al*., 2011).

Ruminant products such as milk and cheese have been criticized for their low content of LC *n*-3 PUFA and high content of SFA (Kliem and Shingfield, 2016). Despite this, one of the most effective means of increasing the content of LC *n*-3 PUFA in the human diet is via dairy products, particularly cheese (Givens and Gibbs, 2006). In the majority of studies that have attempted to improve the health attributes of milk and cheese, the main dietary source of LC *n*-3 PUFA has been FO (Chilliard *et al*., 2001; Palmquist and Grinnari, 2006). However, the primary producer of LC *n*-3 PUFA at the base of the food chain is ALG (Givens and Gibbs, 2006), and feeding ALG has been proposed as a more effective means of manipulating the FA composition of ruminant products, partly due to its high concentration of LC *n*-3 PUFA, but also due to the lower extent of biohydrogenation in the rumen compared to FO (Sinclair *et al*., 2005). Adding ALG to the diet of ruminants has resulted in an increase the content of LC *n*-3 PUFA and CLA in milk (Franklin *et al*., 1999; Stamey *et al*., 2012) and beef (Rodriguez-Hernandez *et al*., 2017), although little work has been conducted on the effect of rate of inclusion on milk or cheese FA composition or more importantly, the organoleptic properties of cheese.

Despite the potential advantages of including sources of LC *n*-3 PUFA such as ALG in the diet of dairy cows, their inclusion has often been associated with negative effects on performance and milk composition, particularly when included at high levels. For example, a substantial decline in milk fat content has been reported in some studies, which has often been linked to the production of *trans* isomers such as *trans*-10, *cis*-12 CLA in the rumen (Franklin *et al*., 1999; Boeckaert *et al*., 2008; Bichi *et al*., 2013). Additionally ALG may affect whole tract digestibility, as UFA have been suggested to be

toxic to rumen bacteria reducing fibre digestibility (Maia *et al*., 2007). Little work has been conducted on the effect of ALG supplementation on diet digestibility, and no previous work has been done on the effect of feeding ALG to dairy cows on cheese properties and taste.

Hypothesis

Feeding DHA enriched ALG at an increasing rate of inclusion will increase the concentration of LC *n*-3 PUFA in milk and cheese but will not affect cow performance.

Objectives and aims

The objectives of this study were to determine the effect of rate of inclusion of DHA enriched ALG on milk and cheese FA profile, cheese taste, and cow performance.

4.2 Material and methods

The study was conducted in accordance with the requirement of the Animals (Scientific Procedures) Act 1986 (amended 2013) and received approval by the Harper Adams University Ethical Committee.

4.2.1 Animals and treatments

Twenty early lactation (77 ± 17.0 d in milk) Holstein-Friesian dairy cows yielding 44 ±1.9 kg/d of milk, with a live weight of 654 ± 42.4 kg, and body condition score (Ferguson *et al*., 1994) of 3.0 ± 0.2 at the beginning of the study were used. The study design was a 4 x 4 Latin square, with each period consisting of a 21 d adaption period followed by 7 d of sampling. All cows were fed the same basal diet (Table 4.1) which was supplemented with one of four inclusion levels of ALG (*Schizochytrium imancinum sp.,* Alltech, Kentucky, USA) during each period. The ALG contained 135 g/kg crude protein, 580 g/kg oil and 0.28 g/100 g FA as EPA and 25.7 g/100 g FA as DHA. Treatment diets were; control (C) no ALG inclusion, 50 g ALG/cow per day (L-ALG), 100 g ALG/ cow per day (M-ALG) and 150 g ALG/cow per day (H-ALG). A 50:50 (DM basis) wheat/dried sugar beet feed mix replaced the ALG in the C, L-ALG and M-ALG diets, fed at 150, 100 and 50 g/cow per day respectively. The diets were formulated to produce approximately 37 kg/d (Thomas, 2004) and were fed as a TMR once daily at 1.05 of ad-libitum intake, with feed refusals collected 3 times per week. The forages and straight feeds were mixed along with the ALG (or wheat/sugar beet feed) using a forage mixer wagon (HiSpec, County Carlow, Ireland), calibrated to ±1 kg, and fed through roughage intake feeders (Insentec B.V., Marknesse, The Netherlands) fitted with an automatic animal identification and forage weighing system calibrated to ±0.1 kg. Cows had continual access to fresh water.

Cows were housed together in the same portion of a building containing cubicles fitted with foam mats, which were bedded twice weekly with sawdust, limed weekly and

scraped every 2 h by automatic scrapers. Cows were milked twice daily at approximately 0615 and 1600 h.

Table 4.1. Diet composition (kg/kg DM) of the basal diet and chemical composition (g/kg DM) of total mixed rations that contained no ALG (Control (C)), 50 g/ALG per cow/d (Low algae (L-ALG)); 100 g/ALG per cow/d (Medium algae (M-ALG)), or 150 g/ALG per cow/d (High algae(H-ALG))

Item		Treatment			
Ingredient	kg/kg DM	C	L-ALG	M-ALG	H-ALG
Maize silage	0.436				
Grass silage	0.118				
Rape seed meal	0.077				
Wheat distillers grains	0.077				
Hipro soybean meal	0.045				
Palm kernel meal	0.022				
Molasses	0.006				
Molassed sugar beet feed	0.051				
Wheat	0.051				
Soy hulls	0.094				
Megalac[1]	0.015				
Urea	0.003				
Minerals and vitamins[2]	0.005				
Chemical composition (g/kg)					
DM		372	374	369	371
Ash		64	73	66	70
OM		936	927	934	930
CP		166	170	165	164
NDF		452	455	452	460
Fatty acid (g/kg DM)					
C16:0		6.06	5.10	6.82	5.65
C18:0		0.49	0.38	0.46	0.40
C18:1 *cis*-9		4.51	3.55	4.26	3.30
LA		5.74	4.53	4.94	4.03
ALA		0.76	0.73	0.80	0.66
EPA		nd	nd	nd	nd
DHA		nd	0.33	0.68	1.00

[1]Protected fat. Volac International Ltd, UK
[2]Mineral/vitamin premix. Major minerals (g/kg): Ca 220; P 30; Mg 80; Na 80; trace minerals (mg/kg) Cu 760; Se 30.3, I 200; Co 70; Mn 5000; Zn 6350; vitamins (mg/kg) retinol 300; cholecalciferol 7.5; all *rac* α-tochopherol acetate 2000; B_{12} 2.50; biotin 135.
[3]Not detected

4.2.2 Sampling and measurements

Feed intake was recorded daily during the sampling week of each period, and sub-samples of each TMR and the two forages collected daily and stored at -20 °C for subsequent analysis. Further forage samples were collected weekly, oven dried at 105°C

and the ratio of maize:grass silage adjusted to the desired level on a DM basis. Milk yield was recorded daily and samples collected on four occasions during the sampling week of each period, a preservative added (Microtabs II, Advanced Instruments, Inc., Massachusetts, USA) and stored at 4 °C prior to subsequent analysis. Additional samples were collected on successive milkings for FA analysis (Hara and Radin, 1978). Cows were weighed and body condition score recorded at 11 00 h prior to the start of the study, and on the final day of each period. Blood samples were collected from the jugular vein from 3 cows per treatment per period over two days at 09 00, 07 00 and 13 00 h into vacutainers containing sodium heparin for the subsequent determination of albumin, β hydroxybutyrate (3-OHB), total protein and urea, or vacutainers containing potassium oxalate for the determination of glucose and NEFA. Samples were centrifuged at 1000 xg for 15 min (Refrigerated Centrifuges SIGMA 3-16PK), and the plasma separated and stored at -20 °C prior to subsequent analysis. Faecal samples were collected twice daily at 0 800 and 14 30 h for 5 d during the sampling week from 12 cows (3 per treatment), and stored at -20 °C prior to subsequent analysis.

4.2.3 Cheese production

Milk was collected for cheese making during each sampling week from 4 cows per treatment at consecutive pm and am milkings into 50 L buckets. The pm milk was bulked, rapidly cooled to 4°C and stored overnight in a mini bulk milk tank (Frigomilk milk cooler G1, Via Trivulzia, Italy), and stirred continuously. Milk from the morning mixing was mixed with the pm milk for 30 min before 50 L was transferred into a cheese vat (Jongia, UK). Cheese was made following a cheddar recipe as described by Robinson and Wilbey (1998). The milk was pasteurized by heat-treating to 63 °C for 30 min, with temperature and titratable acidity % (TA) measured every 15 min by titration with 0.1 N NaOH. When the milk had cooled to 29.5 °C, 3 g of a starter culture of mixed lactic bacteria (single shot culture OV26, Orchard Valley Dairy Supplies, Worcestershire, UK) was added. Ripening continued until the TA reached 0.20-0.22 % (up to 1 h), and vegetarian marzyme rennet (Orchard Valley dairy supplies, Worcestershire, UK) added as a clotting agent at a rate of 25 ml diluted in 175 ml of water per 100 L of milk, and the temperature held at 29.5 °C. The curd was then allowed to set over 50 min before being cut into 3 to 5 mm cubes. The temperature was then raised to 40 °C over 40 min with stirring, the whey drained off, and the curd cut and blocked every 20 min until dry. The curd was then milled by chopping into finger size pieces, and cooled to 25.5 °C. Salt was then added (100 g per 5 kg of curd) and mixed into the curd before being transferred into 3 cheese moulds, and pressed overnight at 75 kN/ m^2. The cheese was turned the following day in the molds and re-pressed at 200 kN/ m^2 for 24 h. The cheese wheels were then vacuum packed in

individual embossed vacuum bags and stored at 4 °C for 120 d to mature until subsequent analysis.

4.2.4 Chemical analysis

Milk compositional analysis was conducted using a Milkoscan Minor 78110 (Foss Electric, Denmark), calibrated using standards according to AOAC (2012). Milk FA analysis followed the method described by Hara and Radin, (1978) for lipid extraction and Chouinard *et al.,* (1999) for methylation as described in section 2.4.2. Cheese FA analysis followed the method described by Coakley *et al*., (2007) for lipid extraction and followed the same method as the milk for methylation as described in section 2.7.2. The TMR samples for each diet were bulked within each period and a sub-sample analysed according to AOAC (2012) for DM (934.01), CP (988.05) and ash (924.05), whilst NDF was analysed according to Van Soest *et al*., (1991) as described in sections 2.1.1 to 2.1.4. Fatty acid analysis of the TMR samples was determined using a modified protocol of Sukhija and Palmquist (1988) as described by Jenkins (2010), described in section 2.2. Fatty acids were identified using a GC as described in section 2.4.4.

Plasma samples were analysed for albumin, 3-OHB, total protein, urea, glucose and NEFA as described in section 2.5. Faecal samples were bulked within days and sampling times for each cow for each period, dried at 65 °C until consistent weight and ground prior to subsequent analysis of AIA (Van Keulen and Young, 1977), ash and NDF as described in section 2.3.

4.2.5 Sensory analysis

For the sensory assessment a descriptive sensory analysis was used (Drake, 2007). A group of 8 individuals was trained to identify and quantify specific sensory attributes of the cheese. The panellists were screened and trained for a total of 40 h to establish descriptive terms for cheese texture and flavour (Table 4.2), and were monitored to track the discriminatory ability of the panel. A 15-point product-specific scale was used for each attribute (Drake, 2007), and references were used to aid panellists in training and attribute identification and scale usage. Panellists received an additional 4 h of ‘refresher’ training prior to the initiation of the assessment to ensure that they were familiar with the attributes.

Each cheese was prepared for sensory analysis as described by Brown *et al*., (2003). Briefly, the matured cheese samples were trimmed of all external surfaces and cut into 2 cm^3 cubes. Each panellist was provided with four cubes per sample per replication. The samples were presented in lidded plastic sample pots and maintained at 12 °C and evaluated under white light in a room dedicated to sensory analysis and free from external

aromas and noise. Each panellist evaluated each cheese in duplicate on odour, appearance, flavour, aftertaste, and texture attributes.

Table 4.2. Definitions and scaling magnitudes used for the sensory evaluation of the experimental cheese

Attribute	Description	0	15
Odour			
Fruity	Smell associated with fruits	Nil	Extreme
Sweet smell	Overall sweet smell	Nil	Extreme
Acidic/ sharp note	Smell associated with acid	Nil	Extreme
Farmyard	Smell associated with a farm	Nil	Extreme
Creamy	Smell associated with dairy richness	Nil	Extreme
Appearance			
Edge cut	How clean/smooth is the knife cut	Firm	Crumbly
Air holes	Number of round holes on the surface	Nil	Extreme
Colour	Colour in white to yellow shade	White	Dark yellow
Glossiness	Shiny appearance	Dull	Shiny
Flavour			
Sweetness	Taste associated with sugar	Nil	Extreme
Tangy	Tastes bright, clean and acidic	Nil	Extreme
Acidic	Taste associated with acids	Nil	Extreme
Creaminess	Amount of dairy richness	Nil	Extreme
Pleasant nutty flavour	Distinctive taste	Nil	Extreme
Savoury	Presence of Glutamates	Nil	Extreme
Bitterness	Particular pungent taste	Nil	Extreme
Metallic	Taste associated with metal	Nil	Extreme
Salty	Taste associated with salt	Nil	Extreme
Aftertaste			
Acidity	Taste associated with acids	Nil	Extreme
Bitter	Particular pungent taste	Nil	Extreme
Dry mouth	Dry mouth	Moist	Dry
Dry throat	Dry throat	Moist	Dry
Metallic	Taste associated with metal	Nil	Extreme
Creamy	Amount of dairy richness	Nil	Extreme
Texture			
Firmness	Fore required to bite through sample	Soft	Firm
Dryness	Perceived degree of water in sample during chewing	Moist	Dry
Crumbliness	Ease sample breaks into small crumbs	Cohesive	Very crumbly
Grittiness	Amount of small crystals in the sample	Nil	Extreme
Stickiness	Sticks to the roof of the mouth	Nil	Extreme
Emulsify	The presence of fat lumps	Lumpy	Dissolved

4.2.6 Statistical analysis

Energy corrected milk (ECM) was calculated according to Moate *et al*., (2013) as:

(0.327 x milk kg/d) + (12.95 x fat kg/d) + (7.65 x protein kg/d) Equation 14

Data was analyzed by ANOVA as a Latin square design using Genstat 17th edition (VSN. Ltd, Oxford, UK) using the following model:

$$Y_{ijk} = \mu + T_i + P_j + A_k + \varepsilon_{ijk}$$

Where Y_{ijk} is the observation, μ is the overall mean, T_i is treatment, P_j is period, A_k is animal and ε_{ijk} is the residual error. Treatment effects were split into orthogonal polynomial contrasts (linear, quadratic and cubic). Blood metabolites were analyzed as repeated measures analysis of variance. Results are presented as treatment means with standard error of the difference of means (SED).

4.3 Results

4.3.1 Feed analysis

All diets had a similar DM content, with a mean of 372 g/kg (Table 4.1). Ash and OM content was similar in all diets (mean of 68 and 932 g/kg DM respectively). The L-ALG diet had a CP content that was 6 g/kg DM higher than the H-ALG diet, which had the lowest value, with C and MA being intermediate. The NDF content was similar between treatments with a mean value of 455 g/kg DM. The content of C16:0, C18:0, C18:1*n*-9, LA and ALA were similar in all four diets, with mean values of 5.90, 0.43, 3.91, 4.77 and 0.74 g/kg DM respectively. No DHA was detected in C, with the content increasing as the dietary inclusion level of ALG increased.

4.3.2 Animal performance

There was no effect ($P > 0.05$) of dietary treatment on DM intake, with a mean value of 23.4 kg/d (Table 4.3), and there was no effect ($P > 0.05$) of treatment on milk yield, which averaged 38.5 kg per day, but there was a trend ($P = 0.064$) for a linear decrease in ECM as the inclusion rate of ALG increased. In contrast there was a linear decrease ($P < 0.001$) in milk fat content and yield with increasing dietary inclusion rate of ALG, with cows fed H-ALG producing 3.7 g/kg and 0.15 kg/d less than those receiving C. Milk protein content and yield, and lactose yield were not affected by dietary treatment ($P > 0.05$), with mean values of 32.4 g/kg, 1.24 kg/d and 1.78 kg/d respectively. In contrast milk lactose concentration decreased linearly ($P = 0.007$) with increasing dietary inclusion of ALG, from 46.5 g/kg in cows receiving C to 45.8 g/kg in HA. There was no effect ($P > 0.05$) of dietary treatment on mean live weight, live weight change or body condition score, with mean values of 667 kg, 0.34 kg/d, and 2.94 units respectively.

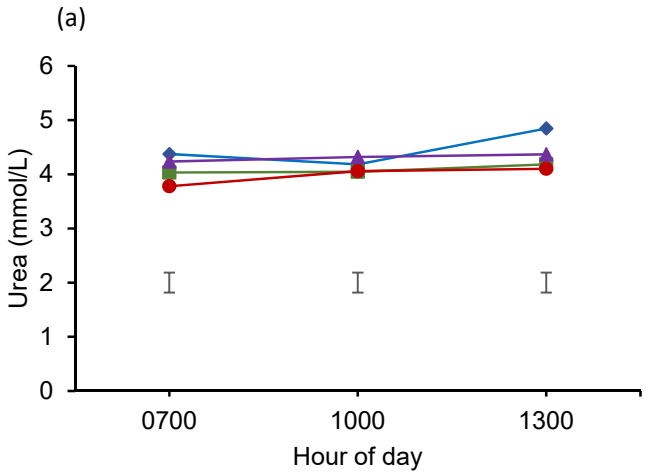
Table 4.3. Milk performance, live weight and body condition of dairy cows fed no ALG (Control (C)), 50 g/ALG per cow/d (Low algae (L-ALG)); 100 g/ALG per cow/d (Medium algae (M-ALG)), or 150 g/ALG per cow/d (High algae (H-ALG))

	Treatment					*P*-value		
	C	L-ALG	M-ALG	H-ALG	s.e.m	Lin	Quad	Cub
DM intake (kg/d)	23.7	23.3	23.1	23.3	0.323	0.162	0.281	0.926
Milk yield (kg/d)	38.1	38.8	38.6	38.4	0.305	0.770	0.360	0.629
ECM[1] (kg/d)	41.3	41.3	40.5	39.4	1.041	0.064	0.440	0.904
Milk fat (g/kg)	39.6	38.4	37.1	35.9	1.105	<.001	0.970	0.968
Fat yield (kg/d)	1.50	1.47	1.41	1.35	0.055	0.007	0.647	0.849
Milk protein (g/kg)	32.2	32.2	32.8	32.2	0.399	0.623	0.235	0.141
Protein yield (kg/d)	1.22	1.24	1.26	1.22	0.029	0.972	0.181	0.670
Milk lactose (g/kg)	46.5	46.6	45.9	45.8	0.305	0.007	0.442	0.160
Lactose yield (kg/d)	1.77	1.81	1.77	1.78	0.036	0.816	0.552	0.279
Live weight (kg)	668	663	667	669	4.140	0.595	0.241	0.351
Live weight change (kg/d)	0.56	0.06	0.37	0.37	0.222	0.731	0.118	0.122
Body condition	2.91	2.94	2.92	2.99	0.050	0.165	0.560	0.430

[1]Energy corrected milk

4.3.3 Plasma metabolite concentrations

There was no effect ($P > 0.05$) of dietary treatment or time on the mean plasma concentration of urea (Figure 4.1a). In contrast, plasma 3-OHB increased with time ($P < 0.001$) but there was no effect ($P > 0.05$) of dietary treatment (Figure 4.1b). Similarly plasma glucose was not affected by dietary treatment ($P > 0.005$) but there was an effect ($P = 0.002$) of time, with concentrations decreasing post feeding (Figure 4.1c). There was no effect ($P > 0.05$) of dietary treatment on plasma NEFA, which decreased with time ($P < 0.001$; Figure 4.1d).



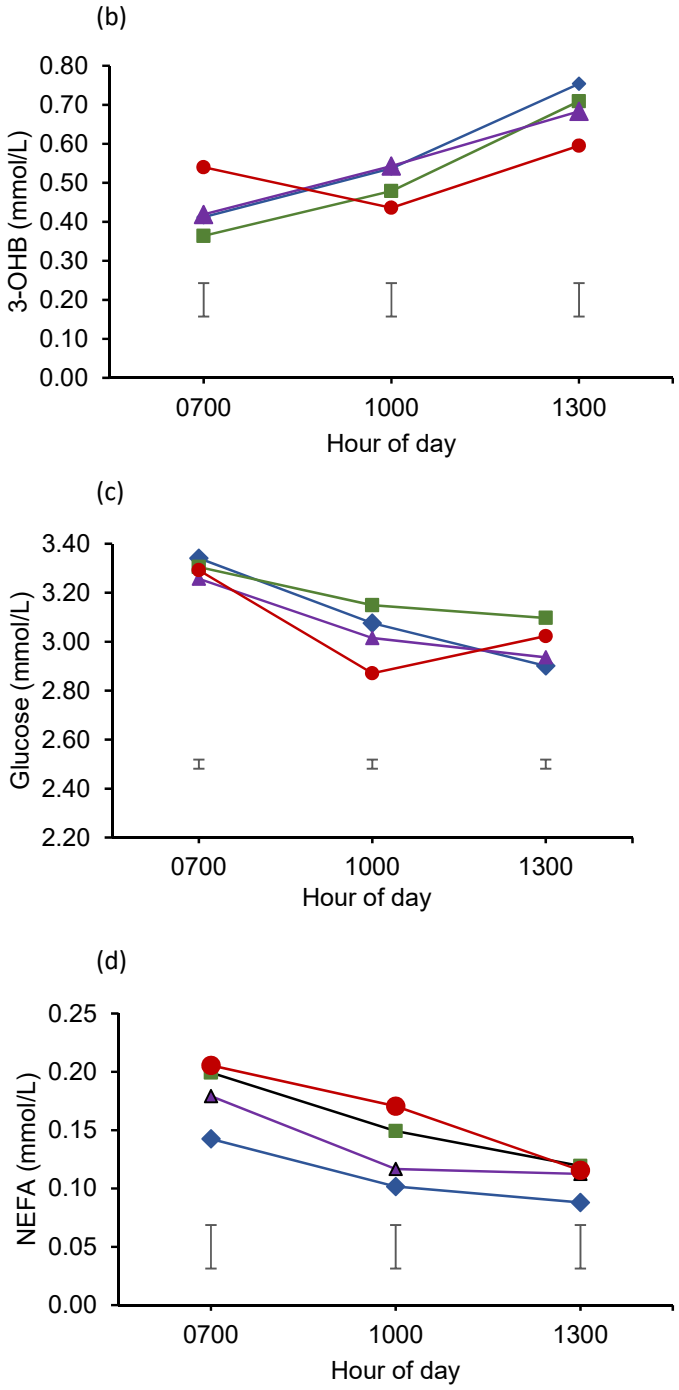


Figure 4.1. Plasma urea (a) β hydroxybutyrate (3-OHB) (b) glucose (c) and non esterified fatty acids (NEFA) (d) of dairy cows fed no algae (Control; C ♦); 50 g/ALG per cow/d (Low algae; L-ALG ■); 100 g/ALG per cow/d (Medium algae; M-ALG ▲); and 150 g/ALG per cow/d (High algae; H-ALG ●). Error bars indicate s.e.d.

4.3.4 Whole-tract apparent digestibility

The mean DM intake of cows selected for the determination of digestibility was 22.8 kg/d, and there was no effect of treatment ($P > 0.05$; Table 4.4). There was a

tendency for faecal DM output to increase linearly (P = 0.054) with inclusion rate of ALG, and as a consequence DM digestibility decreased linearly with the addition of ALG in the diet (P = 0.015). Organic matter digestibility followed a similar pattern to DM, with no effect of treatment on OM intake (P = 0.603), a trend for a linear increase in faecal output (P = 0.057), and a linear decrease (P = 0.015), in digestibility with increasing dietary inclusion of ALG. There was no effect of diet on NDF intake (P > 0.05), but NDF output increased linearly with the addition of ALG in the diet, being highest in cows when fed H-ALG, whereas the digestibility of NDF decreased linearly (P = 0.03) with rate of inclusion of ALG from 0.45 kg/kg when fed C to 0.36 kg/kg when fed HA.

Table 4.4. Digestibility of DM, OM and fibre of dairy cows fed no algae (Control (C)), 50 g/ALG per cow/d (Low algae (L-ALG)); 100 g/ALG per cow/d (Medium algae (M-ALG)), or 150 g/ALG per cow/d (High algae (H-ALG))

	Treatment					*P* value		
	C	L-ALG	M-ALG	H-ALG	s.e.d	Lin	Quad	Cubic
Dry matter (kg/d)								
Intake	22.7	22.8	23.0	22.6	0.697	0.98	0.72	0.75
Faecal output	5.67	6.66	6.45	6.72	0.461	0.05	0.28	0.26
Digestibility (kg/kg)	0.750	0.710	0.720	0.700	0.018	0.02	0.44	0.18
Organic matter (kg/d)								
Intake	21.3	21.3	21.4	20.9	0.46	0.60	0.64	0.70
Fecal output	5.05	5.93	5.76	5.98	0.295	0.06	0.28	0.28
Digestibility (kg/kg)	0.764	0.726	0.733	0.714	0.0120	0.02	0.45	0.21
NDF (kg/d)								
Intake	10.0	10.3	10.3	10.3	0.23	0.50	0.48	0.83
Fecal output	5.52	6.27	6.28	6.55	0.314	0.04	0.45	0.48
Digestibility (kg/kg)	0.449	0.401	0.395	0.358	0.0264	0.03	0.82	0.55

4.3.5 Milk FA profile

There was no effect (P > 0.05) of dietary treatment on milk fat content of C4:0, C14:0 to C17:1, C20:0 or EPA (Table 4.5). In contrast there was a linear decrease (P < 0.05) in the milk fat content of C6:0, C8:0, C10:0, C18:0, C18:1*cis*-9, and C22:0, as the inclusion level of ALG increased in the diet. The milk fat concentration of C18:1 *trans*-8 to C18:1 *trans*-12, LA, ALA, C18:2 *cis*-9 *trans*-11 CLA, C18:2 *trans*-10, *cis*-12 CLA, C20:3*n*-6 and C20:3*n*-3 increased linearly (P < 0.05) as the inclusion level of ALG increased in the diet. Milk fat DHA content also increased linearly (P < 0.001) from 0.08 g/100 g in cows fed C diet to 0.37 g/100 g FA when fed H-ALG.

There was a linear decrease (P = 0.02) in the proportion of milk FA of chain length less than C16, and increase in FA more than C16 as the dietary inclusion rate of ALG increased, but there was no effect of treatment on the proportion of C16:0 plus C16:1 (P >

0.05). Increasing the inclusion level of ALG had a linear effect ($P < 0.001$) on milk fat content of SFA, being highest in cows when offered C, and lowest when offered H-ALG. In contrast both MUFA and PUFA content in milk fat increased linearly ($P < 0.001$) as the dietary inclusion level of ALG increased. There was a linear increase ($P < 0.001$) in total *n*-3 and *n*-6 FA in milk fat as ALG inclusion increased, and a linear decrease ($P < 0.001$) in the ratio of *n*-6 to *n*-3 was observed, being highest in cows offered C and lowest in those offered H-ALG. The Δ^9 desaturase index calculated using C16:1/C16:0 or C18:2 *cis*-9, *trans*-11 CLA/ C18:1 *trans*-11 was similar between treatments ($P > 0.05$). However, there was a linear increase ($P < 0.001$) in the Δ^9 desaturase index when calculated using C18:1 *cis*-9/ C18:0, being lowest in cows when offered C and highest when offered H-ALG.

Table 4.5. Milk fatty acid composition (g/100 g of FA) of dairy cows fed no ALG (Control (C)), 50 g/ALG per cow/d (Low algae (L-ALG)); 100 g/ALG per cow/d (Medium algae (M-ALG)), or 150 g/ALG per cow/d (High algae (H-ALG))

	Treatment					*P* value		
Fatty acids (g/ 100 g)	C	L-ALG	M-ALG	H-ALG	s.e.d	Lin	Quad	Cubic
C4:0	1.43	1.44	1.39	1.39	0.036	0.20	0.82	0.25
C6:0	1.24	1.27	1.19	1.17	0.033	0.01	0.31	0.12
C8:0	0.900	0.900	0.840	0.820	0.026	<.001	0.42	0.21
C10:0	2.23	2.24	2.09	2.04	0.067	<.001	0.55	0.23
C12:0	3.11	3.03	2.96	2.90	0.089	0.02	0.81	0.97
C14:0	11.2	11.1	11.0	10.9	0.177	0.14	0.62	0.70
C14:1 *cis*-9	0.950	0.930	1.02	0.991	0.042	0.16	0.79	0.08
C15:0	1.03	0.982	0.971	0.983	0.033	0.18	0.23	0.94
C16:0	37.5	36.9	37.5	36.9	0.395	0.38	0.87	0.07
C16:1 *cis*-9	1.59	1.51	1.44	1.62	0.111	1.00	0.10	0.49
C17:0	0.398	0.394	0.387	0.404	0.007	0.65	0.05	0.23
C17:1 *cis*-9	0.223	0.238	0.234	0.240	0.011	0.21	0.56	0.46
C18:0	9.70	9.60	8.58	8.73	0.239	<.001	0.47	0.01
C18:1 *trans*-8	0.325	0.389	0.387	0.491	0.049	0.003	0.57	0.27
C18:1 *trans*-9	0.294	0.365	0.556	0.538	0.044	<.001	0.17	0.02
C18:1 *trans*-10	0.614	0.779	0.825	0.869	0.090	0.01	0.35	0.69
C18:1 *trans*-11	1.15	1.28	1.63	1.84	0.173	<.001	0.85	0.18
C18:1 *trans*-12	0.459	0.537	0.900	0.819	0.106	<.001	0.29	0.03
C18:1 *cis*-9	21.3	21.2	20.6	20.7	0.278	0.01	0.58	0.09
LA	2.61	2.66	2.75	2.78	0.046	<.001	0.90	0.50
C20:0	0.067	0.069	0.065	0.068	0.002	0.92	0.98	0.05
ALA	0.452	0.461	0.489	0.496	0.009	<.001	0.72	0.07
C18:2 *cis*-9, *trans*-11 CLA	0.606	0.756	0.856	0.900	0.031	<.001	0.02	0.96
C18:2 *trans*-10, *cis*-12 CLA	0.033	0.031	0.044	0.048	0.005	<.001	0.35	0.17
C22:0	0.042	0.037	0.033	0.032	0.002	0.01	0.52	0.31
C20:3*n*-6	0.050	0.055	0.055	0.057	0.002	0.01	0.52	0.31
C20:3*n*-3	0.132	0.137	0.136	0.160	0.005	<.001	0.01	0.07
EPA	0.073	0.071	0.063	0.068	0.006	0.24	0.40	0.38
DHA	0.077	0.148	0.249	0.371	0.017	<.001	0.05	0.86
Indices								
<C16:0	22.0	21.9	21.5	21.2	0.381	0.02	0.64	0.56
16:0 + C16:1	39.1	38.4	38.9	38.6	0.429	0.42	0.56	0.14
>C16:0	40.5	41.2	41.1	41.5	0.494	0.03	0.84	0.37
ΣSFA[1]	68.7	68.0	67.0	66.7	0.435	<.001	0.85	0.62
ΣMUFA[2]	26.5	27.1	27.9	27.9	0.402	<.001	0.30	0.52
ΣPUFA[3]	4.48	4.79	5.21	5.43	0.084	<.001	0.54	0.22
Σ*n*-3[4]	0.730	0.822	0.937	1.10	0.025	<.001	0.06	0.79
Σ*n*-6[5]	3.12	3.18	3.34	3.39	0.051	<.001	0.92	0.20
n-6:*n*-3	0.810	0.794	0.780	0.756	0.004	<.001	0.14	0.30
Estimates of mammary Δ^9-desaturase activity								
C14:1:(C14:0 + C14:1)	0.079	0.077	0.085	0.083	0.003	0.02	0.90	0.03
C16:1:(C16:0 + C16:1)	0.041	0.039	0.037	0.042	0.003	0.89	0.08	0.37
C18:1*c*9: (C18:0 + C18:1*c*9)	0.686	0.689	0.705	0.706	0.004	<.001	0.78	0.12
C18:2 *c*9 t11 CLA: (C18:1*t*11 + C18:2 *c*9 *t*11 CLA)	0.375	0.378	0.354	0.355	0.022	0.24	0.94	0.47

4.3.6 Cheese composition, FA profile and taste

There was no effect ($P > 0.05$) of treatment on cheese yield, which averaged 0.26 kg/kg (Table 4.6). In contrast, cheese moisture content increased linearly ($P < 0.001$) with dietary inclusion rate of ALG, whereas the fat content decreased linearly ($P < 0.05$). There was a linear decrease ($P < 0.05$) in cheese C6:0, C18:0, C18:1 *cis*-9 and C22:0 as the inclusion level of ALG increased in the diet, but there was no effect ($P > 0.05$) on any of the other FA below C18:0, or on LA, C20:0, C18:2 *trans*-10 *cis*-12 CLA and C20:3*n*-3. Cheese FA content of C18:1 *trans*-10, 11 and 12, ALA, C18:2 *cis*-9 *trans*-11 CLA and C20:3*n*-6 increased linearly ($P < 0.05$) as the supplementation of ALG increased. Cheese content of DHA increased quadratically with dietary inclusion of ALG ($P < 0.001$), being highest in cheese made from cows fed H-ALG. There was a linear increase ($P < 0.05$) in cheese content of EPA from 0.05 g/100g in cheese from cows when fed C to 0.06 g/100g in those receiving H-ALG. There was no effect ($P > 0.05$) of treatment on the sum of cheese FA of chain length less than C16:0 or chain length more than C16:0, MUFA or total *n*-6. However increasing the dietary supplementation of ALG had an effect ($P< 0.05$) on the total SFA in cheese, which decreased linearly from 67.9 in C to 66.2 g/100 g FA in H-ALG, and on total PUFA, which increased from 3.92 in C to 4.61 g/100 g in H-ALG. A cubic decrease ($P < 0.001$) in the ratio of *n*-6:*n*-3 was observed in cheese as the inclusion level of ALG increased in the diet, being lowest in cheese from cows fed L-ALG and highest in those fed C.

Supplementation with ALG affected 20 out of the 32 sensory attributes ($P < 0.05$; Table 4.7). There was a linear increase ($P < 0.05$) in the appearance of air holes, initial sweetness, nutty flavour, and acidic, and dry throat aftertaste, and a linear decrease ($P < 0.05$) in the creamy flavour of the cheese as the inclusion level of ALG increased in the diet. There was also a quadratic effect ($P < 0.05$) on the fruity odour, which was highest in cheese from cows when fed H-ALG and lowest in those receiving L-ALG, edge cut appearance ($P < 0.001$) which was highest in H-ALG and lowest in cheese made from cows fed M-ALG, and firmness and crumbliness texture ($P < 0.05$) being highest in cheese from cows when fed M-ALG, with H-ALG fed cows producing crumblier and less firm cheese. There were cubic effects of treatment ($P <0.05$) on farm yardy odour, stickiness, acid flavour, bitterness and dry mouth aftertaste.

Table 4.6. Cheese composition, yield and fatty acid composition (g/100 g of FA) of dairy cows fed no ALG (Control (C)), 50 g/ALG per cow/d (Low algae (L-ALG)); 100 g/ALG per cow/d (Medium algae (M-ALG)), or 150 g/ALG per cow/d (High algae (H-ALG))

	Treatment					*P* value		
Cheese composition	C	L-ALG	M-ALG	H-ALG	s.e.d	Lin	Quad	Cubic
Weight (kg)	5.24	5.13	5.23	5.10	0.366	0.791	0.946	0.719
Yield (kg cheese/kg milk)	0.262	0.257	0.262	0.255	0.018	0.791	0.946	0.719
Moisture (g/kg)	41.4	41.5	42.9	42.9	0.46	<.001	0.746	0.08
Fat (g/ kg)	24.6	23.7	20.8	21.3	1.32	0.005	0.505	0.198
Fatty acids (g/100 g)								
C4:0	0.486	0.473	0.464	0.47	0.014	0.18	0.31	0.8
C6:0	1.72	1.68	1.63	1.59	0.063	0.05	0.95	0.99
C8:0	0.823	0.804	0.780	0.754	0.036	0.06	0.9	0.98
C10:0	2.27	2.26	2.18	2.12	0.113	0.16	0.76	0.81
C12:0	3.32	3.32	3.27	3.20	0.135	0.35	0.71	0.95
C14:0	11.7	11.8	11.9	11.8	0.186	0.58	0.49	0.86
C14:1 *cis*-9	1.11	1.15	1.21	1.09	0.092	0.98	0.24	0.5
C15:0	1.06	1.10	1.12	1.06	0.036	0.85	0.05	0.56
C16:0	37.4	37.1	36.8	36.8	0.582	0.22	0.76	0.96
C16:1 *cis*-9	1.84	1.79	1.95	1.86	0.088	0.49	0.72	0.1
C17:0	0.372	0.381	0.381	0.380	0.009	0.42	0.4	0.78
C17:1 *cis*-9	0.256	0.236	0.244	0.236	0.009	0.07	0.32	0.13
C18:0	8.61	8.67	7.9	7.98	0.151	<.001	0.94	0.002
C18:1 *trans*-9	0.363	0.523	0.636	0.631	0.036	<.001	0.004	0.53
C18:1 *trans*-10	0.269	0.306	0.408	0.458	0.058	0.002	0.88	0.54
C18:1 *trans*-11	0.680	1.06	1.51	1.75	0.316	0.001	0.77	0.79
C18:1 *trans*-12	0.914	1.19	1.33	1.48	0.089	<.001	0.35	0.59
C18:1 *cis*-9	22.7	21.9	21.8	21.8	0.455	0.05	0.21	0.77
LA	2.62	2.63	2.67	2.70	0.082	0.28	0.88	0.83
C20:0	0.067	0.070	0.068	0.070	0.001	0.08	0.95	0.01
ALA	0.440	0.434	0.459	0.471	0.016	0.03	0.44	0.39
C18:2 *cis*-9, *trans*-11 CLA	0.600	0.704	0.834	0.865	0.032	<.001	0.12	0.22
C18:2 *trans*-10, *cis*-12 CLA	0.016	0.025	0.026	0.024	0.005	0.17	0.18	0.82
C22:0	0.035	0.034	0.029	0.027	0.004	0.03	0.91	0.61
C20:3*n*-6	0.042	0.056	0.056	0.058	0.006	0.02	0.17	0.46
C20:3*n*-3	0.089	0.100	0.091	0.095	0.010	0.79	0.62	0.33
EPA	0.050	0.050	0.049	0.056	0.002	0.03	0.06	0.36
DHA	0.062	0.128	0.230	0.352	0.010	<.001	<.001	0.59
Indices								
<C16:0	22.5	22.6	22.5	22.1	0.481	0.41	0.43	0.87
16:0 + C16:1	39.3	38.9	38.8	38.6	0.605	0.28	0.81	0.85
>C16:0	40.1	40.3	40.6	41.2	0.761	0.15	0.78	0.95
ΣSFA[1]	67.9	67.7	66.6	66.2	0.812	0.02	0.91	0.5
ΣMUFA[2]	28.2	28.2	29	29.2	0.746	0.11	0.89	0.52
ΣPUFA[3]	3.92	4.12	4.42	4.61	0.133	<.001	0.96	0.65
Σ*n*-3[4]	0.641	0.712	0.830	0.969	0.028	<.001	0.09	0.75
Σ*n*-6[5]	2.66	2.68	2.73	2.75	0.082	0.21	0.97	0.87
n-6:*n*-3	0.806	0.740	0.790	0.767	0.003	<.001	<.001	<.001

Table 4.7. Sensory attribute ratings of cheese made from dairy cows fed no algae (Control (C)), 50 g/ALG per cow/d (Low algae (L-ALG)); 100 g/ALG per cow/d (Medium algae (M-ALG)), or 150 g/ALG per cow/d (High algae (H-ALG))

	Treatment					P value		
Item	C	L-ALG	M-ALG	H-ALG	s.e.d	Lin	Quad	Cubic
Odour								
Fruity	4.71	3.43	4.52	4.76	0.468	0.27	0.02	0.03
Sweet	3.94	3.31	3.71	3.83	0.370	0.83	0.15	0.25
Acidic note	4.12	4.95	3.73	5.60	0.400	0.001	0.04	<.001
Farmyardy	1.09	1.36	0.839	1.48	0.217	0.18	0.17	0.01
Creamy	3.16	3.50	3.35	2.81	0.347	0.15	0.06	0.91
Appearance								
Edge cut	7.08	6.38	6.15	7.81	0.458	0.04	<.001	0.33
Air holes	1.78	1.69	2.05	2.39	0.271	0.004	0.25	0.57
Colour	1.59	1.86	1.76	1.69	0.080	0.59	0.002	0.11
Glossiness	5.19	5.76	6.10	5.64	0.367	0.20	0.04	0.63
Texture on eating								
Firmness	5.05	5.67	5.92	3.98	0.319	<.001	<.001	0.07
Dryness	6.35	6.31	5.81	6.41	0.393	0.98	0.21	0.21
Crumbliness	5.20	5.43	5.58	4.14	0.315	<.001	<.001	0.14
Grittiness	1.05	0.983	0.847	1.62	0.273	0.02	0.02	0.26
Stickiness	9.34	10.3	9.47	9.56	0.357	0.84	0.11	0.02
Emulsifying	11.2	11.1	10.7	11.2	0.403	0.83	0.22	0.25
Flavour								
Initial sweetness	1.16	1.47	1.56	1.83	0.298	0.02	0.93	0.67
Fruity	1.25	1.45	1.63	1.64	0.266	0.09	0.60	0.86
Tangy	5.62	5.78	5.89	5.96	0.410	0.35	0.87	1.00
Acidic note	6.49	6.83	5.66	7.11	0.497	0.40	0.08	0.01
Creaminess	2.52	2.45	2.44	1.87	0.287	0.01	0.19	0.49
Saltiness	2.15	2.47	2.23	2.31	0.193	0.66	0.38	0.14
Pleasant nutty flavour	0.910	1.37	1.06	2.04	0.347	0.001	0.23	0.06
Savoury	0.679	0.779	0.809	0.824	0.097	0.11	0.52	0.86
Bitterness	4.10	4.74	3.70	5.25	0.539	0.06	0.18	0.01
Metallic	0.696	0.978	0.649	0.935	0.194	0.41	0.93	0.05
Aftertaste								
Salty	1.97	2.21	2.05	2.22	0.21	0.34	0.84	0.28
Acidic	5.09	5.57	5.09	6.25	0.464	0.01	0.25	0.07
Bitter	5.24	5.61	5.51	6.91	0.548	<.001	0.16	0.25
Dry mouth	5.55	6.12	5.49	6.63	0.346	0.02	0.28	0.03
Dry throat	3.37	3.7	3.56	4.46	0.374	0.002	0.25	0.19
Metallic	1.25	1.65	1.17	1.60	0.292	0.41	0.88	0.05
Creamy	1.58	1.55	1.75	1.33	0.255	0.33	0.24	0.29

4.4 Discussion

4.4.1 Feed analysis, animal performance and diet digestibility

The ALG supplement used in this study was high in DHA, and as the inclusion level of ALG increased the supply of DHA increased to provide approximately 0, 8, 16 and 24 g/cow per d. These dietary inclusion levels were selected as higher amounts have been associated with a decrease in animal performance and milk fat content (Franklin *et al*., 1999; Boeckaert *et al*., 2008). For example, supplementation with marine lipids at high

rates has often been reported to decrease DMI in both dairy cows (Franklin *et al*., 1999; Moate *et al*., 2013) and sheep (Toral *et al*., 2010). In the current study there was no effect of treatment on DMI, which averaged 23.3 kg/d, a finding in accordance with both Stamey *et al*., (2012) and Vahmani *et al*., (2013) who reported no effect of feeding 200 g/d of ALG or FO to Holstein cows. Similarly, Bichi *et al*., (2013) also reported no effect of feeding ALG on DMI in lactating ewes when supplemented at 8 g/kg DM. In the current study the highest inclusion of ALG provided a similar DHA supply to that used in study of Moate *et al*. (2013), who also observed no effect on DMI. However, at a higher inclusion level of 50 g DHA/cow per d resulted in a 6 % decrease in DMI, with an 11 % decrease at an inclusion level of 75 g/cow per day (Moate *et al*., 2013), and it would therefore appear that supplying DHA from ALG at up to 25 g/d can be achieved without a negative impact on intake.

It has been reported that supplementation with ALG at the rate of 43 g/kg DMI decreased milk yield by 45 % when administered directly through a rumen fistula, (Boeckaert *et al*., 2008), mainly as a consequence of reduced DMI. In contrast there was no effect of ALG supplementation on milk yield in the current study, a finding similar to several others (AbuGhazaleh *et al*., 2009; Stamey *et al*., 2012; Vahmani *et al*., 2013). In contrast, Hostens *et al*., (2011) and Sinedino *et al*., (2017) reported an increase in milk yield when 224 g of ALG containing 44 g DHA and 100 g ALG containing 10 g DHA was fed daily to dairy cows for 46, and 120 d postpartum respectively. This difference may be explained by the longer term feeding of ALG in both studies, whereas in the current study the level of ALG inclusion was changed every 4 weeks.

Milk fat depression induced by ALG supplementation has been reported in both dairy cows (Sinedino *et al*., 2017; Moate *et al*., 2013; Vahmani *et al*., 2013) and sheep (Bichi *et al*., 2013). The exact mechanism behind milk fat depression following supplementation with marine oils such as ALG or FO is however, unclear (Bichi *et al.,* 2013). Bauman and Griinari (2003) described how unique FA intermediates that are produced through the biohydrogenation of PUFA can cause an inhibitory effect on milk fat synthesis, with one intermediate identified as a potent inhibitor of milk fat synthesis being *trans*-10 *cis*-12 CLA (Hussein *et al*., 2013; Peterson *et al*., 2003; Sinclair *et al*., 2007). However, other intermediates such as C18:1 *trans*-10 are also involved, and are often elevated in milk fat when milk fat depression is observed (Chilliard *et al*., 2001). Supplementation of oil mixtures rich in PUFA or intermediaries of biohydrogenation in the rumen can strongly inhibit *de novo* synthesis and uptake of circulating FA by the mammary gland (Hussein *et al*., 2013), and may therefore explain the results reported in the current study. For example, it has been reported in cell culture and rodent models that sterol regulatory element binding protein (SREBP) signaling is inhibited by PUFA (Harvatine *et al*., 2006), and Vahmani *et al*, (2013) reported a 15 % reduction in the

expression of SREBP in the mammary tissue of cows fed FO or ALG compared to a control diet. Other authors have also observed that CLA causes a down-regulation in SREBP mRNA abundance and enzymatic activity in mammary tissue of dairy cows and sheep, which affects genes involved in the uptake, *de novo* synthesis, desaturation and esterification of FAs (Peterson *et al*., 2003; Hussein *et al*., 2013). In the current study there was a linear increase in both *trans*-10 *cis*-12 CLA, and C18:1 *trans*-10 as daily milk fat content and yield decreased with the addition of ALG in the diet, supporting the findings that *trans*-10 *cis*-12 CLA is involved in milk fat depression and that C18:1 *trans*-10 may also be a contribute in dairy cows fed sources of marine oil.

Milk protein content and yield, as well as lactose yield were unaffected by ALG supplementation in the current study, a finding consistent with previous observations in cows fed ALG or FO (AbuGhazaleh *et al*., 2003; Stamey *et al*., 2012; Vahmani *et al*., 2013), and in sheep fed ALG (Bichi *et al*., 2013). Milk lactose content decreased linearly with the addition of ALG, a finding that contrasts with previous observations that reported that milk lactose content was unaffected by ALG supplementation (AbuGhazaleh *et al*., 2009; Vhamani *et al*., 2013), although the reason for this difference is unclear. There was no effect of dietary treatment on BCS or live weight, a finding in agreement with Glover *et al*. (2012), but there was a linear decrease in ECM as the level of ALG increased in the diet, which in combination with the similar DM intake between treatments, indicates that less energy may have been digested.

Few studies have evaluated the effect of ALG on whole tract digestibility, and making comparisons between studies is problematic as different sources of ALG have a diverse nutrient profile (Stokes *et al*., 2015). In the current study there was no difference in DM, OM or NDF intake between treatments, indicating that palatability and feed preference were of minor concern, a finding in accordance with Stokes *et al*., (2015) when feeding ALG meal to sheep. However, similar to that of Stokes *et al*., (2015), there was a linear decrease in DM, OM and NDF digestibility with increasing rate of dietary inclusion of ALG. Diets high in PUFA have been shown to suppress the protozoal community in the rumen of cows and can also alter the *Butyrivibrio* related bacterial community, leading to the loss of some strains which are actively involved in biohydrogenation (Lourenco *et al*., 2010). In contrast Moate *et al*., (2013) reported an increase in the number of protozoa with the addition of ALG high in DHA in the diet of dairy cows, and concluded that when DHA is fed at a level that does not affect DMI, it does not alter rumen volatile fatty acid proportions, or enteric CH_4 emissions, a finding supported by Klop *et al*., (2016). In contrast Maia *et al*., (2007) reported that the activity of cellulolytic bacteria may be reduced by long chain PUFA, as these bacteria are inhibited by an accumulation of H_2 in the rumen, which can occur when methanogenesis is impeded (Lourenco *et al*. 2010).

4.4.2 Blood metabolites

Mattos *et al*., (2004) reported a decrease in plasma glucose concentration when FO was fed to cattle which was associated with a decrease in DMI, but in the current study DM intake and plasma glucose concentration were unaffected by dietary treatment. Similar to the present study, Ballou *et al*. (2009) reported no effect of lipid supplementation on plasma NEFA levels in dairy cows fed FO, although 3-OHB concentrations decreased with FO supplementation, which could be related to an improved energy status of the cows. Overall, the lack of an effect of dietary treatment on blood glucose, NEFA or 3-OHB in the current study reflects the lack of a difference in intake, weight change and milk yield.

4.4.3 Milk and cheese FA profile

The primary objective of the current study was to increase milk fat and cheese concentrations of DHA. The similarity between the milk and cheese FA profile across treatments indicates that cheese manufacturing and packaging had little effect on the FA profile, a finding in agreement with Chilliard *and* Ferlay, (2004). The DHA content increased linearly with the addition of ALG in the diet, a finding in accordance with Stamey *et al*., (2012), Vahmani *et al*., (2013) and Boeckaert *et al*., (2008). The DHA content of the cheese from cows fed H-ALG in the current study was however, lower than when Martini *et al*. (2009) fortified reduced-fat cheese with FO. The opportunities for fortification of dairy products with FO is limited however, as oxidative deterioration causes off-flavors, and Kolanowski and Weissbrodt (2007) reported that cheese stability was limited to only 4 weeks, restricting its commercial use.

With a significant increase in DHA and ALA in milk from cows supplemented with ALG, the *n*-6:*n*-3 ratio in both milk and cheese decreased from approximately 0.81 in milk from cows fed the Control to 0.76 at the highest dietary addition of ALG. The recommended daily ratio of *n*-6:*n*-3 FA in the human diet is 2.3:1 (Kris-Etherton *et al*., 2000), but this ratio is often higher in most Western style diets due to a high consumption of *n*-6 FA, and therefore a reduction is attractive for human health (Allred *et al*., 2006). Additionally, in the current study the content of SFA in both milk and cheese decreased with increasing dietary inclusion of ALG, whilst the content of MUFA and PUFA increased. This altered FA profile is in agreement with previously reported responses to ALG (Glover *et al*., 2012; Boeckaert *et al*., 2008). Even in small quantities *n*-3 PUFA can significantly decrease the likelihood of developing coronary heart disease, and in high doses can lower cholesterol and have antithrombotic and anti-inflammatory properties (Marventano *et al*., 2015; Calder, 2014) indicating the importance of *n*-3 PUFA on human health. The European Food Safety Authority (2012) suggested that people should consume at least 250 mg LC *n*-3 FA /d, although a higher intake is required for the prevention of

cardiovascular diseases (Marventano *et al*., 2015). In the European Union (EU) consumption of cheese averages 50 g/d, whereas in the United States it is reported to be 43 g/d (Canadian Dairy Information Centre, 2016). In the current study 50 g of cheese made from cows fed H-ALG would supply a daily intake of 43.5 mg of DHA + EPA, a 2.5 fold increase compared to the 13.8 mg of DHA + EPA in cheese made from cows fed C, and would contribute approximately 17 % of the daily recommendation of DHA and EPA.

Algae supplementation in the current study also increased the concentration of LA and ALA in both milk and cheese, which may either be due to decreased biohydrogenation in the rumen or greater uptake by the mammary gland (AbuGhazaleh *et al*., 2003). Oils high in PUFA have antimicrobial activity against a wide range of microorganisms and can decrease FA biohydrogenation in the rumen by inhibiting the growth of bacteria such as *Butyrivibrio fibrisolvens* (Benchaar *et al*., 2007). Consequently, C18:1 *trans*-11 concentrations in the rumen increase and are subsequently available for uptake into milk and cheese (Chilliard *et al.,* 2001). A decrease in milk and cheese C18:1 *cis*-9 was also observed in the current study, and may be due to its extensive biohydrogenation in the rumen to yield *trans*-8, *trans*-9, *trans*-10 and *trans*-11 isomers as reported by van de Vassenberg and Joblin (2003). Since C18:1 *cis*-9 can be synthesized in the mammary gland from C18:0 by Δ^9 desaturase (Palmquist and Griinari, 2006), a decrease in the amount of C18:0 entering the mammary gland, or in the activity of this enzyme, would also lead to less C18:1 *cis*-9 being present in milk.

In order to measure the activity of Δ^9 desaturase, previous studies have estimated ratios of FA dependent on the activity of this enzyme (Soyeurt *et al*., 2008). In the current study the ratio of C16:0 to C16:1 was similar between treatments. In contrast the ratio of C14:1 to C14:0 increased linearly with the addition of ALG in the diet. Moate *et al*. (2013), also observed an increase in C14:1 to C14:0 and C16:1 to C16:0 ratios when feeding 25 g/cow per day of DHA, a dose similar to H-ALG diet in the current study. At higher inclusion levels of 50 and 75 g DHA/cow per d no additional effect was reported (Moate *et al*., 2013). In the current study an increase in the ratio of C18:1 *cis*-9 to C18:0 was observed, which is in accordance with Allred *et al*., (2006) who fed FO to dairy cows. In contrast there was a decrease in the ratio of *cis*-9 *trans*-11 CLA to C18:1 *trans*-11, whilst an increase was reported by Allred *et al.,* (2006). It is difficult however to determine whether the increase in CLA in milk FA was due to Δ^9 desaturase activity or an increase in flow from the rumen.

4.4.4 Cheese composition and sensory evaluation

The secondary objective of the current study was to determine the effect of ALG inclusion in the diet on cheese composition and taste, as ultimately this will influence consumer preference and cheese consumption. There was no effect of dietary treatment

on cheese yield despite the decrease in milk fat content that occurred with increasing inclusion levels of ALG, a finding similar to Sinclair *et al*. (2007) who reported no difference in cheese yield when a CLA supplement was fed to sheep, despite a significant reduction in milk fat content.

Sensory evaluation of dairy products made from milk from cows fed ALG has not previously been reported and, overall, there was a number of differences in cheese flavour. It is however, well established that the high content of LC *n*-3 PUFA in FO makes it particularly susceptible to oxidation, which can significantly decrease the sensory quality of milk and cheese due to the development of fishy off-flavours (Kolanowski and Weissbrodt, 2007; Damodaran and Park in, 2017), but there was no such flavours in the current study. There was however, a slight linear increase in acidic and bitter aftertaste, although the highest score of 6.9 for H-ALG was still well within the 15 point scale, suggesting that LC *n*-3 PUFA oxidation may not have been a major factor. A softer structure of cheese has been reported in some studies when cheese was made from milk from cows fed diets rich in PUFA (Chen *et al*., 2004). Similarly, cheese made from cows fed H-ALG in the current study was less firm and more crumbly, and may therefore be used to produce dairy products with a softer structure. There was also a linear decrease in the creamy flavour of the cheese as the level of PUFA increased in the cheese, a finding consistent with Chen *et al*. (2004) who stated that PUFA can inhibit lipases that are important for the generation of a cultured dairy product flavour by releasing free FA. Others have reported an increase in a pleasant nutty flavour which was related to content of LA (Stuchlik and Zak, 2002), a finding consistent with that reported here where there was a linear increase in a nutty flavour with ALG inclusion, which was associated with an increase in the cheese fat content of LA.

4.5 Conclusion

Feeding DHA-enriched ALG to dairy cows linearly increased the milk and cheese concentration of DHA, *cis*-9 *trans*-11 CLA and *trans*-10 *cis*-12 CLA and decreased concentrations of SFA, with potential human health benefits. The modified FA composition was associated with a decrease in milk fat content and yield when fed at the highest level, but there was no effect on DMI or milk yield, although NDF digestibility decreased. Despite the decrease in milk fat content there was no detrimental effect on cheese yield. There was an increase in crumbliness and decrease in firmness of cheddar cheese as well as an increase in nutty flavour at the highest ALG inclusion level. It is therefore recommended that ALG may be fed at 100 g/cow per day, as this will improve milk and cheese FA quality without negatively impacting animal performance, whilst having a beneficial impact on the milk and cheese FA profile.

CHAPTER 5: Experiment 3 - Effect of supplementation of DHA enriched ALG in the diet of dairy cows on milk FA profile over time and indicators of fertility

5.1 Introduction

Including *n*-3 PUFA in the diet of the dairy cow can increase the energy density of the diet, and subsequently energy intake if DMI is not reduced (Wullepit *et al*., 2012), helping the dairy cow to cope with metabolic challenges during lactation. Feeding *n*-3 PUFA can alter milk FA composition, resulting in milk fat depression, which may reduce the energy output post-partum (Wullepit *et al*., 2012). Previous studies have examined the effect of ALG supplementation on milk FA of dairy cows (Chapter 4; Boeckaert *et al*., 2008; Glover *et al*., 2012; and Vahmani *et al*., 2013) and have successfully increased the milk content of beneficial PUFA when ALG is supplemented in the diet. Despite an increase in milk PUFA the transfer efficiency of DHA to milk from marine oil sources added to the diet is low (Chilliard *et al*., 2001), as the majority of the PUFA are biohydrogenated in the rumen and not incorporated into milk intact (Vahmani *et al*., 2013). Polyunsaturated FA have been shown to be toxic towards rumen microorganisms, altering the biohydrogenation of FA and the rumen ecosystem (Benchaar *et al*., 2007). Ruminal adaption to high levels of lipid in the diet can occur, altering the formation of specific biohydrogenation intermediates, reflecting a time-dependant effect on lipid supplementation (Shingfield *et al*., 2006). Boeckaert *et al*., (2008) reported a rapid increase in milk C18:1 *trans*-11 following continual ALG supplementation in the diet of dairy cows, but after 6 days the concentrations of C18:1 *trans*-11 decreased and stabilized. A more gradual increase of DHA was reported when ALG was fed continuously to dairy cows and after 20 days no decline was seen (Boeckaert *et al*., 2008). Shingfield *et al*., (2006) examined milk fatty acid composition responses to FO and sunflower oil over a period of 28 days and also reported a rapid increase in milk C18:1 *trans*-11 FA, which decreased and stabilised at day 16, *cis*-9 *tran*s-11 CLA followed a similar pattern. No studies have previously examined the effect of ALG supplement over a prolonged period of time, therefore further work is required in order to understand if rumen adaptation will occur during ALG supplementation.

Fertility in dairy cows has declined over the past five decades which has been associated with an intensification of production and higher milk yields (Rodney *et al*., 2015). Reduced fertility includes delayed resumption of oestrous post-partum, greater incidence of abnormal oestrous cycles and poorer conception rates to first and subsequent inseminations (Pryce *et al*., 2004). High reproductive efficiency in dairy cows requires a reduction in disease during the transition period, high submission rate to AI and

high pregnancy rates per service (Roche *et al.,* 2000). The reproductive performance of cows is influenced by nutritional and metabolic status (Elis *et al*., 2016). In high-yielding dairy cows nutrition can have a significant impact on the resumption of ovarian cycle's post-partum and on subsequent conception rates (Fouladi-Nashta *et al*., 2009). Elis *et al*., (2016) investigated the effect of FO supplementation post calving on the production and reproduction in post-partum dairy cows. Feeding of LC *n*-3 PUFA in FO increased the number of large follicles and decreased non-fertilization and early embryo mortality rate, suggesting an effect on oocyte quality (Elis *et al*., 2016). Oseikrina *et al*., (2016) investigated *in-vitro* the effect of DHA supplemented at a rate of 1 µM on cattle oocyte development and reported an increase in the rate of embryo development and an increase in blastocyst cell number.

Polyunsaturated FA have major roles in the endocrine system, metabolism and disease control in various tissues, influencing the reproductive status of dairy cows in various ways. The 1 and 2 series, of prostaglandins are derived from *n*-6 FA and are involved in uterine involution and subsequent sequential ovulation post-partum (Otto *et al*., 2014). The 3- series prostaglandins are derived from *n*-3 FA and are involved in improving the environment for embryo implantation and survival by decreasing the secretion of PG metabolites, resulting in increased lifespan of the CL (Dong Hyeon *et al*., 2016). $PGF_{2\alpha}$ is secreted during the oestrous cycle, and the pulsatile secretion mediates the regression of the CL (Binelli and Thatcher, 1999). Feeding LC *n*-3 PUFA has been reported to reduce uterine $PGF_{2\alpha}$ production and improve embryo quality and pregnancy maintenance (Otto *et al*., 2014). Only a few studies have looked at the effect of ALG supplementation on fertility parameters in cows (Sinedino *et al*., 2017; Moran *et al*., 2017), with results being inconsistent, and no work has been done on the effect ALG supplementation may have on $PGF_{2\alpha}$ production.

Hypothesis

The hypothesis of this study was that feeding ALG would increase the concentration of health promoting LC *n*-3 PUFA in milk which would be maintained over time, and improve fertility by reducing the uterine secretions of $PGF_{2\alpha.}$

Objectives and aims

The objective of the study was to determine the effect of supplementation with ALG on milk DHA concentration over time and to determine the effect on indicators of fertility.

5.2 Material and methods

The study was conducted in accordance with the requirement of the Animals (Scientific Procedures) Act 1986 (amended 2013) and received approval by the Harper Adams University Animal Welfare and Ethical Review Body.

5.2.1 Animals, diets and experimental design

The study was conducted at Harper Adams University, Edgmond, Shropshire over a period of 6 months from September to February 2016-2017. Sixty Holstein-Friesian dairy cows were randomly allocated to one of two dietary treatments 25 ± 0.53 days post calving based on parity, calving date, 305 day milk yield and milk yield 1 week prior to the start of the study. The experiment was a continuous design, with the diets fed from 3 weeks post calving for 14 weeks. The two diets (30 cows per treatment), were either unsupplemented (Control) or supplemented with 105 g of DHA enriched ALG per cow/ day (*Schizochytrium imancinum sp.,* Alltech, Kentucky, USA). Cows on the Control treatment received an additional 105 g/cow per day of a rolled wheat/ sugar beet feed mixture. Both treatment contained the same basal diet (Table 5.1). Prior to the start of the study the cows were fed the same basal ration. Diets were formulated according to Thomas (2004) to be isonitrogenous and isoenergetic.

Cows were fed the TMR daily at 09 00 h to provide daily refusals of approximately 5% and had continual access to fresh water. The forages and straight feeds were mixed for 5 min before the addition of the ALG supplement, with a further 5 min of mixing using a commercial forage mixer (HiSpec mixer, County Carlow, Ireland), calibrated to ±1 kg, and fed through roughage intake feeders (Insentec B.V., Marknesse, The Netherlands) fitted with an automatic animal identification and forage weighing system calibrated to ±0.1 kg. Refusals were collected three times per week on Monday, Wednesday and Friday.

Cows were housed together in the same portion of a building containing cubicles fitted with foam mats. The area was scraped every 2 h with automatic scrapers, cubicles were bedded twice weekly with sawdust and limed weekly. All cows had free access to salt blocks, and were milked twice daily at approximately 0615 and 1600 h.

Table 5.1. Diet composition (kg/kg DM) of the pre study and basal diet

Ingredient	Pre-study	Basal
Maize silage	0.350	0.413
Grass silage	-	0.130
Lucerne	0.152	-
Chopped wheat straw	0.019	-
Rapeseed meal	0.059	0.065
Wheat distillers dark grains	0.071	0.078
Soya bean meal	0.030	0.065
Palm kernel meal	0.020	0.022
Molasses	0.006	0.007
Sodawheat[1]	0.114	0.109
Spey syrup[2]	0.040	-
Soya hulls	0.060	0.078
Sweetstarch[3]	0.039	-
Megalac[4]	0.007	0.013
Butterfat Extra	0.007	-
Minerals and Vitamins[5]	0.007	0.006
Acid Buf[6]	0.004	-
Salt	0.003	-
SC Gold 25g[7]	0.001	-
ME[8] (MJ/ kg DM)	11.8	12.3
MPN[9] (g/ kg DM)	116	113
MPE[10] (g/ kg DM)	102	102
MPB[11] (g/ kg DM)	47	44

[1]Alkaline buffer. [2]Distillery syrup. [3]Blend of products from the bakery, confectionary, pastry and breakfast cereal industries on a friable vegetable protein carrier - KW alternative feeds, Ternhill, UK. [4]Protected fat: Calcium salt of palm fatty acids- Volac, Royston, UK. [5]Mineral/vitamin premix. Major minerals (g/kg): Ca, 220; P, 30; Mg, 80; Na, 80; Cu (total), 7600 and Se (total) 302.9. Additives (per kg) vit A 1000000 IU; vit D3, 300000 IU; vit E, 3000 IU and vit B12, 2500 mcg. [6]Natural seaweed based minerals: Maerl (calcareous marine algae) – KW alternative feeds, Ternhill, UK. [7]Live yeast: Saccharomyces cerevisiae (I -1077) – Biotal, Worcestershire, UK. [8]Metabolisable energy [9]Metabolisable protein when rumen nitrogen is limiting. [10]Metabolisable protein when rumen energy is limiting. [11]Metabolisable protein from by-pass protein

5.2.2 Animal Performance

Feed intake was recorded daily and the TMRs were sampled weekly throughout the experiment, and stored at -20 °C for subsequent analysis. Further forage samples were taken weekly, oven dried at 105 °C for 24 h and the ratio of maize: grass silage adjusted to the desired level on a DM basis. The TMR samples were bulked within each month and a sub-sample analysed according to AOAC (2012) for DM (934.01), CP (988.05) and ash (924.05), whilst NDF was analysed according to Van Soest *et al*., (1991) as described in sections 2.1.1 to 2.1.4. Fatty acid analysis of the TMR samples was determined using a modified protocol of Sukhija and Palmquist (1988) as described in section 2.2. Fatty acids were identified using a GC as described in section 2.4.4.

Milk yield was recorded daily and cows weighed and body condition scored (Ferguson *et al*., 1994) at 11 00 h 1 week prior to the start of study (week 0) and every other week. Milk samples were collected 3 times per week from 1 week prior to the start of the study until the end of the study and preserved using broad spectrum microtabs® II (Advanced Instruments, inc, Massachusetts, USA) and stored at 4 °C before the determination of progesterone levels, as described in section 2.4.5. Further samples were collected 5 days post artificial insemination (AI) and on days 14-20 post AI. Milk concentration of progesterone were measured by enzyme immunoassay (Ridgeway-M Kit) and inter and intra assay coefficient of variation were 13.97 and 7.54 % respectively. Additional milk samples were taken weekly at consecutive am and pm milkings for subsequent analysis of total fat, protein, lactose and somatic cell count (SCC); all samples were analysed at the National Milk Laboratories (Four Ashes, UK). During weeks 0, 1, 2, 4, 8 and 14 of the study milk samples were collected at 2 consecutive am and pm milkings from 16 pairs of cows per treatment for FA determination. Milk FA analysis followed the method described by Feng *et al.,* (2004) for lipid extraction as described in section 2.4.3, with the methylation of the lipids conducted according to the procedure of Christie (1982) with modifications according to Chouinard *et al*., (1999) as described in section 2.4.4. Milk samples from individual cows were corrected for am and pm yields prior to fat extraction.

5. 2.3 Blood metabolites and PGFM

Blood samples were collected from the jugular vein from 12 pairs of cows per treatment at 1100 h during weeks 0, 2, 4, 8 and 14. The blood samples were collected into sodium heparinised vacutainers for BHB determination and into vacutainers containing potassium oxalate for glucose and NEFA determination. Samples were centrifuged (SIGMA 3-16PK) at 1000 xg for 15 min, and the plasma separated and stored at -20 °C prior to subsequent analysis. Plasma samples were analysed for, 3-OHB, glucose and NEFA, kit catalogue no; RB1008; GU611 and FA115, respectively (Randox Laboratories, County Antrium, UK) using a Cobas Mira Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK) as described in section 2.5.

At 33 ± 0.89 days postpartum, 24 cows were synchronized in pairs using Ceva Prid®Delta (PRIDs) which contained 1.55g of progesterone per device. The PRIDs were removed after 10 d, and on day 17 of the synchronised estrous cycle, a catheter was inserted into the jugular vein following sedation with Sedaxylan (20 mg/ml xylazine solution at 0.5ml/100kg) injected into the tail vein. Blood samples were collected via the jugular catheter into sodium heparinised vacutainers at 15 min intervals for 1 hr prior to the infusion of oxytocin (100 IU), and at 15 mins intervals for a further 3 h, and then at 30 min intervals for 4 h post oxytocin to monitor uterine secretion of 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM). The blood was centrifuged at 1000 xg for 15 min and the plasma frozen at

-20°C prior to subsequent analysis. Plasma concentration of PGFM, a product of $PGF_{2\alpha}$ metabolism was assayed using an ELISA kit (Cayman Chemical, Ann Arbour, MI, USA) as described in section 2.6. Inter and intra assay coefficients of variation were 13.03 and 9.88 % respectively.

5.2.4 Fertility parameters

All cows were observed for signs of oestrus throughout the day and were inseminated (randomly by one of two farm staff) with frozen-thawed semen within 12 h of detected oestrus. Oestrus detection was performed daily until pregnant. Semen came from a single ejaculate of three bulls ensuring that equal numbers of cows from each treatment group were bred to each bull. Ultrasound scanning was performed 4 weeks post AI to confirm pregnancy. Conception rate was defined as the proportion of cows that were detected in oestrus and inseminated that were pregnant at week 4 post AI.

5.2.5 Calculations and statistical analysis

The progesterone concentrations was calculated by subtracting the blank wells from the absorbance reading of the rest of the plate. The standard curve was then plotted and a 4-parameter logistic fit was performed. The concentration of each sample was computed by reading the corresponding values on the x-axis of the standard curve.
To determine the PGFM concentrations the absorbance reading of the blank wells was first subtracted from the absorbance readings of the rest of the plate. The non-specific binding (NSB) wells and maximum binding (B_0) wells were averaged, the NSB average was subtracted from the B_0 average to give the corrected B_0.

%B/ B_0 (% Bound/ Maximum bound) = Equation 10

$$\left(\frac{Standard\ or\ Sample - NSB}{Corrected\ \mathrm{Bo}}\right) \times 100$$

The %B/B_0 for standards S1-S8 were plotted versus their PGFM concentrations using linear (y) and log(x) axes and a 4-parameter logistic fit was performed. The concentration of each sample was identified by the %B/B_0 values on the standard curve and reading the corresponding values on the x-axis.

All data were checked for a normal distribution and were analysed using Genstat 17th edition (VSN. Ltd, Oxford, UK). Variables having more than one observation were analysed using repeated measures ANOVA using data recorded in week 0 as a covariate where appropriate. Results for treatment, time period, their interactions and SED are presented. *P* values < 0.05 were regarded as being statistically significant, and <0.10 were considered to indicate a tendency towards statistical significance.

5.3 Results

5.3.1 Feed analysis

Both treatment diets had a similar DM content, with an average of 378 g/kg (Table 5.2). Ash, OM, crude protein and NDF content were also very similar between the dietary treatments, which averaged 72, 928, 162 and 419 g/kg DM respectively. The pre-study diet had a slightly higher DM content of 442 g/kg, but very similar ash, OM, and crude protein content to the dietary treatments. The NDF content of the pre-study diet was slightly lower at 375 g/kg DM compared to the treatment diets. The FA content of C14:0 – ALA was similar between both dietary treatments, the DHA content was not detectable in the Control or Pre-study diet, but the ALG treatment had a DHA content of 0.71 g/kg DM. The pre-study diet had higher content of C14:0 and C16:0 compared to the treatment diets.

Table 5.2. Chemical composition (g/kg DM) of TMR of the pre-study diet, the control diet that contained no ALG (Control) or with 105 g of ALG per cow/d (Algae)

	Pre-study	Control	Algae
DM (g/kg)	442	375	380
Ash	69.7	72.4	71.5
Organic matter	930	928	928
Crude protein	166	163	161
NDF	375	419	419
Fatty acid (g/kg DM)			
C14:0	0.78	0.48	0.47
C16:0	8.60	5.48	5.62
C18:0	0.67	0.51	0.41
C18:1 *cis*-9	4.75	4.27	3.66
LA	5.35	5.68	4.38
ALA	0.76	0.96	0.64
EPA	Nd	Nd	nd
DHA	Nd	Nd	0.71

5.3.2 Animal performance

There was no effect ($P > 0.05$) of dietary treatment on intake, with mean value of 22.1 kg/d (Table 5.3). Intake was affected by time ($P < 0.001$; Figure 5.1), increasing from 21.1 kg/d in week 1 to 23.4 kg/d at week 3 (week 6 of lactation), before decreasing to 20.9 kg/d at week 14. Similarly there was no effect ($P > 0.05$) of treatment on daily milk yield, which averaged 40.6 kg/d. There was an effect of time ($P < 0.001$; Figure 5.2) on daily milk yield which peaked at week 3 of the study (week 6 of lactation) before declining to 37.2 kg/d at week 14. Milk fat content and yield were not affected by dietary treatment ($P > 0.05$), averaging 37.2 g/kg and 1.49 kg/d respectively. Both milk fat content and milk fat yield decreased with time ($P = 0.048$ and 0.013 respectively; Figure 5.3). Milk protein content and protein yield were unaffected ($P > 0.05$) by dietary treatment. Time had an

effect ($P < 0.001$) on both protein content and yield which increased over the study period. There was no effect ($P > 0.05$) of dietary treatment on live weight, but there was an effect of time ($P < 0.001$) with live weight increasing by 27 kg/ d over the 14 weeks. Body condition score was unaffected ($P > 0.05$) by treatment or time.

Table 5.3. Milk performance, live weight and body condition of dairy cows fed no ALG (Control) or 105 g of ALG per cow/d (Algae)

	Treatment			*P* value[1]		
	Algae	Control	s.e.d	D	T	D x T
DM intake (kg/d)	22.0	22.1	0.861	0.905	<.001	0.791
Milk yield (kg/d)	39.9	39.6	1.023	0.980	<.001	0.729
Milk fat (g/kg)	36.9	37.5	2.090	0.702	0.048	0.912
Fat yield (kg/d)	1.46	1.52	0.200	0.401	0.013	0.738
Milk protein (g/kg)	31.5	31.3	0.682	0.67	<.001	0.681
Protein yield (g/kg)	1.25	1.27	0.044	0.584	<.001	0.799
Live weight (kg)	653	652	13.13	0.97	<.001	0.289
Body condition	2.82	2.67	0.084	0.115	0.837	0.123

[1]Probability of significant effects attributable to the diet (D), time (T), and their interactions (D x T)

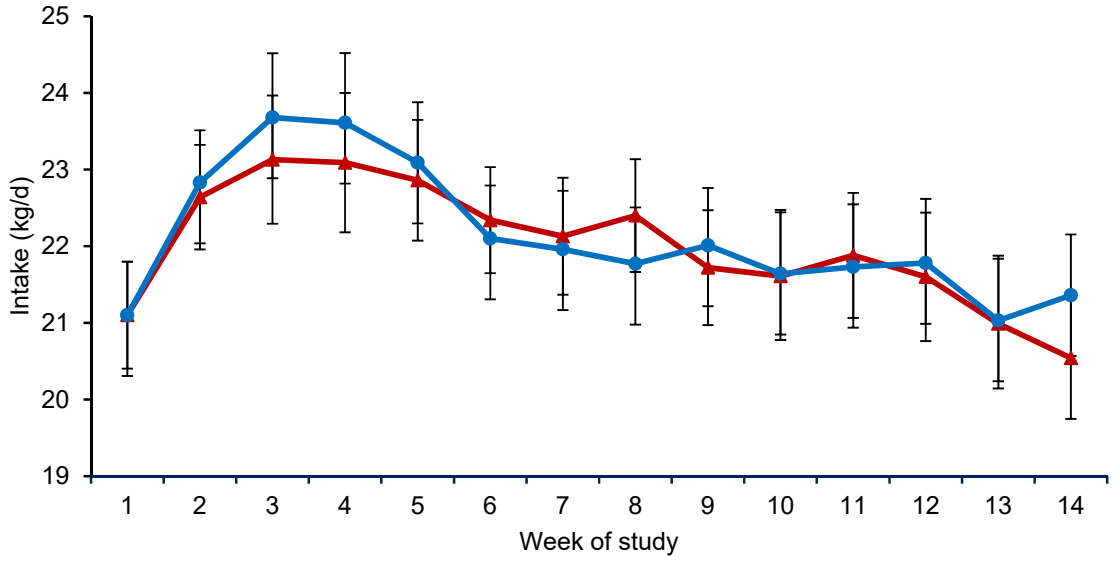


Figure 5. 1. Weekly DM intakes (kg/d) of dairy cows fed no ALG (Control ●) or 100 g of ALG per cow/d (Algae ▲). Error bars indicate SED.

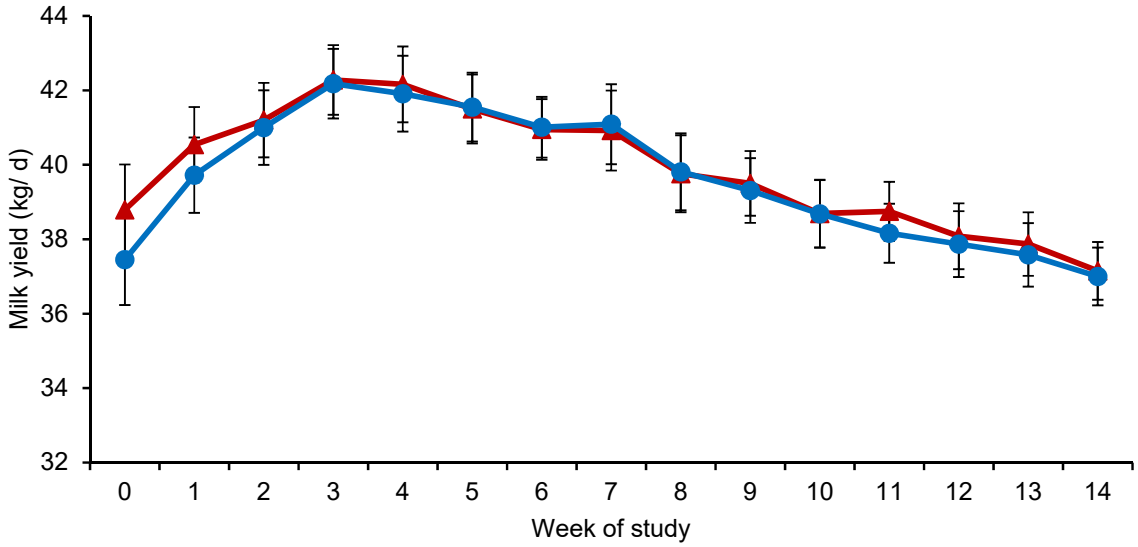


Figure 5. 2. Weekly milk yield (kg/d) of dairy cows fed no ALG (Control ●) or 100 g of ALG per cow/d (Algae ▲). Error bars indicate SED.

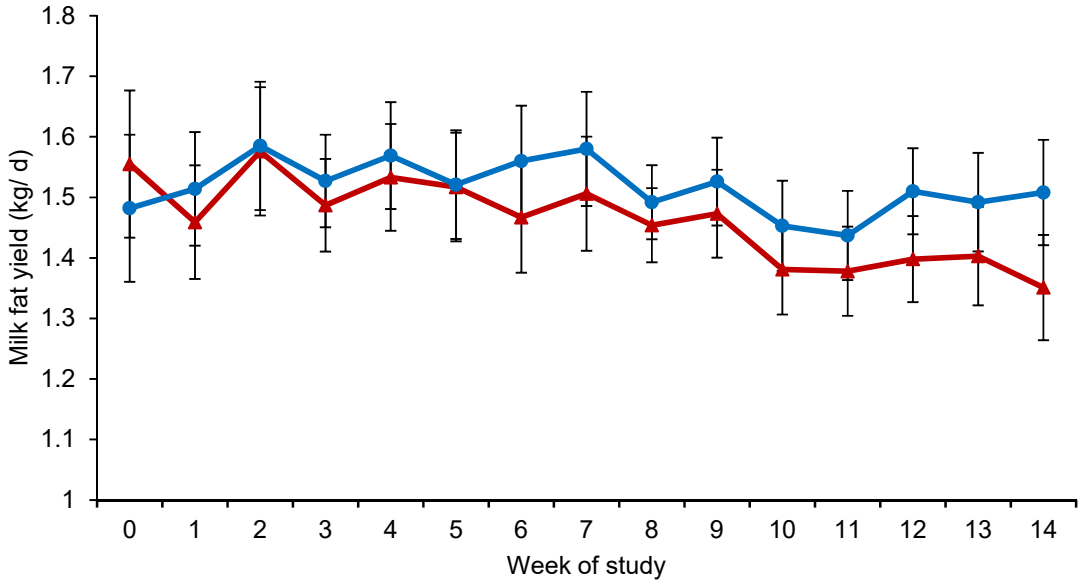


Figure 5. 3. Weekly milk fat yield (kg/d) of dairy cows fed no ALG (Control ●) or 100 g of ALG/cow per day (Algae ▲). Error bars indicate SED.

5.3.3 Plasma metabolite concentrations

There was no effect ($P > 0.05$) of dietary treatment on the mean concentration of plasma 3-OHB, glucose or NEFA (Figures 5.4a-c). Plasma BHB and NEFA were not affected by time ($P = 0.348$ and 0.061 respectively) but plasma NEFA decreased from week 2 to week 14 of the study in both treatments. In contrast there was an effect ($P < 0.001$) of time on plasma glucose, which increased with time in both treatments. There

was also an interaction between diet and time on plasma glucose which decreased in cows receiving ALG at week 2 compared to the Control, whereas at weeks 4 and 8 plasma glucose concentrations were lower in cows receiving the Control diet.

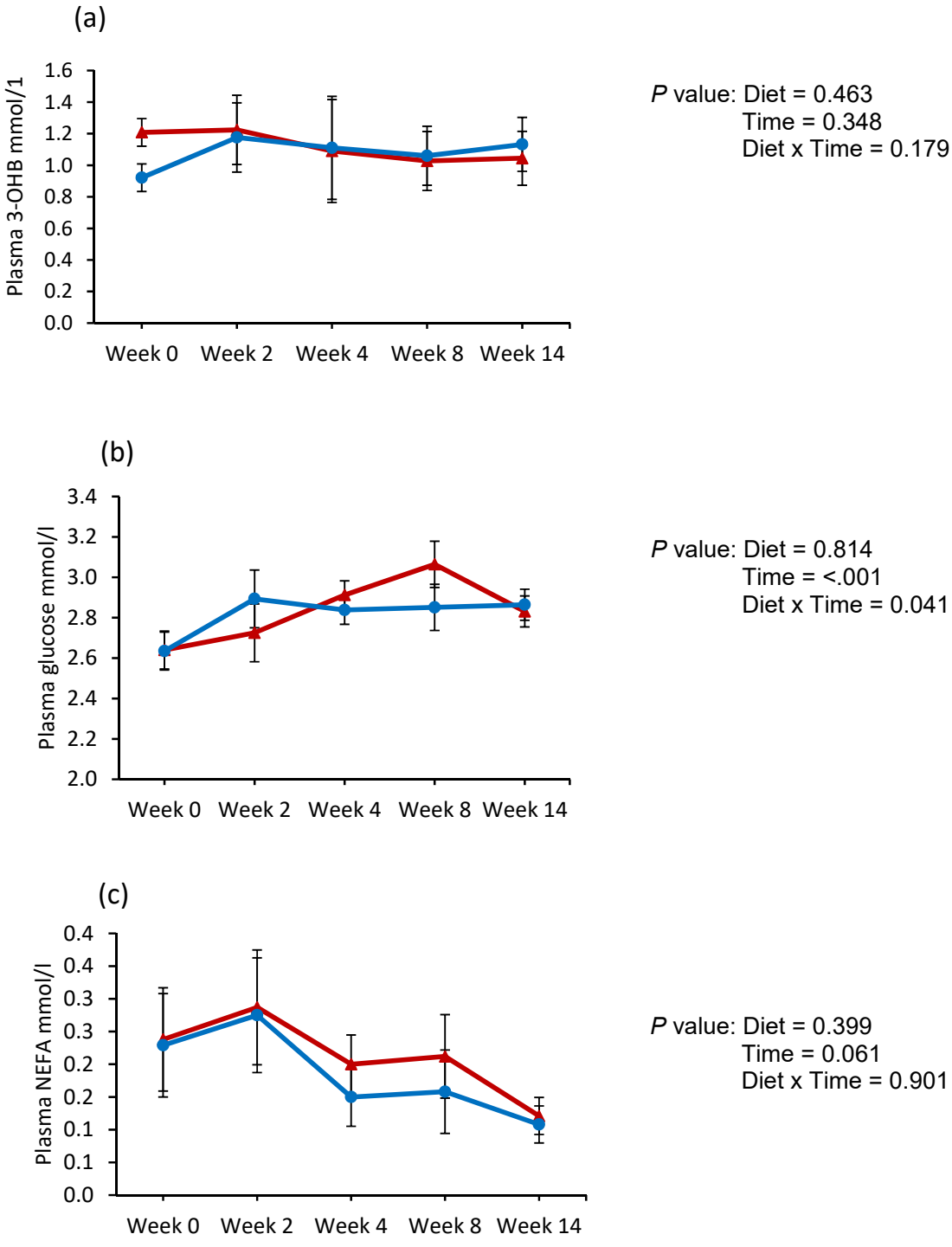


Figure 5.4 Weekly plasma β hydroxybutyrate (3-OHB) (a) glucose (b) and non esterified fatty acids (NEFA) (c) over time of dairy cows fed no algae (Control ●) or 100 g of ALG/cow per day (Algae ▲). Error bars indicate SED.

5.3.4 Milk FA

There was no effect ($P > 0.05$) of dietary treatment on mean milk fat content of C4:0 to C17:1, C18:1 *trans*-12, C18:1 *cis*-9, LA, C20:0, ALA, C18:2 *trans*-10 *cis*-12 CLA, C20:3*n*-3 and EPA (Table 5.4). In contrast there was a decrease ($P < 0.05$) in milk fat content of C18:0 and C22:0 in ALG fed cows compared to Control. In contrast there was an increase ($P < 0.05$) in C18:1 *trans*-8 to *trans*-11, C18:2 *cis*-9 *trans*-11 CLA, C20:3*n*-6 and DHA in cows fed ALG compared to the Control. There was no effect ($P > 0.05$) of dietary treatment on the proportion of FA of chain length less than C16:0, C16:0 plus C16:1 or on FA with a chain length greater than C16:0. There was also no effect of dietary treatment on the total milk fat content of SFA, MUFA or *n*-6. In contrast there was an increase ($P < 0.05$) in milk fat content of PUFA and *n*-3 when cows were fed ALG, and a decrease in the ratio of *n*-6: *n*-3.

There was an effect ($P < 0.05$) of time on most FAs except for C8:0, C16:0, C18:1 *trans*-9, C18:1 *trans*-11, ALA. C22:0 and EPA. The FA content of C18:0 was higher in Control fed cows compared to the ALG fed cows at week 1 and week 2, but was not different ($P > 0.05$) after week 2 (Figure 5.5a). The FA content of C18:1 *trans* 8, 9, 10 and 11 were higher in ALG fed cows from week 2 onwards, with the content of C18:1 *trans*-8 and *trans*-10 also increasing over time, peaking at week 8, whereas the milk fat content of C18:1 *trans*-9 and *trans*-11 remained constant after an initial increase at week 1 (Figure 5.5b-e). In contrast the FA content of C18:1 *trans*-12 was only higher in ALG fed cows compared to the Control fed cows at week 1, 4 and 8, and decreased over time (Figure 5.5f). The FA content of ALA was higher ($P < 0.05$) in cows fed the Control diet at week 1, but there was no difference ($P > 0.05$) from week 2 onwards (Figure 5.5g). Milk fat content of *cis*-9 *trans*-11 CLA was higher ($P < 0.05$) in ALG fed cows compared to those on the Control diet from week 0 onwards and increased over time (Figure 5.5h). In contrast milk fat content of *trans*-10 *cis*-12 CLA was similar for cows on both diets and decreased over time (Figure 5.5i). The milk fat content of DHA was higher ($P < 0.05$) in ALG fed cows from week 2 onwards and increased by 0.34 g/100g FA over the study, peaking at 0.38 g/100g FA at week 14 (Figure 5.5j). Milk fat content of SFA was higher ($P < 0.05$) in Control fed cows compared to those receiving ALG from week 1 onwards (Figure 5.5k). Fatty acid content of PUFA increased over time and was higher ($P < 0.05$) in ALG fed cows compared to those on the Control diet from week 2 onwards (Figure 5.5l). Milk fat content of *n*-3 FA was also higher ($P < 0.05$) in ALG fed cows from week 2 onwards, and increased with time, being 0.26 g/100g FA higher in ALG fed cows compared to those receiving the Control diet at week 14 (Figure 5.5m).

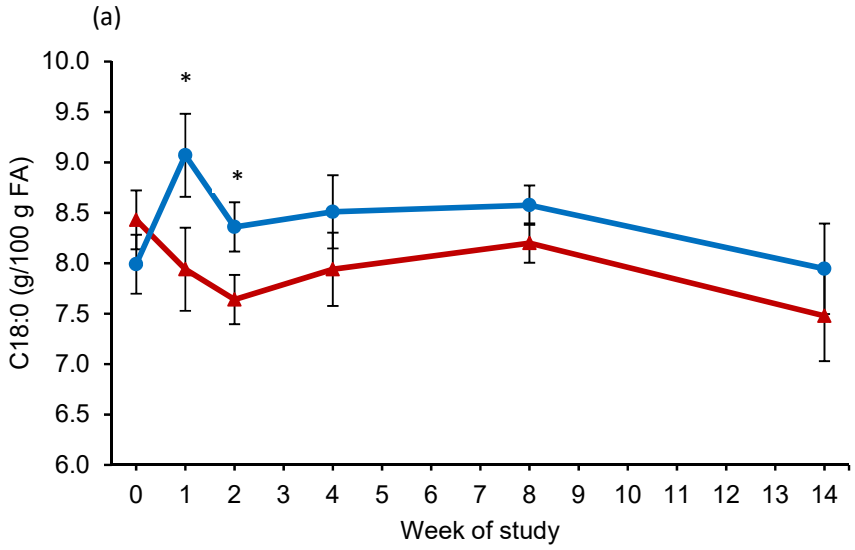
There was an effect of time ($P < 0.05$; Table 5.4) on the proportion of FAs with a chain length of less than C16:0, which increased over time in both treatments, and in

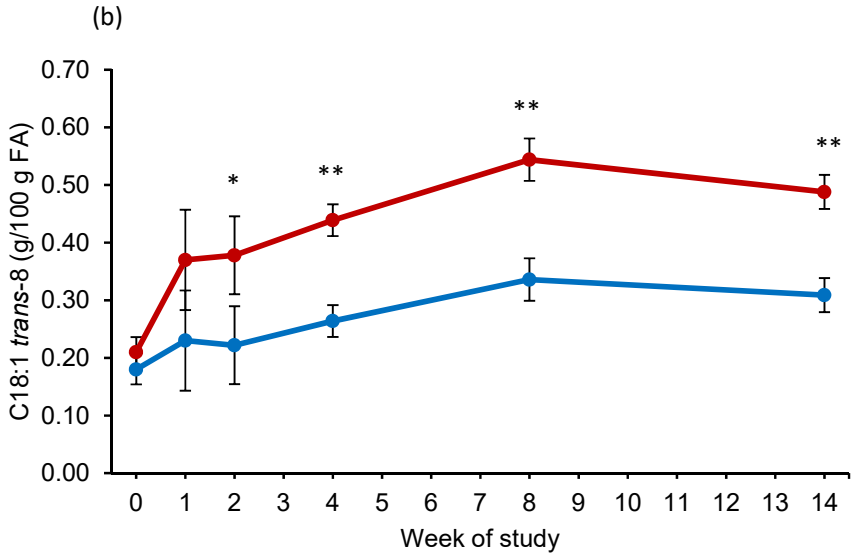
those with a chain length greater than C16:0 which decreased with time in both treatments. The milk fat content of MUFA also decreased over time in both treatment diets, whereas the milk fat content of *n*-6 increased in milk from all cows over time. There was an interaction between diet and time on milk fat content of C18:1 *trans*-10, ALA, C22:0 and DHA.

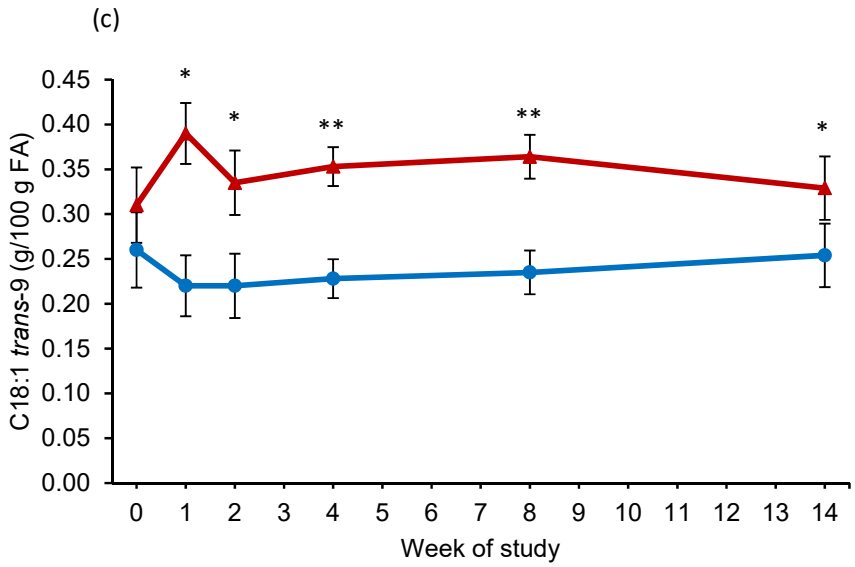
Table 5.4 Mean milk fatty acid composition (g/100g of FA) of dairy cows fed no ALG (Control) or 100 g of ALG/cow per day (Algae).

	Mean			*P* value[1]		
Fatty acid (g/100 g)	Algae	Control	s.e.d	D	T	DxT
C4:0	2.37	2.37	0.148	0.969	<.001	0.727
C6:0	1.67	1.70	0.134	0.877	0.002	0.737
C8:0	1.15	1.18	0.081	0.624	0.052	0.355
C10:0	2.48	2.58	0.192	0.449	0.009	0.299
C12:0	3.08	3.33	0.217	0.174	<.001	0.54
C14:0	9.90	10.4	0.438	0.164	<.001	0.217
C14:1	0.825	0.910	0.086	0.132	<.001	0.43
C15:0	0.978	1.04	0.072	0.146	0.002	0.345
C16:0	30.6	31.0	0.828	0.507	0.124	0.25
C16:1	0.515	0.510	0.053	0.816	0.013	0.361
C17:0	0.515	0.511	0.031	0.845	<.001	0.228
C17:1	0.260	0.258	0.036	0.924	<.001	0.488
C18:0	7.90	8.38	0.420	0.058	0.131	0.215
C18:1 *trans*-8	0.439	0.264	0.059	0.002	<.001	0.13
C18:1 *trans*-9	0.338	0.237	0.035	<.001	0.506	0.176
C18:1 *trans*-10	0.936	0.547	0.226	0.034	0.026	0.033
C18:1 *trans*-11	1.22	0.836	0.163	0.002	0.109	0.356
C18:1 *trans*-12	0.558	0.475	0.094	0.088	0.009	0.152
C18:1 *cis*-9	20.4	21.1	1.24	0.456	<.001	0.069
LA	2.99	2.93	0.148	0.620	0.009	0.205
C20:0	0.129	0.131	0.022	0.876	0.023	0.68
ALA	0.471	0.477	0.034	0.789	0.109	0.012
C18:2 *cis*-9 *trans*-11 CLA	0.752	0.566	0.069	0.038	0.003	0.052
C18:2 *trans*-10 *cis*-12 CLA	0.043	0.047	0.011	0.958	<.001	0.947
C22:0	0.078	0.117	0.020	0.002	0.26	<.001
C20:3*n*-6	0.065	0.052	0.013	0.034	0.008	0.062
C20:3*n*-3	0.172	0.177	0.029	0.648	0.129	0.216
EPA	0.087	0.079	0.019	0.376	<.001	0.242
DHA	0.220	0.039	0.030	<.001	<.001	<.001
Indices						
<C16:0	20.9	21.7	0.916	0.299	<.001	0.321
C16:0 + C16:1	32.1	32.2	0.759	0.423	0.012	0.272
>C16:0	36.3	36.3	1.48	0.976	0.01	0.257
ΣSFA	60.7	62.9	1.48	0.059	0.15	0.423
ΣMUFA	26.7	26.1	1.35	0.57	<.001	0.272
ΣPUFA	4.80	4.37	0.223	0.012	<.001	0.002
Σ*n*-3	1.08	0.828	0.070	0.002	0.121	0.023
Σ*n*-6	3.03	2.96	0.144	0.505	<.001	0.092
n-6:*n*-3	0.758	0.787	0.015	0.005	0.398	0.052

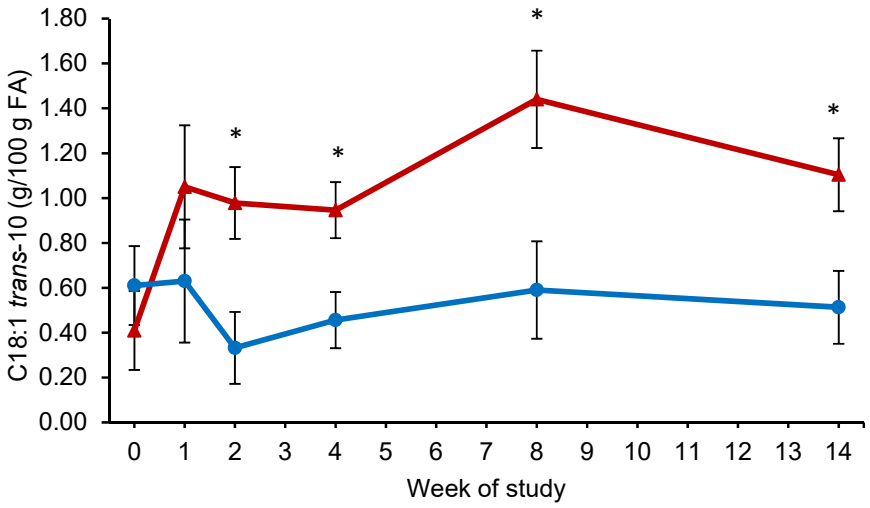
[1]Probability of significant effects attributable to the diet (D), time (T), and their interaction (D x T)


(a)
C18:0 (g/100 g FA)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
*
*
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

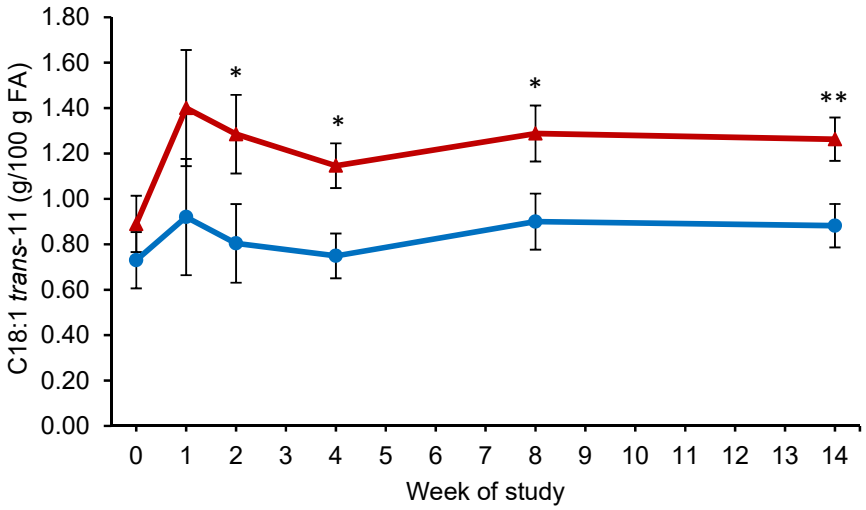

(b)
C18:1 trans-8 (g/100 g FA)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
**
**
**
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study


(c)
C18:1 trans-9 (g/100 g FA)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
**
**
*
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

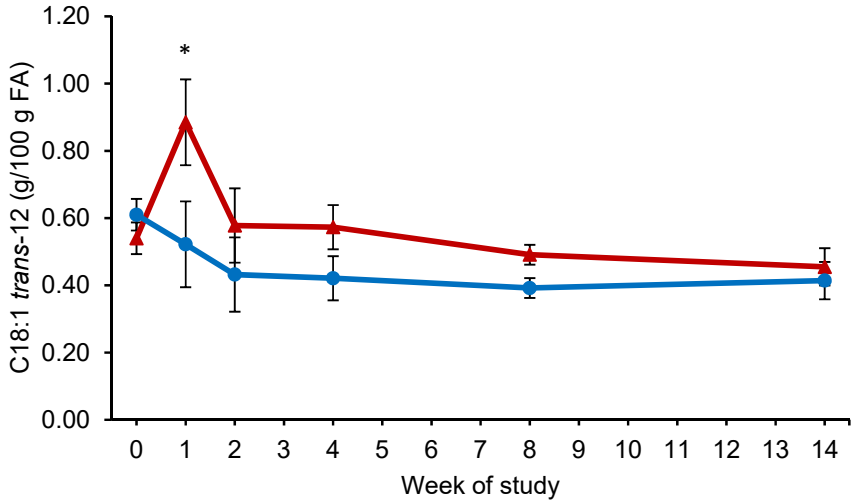
(d)


C18:1 trans-10 (g/100 g FA)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study
* * * * *

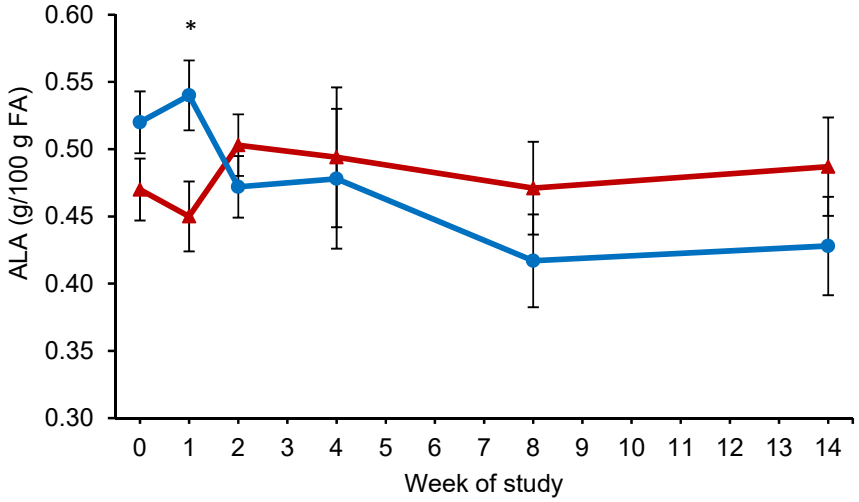
(e)


C18:1 trans-11 (g/100 g FA)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study
* * * **

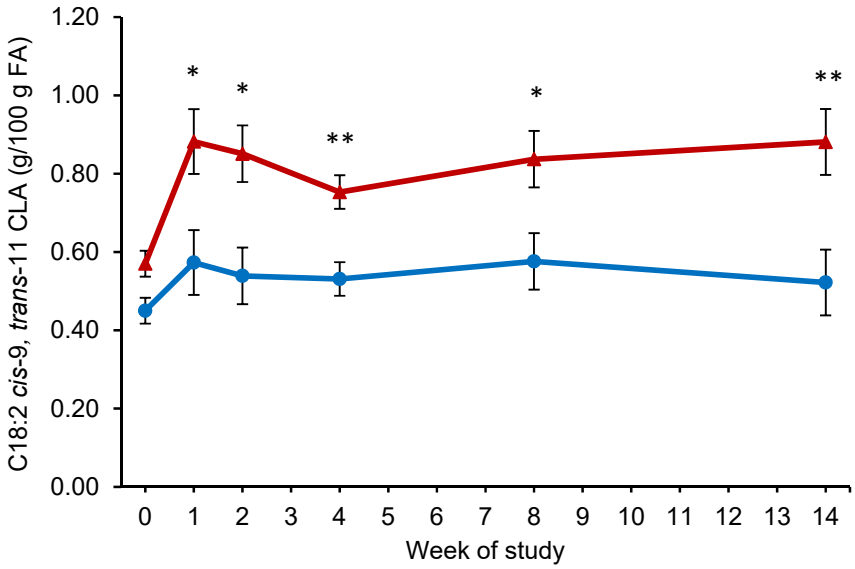
(f)


C18:1 trans-12 (g/100 g FA)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study
*

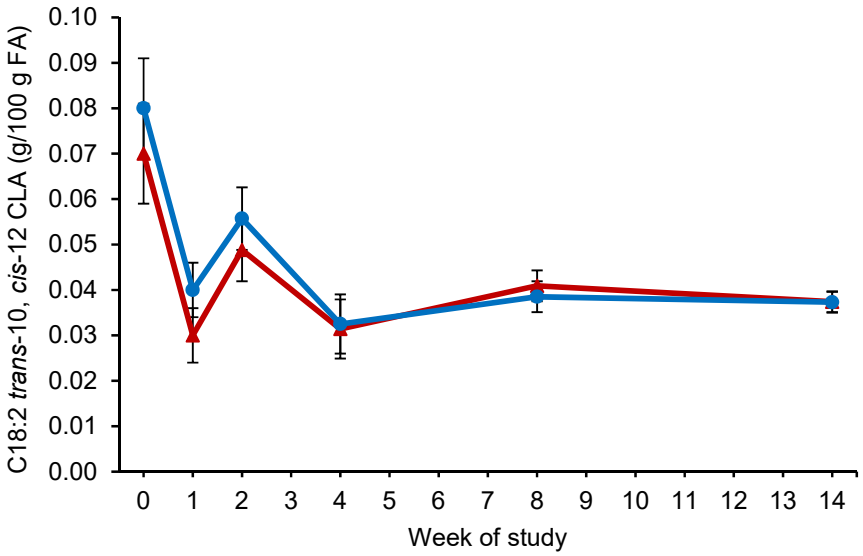
(g)


0.60
0.55
0.50
0.45
0.40
0.35
0.30
ALA (g/100 g FA)
*
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

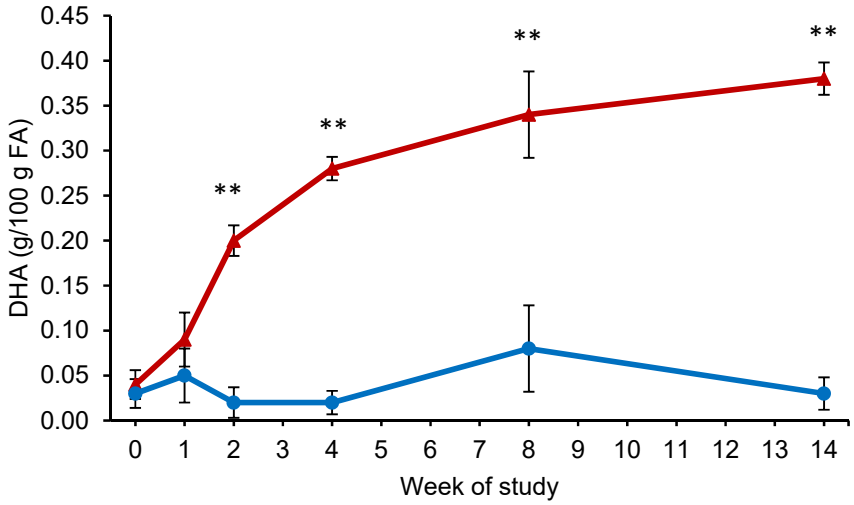
(h)


1.20
1.00
0.80
0.60
0.40
0.20
0.00
C18:2 cis-9, trans-11 CLA (g/100 g FA)
*
*
**
*
**
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

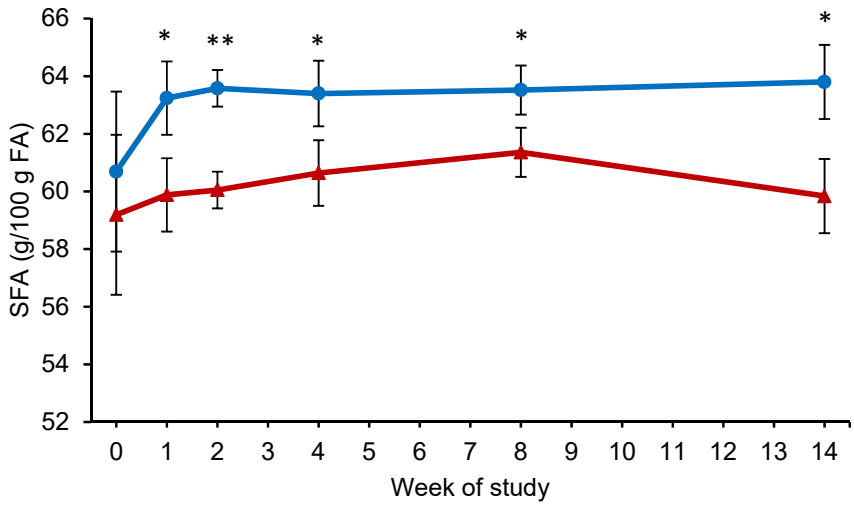
(i)


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
C18:2 trans-10, cis-12 CLA (g/100 g FA)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

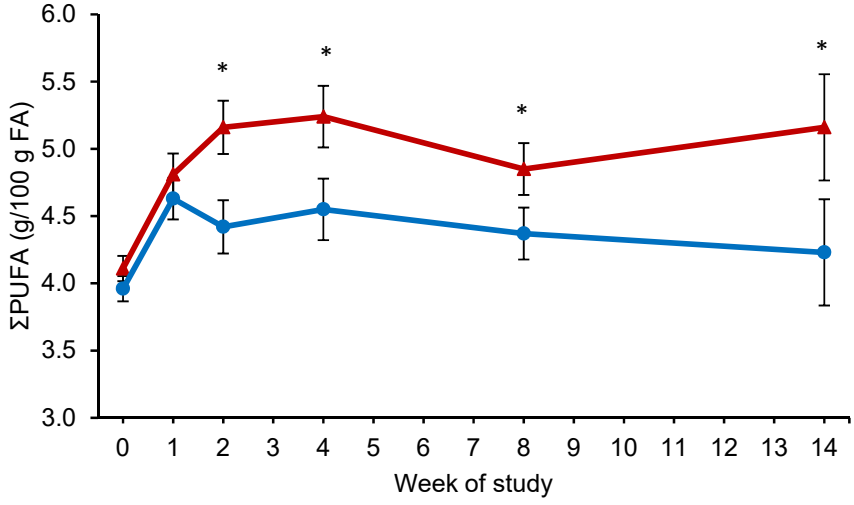
(j)


DHA (g/100 g FA)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
**
**
**
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

(k)


SFA (g/100 g FA)
66
64
62
60
58
56
54
52
*
**
*
*
*
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

(l)


ΣPUFA (g/100 g FA)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
*
*
*
*
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week of study

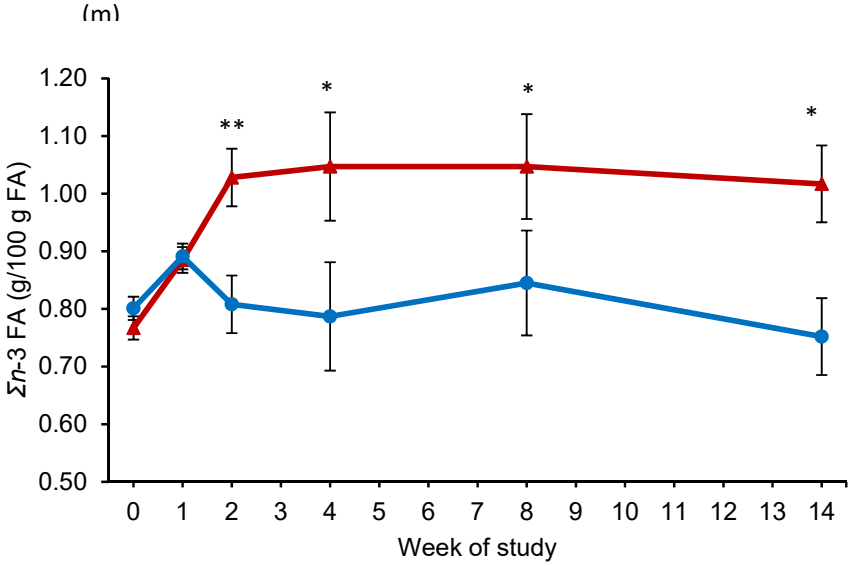


Figure 5.5 Weekly milk fat content of (a) C18:0 (b) C18:1 *trans*-8 (c) C18:1 *trans*-9 (d) C18:1 *trans*-10 (e) C18:1 *trans*-11 (f) C18:1 *trans*-12 (g) ALA (h) C18:2 *cis*-9 *trans*-11 CLA (i) C18:2 *trans*-10 *cis*-12 CLA (j) DHA (k) SFA (l) PUFA and (m) *n*-3 FA over a 14 week period in dairy cows fed no ALG (Control ●) or 100 g of ALG/cow per day (Algae ▲). Error bars indicate SED (n=32). *$P < 0.05$, **$P < 0.001$.

5.3.5 Fertility parameters

The fertility results presented are based on data from 38 cows (algae, n=19; control, n=19) from the original number of cows assigned to the trial. The data from cows that were used for PGFM analysis were excluded from the analyses due to possible confounding effects on oestrus synchronisation. There was no effect ($P > 0.05$; Table 5.5) of treatment on the interval between calving and first AI with a mean value of 67.6 days, or on conception rate to first AI with a mean value of 44.8 %. Conception rate to second AI was numerically higher in ALG fed cows compared to those fed the Control diet, although the difference was not significant ($P = 0.256$). Overall conception rates were also higher in ALG fed cows compared to the Control, but again the improvement in conception rate was not significant ($P = 0.485$). There was no effect ($P > 0.05$) of treatment on milk P_4 levels in pregnant cows at day 5 post AI, but by day 20 post AI, milk P_4 levels in pregnant cows was higher ($P < 0.05$) in ALG fed cows compared to the Control.

Table 5.5. First, second, and accumulated pregnancy (% and number of cows) per artificial insemination (AI), and milk progesterone levels (ng/mL) in pregnant cows at day 5 and 20 post AI of cows fed no ALG (Control) or 100 g of ALG/cow per day (Algae)

Item	Treatment		s.e.d	*P* value
	Algae	Control		
DIM at 1st AI (d)	66.3	68.8	-	0.599
Conception rate to 1st AI (%)	42.1 (8/19)	47.4 (9/19)	-	0.744
Conception rate to 2nd AI (%)	54.5 (6/11)	30.0 (3/10)	-	0.256
Overall conception rate (%)	73.7 (14/17)	63.2 (12/19)	-	0.485
Milk P_4[1] at 5 d post AI (ng/ml)	7.19	6.29	1.54	0.563
Milk P_4[1] at 20 d post AI (ng/ml)	35.1	21.0	5.77	0.024

[1]P_4 = Progesterone

5.3.6 Plasma PGFM concentrations

A total of 16 cows (algae, n=9; control, n=7) were used to determine plasma PGFM concentrations measured during an oxytocin challenge. Mean values, area under the curve, and peak concentrations (Table 5.6) were similar between treatments (*P* > 0.05). The response in PGFM concentration increased after the oxytocin challenge to reach a peak at 15-30 min (Figure 5.6) before slowly returning to basal level at 150 min post oxytocin for both treatments.

Table 5.6. Plasma PGFM concentrations measured at d 17 of the oestrous cycle of non-pregnant dairy cows fed no ALG (Control) or 105 g of ALG/cow per day (Algae)

Item	Treatment		s.e.d	P value[1]		
	Algae	Control		D	T	DxT
Mean value (pg/ml)	39.1	50.3	12.04	0.307	0.003	0.351
Peak value (pg/ml)	67.5	73.9	17.61	0.731	-	-
Area under the curve (pg)	2236	4046	987	0.126	-	-

[1]Probability of significant effects attributable to the diet (D), time (T), and their interactions (D x T)

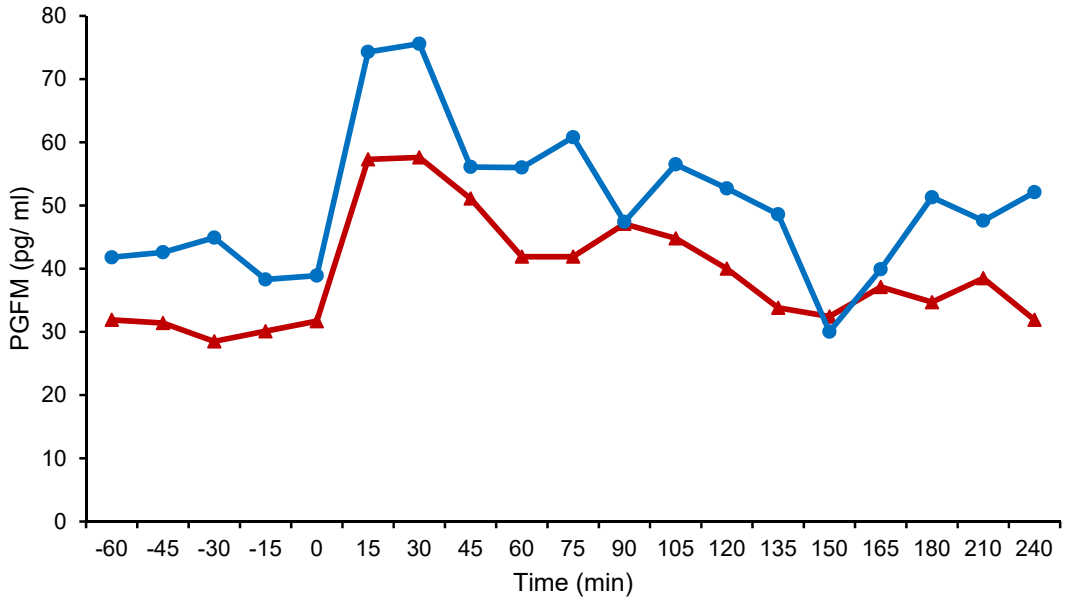


Figure 5.6. Plasma 13,14-dihydro-15-keto $PGF_{2\alpha}$ metabolite (PGFM) concentration, after an oxytocin challenge (time = 0) on day 17 of a synchronised oestrous cycle of cows fed no ALG (Control ●) or 100 g of ALG/cow per day (Algae ▲). Repeated measure analysis.

5.4.0 Discussion

5.4.1 Feed analysis

The current study examined the effect of feeding DHA enriched ALG for 14 weeks on animal performance, milk quality, fertility parameters and plasma concentrations of PGFM. Both TMR's had similar DM, ash, OM, CP and NDF values, which were also similar to other studies (Glover *et al*., 2012; Boeckaert *et al*., 2008). The ALG supplement supplied 15.6 g DHA/cow per day. This dietary inclusion level of 100 g/cow per day of ALG was selected based on the results from Chapter 4, where negative effects of reduced digestibility and reduced milk fat yield were seen at higher inclusion levels.

5.4.2 Animal performance

In the current study there was no effect of dietary treatment on DMI, which averaged 22.1 kg/d over the 14 week period. This intake was similar to the result in Chapter 4, and is also in accordance with Sinedino *et al*., (2017) who reported no effect on DMI when cows were fed 100 g of ALG/ d containing 10 % DHA for 18 weeks. Higher inclusion levels of 50 g and 75 g of DHA have been associated with a decrease in DMI in dairy cows (Moate *et al.,* 2013) and consequently could affect other parameters of animal performance.

Moran *et al.,* (2017) fed DHA enriched ALG at a rate of 6 g/kg DMI for 12 weeks and reported a slightly higher milk yield compared to the control. Similarly Sinedino *et al*., (2017) reported an increase in milk yield of 0.9 kg/d in ALG fed cows compared to the

control. In the current study there was no effect of dietary treatment on milk yield, which is in accordance with several other studies that supplemented ALG to dairy cows (AbuGhazaleh *et al*., 2009; Stamey *et al*., 2012; Vahmani *et al*., 2013). Despite the increase in milk yield reported by Sinedino *et al*., (2017) there was no increase in ECM due to the reduction in milk fat (g/kg). In the current study there was no effect of treatment on milk fat (g/kg) or milk fat yield (kg/d) therefore ECM was similar between treatments. Milk fat depression was reported in Chapter 4 at the highest inclusion level of ALG. Milk fat depression has been reported to be caused by elevated levels of *trans*-10 *cis*-12 CLA, which inhibits milk fat synthesis (Hussein *et al.,* 2013, Sinclair *et al.,* 2007). In the current study milk fat concentrations of *trans*-10 *cis*-12 CLA were similar between dietary treatments, and therefore may support the findings that *trans*-10 *cis*-12 CLA is involved in milk fat depression. It has been suggested that other FA intermediates that are produced through the biohydrogenation of PUFA also have inhibitory effects on milk fat synthesis (Ventto *et al*., 2017). Concentrations of C18:1 *trans*-10 are known to increase in milk from cows fed diets that result in milk fat depression, but in the current study milk concentration of C18:1 *trans*-10 was higher in ALG fed cows compared to the Control. The current finding is in accordance with Lock *et al.,* (2007) who reported that abomasal infusion of C18:1 *trans*-10 had no effect on milk fat synthesis. Milk fat concentration and yield in the current study declined over time from week 0 to week 10 before levelling out towards week 14. Milk fat concentrations vary with stage of lactation (Linn, 1988), and a decline in milk fat is expected in the first two months of lactation which is followed by a slow increase over the course of lactation (Linn, 1988). The total energy output as milk and DMI was similar between treatments in the current study and therefore there was no effect of dietary treatment on BCS or live weight change. Live weight did increase over time, which was mainly due to the primiparous cows, which were still growing.

Milk protein content (g/kg) and protein yield (kg/d) were not significantly affected by dietary treatment in the current study, a finding that is consistent with Chapter 4, where different levels of inclusions of ALG (50, 100 and 150 g/ALG per cow/d) had no effect on milk protein concentration or yield. This finding is also consistent with others who fed ALG to dairy cows (Moran *et al*., 2017 and Stamey *et al*., 2012). In the current study there was an effect of time on milk protein content, which declined initially before increasing. Stage of lactation has a considerable influence on milk protein concentration (Linn, 1988). Total milk protein is very high in colostrum milk and declines rapidly during the first few days of transition from colostrum to normal milk (Linn, 1988). Milk protein concentration then generally declines to a minimum at around 5-10 weeks into lactation, which correlates with maximum milk yield, before increasing gradually as lactation progresses (Linn, 1988). In the current study maximum milk yield occurred at week 3 of the study (week 6 of lactation) the same week as protein concentration reached its minimum.

The plasma metabolites measured in the current study were not significantly affected by dietary treatments which is in accordance with results from Chapter 4. Plasma 3-OHB remained constant throughout the study, whereas plasma concentration of glucose increased from week 0 to week 14, and plasma concentration of NEFA decreased. Plasma NEFA concentrations are closely related to cow energy status, and in early lactation (beginning of the current study) higher NEFA concentrations reflect the mobilisation of lipid reserves to compensate for the imbalance between energy consumed, and energy secreted in milk (Cozzi *et al*., 2011). As cows progress through lactation, NEFA levels generally drop because energy balance becomes positive and the tissue reserves are replenished (Cozzi *et al*., 2011). Low glucose level is another indicator of negative energy balance, and cows in the current study could have experienced some degree of negative energy balance at week 0 when glucose levels were at their lowest, which is expected in early lactation (Adewuyi *et al*., 2005).

5.4.3 Milk FA profile

In Chapter 4, added dietary ALG significantly altered the concentration of individual milk FA, especially DHA content, which increased as the inclusion level of ALG increased in the diet. The aim of the current study was to investigate the long term effect and potential adaptation of the rumen microbial ecosystem when cows are supplemented with ALG for a longer period of time, by measuring milk FA over a 14 week period. In the current study cows that were fed ALG had significantly higher concentration of milk DHA compared to the Control from week 2 onwards, with levels peaking at week 14, with an increase of 0.35 g/100 g FA compared to the Control. This finding is in accordance with Moran *et al*., (2017), who reported an increase in milk DHA in ALG fed cows, with concentrations increased rapidly for the first 30 days of supplementation, with no decrease observed over the experimental period of 12 weeks. Similarly Vlcek *et al*., (2017) reported an increase in milk DHA when dairy cows were supplemented with ALG containing 10 % DHA, although the feeding period was shorter at 6 weeks, and the increase in milk DHA had started to plateau by day 42. Similarly Franklin *et al*. (1999) fed both unprotected and protected ALG for 6 weeks to mid lactating dairy cows, and reported an initial increase in milk DHA content before gradually decreasing at day 28 and then remained constant through to 42 days for both treatments in comparison to the Control. Other studies that have fed ALG to dairy cows for a longer period of time include Sinedino *et al*., (2017) and Vahmani *et al*., (2013) who reported an increase in milk DHA concentration, but only measured milk FA at one time point and therefore couldn't report the change in DHA over time. In the current study there was also a significant increase in milk fat content of total PUFA in ALG fed cows compared to the Control, with an initial increase at week 1 before persisting at a constant level for the remaining 13 weeks. Milk

concentration of total *n*-3 followed a similar pattern to that of DHA, increasing in ALG fed cows from week 2 onwards, and then remaining at a constant level for the duration of the study. Sinedino *et al*., (2017) also reported an increase in PUFA and total *n*-3 when ALG was fed to cows for 19 weeks, but again no change over time was reported.

Inclusion of LC-PUFA in the diet of ruminants typically lowers short and medium chained FA concentration in milk, due to their inhibitory effects on mammary *de novo* FA synthesis (Shingfield *et al*., 2006). In the current study the concentration of FA with a chain length < 16, and > 16 were unchanged, suggesting that ALG supplementation did not affect *de novo* FA synthesis. A reduction in the concentration of C18:0 was observed in the previous study (Chapter 4) when cows were fed ALG, but in the current study the reduction was only significant at weeks 1 and 2 of supplementation. The reduction in milk concentration of C18:0 in ALG fed cows can be attributed to the inhibitory effect of ALG on the biohydrogenation of C18-UFA to C18:0 in the rumen. The recovery in C18:0 concentration at week 4 occurred at the same time when milk yield and milk fat content started to decline, which is consistent with the findings of Shingfield *et al*., (2006). It was suggested by Shingfield *et al*., (2006) that the changes in milk C18:0 content reflect an adaptation to an acute reduction in mammary C18:0 supply, that initiated a decrease in mammary lipid synthesis of C18:1 *cis*-9 (which is synthesised from C18:0 via Δ^9-desaturation) which plays an important role in the maintenance of fluidity of milk fat and their secretion (Bichi *et al*., 2013). This is hard to conclude from the current study as milk concentrations of C18:1 *cis*-9 were similar between dietary treatments and both milk yield and milk fat content of cows fed the Control diet also decreased at week 4, suggesting that stage of lactation had a greater effect on the temporal changes in milk yield and milk fat rather than changes in individual FA concentrations.

Many studies have investigated the temporal changes in milk fat content of *cis*-9 *trans*-11 CLA, *trans*-10 *cis*-*12* CLA and C18:1 *trans*-10, in order to help understand the cause of milk fat depression when oils containing PUFA are supplemented to dairy cows and sheep (Bichi *et al*., 2013; AbuGhazaleh, 2008; Shingfield *et al*., 2006). In the current study milk fat content of *cis*-9 *trans*-11 CLA increased during the first week of supplementation with ALG and then remained constant throughout the study, a finding in agreement with AbuGhazaleh (2008) who reported an increase in milk fat content of *cis*-9 *trans*-11 in dairy cows supplemented with a combination of FO and sunflower oil, that peaked at day 3 of supplementation and then remained constant. In contrast both Bichi *et al*., (2013) and Shingfield *et al*., (2006) reported an initial increase in *cis*-9 *trans*-11 CLA within 6 days of supplementing dairy ewes with ALG and within 5 days of supplementing dairy cows with a mixture of FO and sunflower oil respectively, before the concentration of the FA declined in both studies. The milk fat content of C18:1 *trans*-11 in the current study followed a similar temporal pattern to that of *cis*-9 *trans*-11 CLA, increasing during the first

week of supplementation in ALG fed cows before remaining constant. Similarly Bichi *et al*., (2013) also reported an increase in milk content of C18:1 *trans*-11 in ALG fed sheep, peaking at day 6 of supplementation and persisting at a relatively constant level during the experimental period. In contrast Shingfield *et al*., (2006) reported an initial increase in C18:1 *trans*-11 at day 5 of supplementation before declining and remaining constant from day 16 onwards, a pattern very similar to the milk fat content of *cis*-9 *trans*-11 CLA in the same study. The close linear relationship between C18:1 *trans*-11 and *cis*-9 *trans*-11 CLA in the current study was observed due to milk *cis*-9, *trans*-11 CLA being synthesised mainly endogenously from C18:1 *trans*-11 by steaoryl-Co A desaturase in the mammary gland (Roy *et al*., 2006). In the current study milk fat content of C18:1 *trans*-8 and *trans*-10 increased over the 14 week period in ALG fed cows, a finding similar to Bichi *et al*., (2013) who reported a continuous increase in milk content of C18:1 *trans*-10 in dairy ewes fed ALG. Shingfield *et al*., (2006) also reported an increase in C18:1 *trans*-6+7+8 and *trans*-10 over a 28 day period. The increase in C18:1 *trans*-10 could be explained by a shift in rumen biohydrogenation, but with no temporal decrease in C18:1 *trans*-11 concentrations, it is difficult to conclude this in the current study. There is also evidence that milk FA composition responses to lipid supplement are dependent on the composition of the basal diet (Roy *et al*., 2006), with a greater shift towards C18:1 *trans*-10 in low forage diets (Shingfield *et al*., 2006).

The finding that most of the FA temporal changes in the current study persisted throughout the 14 week period supports that the rumen microbial ecosystem did not adapt to the consumption of ALG, a finding similar to Bichi *et al*., (2013). It is therefore possible to enrich milk with increased levels of DHA by feeding dairy cows ALG, with little need to be concerned for rumen adaptation.

5.4.4 Fertility parameters

The secondary aim of the current study was to investigate the effect of feeding DHA enriched ALG on indicators of fertility, especially blood concentration of $PGF_{2\alpha}$. Diets high in *n*-3 may reduce $PGF_{2\alpha}$ synthesis and consequently prevent the regression of the CL, allowing continued secretion of P_4 that may help improve embryo survival (Gulliver *et al*., 2012). Previous studies on feeding FA to cattle have shown a number of effects on reproductive function, although responses have not always been consistent. To date only two other studies have reported the effects of feeding DHA enriched ALG on reproduction in dairy cows. Sinedino *et al*., (2017) increased overall conception rates, and reported an upregulation of the interferon-stimulated gene RTP4 in ALG fed cows, whereas Vleck *et al*., (2017) reported that including ALG in the diet of dairy cows did not influence ovarian activity, although neither study determined the concentration of plasma PGFM. In the current study ALG supplementation had no

significant effect on mean, peak or area under the curve of plasma $PGF_{2\alpha}$, although in all three cases the plasma PGFM concentrations were numerically lower in ALG fed cows compared to the Control. These results are consistent with that of Moussavi *et al*., (2007) who fed different inclusion levels of fish meal as a source of *n*-3 to dairy cows from 5 – 50 days in milk, and reported no effect of *n*-3 supplementation on plasma PGFM concentration. Despite having no significant decrease in plasma PGFM concentration Moussavi *et al*., (2007) did report a decrease in the ratio of *n*-6*:n*-3 FA in the uterine endometrial. In contrast Mattos *et al*., (2004), fed FO to dairy cows from 21 days pre-partum until 21 days post-partum, and found a significant decrease in plasma PGFM concentrations at days 0, 0.5, 2 and 2.5 post-partum in cows fed FO. More recently Dirandeh *et al*., (2013) investigated the effect of feeding linseed as a source of *n*-3 on plasma concentration of PGFM compared to a control diet and a diet high in *n*-6 from calving to 70 days post calving and reported that the linseed diet reduced plasma PGFM concentration following an oxytocin challenge on day 15 of a synchronized oestrous cycle, whereas the *n*-6 diet increase plasma PGFM concentrations compared to the control. One difference between the current study and that of Mattos *et al*., (2004) and Dirandeh *et al*., (2013) is the feeding period. In the current study the ALG wasn't fed until 21 days post-partum, whilst Mattos *et al*., (2004) fed from 21 days before calving and Dirandeh *et al*., (2013) fed from calving. In the current study, by day 15 of the synchronized oestrus cycle, the cows selected for PGFM analysis had received the ALG supplement for 39 ± 0.89 days. Results from other studies suggest that this period of feeding was sufficient to allow incorporation of dietary EPA and DHA into membrane phospholipids of the uterine caruncles (Howie *et al*., 1992). In the current study the ALG supplement was not fed pre-partum or in the first 3 weeks post-partum as $PGF_{2\alpha}$ plays an important role in enhancing uterine defence mechanisms, reducing uterine infections, and assist normal uterine involution post-partum (Richardson *et al*., 2013; Santos *et al*., 2008) and therefore it would not have been advantageous to decrease its concentration at this time in the cows cycle. Another reason behind the difference in PGFM concentrations between the current study and others may be the type of *n*-3 FA used, as ALG is rich in DHA whilst the FO used by Mattos *et al*., (2004) was richer in EPA, and the linseed used by Dirandeh *et al*., (2013) was rich in ALA. Both EPA and DHA act differently, with EPA in membrane phospholipids displacing AA, leading to increased synthesis of prostanoids of the 3 series (Mattos *et al.,* 2004). In contrast DHA reduces the expression of the PGHS enzymes (Mattos *et al*., 2003), making these enzymes less available, and reducing prostanoid synthesis. The lack of a significant decrease in PGFM in the current study may also be due to an insufficient numbers of cows.

It is well established that early embryo loss in the dairy cows is associated with the failure of the CL to produce sufficient P_4 (Yann *et al*., 2018). During the early stages of

pregnancy, elevated P_4 concentration stimulates luteotropic and antiluteolytic signals (Arosh *et al*., 2004), which are fundamental in maternal recognition of pregnancy which occurs prior to the implantation of the conceptus (Cargile and Tracy, 2014). Yann *et al*., (2018), reported that low P_4 concentration at day 5 post AI has been associated with poor embryo development. Other studies (McNeill *et al*., 2006; Green *et al*., 2005) have reported a similar relationship between milk P_4 at days 4-5 post AI and embryo survival, and milk P_4 at day 5 post AI can be used to monitor reproductive function. In the current study milk P_4 levels were numerically higher in pregnant cows that were fed ALG compared to the Control at day 5 post AI, and by day 20 post AI milk P_4 concentration was higher in ALG fed cows, a finding similar to Petit and Twagiramungu, (2006), who reported higher plasma P_4 concentration at days 17-21 of gestation from cows fed linseed compared to megalac or soyabean meal. A higher P_4 at day 5 post AI were associated with a numerically lower PGFM concentration in ALG fed cows in the current study, since elevated P_4 concentration along with IFN_τ leads to an increase in the ratio of PGE_2 to $PGF_{2\alpha}$ (Asselin *et al*., 1997). Feeding *n*-3 FA has been reported to induce granulosa cell proliferation and increase follicular size, generating a larger CL and P_4 secretion (Petit and Twagiramungu, 2006), and in the current study could be the reason behind higher P_4 concentration at day 20 post AI in the ALG fed cows. Garcia-Ispierto and Lopez-Gatius, (2017) treated cows with P_4 at either days 3-5 post AI or at days 15-17 post AI, and reported that cows treated earlier were 1.71 times more likely to conceive compared to the control group who were untreated. Diskin and Morris, (2008) also reported that animals that have an earlier increase in P_4 concentration between day 4-7 after insemination have a greater chance of maintaining pregnancy than animals with a slower rise. This suggests that adequate P_4 levels at day 5 post AI brings about better fertility. In the current study overall conception rate was 73.7 % in ALG fed cows compared to 63.2 % in Control fed cows, suggesting that higher P_4 levels at day 5 post AI, and numerically lower PGFM could improve fertility in the dairy cow although it was not statistically significant which could be down to an insufficient number of cows used. The conception rates to first service in the current study was 42.1 and 47.4 % for ALG and Control respectively, which is higher than the average UK herd, where pregnancy rates to first service is usually 40 – 42 % (AHDB, 2013). The unexpected high pregnancy rates to 1st service, especially in Control cows affected the overall pregnancy %, and it is hard to conclude whether this was due to better oestrus detection or if oestrus behaviour was affected by diet as neither of these factors were recorded.

5.5.0 Conclusion

The rapid increase in milk DHA content as well as C18:1 *trans* isomers that persisted until the end of the monitored period (14 weeks) suggests that the rumen

microbial ecosystem did not adapt to dietary supplementation of very LC-PUFA when dairy cows were supplemented with 100 g/d of ALG. The increase in milk DHA content as well as *cis*-9, *trans*-11 CLA improved the milk quality for human consumption without affecting milk performance. Furthermore, supplementing dairy cows with ALG has the potential to decrease plasma PGFM concentrations and increase milk P_4 levels at day 5 post AI, and did increase milk P_4 levels at day 20 post AI, which correlated with an improvement in overall conception. This suggests that ALG supplementation may have led to the formation of a larger CL that was able to secrete more P_4, reducing uterine secretion of $PGF_{2\alpha}$ leading to improved maternal recognition of pregnancy. Further research is required to confirm this as CL size was not reported in the current study, and greater number of cows per treatment are required to detect the possibility of significant differences.

CHAPTER 6: General discussion

6.1 Introduction

Public awareness of the healthiness of food, and the impact of agriculture on the environment has increased over recent years, giving rise to the need for public and policy engagement to enable a secure and sustainable food supply (Lang and Heasman, 2015). With the global population increasing, there is an urgent need for alternative feeds which cause less adverse effects on the environment. Microalgae has a very attractive nutrient profile, compared to conventional feeds (Lum *et al*., 2013). Public health policies implemented in most countries recommend a decrease in SFA consumption to lower the risk of developing CVD, with guidelines advising SFA intake of 10 -11 % of total energy intake (Kliem and Shingfield, 2016). But intakes of SFA exceeds these recommended levels in most countries (Kliem and Shingfield, 2016). In the EU dietary guidelines also acknowledge the importance of PUFA in preventing CVD, and it has been recommended that the general public consume 250 mg/d of EPA + DHA, with increased levels for pregnant women (Food and Agriculture Organization of the United Nations, 2010). Dairy products have been an important food component in the diet of humans for thousands of years (Rozenberg *et al*., 2016), but despite being rich in calcium, protein, potassium, phosphorus and iodine, dairy products have been criticised for their high levels of SFA (Rozenberg *et al*., 2016). Milk fat from cows contain higher concentrations of SFA and lower concentrations of PUFA compared to their diet, due to extensive lipolysis and biohydrogenation of PUFA to their saturated form in the rumen (Kliem and Shingfield, 2016). The FA composition of milk can be altered by dietary manipulation, and dietary supplementation of dairy cows with vegetable or marine oils have shown to increase the milk content of *n*-3 FAs and decrease the content of SFA (Moran *et al*., 2017; Shingfield *et al*., 2006; Sinedino *et al*., 2017). However, fat supplementation at high inclusion levels may have a negative impact on cow performance, decreasing DMI and causing milk fat depression (Franklin *et al*., 1999; Moate *et al*., 2013). Algae offers an alternative source of *n*-3 supplementation to ruminants, although its effects on rumen metabolism and uptake into milk and cheese have not been fully determined.

The hypothesis tested in this thesis was that the LC *n*-3 PUFA in ALG would partially resist biohydrogenation *in vitro*, increasing their uptake into milk, and would reduce plasma PGFM levels as an indicator of fertility. Dietary manipulation of dairy cows with ALG improved the FA profile of milk and cheese, with a greater effect at higher inclusion levels. There was no evidence of ruminal adaptation to the LC *n*-3 PUFA in ALG over time, and indicators of fertility were improved.

6.2 Effect of ALG on biohydrogenation *in-vitro*

The objective of the first study was to determine the effect of different inclusion levels of both ALG and FO on the biohydrogenation of EPA and DHA *in-vitro,* and to establish the effect of supplementation on CH_4 output. Both ALG and FO reduced CH_4 output at 30 h of incubation *in-vitro* compared to the control at all inclusion levels (20, 40, 60 and 80 mg DM). CH_4 production increased over time for all treatments, and at 72 hr of incubation CH_4 output was lowest at the highest inclusion level of ALG. These results demonstrate that ALG supplementation affects rumen fermentation, and may alter the activity of celluolytic bacteria due to an inhibitory effect on methanogenesis. This would explain the lower rate of biohydrogenation of both EPA and DHA when vessels were supplemented with ALG compared to FO and the control, and the lower extent of DHA biohydrogenation as the inclusion level of ALG increased. Biohydrogenation of both EPA and DHA was however extensive at all-time points (6, 12, 24 and 48 h). These time points were selected since previous studies have reported a lower biohydrogenation rate of EPA and DHA after 6 h of *in-vitro* incubation of rumen fluid with FO (Chow *et al*., 2004; Wa˛sowska *et al*., 2006). Since the completion of the *in-vitro* study, more recent research has been conducted on the biohydrogenation of DHA, and a study by Aldai *et al*., (2018) reported that biohydrogenation intermediates of DHA were formed as early as 1 hr after incubation, and by 2.5 h of incubation half of the DHA was biohydrogenated, with 80 % of the DHA having disappeared by 6 h. It therefore would have been interesting to include earlier time points in the current study to identify possible biohydrogenation intermediates of DHA.

6.3 Effect of dietary supplementation of ALG on cow performance and product FA profile

The 1st cow study was a 4 x 4 latin square, with four different inclusion levels of ALG of 0, 50, 100 and 150 g/ALG per cow/d. The objective of the study was to determine which inclusion level of ALG was most suitable to supplement dairy cows in order to increase the DHA content of both milk and cheese without affecting cow performance or cheese taste. The dietary treatments supplied 0, 8, 16 and 24 g of DHA per cow/d. The study demonstrated that DMI and milk yield were unaffected by ALG supplementation, but that milk fat content and yield decreased linearly as the inclusion level of ALG increased in the diet, which was associated with an increase in milk *trans*-10 *cis*-12 CLA, a potent inhibitor of milk fat synthesis (Hussein *et al*., 2013; Peterson *et al*., 2003; Sinclair *et al*., 2007). Some research has been conducted to determine other biohydrogenation intermediates that cause milk fat depression, with C18:1 *trans*-10 having been reported to reduce milk fat content (Chilliard *et al*., 2001), a finding that is supported by the 1st dairy cow study as there was a linear increase in milk fat content of C18:1 *trans*-10 as the

feeding level of ALG increased. Added dietary ALG altered individual milk FA, similar to other studies that have fed marine oils to dairy cows (Franklin *et al*., 1999; Sinedino *et al*., 2017). As the dietary inclusion level of ALG increased a linear decrease in SFA was observed, along with a linear increase in MUFA and PUFA, suggesting that supplementation with LC-PUFA reduced the biohydrogenation rate of PUFA to their saturated form, as observed in the *in-vitro* study. The main outcome of this study was to increase milk and cheese DHA concentration, with an increase of 0.29 mg/100 g FA in both milk and cheese achieved when feeding 150 g/ALG per cow/d, improving the quality of these dairy products for human consumption.

Dairy products with high concentrations of PUFA are sensitive to oxidation, which has been associated with the development of undesirable flavours (e.g rancidity), spoiling their sensory properties (Fauteux *et al*., 2016). Sensory evaluation of the cheddar cheese during the 1st cow study was conducted when the cheese had reached maturity (>6 months old), and a trained sensory panel was used to test the cheese over 32 different sensory attributes, ranging from odour, appearance, flavour and aftertaste using a 15-point scale. Increasing dietary inclusion of ALG resulted in a linear increase in an acidic note odour, air holes and a pleasant nutty flavour, and resulted in an acidic and bitter aftertaste, whilst decreasing the firmness and creaminess of the cheese. Despite having a few significant sensory characteristics between the cheese samples, the scores within the 15-point scale were very similar. No research has previously been conducted on cheese sensory quality made from cows milk that were fed ALG, therefore the results from the 1st dairy cow study provide valuable information for future work on increasing DHA content in dairy products without adverse effect on sensory quality. Cheese weight and yield were recorded, and despite a reduction in milk fat content with increasing feeding level of ALG, there was no difference between the yield and weight of cheese made from milk with a lower fat content, although further work is required in order to confirm that milk fat reduction is not important for the production of cheese. These results demonstrate that it is possible to include ALG in the diet of dairy cows to increase the healthiness of dairy products, without having an impact on cheese yield, and only having a minor influence on cheese taste. Feeding ALG appeared to affect rumen microbial metabolism leading to a decrease in DM, OM and NDF digestibility as the dietary inclusion level of ALG increased, and therefore high amounts of LC-PUFA in the diet of dairy cows is not advised. No previous work has been conducted on the effects of ALG supplementation on diet digestibility, the results from this study provides evidence that high inclusion levels should be avoided. Due to the effect on digestibility at the highest inclusion level of ALG, 100 g/ ALG per cow/ d was used for the 2nd dairy study.

The 2nd dairy cow study investigated the impact of supplementation of ALG to dairy cows from 3 weeks post calving for 14 weeks on milk FA profile to observe any shift in

biohydrogenation or rumen adaptation over time. Again the results of the study demonstrated that ALG supplementation had no effect on DMI or milk yield. In contrast to the 1st study there was no effect of feeding ALG on milk fat content or yield. In line with this observation there was no difference in milk fat content *trans*-10 *cis*-12 CLA. Results from both dairy cow studies support the role of *trans*-10 *cis*-12 CLA in milk fat depression, with a relative high correlation (r^2) of 0.72 (Figure 6.1). Similarly Baumgard *et al*., (2001) reported a similar relationship between milk fat yield and milk *trans*-10 *cis*-12 CLA when increasing concentration of *trans*-10 *cis*-12 CLA was included in the diet of cows, with increasing milk concentration of *trans*-10 *cis*-12 correlating with a reduction in milk fat synthesis. Milk FA profiles were significantly altered by feeding ALG, similar to results from the 1st dairy cow study, with C18:1 *trans*-10 being increased in milk from cows fed the ALG. Figure 6.2 illustrates the low correlation (r^2) of 0.14 between C18:1 *trans*-10 concentration and milk fat content (g/kg) which does not support the finding that this FA is primarily involved in milk fat depression, a finding in accordance with Lock *et al*., (2007). Milk FAs were measured at weeks 0, 1, 2, 4, 8 and 14 of the study as it was hypothesised that a rapid change in milk FA profile would occur during the first few weeks of supplementation, and that rumen adaptation would occur, decreasing milk DHA content and some biohydrogenation intermediates by week 5, as reported by others (Bichi *et al*., 2013; Shingfield *et al*., 2006). Cows fed the ALG had a higher milk fat content of C18:1 *trans* 8, 9, 10, 11 and 12, with C18:1 *trans* 8, 9, 10 and 11 remaining higher in the ALG fed cows compared to the control animals throughout the study. Previous studies have reported biohydrogenation shifts when supplementing ruminants with oils (Shingfield *et al*., 2006), where C18:1 *trans*-11 concentrations decrease as the content of C18:1 *trans*-10 increases. The results obtained in the 2nd dairy cow study demonstrate that a shift in biohydrogenation did not occur. Some studies have attempted to investigate rumen adaptation when supplementing marine oils to dairy cows (Franklin *et al*., 1999; Vlcek *et al*., 2017), but these studies were comparably short, lasting only 6 weeks. Other studies that have supplemented marine oils for a longer period of time did not report milk FA profile over time (Sinedino *et al*., 2017). The results of the 2nd dairy cow study demonstrated that rumen adaptation did not occur when supplementing LC-PUFA to dairy cows, that the milk fat content of DHA, *cis*-9 *trans*-11 CLA and total *n*-3 increased rapidly in the first few weeks of ALG supplementation and remained high over the 14 week period, whereas the milk fat content of SFA remained higher in the control fed cows. There is relatively little research on the change in individual milk FA profiles over time, and this thesis provides new information on the effects of feeding of LC-PUFA on individual milk FA profiles for a 14 week period.

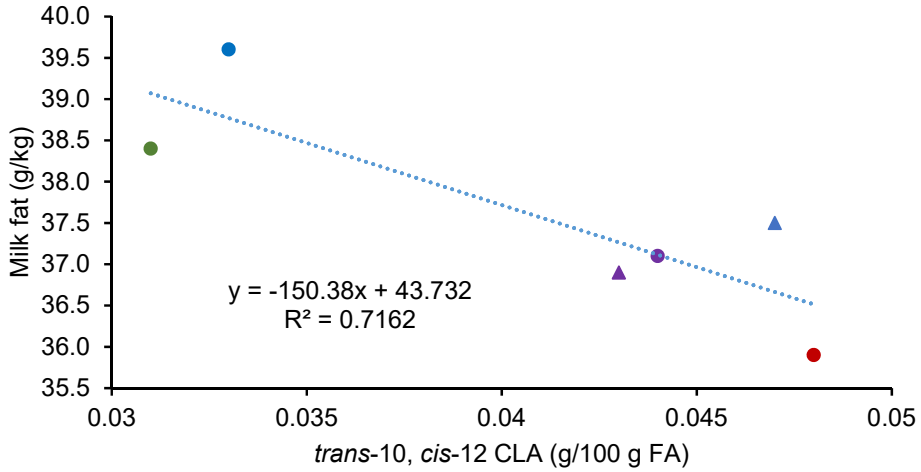


Figure 6.1. Effect of supplementing dairy cows with various levels of ALG on the correlation of milk fat content (g/kg) to milk fat content of *trans*-10, *cis*-12 CLA (g/100 g FA). ●▲ no ALG, experiment 1 and 2; ● 50 g/ALG per cow/d; ●▲100 g/ALG per cow/d, experiment 1 and 2; ●150 g/ALG per cow/d.

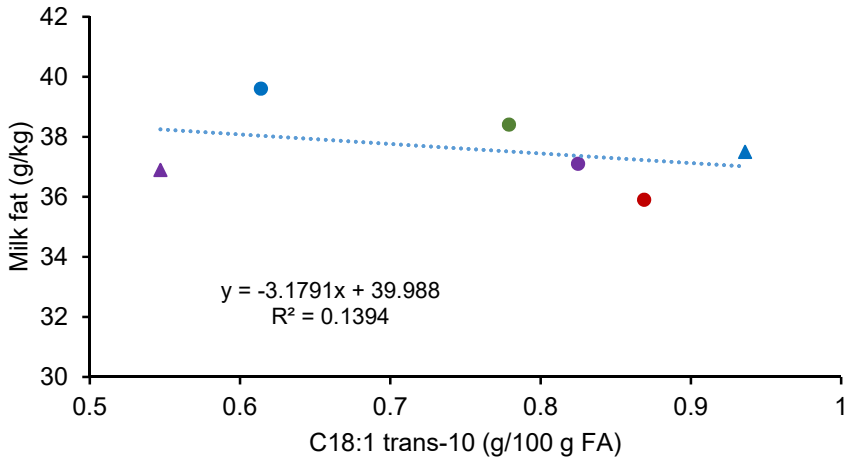


Figure 6.2. Effect of supplementing dairy cows with various levels of ALG on the correlation of milk fat content (g/kg) to milk fat content of C18:1 *trans*-10 (g/100 g FA). ●▲ no ALG, experiment 1 and 2; ● 50 g/ALG per cow/d; ●▲100 g/ALG per cow/d, experiment 1 and 2; ●150 g/ALG per cow/d.

6.4 Effect of dietary supplementation of ALG on indicators of fertility

The effect of ALG supplementation on indicators of fertility was also determined in the 2nd dairy cow study. Previous studies have reported an improvement in conception rate when feeding linseed (Petit *et al*., 2001) or ALG (Siedino *et al*., 2017), and a reduction in early embryo mortality when feeding FO (Elis *et al*., 2016) to dairy cows. The secondary objective of the 2nd dairy cow study was to measure plasma concentrations of PGFM, a metabolite of $PGF_{2\alpha}$, as an indicator of fertility as low concentrations of $PGF_{2\alpha}$

are important for maternal recognition of pregnancy (Binelli and Thatcher, 1999). The $PGF_{2\alpha}$ secretion is inhibited by P_4, indicating the importance of a large CL for adequate secretion of P_4 (Asselin *et al*., 1997). Plasma PGFM was measured following a synchronised programme, and on d 17 of the oestrus cycle blood was collected from a jugular catheter every 15 min for 4 h following an oxytocin challenge. A reduction in plasma PGFM mean, peak and area under the curve was reported, although the reduction did not reach statistical significance, possibly due to the comparably low number of animals or the sensitivity of PGFM to day of sampling, as reported by others (Childs *et al*., 2008; Robinson *et al*., 2002). Milk P_4 were also measured at d 5 and d 20 post AI and it was demonstrated that feeding ALG from 3 weeks post calving increased milk P_4 in pregnant cows, suggesting that a larger CL was produced. In order to confirm that the CL was larger in ALG fed cows, ultrasound scanning would have been required, as previous studies have reported a larger CL when feeding FO (Childs *et al*., 2008), and a larger dominant follicle when feeding fish meal (Moussavi *et al*., 2007) to dairy cows. Overall conception rates were demonstrated to be higher in ALG fed cows (73.7 v 63.2 %), but the difference were not significant, and the conception rates were higher for both treatments compared to the UK average (40-42 %) (AHDB, 2013). Higher conception rates could also be a result of better oestrus detection, due to a stronger and longer duration of oestrus behaviours demonstrated by the cows. Zachut *et al*., (2011) reported an increase in the duration of oestrus behaviour in cows fed linseed compared to a control diet, and cows supplemented with *n*-3 rich linseed also exhibited stronger intensity of oestrus behaviour. No studies have investigated the effect of ALG supplementation on oestrus behaviour in dairy cows, and this is an area that requires further investigation.

6.5 Conclusions

Results obtained in this thesis over the three experiments support the hypothesis that LC *n*-3 PUFA in ALG can partially resists biohydrogenation in the rumen, and can increase milk and cheese DHA content, improving the healthiness of dairy products for human consumption, whilst also improving indicators of fertility in the dairy cow. Supplementation of 100 g/ALG per cow/d is the most appropriate inclusion level, due to negative effects on cow performance and diet digestibility at higher inclusion levels. Supplementing 100 g of ALG/d will also increase milk DHA content, and there is no evidence of rumen adaptation over time. Algae may also be supplemented to dairy cows to improve fertility, by decreasing plasma PGFM concentration and increasing milk P_4 levels, helping with early maternal recognition and reducing early embryo losses, although further research is required.

CHAPTER 7: References

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