BOVINE MILK FAT TRIACYLGLYCEROLS: Factors influencing their composition and structure, and its effect on milk fat solid fat content

Sara Liliana Pacheco Pappenheim

Propositions

1. By changing the cow's diet, bovine milk fat triacylglycerol structures can be made more similar to human milk.
   (This thesis)

2. Insights in milk triacylglycerol composition and its saturation degree can be used to predict the changes in milk solid fat content.
   (This thesis)

3. In order to avoid repeating mistakes in scientific research, it is important to publish failed experiments.

4. It is a pity that today one of the roles of science is to report on how human lifestyle destroys the earth.

5. Without fears, individuals would be completely free.

6. Music is the best comfort for the heart and soul.

Propositions belonging to the thesis, entitled:

"Bovine milk fat triacylglycerols: Factors influencing their composition and structure, and its effect on milk fat solid fat content."

Sara L. Pacheco Pappenheim

Wageningen, 27th of October 2021

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Thesis

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To my Family

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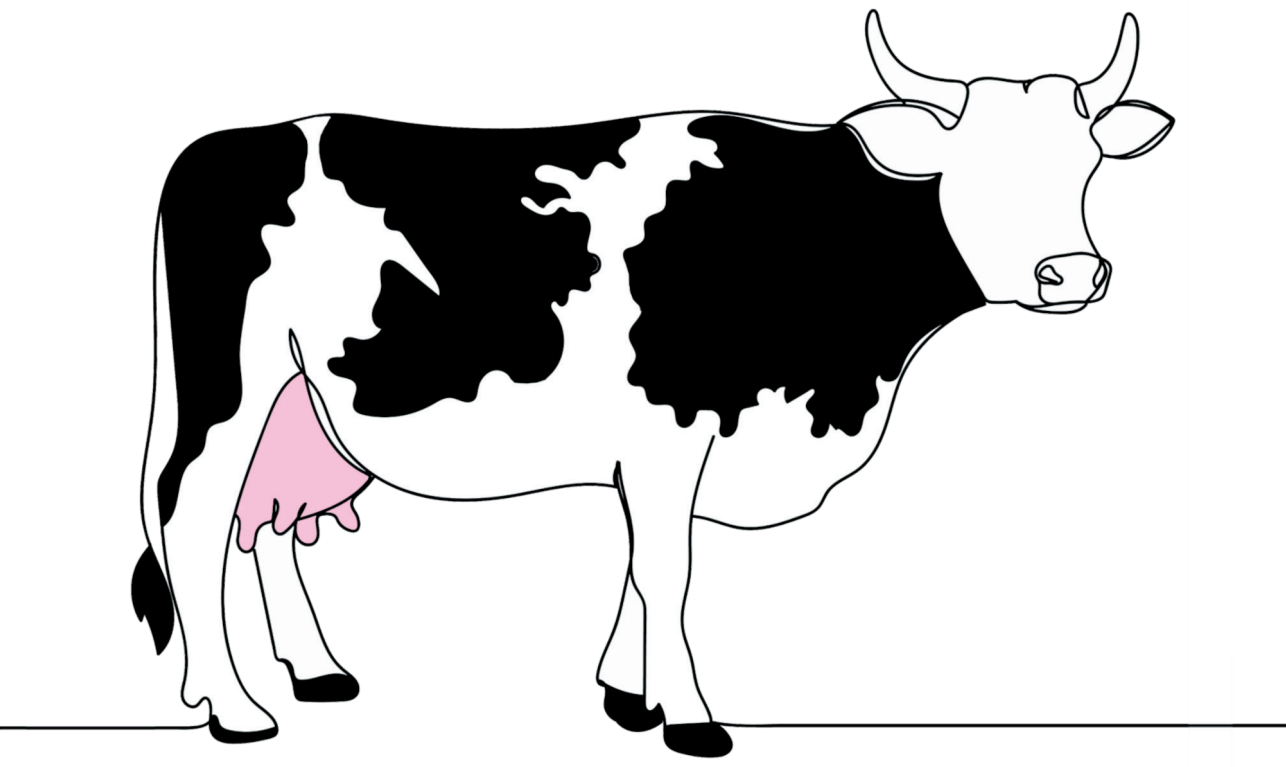
List of abbreviations

ACACA	Acetyl-CoA carboxylase alpha
ACSL	Acetyl CoA synthase-long chain
ACSM	Acetyl CoA synthase-medium chain
ACSS	Acetyl CoA synthase-short chain
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
ALA	Linolenic acid
BCFA	Branched-chain fatty acids
BTN	Butyrophilin
CLA	Conjugated linoleic acid
CN	Carbon number
DAG	Diacylglycerol
DB	Double bonds
DGAT	Diacylglycerol O-acyltransferase
DHA	Docosahexaenoic acid
DIM	Days in milk
EPA	Eicosapentaenoic acid
FA	Fatty acids
FABP	Fatty acid binding proteins
FAEE	Fatty acid ethyl esters
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FFA	Free fatty acids
G3P	Glycerol-3-phosphate
GC-FID	Gas chromatography - flame ionization detector
GPAT	Glycerol-3-phosphate acyltransferase
HF	High fat
HMW	High molecular weight
HP	High protein
LA	Linoleic acid
LCFA	Long-chain fatty acids
LF	Low fat
LIPIN	Lipid phosphate phosphatases
LMW	Low molecular weight
LP	Low protein
LPA	Lysophosphatidic acid
LPL	Lipoprotein lipase
2-MAG	2-Monoacylglycerol
MAG	Monoacylglycerol
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization - time of flight - mass spectrometry
MCFA	Medium-chain fatty acids
MEC	Mammary epithelial cell
MFGM	Milk fat globule membrane
MMW	Medium molecular weigh
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids

MW	Molecular weight
NEB	Negative energy balance
NEFA	Non-esterified FA
NMR	Nuclear magnetic resonance
OBCFA	Odd-branch chain fatty acids
OCFA	Odd-chain fatty acids
PA	Phosphatidic acid
PLIN2	Adipophilin (protein)
PUFA	Polyunsaturated fatty acids
SCD	Stearoyl-CoA desaturase
SCFA	Short-chain fatty acids
SER	Smooth endoplasmic reticulum membrane
SFA	Saturated fatty acids
SFC	Solid fat content
SLCFA	Saturated long-chain fatty acids
sn	stereospecific numbering
TAG	Triacylglycerols
TFA	Trans fatty acids
TLC	Thin layer chromatography
UFA	Unsaturated fatty acids
VFA	Volatile fatty acids
VLDL	Very-low-density lipoproteins
VLDL-R	Very low-density lipoproteins-Receptor
XDH	Xanthine dehydrogenase

Chapter 1

General introduction



1. Thesis overview:

The volume of bovine milk produced globally has increased steadily in the last years. According to FAO, by the end of 2019, 522 million metric tons of milk were produced worldwide (FAOSTAT 2019). This large milk production is expected to further increase up to 1000 million tons by 2025 (IDF 2015). There is thus a need for a constant update of knowledge on how to make better use of milk to improve (dairy and non-dairy) food products for the benefit of human well-being.

Fat is one of the most valuable components of milk. It determines its quality and influences its technological, sensorial, and nutritional properties (Cozma et al., 2013). The triacylglycerols (**TAG**; 98.3%), which are the main fractions in milk fat (Walstra et al., 2005), vary in their composition depending on both animal (genetics, lactation stage, etc.) and feed related factors (Jensen, 2002). Variation in TAG composition directly influences milk fat properties, such as its digestibility, melting point, and solid fat content (**SFC**; Jensen, 2002; Karupaiah and Sundram, 2007; Tzompa-Sosa et al., 2016a; Mohan et al., 2020). In turn, these variations in milk fat properties influence essential characteristics of dairy products rich in fat, such as the spreadability of butter and the creaminess of ice-cream (Ashes et al., 1997; Mohan et al., 2020). Therefore, understanding the factors that affect TAG composition in milk fat is essential in order to predict its properties, so as to determine its suitability for different food product applications.

The structure of TAG is composed of three fatty acids (**FA**) bound to a glycerol molecule by means of an ester bond. Hence, the characteristics and properties of these three FA determine the structural characteristics and properties of TAG. Until now, milk FA composition and the factors that affect it have been extensively studied (Jensen, 2002; Elgersma et al., 2006). It is well known that bovine milk fat content and FA composition can be influenced by variations in cattle feeding, genetics and selective breeding, as well as in the cows' lactation stage (Jensen, 2002; Harvatine et al., 2009; Samková et al., 2012). However, scarce information can be found on the effect of these factors on TAG composition and its structure.

This chapter will give an overview of key concepts to understand how variations in dairy cattle feeding, genetics, and cows' lactation stage affect TAG composition in bovine milk fat. In order to do so, first a general overview of milk fat composition will be given (Section 1.1). Next, the biosynthesis of milk fat in the mammary epithelial cell (**MEC**) will be explained as background information for understanding the impact of genetic variation on milk fat composition (Sections 1.2, 1.3 and 1.4). Then, detailed information on the TAG composition and structure in bovine milk fat will be given to comprehend its variation and to relate it to the different factors (Sections 1.5 and 1.6). Last, the link between the variation in the TAG composition and SFC will be explained (Section 1.7).

1.1. Milk lipids: General overview

Milk lipids, most commonly referred to as milk fat, are amongst of the most complex fats in biological lipids (Palmquist, 2006). Milk fat in bovine milk (2.5 to 5.5%) occurs as globules emulsified in the aqueous phase (87%) (Jensen, 2002; Walstra et al., 2005). The milk fat globule consists of a trilayer membrane formed during fat synthesis and secretion in the MEC and encloses mainly TAG. The membrane itself is composed of phospholipids, sphingolipids, cerebrosides, gangliosides and sterols (**Figure 1.1**) with proteins embedded in it. These components are polar and surface active; hence, they contribute to the milk fat globule membrane (**MFGM**) stability. Sterols, mainly cholesterol in milk fat, are present in the MFGM as well as in the core of the fat globule. The MFGM (10 to 20 nm thick) acts as an emulsifying agent and protects the fat globules from coalescence and enzymatic degradation (Dewettinck et al., 2008).

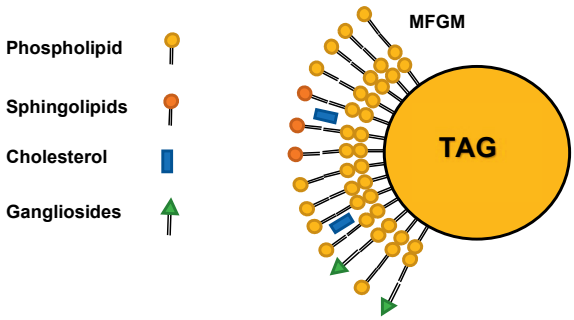
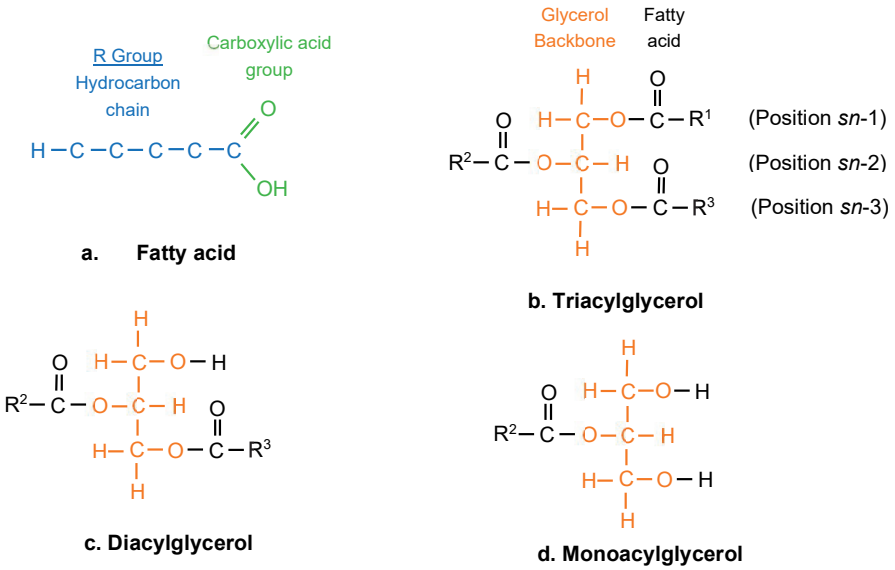


Figure 1.1. Structure of the milk fat globule membrane. Adapted from Kwak et al. (2012). MFGM: Milk fat globule membrane, TAG: Triacylglycerol.

Fatty acids compose the structure of the major component in milk fat, the acylglycerols. Among the acylglycerols, TAG constitute almost 98.3%, whereas diacylglycerols (**DAG**) and monoacylglycerols (**MAG**) make up only 0.3%. **Figure 1.2** presents the structure of a FA and different acylglycerols. The FA structure is a hydrocarbon chain with a carboxylic acid group at its end, whereas the acylglycerols structures are one (mono), two (di), or three (tri) FA bound through an ester bond to a glycerol molecule (**Figure 1.2**). The positions at which the FA are esterified to the glycerol molecule are identified by their stereospecific numbering (***sn***) as *sn*-1, *sn*-2 and *sn*-3 (**Figure 1.2b**). The positional distribution of the FA is non-random and is defined by the enzymes involved in the TAG synthesis in the mammary gland (see Section 1.2). Jensen et al. (2002) reported the presence of 416 different fatty acids in bovine milk fat that include a wide array of structures, ranging from 4 to 22 carbon atoms, and varying in number of double bonds, double bond configuration (*cis*

and *trans*), and inclusion of methyl branches on the hydrocarbon chain (*iso* and *anteiso*). Considering the large variety of fatty acids in milk fat, combined with the many possible combinations of different fatty acids within the acylglycerol structure, this results in a huge range of acylglycerol with different compositions and properties.



a. Fatty acid

b. Triacylglycerol

c. Diacylglycerol

d. Monoacylglycerol

Figure 1.2. Structure of fatty acids and acylglycerols.

Fatty acids can be grouped according to their structure characteristics. **Table 1.1** below presents the FA groups defined in this thesis. The FA groups are based on their saturation degree and on the length of their hydrocarbon chain. The saturation degree refers to the number of double bonds, whereas the length of the hydrocarbon chain refers to the number of carbon atoms present in their structure. The saturation degree is defined as either saturated or unsaturated. Saturated FA (**SFA**) structures do not contain double bonds, while unsaturated FA (**UFA**) structures do include one or more double bonds in their structures. Within the unsaturated FA group, two subgroups are generally defined: monounsaturated and polyunsaturated. The monounsaturated FA structures contain one double bond, and the polyunsaturated FA structures contain two or more double bonds. Considering the definition of FA according to their hydrocarbon chain length, the FA structures are generally defined as short (4-11 carbon atoms), medium (12-17 carbon atoms), long (>18 carbon atoms) and odd-branched chain (uneven carbon number and *iso* and *anteiso* structures).

Table 1.1. Definitions of fatty acids (FA) groups.

Fatty acid groups according to saturation degree		
Group name	**Acronym**	**Formula**
Saturated FA	SFA	C4:0, C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0*iso*, C14:0, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, Pristanic-acid, C16:0, C17:0*iso*, C17:0*anteiso*, Phytanic-acid, C17:0, C18:0, C19:0, C20:0, C22:0, C24:0
Unsaturated FA	UFA	C10:1*cis*9, C12:1*cis*9, C14:1*cis*9, C16:1*trans*9, C16:1*cis*9, C17:1*cis*9; C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*cis*9, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:1*cis*14, C18:1*cis*15, C18:2*cis*9,12 (LA), C18:3*cis*6,9,12 (GLA), C18:3*cis*9,12,15 (ALA), C18:2*cis*9,*trans*11 (CLA), C20:3*cis*8,11,14 (DGLA), C20:4*cis*5,8,11,14 (AA), C20:4*cis*8,11,14,17 (ETA), C20:5*cis*5,8,11,14,17 (EPA), C22:5*cis*7,10,13,16,19 (DPA)
Monounsaturated FA	MUFA	C10:1*cis*9, C12:1*cis*9, C14:1*cis*9, C16:1*trans*9, C16:1*cis*9, C17:1*cis*9; C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*cis*9, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:1*cis*14, C18:1*cis*15
Polyunsaturated FA	PUFA	C18:2*cis*9,12 (LA), C18:3*cis*6,9,12 (GLA), C18:3*cis*9,12,15 (ALA), C18:2*cis*9,*trans*11 (CLA), C20:3*cis*8,11,14 (DGLA), C20:4*cis*5,8,11,14 (AA), C20:4*cis*8,11,14,17 (ETA), C20:5*cis*5,8,11,14,17 (EPA), C22:5*cis*7,10,13,16,19 (DPA)
Fatty acid groups according to chain length		
Groups	**Acronym**	**Formula**
Short-chain FA	SCFA	C4:0, C5:0, C6:0, C 7:0, C8:0, C9:0, C10:0, C10:1*cis*9, C11:0
Medium-chain FA	MCFA	C12:0, C12:1*cis*9, C13:0, C14:0*iso*, C14:0, C14:1*cis*9, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, Pristanic acid, C16:0, C16:1*trans*9, C16:1*cis*9, C17:0*iso*, C17:*0anteiso*, Phytanic acid, C17:0, C17:1*cis*9
Long-chain FA	LCFA	C18:0, C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*cis*9, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:1*cis*14, C18:1*cis*15, C18:2*cis*9,12 (LA), C19:0, C18:3*cis*6,9,12 (GLA), C18:3*cis*9,12,15 (ALA), C18:2*cis*9,*trans*11, C20:0, C20:3*cis*8,11,14 (DGLA), C20:4*cis*5,8,11,14 (AA), C22:0, C20:4*cis*8,11,14,17 (ETA), C20:5*cis*5,8,11,14,17 (EPA), C24:0, C22:5*cis*7,10,13,16,19 (DPA)
Odd-branched chain FA	OBCFA	C5:0, C7:0, C9:0, C11:0, C13:0, C14:0*iso*, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, C17:0*iso*, C17:0*anteiso*, C17:0, C17:1*cis*9, C19:0

1.2. Milk fat biosynthesis in the mammary gland

Milk fat biosynthesis and secretion in the bovine mammary gland has been studied in great detail in the past years, with special focus on the MEC, where all the processes take place (Clegg et al., 2001; Lu et al., 2014; Osorio et al., 2016). **Figure 1.3** presents a schematic overview of the milk fat biosynthesis pathways in the MEC (Lu et al., 2014; Osorio et al., 2016).

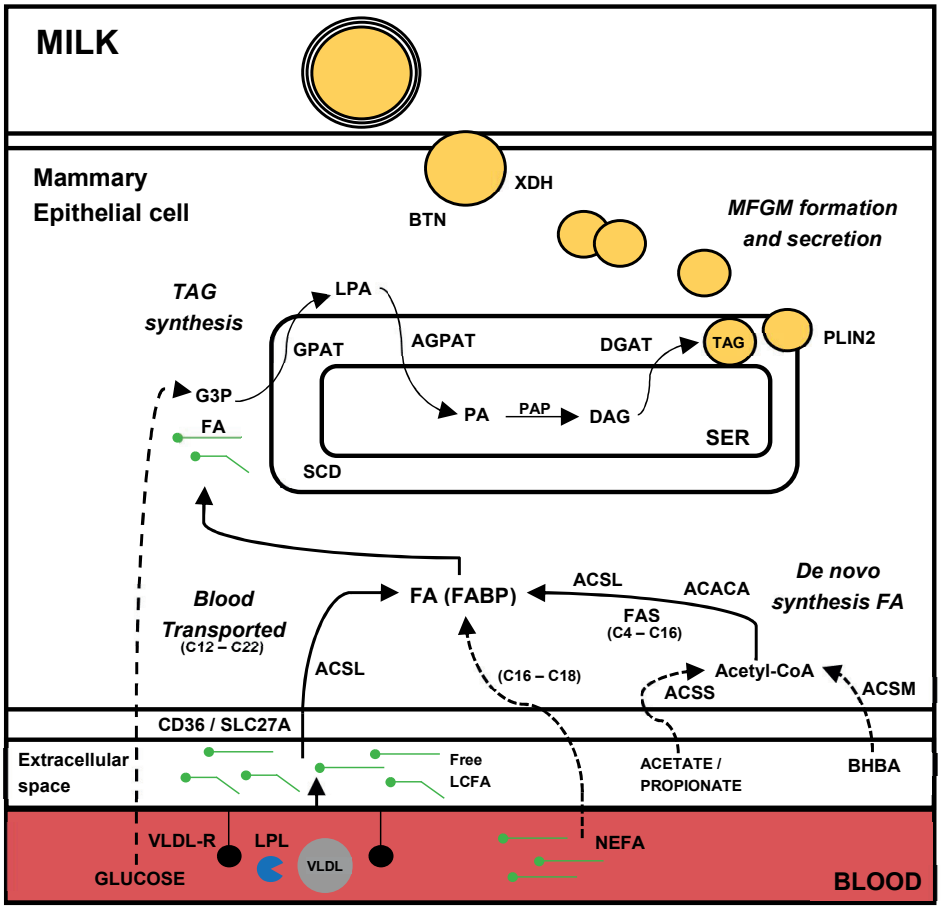


Figure 1.3. Schematic representation of milk fat synthesis. Adapted from Osorio et al. (2016) and Lu et al. 2014). ACACA: Acetyl CoA carboxylase alpha, ACSL: Acetyl CoA synthase-long chain, ACSM: Acetyl CoA synthase-medium chain, ACSS: Acetyl CoA synthase-short chain, AGPAT: 1-acylglycerol-3-phosphate-O-acyltransferase, BHBA: β-hydroxybutyrate, BTN: Butyrophilin, CD36: FA translocase, DAG: Diacylglycerol, DGAT: Diacylglycerol-O-acyltransferase, FABP: FA binding protein, G3P: Glycerol-3-phosphate, GPAT: Glycerol-3-phosphate acyltransferase, LCFA: Long chain FA, LPA: Lysophosphatidic acid, LPL: Lipoprotein lipase, MEC: Mammary epithelial cell, MFGM: Milk fat globule membrane, NEFA: Non esterified FA, PA: Phosphatidic acid, PAP: Phosphatidate phosphatases, PLIN: Adipophilin, SCD: Δ9 stearoyl CoA desaturase, SER: Smooth endoplasmic reticulum, SLC27A: FA transporter protein, TAG: Triacylglycerol, VLDL: Very-low-density lipoproteins, VLDL-R: Very low-density lipoproteins-Receptor, XDL: Xanthine dehydrogenase.

The first step towards milk fat biosynthesis is the absorption of different types of FA from the blood for lipid synthesis in the MEC. These types of FA include volatile fatty acids (**VFA**), which are used as precursors for FA synthesis in the MEC (also called *de novo* synthesized FA), as well as non-esterified FA (**NEFA**), which can be directly used for TAG synthesis. The FA with 16 and less carbon atoms can originate from blood and be *de novo* synthesized in the MEC, whereas the fatty acids with 18 and more carbon atoms are only derived from blood. The precursors taken up for *de novo* synthesized FA, mainly being VFA acetate (C2), propionate (C3) and β-hydroxybutyrate (**BHBA**, formed out of butyrate; C4), have been largely produced during microbial fermentation of feed in the rumen (see Section 1.3). Acetate and BHBA are activated by acetyl-CoA synthase-short chain (**ACSS**) and acetyl-CoA synthase-medium chain (**ACSM**) to form acetyl-CoA. Next, the enzyme acetyl-CoA carboxylase alpha (**ACACA**) catalyzes the formation of malonyl-CoA used later by fatty acid synthase (**FAS**) for FA chain elongation, forming FA with 4 to 16 carbons. These newly formed FA are further activated by ACSL and bound to FABP to be transported to the SER for TAG synthesis. Parallel to this pathway, the FA taken up by the MEC from blood are obtained by the hydrolysis of very-low-density lipoproteins (**VLDL**) in the blood by lipoprotein lipase (**LPL**). After hydrolysis, these FA (ranging from C12 to C22) are transported by the VLDL-Receptor (**VLDL-R**) and transferred to the extracellular space, where they are taken up either by FA translocase CD36, FA transporter protein SLC27A, or the acetyl-CoA synthase-long chain (**ACSL**), after which they are bound intracellularly to fatty acid binding proteins (**FABP**). The FABP transport the LCFA to the smooth endoplasmic reticulum membrane (**SER**) for TAG synthesis.

Once the FA reach the SER, part of the FA (mainly the SFA) will be desaturated by Δ9 stearoyl-CoA desaturase (**SCD**), thus creating a *cis*-9 bond in the FA structure. At the same time, the first step for TAG synthesis occurs. A FA is esterified at the *sn*-1 position to the precursor glycerol-3-phosphate (**G3P**; mainly produced by glycolysis). This reaction is catalyzed by glycerol-3-phosphate acyltransferase (**GPAT**), thus forming lysophosphatidic acid (**LPA**). The LPA is acylated by the enzyme 1-acylglycerol-3-phosphate O-acyltransferase (**AGPAT**) in the *sn*-2 position, forming either phosphatidic acid (**PA**), which is later dephosphorylated by phosphatidate phosphatases (**PAP**), or lipid phosphate phosphatases (**LIPIN**), forming a diacylglycerol (**DAG**; 1,2-diacyl-*sn*-glycerol). Finally, the enzyme diacylglycerol O-acyltransferase (**DGAT**) catalyzes the esterification of a third FA to the DAG, forming a TAG. Once the TAG species are formed in the SER membrane, the formation of the lipid droplet starts with the support of the protein adipophilin (**PLIN2**) and the phospholipids surrounding the lipid droplet. The lipid droplets intracellularly coalesce to form bigger fat droplets, followed by active exocytosis from the MEC into the milk, assisted by the proteins xanthine dehydrogenase (**XDH**) and butyrophilin (**BTN**).

1.3. Volatile FA (VFA): Precursors of *de novo* FA formed in the rumen

One particular characteristic of ruminants is the fermentation of feed in the rumen, a process that makes it possible for cows to partially digest plants (*e.g.* grass). During this process, VFA are formed, which are subsequently absorbed by the papillae of the rumen wall into the blood. The VFA in the blood are absorbed into the MEC for further use as precursors for, amongst others, *de novo* FA synthesis (Figure 1.3). The microorganisms present in the rumen are bacteria, protozoa and fungi, of which bacteria compose most of the biomass. The main VFA formed by the microbiota in the rumen are acetic, propionic and butyric acid, and their production is determined by the cows' diet. In general, the diet of the cows is composed of carbohydrates (75-80%), protein (15-20%), and fat (<6%). Within the carbohydrates, a high intake of fibers (*e.g.* forages such as grass) increases greatly the formation of acetic acid, whereas a high consumption of sugars (*e.g.* molasses) increases the formation of butyric acid. Next to this, a high intake of starch (*e.g.* corn), results in increased formation of propionic acid. Protein fermentation in the rumen can also lead to the formation of acetic, propionic and butyric acid as well as branched-chain FA and valeric acid (C5:0). Propionic and valeric acid and branched-chain FA are precursors for *de novo* synthesis of OBCFA in the MEC (Dijkstra, 1993; Vlaeminck et al., 2006; Fievez et al., 2012).

1.4. Mono- and polyunsaturated fatty acids metabolism

1.4.1. Rumen biohydrogenation

Rumen microorganisms are responsible for biohydrogenation processes that transform dietary PUFA into SFA, forming *trans* FA as intermediate products (Chilliard et al., 2000; Jensen, 2002). During this process, first isomerization takes place, forming the *trans* configuration, followed by hydrogenation of the *cis* parts (Lourenço et al., 2010). The major plant PUFA available in the cows' diet is linoleic acid (C18:2*cis*9,12, **LA**), present at high concentration in corn silage, and linolenic acid (C18:3*cis*9,12,15, **ALA**), present at high concentration in fresh grass (Palmquist, 2006). The biohydrogenation of these two PUFA form C18:1 *trans* MUFA and C18:0 as products, which are absorbed in the MEC for fat synthesis (Chilliard et al., 2000; Shingfield et al., 2008).

1.4.2. Desaturation and elongation pathways in the mammary gland

Desaturation and elongation pathways in the MEC are responsible for the formation of LCFA and PUFA. The elongation of *de novo* synthesized SCFA up to 16 carbons is performed by FAS (Figure 1.3), whereas the elongases synthesize saturated and unsaturated LCFA (*e.g.* C20 to C24 FAs; Leonard et al., 2004; D.B. Jump, 2009). Elongases are enzymes present in the MEC that synthesize saturated and unsaturated LCFA, adding two carbon atoms to the carboxyl end of a FA chain. The FA elongases work together with FA desaturases, to form MUFA and PUFA, which are later taken up for TAG synthesis by the MEC (D.B. Jump, 2009). Examples of collaboration of FA desaturase and elongase are the Δ9 desaturase (SCD) / elongase pathway forming *cis*-9 MUFA and the combined association of Δ5 and Δ6 desaturases and elongase in the n-3 and n-6 pathways responsible for the formation of n-3 and n-6 PUFA (*e.g*. docosahexaenoic acid (**DHA**); Leonard et al., 2004; Jump D.B, 2009; Russo, 2009).

1.5. Triacylglycerol (TAG) composition in milk fat

1.5.1. Triacylglycerol profile and structure of milk fat

The major TAGs in milk fat are presented in **Table 1.2**, where the total carbon number (**CN**) of a TAG represents the total summed carbon atoms of the three FA esterified on the glycerol backbone. Triacylglycerols are composed of several molecular species with the same CN and different saturation degree (sum of number of double bonds; Gresti et al., 1993; Liu et al., 2020). For example, CN32 includes the TAG species with 1 to 5 double bonds in its structure, referred as CN32:1, CN32:2, CN32:3, CN32:4 and CN32:5 (Liu et al., 2020). Liu et al. (2020) reported the most updated list of TAG present in milk fat with a total of 3452 TAG species. These TAG species can be an even-chain (*e.g.* CN28, CN30) or an odd-chain (*e.g.* CN37, CN41). The even-chain TAG species are the most abundant ones, whereas the odd-chain TAG species vary in total concentration from 5 to 15 g/100 g milk fat (Gresti et al., 1993; Liu et al., 2020). Among the even chain TAG, the TAG with the highest concentrations are CN36, CN38, CN40, CN50 and CN52, whilst the TAG with the lowest concentrations are CN26, CN28, CN30 and CN32 (Table 1.2). Next to the even TAGs, milk fat also contains minor (≤0.1%) TAG species <CN24 and TAG >CN54, which are not included in Table 1.2.

According to the FA composition in the TAG structures, different categories can be distinguished, based on the molecular weight (**MW**; the summed chain lengths of FA) and the saturation degree. Based on the MW, TAG species are defined as low, medium and high MW TAG groups. The low MW (**LMW**) TAG group contains TAG species with 26 to 36 carbons, the medium MW (**MMW**) TAG group contains TAG species with 38 to 48 carbons, and the high MW (**HMW**) TAG group contains TAG species with 50 to 54 carbons. The FA mainly esterified in each of these TAG group structures are: SCFA in the LMW, SCFA and

MCFA and C18 FA in the MMW, and C16:0 and LCFA in the HMW TAG. Regarding the categorization based on saturation degree, the TAG species can also be classified into saturated, monounsaturated, and polyunsaturated. Saturated TAG species contain only SFA, the monounsaturated TAG species one MUFA, and the polyunsaturated TAG encompass MUFA and/or PUFA in their structure.

Table 1.2. Triacylglycerol (TAG) composition of bovine milk fat measured by gas chromatography – flame ionization detector adapted from Jensen (2002).

Triacylglycerol (carbon number, CN)	Concentration Range (g/100 g milk fat)
CN26	0.1 – 1.0
CN28	0.3 – 1.3
CN30	0.7 - 1.5
CN32	1.8 – 4.0
CN34	4.0 – 8.0
CN36	9.0 – 14.0
CN38	10.0 – 15.0
CN40	9.0 – 13.0
CN42	6.0 – 7.0
CN44	5.0 – 7.5
CN46	5.0 – 7.0
CN48	7.0 – 11.0
CN50	8.0 – 12.0
CN52	7.0 – 11.0
CN54	1.0 – 5.0

The structure of the TAG species varies according to the position in which each FA is esterified to the glycerol backbone. The positional distribution of a FA in the TAG structure is known to be non-random (Jensen, 2002; Blasi et al., 2008; Tzompa-Sosa et al., 2014). **Table 1.3** presents the stereospecific distribution of the major FA in the TAG structure of milk fat. Overall, the FA that are preferably esterified at the *sn*-1(3) positions (primary positions) are C4:0, C6:0, C8:0 and C18:0, whereas C10:0, C12:0 C14:0 are more frequently esterified at the *sn*-2 position (secondary position). The FA C16:0, which is the most abundant FA in milk fat, is equally esterified at the *sn*-1 and *sn*-2 positions of the TAG (Jensen, 2002; Tzompa-Sosa et al., 2014; Kemppinen, 2018).

Table 1.3. Stereospecific distribution of major fatty acids (FA) in the triacylglycerol (TAG) structure in milk fat; adapted from Kemppinen (2018). The table presents the relative concentrations in mol percentage (%) of the major FA in bovine milk fat in the overall TAG structure and in each *sn*-position.

FA	TAG (mol%)	*sn*-1 (mol %)	*sn*-2 (mol %)	*sn*-3 (mol%)
C4:0	10.5 ± 3.3	2.8 ± 3.6	0.8 ± 1.1	96.4 ± 4.7
C6:0	4.4 ± 1.0	10.2 ± 15.0	6.6 ± 3.8	83.2 ± 12.2
C8:0	2.1 ± 0.2	13.4 ±10.0	24.2 ± 27.1	62.4 ± 27.6
C10:0	4.0 ± 0.2	14.2 ± 3.5	31.6 ± 23.1	52.3 ± 25.2
C12:0	4.2 ± 0.5	28.4 ±11.8	50.7 ± 13.4	20.8 ± 20.4
C14:0	12.8 ± 2.1	27.9 ± 0.8	56.7 ± 7.7	15.2 ± 7.1
C16:0	28.4 ± 6.8	45.6 ± 1.7	44.6 ± 1.1	9.8 ± 2.0
C18:0	9.6 ± 3.5	51.5 ± 2.5	28.5 ± 15.0	20.0 ± 12.7
C18:1*cis*9	24.0 ± 3.0	38.0 ± 3.4	24.2 ± 2.7	37.8 ± 5.0

These TAG structures are of great importance for fat digestion and absorption (Mu and Høy, 2004). During fat digestion in humans, the major TAG breakdown takes place in the small intestine. The degradation of TAG is regiospecific, where the FA at the *sn*-1 and *sn*-3 positions are hydrolyzed by pancreatic lipase resulting in two free FA (**FFA**) and a *sn*-2 monoacylglycerol. The FA enzymatic hydrolysis of a TAG structure may vary by the specificity of the enzyme involved. Several studies on fat absorption refer to the particular structure of human milk, characterized by the great abundance of C16:0 at the *sn*-2 position in the TAG structures (almost 70%) (Innis, 2011). It has been shown that increased content of C16:0 at *sn*-2 position enhances FFA and calcium absorption. On the contrary, C16:0 esterified at the *sn*-1 and/or *sn*-3 positions are hydrolyzed into free C16:0. These free C16:0 molecules can subsequently react with calcium and other dietary minerals and form insoluble and indigestible molecular structures that cause reduced FFA and calcium absorption, hard stools and constipation in infants (Kennedy et al., 1999; Mu and Høy, 2004; Yaron et al., 2013). Hence, the efforts of the dairy industry to produce infant formula with an increased proportion of C16:0 at the *sn*-2 position, by altering the FA positional distribution in the TAG structure (Kloek et al., 2020). There is, however, only limited information available on how to increase the contents of C16:0 at the *sn*-2 position in the TAG structure (Tzompa-Sosa et al., 2014; Kloek et al., 2020). Interesting questions that still need to be answered would be, for example, which are the factors that affect the FA positional distribution in the TAG structure and which is the best way to increase the esterification preference of C16:0 at the *sn*-2 position in the TAG structure.

1.6. Factors affecting triacylglycerol composition in milk fat

The main factors that affect milk fat composition can be categorized into two groups: animal related and feed related factors. These factors in turn affect the formation of FA in the MEC (*de novo* synthesis) and modify the LCFA present in blood. For many years, most studies have focused on the FA composition to assess the effect of these factors on milk fat composition, but little attention has been paid to the effect on TAG composition. Variations in the supply of FA available for TAG synthesis result in changes in the overall milk fat TAG composition and the TAG *sn*-positional distribution (Capuano et al., 2014; Tzompa-Sosa et al., 2014; Liu et al., 2017). It is only recently that variation in TAG composition has become of interest.

1.6.1. Animal related factors

1.6.1.1. Cow genetic variants

Selective breeding and genetic variation in cows have been proven to modify milk fat content and composition (Schennink et al., 2008; Duchemin et al., 2013; Strucken et al., 2015). Numerous gene products have been identified to have key roles in milk fat synthesis, with AGPAT and DGAT being the most important ones. Both of them are involved in TAG synthesis in the MEC (Figure 1.3; Coleman and Lee, 2004; Bionaz and Loor, 2008; Cozma et al., 2013; Strucken et al., 2015). Special interest has been drawn to the DGAT enzyme responsible for catalyzing the esterification of FA at the *sn*-3 position in the TAG (Figure 1.3). The DGAT enzymes were identified to have two different isoforms DGAT1 and DGAT2 (Yen et al., 2008). So far, the roles of DGAT1 and DGAT2 in TAG synthesis are not clearly understood (Bovenhuis et al., 2015). A recent study of Chitraju et al. (2019) assessed the role of both isoforms in mice in the TAG synthesis in the liver and in bones. It was found that either DGAT catalyzes TAG synthesis for energy storage. Moreover, several studies reported that DAGT1 has a broader substrate specificity, but it also requires higher substrate concentrations compared to DGAT2 (Cases et al., 2001; Bhatt-Wessel et al., 2018). Hence, if concentrations of FA available for TAG synthesis are high, the suggestion is that DGAT1 would be the main DGAT isoform performing TAG synthesis. Also, the DGAT2 enzyme expressed in the liver has been identified to have a link with *de novo* FA synthesis, meaning that it would be expected to esterify more *de novo* synthesized FA compared to DGAT1 (Chitraju et al., 2019). However, no evidence was found for this statement regarding DGAT2 activity in the mammary gland.

The DGAT1 isoform was identified to have the K232A polymorphism in bovine species. This polymorphism was recognized to increase milk fat content (by 1.5 percentage point), fat yield, and modify milk fat FA composition (Cases et al., 1998; Schennink et al., 2007). The K allele (DGAT1 KK) compared to A allele (DGAT1 AA) was associated with increased concentrations of SFA and C16:0 and also with decreased concentrations of C14:0,

unsaturated C18 FA and C18:2*cis*9,*trans*11 (**CLA**; Schennink et al., 2007). Regarding the effect of the DGAT1 K232A polymorphism on the TAG composition in milk fat, Tzompa-Sosa et al. (2016) identified a significant increase of CN38 for the KK genotype and suggested that this could be caused by the increased concentration of C16:0 identified in KK milk fat. According to current knowledge, no further studies have been done to analyze the effect of the DGAT1 K232A polymorphism on TAG composition in milk fat. Thus, it would be useful to further explore how the FA preferences of the DGAT1 K232A polymorphisms modify the milk fat TAG profile, as well as the structure of the TAG species.

1.6.1.2. Lactation stage

The lactation stage of the cow is an important animal related factor known to modify milk fat FA composition. The lactation cycle, measured in days in milk (**DIM**), can be divided into three different stages: early (<100 DIM), mid (100–200 DIM) and late lactation (>200). During early lactation, the cows are usually in a negative energy balance (**NEB**). When the cows are in NEB, FA from body fat are mobilized causing increased incorporation of LCFA and UFA in the MEC, later used for TAG synthesis. Specifically, Stoop et al. (2009) reported a decreased concentration of *de novo* synthesized FA (C4 to C16) and increased concentrations of C18:0 and UFA in early lactation (after 63 days) of Holstein-Friesian cows. The reviews of Jensen (2002) and Samková et al. (2012) summarize several studies that have reported similar FA composition in the early lactation stage of cows. Moving forward in the lactation cycle, the FA composition starts to vary week after week, where *de novo* synthesized FA (C6:0-C14:0) and odd-chain FA (C5:0 to C15:0) increase and C18:0, LCFA, and UFA decrease (C18 unsaturated FA). The concentration of C16:0 increases and remains constant until the end of the lactation cycle (Stoop et al., 2009; Samková et al., 2012). According to the variation in FA composition of milk fat in the different lactation stages, changes are expected in the TAG composition, as well as in its structures. So far, no studies have reported on the effect of lactation stage on the TAG composition and structure in milk fat.

1.6.2. Feed related factors affecting triacylglycerols composition: Dietary variation in cows' feed and seasonal feeding regimes

The FA composition of milk fat can be modified by changing the feed of the cows (Palmquist et al., 1993; Jensen, 2002; Lock and Bauman, 2004; Elgersma et al., 2006). Many feeding trials have been performed over the years to understand the effect of different feeding regimes on the variation in milk FA composition. Most of these studies focused on changing the milk fat FA composition by increasing the cow's intake of pasture and supplementing cows' feed with oilseeds and oils (Elgersma et al., 1998, 2006; Mosley et al., 2007; Patel et al., 2013; Puppel et al., 2013). The variation in cows' feed FA composition is known to affect the milk fat content and its FA composition. These modifications are usually done with the

purpose of increasing the UFA and decreasing the SFA contents of milk fat. Special interest for PUFA such as eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) has been expressed in view of their health benefits for humans (Jensen, 2002; Singh et al., 2018). The variation in the FA composition due to alterations in cows' feed was identified as a means to modify the concentrations of the overall TAG composition. Variations in milk fat TAG is dependent on the FA composition of the feed (Banks et al., 1989; DePeters et al., 2001). Because of the health implications of increased PUFA levels in milk fat, most studies have analyzed the effect of diets rich in UFA on the FA and TAG composition, whereas little information is available on the effect of diets high in SFA in the TAG composition (Bhaskar et al., 1998; Couvreur et al., 2006).

Part of the effect of feed on milk FA and TAG composition has been observed as seasonal variation (Elgersma et al., 2006; Heck et al., 2009; Kliem et al., 2013; Liu et al., 2017). The differences in the cows' diets between seasons, especially between summer and winter, has been studied throughout the years (Corl et al., 2001; Jensen, 2002; Heck et al., 2009). In spring and summer, cows' feed has greater proportions of fresh grass, whereas in autumn and winter the diet is mainly based on silage and concentrates. Fresh grass has a high content of ALA and other PUFA. These FA were present in increased concentrations in summer milk fat. Hence, fresh grass feeding can be considered as a practical feeding strategy to increase PUFA content in milk fat. High concentrations of ALA and PUFA in summer milk fat were correlated to lowered concentrations of saturated TAG species and increased concentrations of unsaturated TAG species in comparison to winter milk fat (Capuano et al., 2014; Liu et al., 2017; Tzompa-Sosa et al., 2018). Considering that these results were based on a maximum period of 8 months, these studies did not show the seasonal variation on the TAG composition over a full year.

In general, most of the available research on the effect of feeding regimens on milk fat composition has focused on the FA composition. Information on the ensuing effects on TAG composition is scarce. Therefore, the research described in this thesis aims at providing insights on the effect of changes in feeding regimes of dairy cows on the TAG composition and structure in milk fat.

1.7. Triacylglycerols variation and its effect on the solid fat content

The characteristics of the three FA that compose a TAG structure (Section 1.1, Figure 1.1) determine the melting point, SFC, and crystallization behavior of milk fat (De Graef et al., 2012; Kaufmann N, Andersen U, 2012; Mohan et al., 2020). The FA chain length and the number of double bonds are key factors that define its melting point. Knothe and Dunn (2009) reported a complete overview on the melting points of the FA from C8 to C24, which includes their saturated, monounsaturated *cis*/*trans* and branched (*iso*/*anteiso*), and polyunsaturated configurations. In general, the shorter the chain length and the larger the number of double bonds, the lower the melting point of the FA. The combination of the three FA in the TAG structure determines the overall melting point of the TAG, which aims for an overall melting point of the milk fat of 39ºC, to facilitate the secretion of milk (Jensen, 2002). The wide range of FA available for TAG synthesis in the MEC generates an even larger amount of possible TAG species in milk fat with different melting points. The variation in the melting points of TAG further determines the SFC of milk fat. The SFC profile is commonly used in the food industry as a parameter to judge the suitability of fats and oils for different applications (De Graef et al., 2012; Mohan et al., 2020). Therefore, changes in the TAG composition of milk fat will determine its SFC profile, thereby influencing its applicability in dairy and non-dairy food applications.

Few studies analyzed the effect of dietary variation in cows' feed on the TAG composition, the melting properties, and the SFC of milk fat (Couvreur et al., 2006; Smet et al., 2010; Larsen et al., 2014). In these studies, it was found that high proportions of UFA in the diet of cows lowered the melting temperature and SFC of milk fat, due to the increased concentrations of low melting TAG. Therefore variation in the milk fat FA profile due to changes in the diet may influence the formation of TAG species with different melting properties, further changing the SFC in milk fat.

Based on current literature, there is still a lack of information on the effects of feed on the variation in TAG composition and SFC in milk fat. Therefore, our research aims to fill this knowledge gap, providing insights in the overall influence of the variations in FA and TAG composition on the SFC of milk fat.

1.8. Aim and outline of this thesis

Both TAG composition and structure influence the physical, nutritional and functional characteristics of milk fat. Therefore, understanding the variation in milk fat TAG composition and structure is essential in order to improve and customize its use as an ingredient for food applications. Until now, most of the studies on changes in milk fat composition have focused on FA variation, whereas little is known about the influence of this variation on the TAG composition and structure. Hence, the aim of the research described in this thesis was to assess the effect of season, feeding regimes, genetics and lactation stage on the TAG composition and structure in bovine milk fat. Additionally, the influence of the variation in TAG composition on the SFC in bovine milk fat was also investigated in this thesis. The schematic overview of this thesis is presented in **Figure 1.4.** The analysis of the different factors that affect the FA and TAG composition in bovine milk fat were categorized in two groups, being feed related factors and animal related factors. **Chapters 2 to 4** address the feed related factors, and **Chapters 5 and 6** address the animal related factors. Most chapters share a similar approach: first, an analysis of the milk fat compositional variations of FA and TAG, second, an exploration of how the variation in the FA composition influences the FA positional distribution in the TAG structure, and third, an assessment of the effect of the FA and TAG composition on the SFC in milk fat.

Chapter 2 presents the effect of seasonal feeding regimes on the FA and TAG composition in bovine milk fat. **Chapter 3** complements this information with the analysis of the FA positional distribution in the TAG structure over a year. These studies were based on weekly-pooled milk samples from fourteen dairy factories in the Netherlands, considered as representative samples for the average Dutch bovine milk. **Chapter 4** is an analysis of the effect of hydrogenated palm FA and protein supplementation in cow's feed on the TAG composition, the FA positional distribution, and the SFC of bovine milk fat. This study provides insights on the implications of palm oil-based high energy feed for dairy cattle. Addressing the animal related factors, **Chapter 5** examines the effect of the lactation stage on the FA and TAG composition, the FA distribution in the TAG structure, and the SFC in bovine milk fat. For this study, we examined milk fat of a set of 11 cows sampled in early and late lactation. Finally, in **Chapter 6** both the effect of the DGAT1 K232A polymorphism (AA and KK), as the major genetic variation of dairy cattle influencing milk fat, and the seasonal variation in feeding, on the TAG composition in bovine milk fat were assessed. This study was based on milk of 25 DGAT1 KK cows and 25 DGAT1 AA cows collected in both summer and winter. In this chapter we were able to assess the individual effect of the DGAT1 K232A (AA and KK) polymorphism and seasonal feeding regimes as well as their combined effect on the TAG composition in bovine milk fat.

To conclude, **Chapter 7** summarizes the main findings of all the chapters and provides a general discussion by comparing the effect of all the analyzed factors on the variations in

FA and TAG composition, the FA regiospecific positional distribution in the TAG structure, and the SFC of bovine milk fat. Finally, future research perspectives are suggested, based on the main conclusions of this thesis.

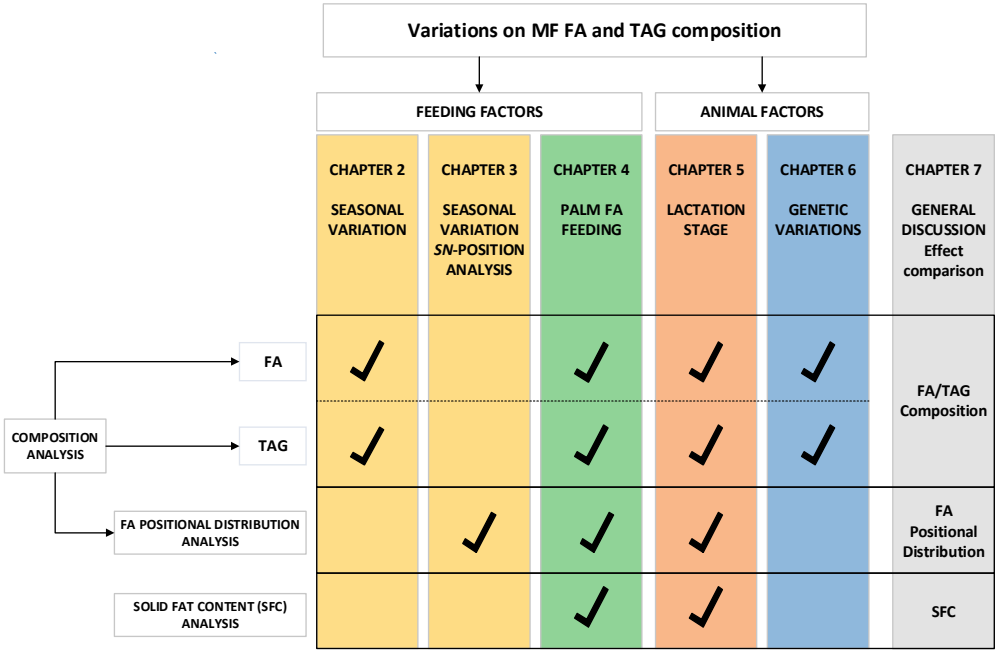


Figure 1.4. Schematic overview of the thesis chapters. The factors analyzed are presented in different colors. The information included in each chapter is indicated by a "check" mark. Chapters 2 to 4 analyze the feed related factors, and chapter 5 and 6 analyze the animal related factors. Chapter 7 compares the effect observed in both feed and animal related factors on the fatty acids (FA) and triacylglycerols (TAG) composition, fatty acid (FA) positional distribution in the TAG structure, and milk fat solid fat content (SFC).

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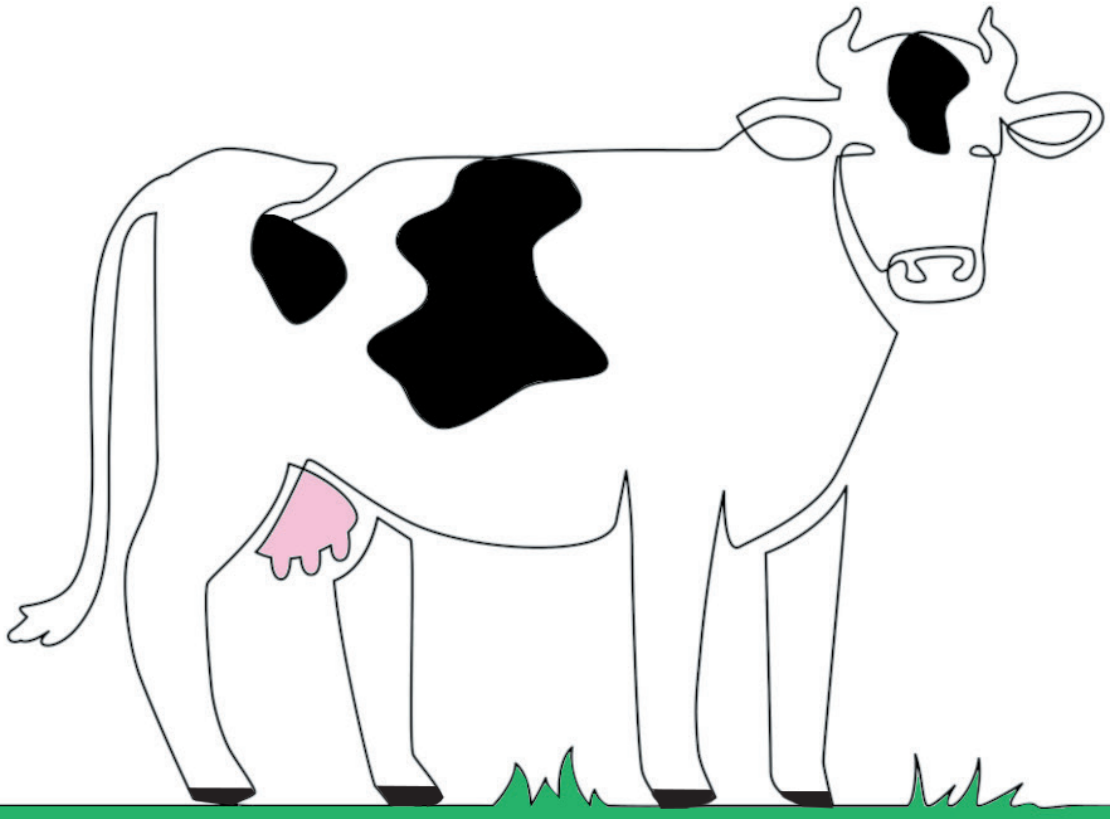

Chapter 2

Seasonal variation in fatty acid and triacylglycerol composition of bovine milk fat

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Abstract

The aim of this study was to assess the effect of seasonal variation on the changes of the fatty acids (**FA**) and triacylglycerols (**TAG**) composition of bovine milk fat in a non-seasonal milking system. Weekly milk samples were collected from fourteen dairy factories and pooled per week as representative samples of the average Dutch bovine milk. The sample collection started in May 2017 and finished in April 2018, resulting in a total of 52 samples corresponding to each week of the year. The samples were analyzed for milk fat content (%), FA and TAG composition using gas chromatography with flame-ionization detection (**GC-FID**). The increased intake of C18:3*cis*9,12,15 through grass feeding in spring and summer was associated with major changes in milk fat FA composition, including reduced proportions of *de novo* synthesized FA and presence of several rumen biohydrogenation products and CLA isomers in milk fat. These changes in the seasonal FA composition had an effect on the TAG seasonal variation. The TAG seasonal variation showed that all TAG groups were significantly different between months. The low molecular weight (**LMW**) and the medium molecular weight (**MMW**) TAG groups increased in winter and decreased in summer, whereas the high molecular weight (**HMW**) TAG groups increased in summer and decreased in winter. Based on pooled monthly samples, MALDI-TOF-mass spectrometry (**MS**) allowed the analysis of even- and odd-chain TAG species in milk fat based on their total carbon number (**CN**) and number of double bonds. These analyses indicated saturated TAG species to be greatest in winter, whereas monounsaturated, polyunsaturated and odd-chain TAG species were greatest in summer. Our study showed that TAG seasonal variation in a non-seasonal milking system is influenced by the variation in FA composition throughout the seasons.

2.1. Introduction

Milk fat is mainly composed of triacylglycerols (>95% of total lipids) with about 400 fatty acid (**FA**) with different chain lengths, degrees of saturation and stereospecific numbering (***sn***)-positional distribution (Jensen, 2002). The TAG composition of milk fat changes according to the stage of lactation, feeding regimes, genetic factors and season (Jensen, 2002; Capuano et al., 2014; Tzompa-Sosa et al., 2014). In dairy farming systems with indoor housing during winter and pasture-based systems during summer, seasonal variation in milk fat composition mainly occurs due to the changes in feed according to the season. In such systems, increased intake of fresh pasture in spring and summer occurs with or without supplementary feed including corn silage and concentrate, whereas in winter the diet mainly includes ensiled feed and concentrate. Overall, the variation in feeding regimes affects the FA composition (*e.g.* Couvreur et al., 2006) and consequently leads to changes in milk fat TAG composition. It has previously been shown that the TAG profile and structure of milk fat determine its physicochemical and functional properties when being applied in a food product (Wright et al., 2000, 2008) and may affect the fatty acid absorption efficiency in infants (Zou et al., 2013). Specifically, TAG seasonal composition variation in milk fat results in different crystallization behavior throughout the year known to affect the texture properties of milk fat (*e.g.* hardness and spreadability) (Mohan et al., 2020). Hence, the identification of TAG composition and saturation degree at a certain time of the year is important for understanding the functionality of milk fat as a dairy ingredient and for reducing the limits that might be encountered by the dairy industry due to large variations in milk fat characteristics during the year (O'Brien and Guinee, 2016).

In a non-seasonal milking system, where calving occurs all year round, changes in FA composition were linked to a cow's diet rich in fresh pasture in spring, the period of the year when the cows start grazing (Baumgard et al., 2000; Lock and Garnsworthy, 2003; Heck et al., 2009). Specifically, proportions in milk fat of conjugated linoleic acid (**CLA**), C18:3*cis*9,12,15, n-3 FA, and *trans* FA increased in spring/summer, whereas proportions of saturated FA and *de novo* synthesized FA increased in autumn/winter (Baumgard et al., 2000; Heck et al., 2009; Van Valenberg et al., 2013). These aforementioned seasonal variations in the FA composition induce changes in TAG composition (Tzompa-Sosa et al., 2018; Pacheco-Pappenheim et al., 2019). There are limited studies on the variation in TAG composition throughout the year (Larsen et al., 2014; Liu et al., 2017; Tzompa-Sosa et al., 2018). Overall, these studies show that diets rich in forages with high contents of C18:3*cis*9,12,15 decrease the relative concentration of saturated TAG species and increase the relative concentrations of unsaturated TAG species. Although these three studies assessed the seasonal variation in TAG composition and saturation degree, they were based on a limited number of cows or dairy farms. To the best of our knowledge, information regarding the TAG composition variations in spring and autumn and a year-

round analysis of the TAG composition and its saturation degree in milk delivered to dairies is lacking. This study aims to investigate the effect of seasonal variation in a non-seasonal milking system on the changes of the FA and TAG composition in Dutch bovine milk fat. In order to do so weekly bulk milk samples from a whole year as representative of the seasonality in the Netherlands were collected for this study. Weekly milk samples were analyzed for FA and TAG composition. These samples provide a detailed overview of the seasonal variation of FA and TAG composition of bovine milk fat as used by dairy producers, important for the assessment of functional and nutritional properties of dairy products.

2.2. Materials and Methods

2.2.1. Sample selection

Weekly pooled milk samples were collected from fourteen dairy factories by the Dutch Milk Control Institute Qlip (Quality, laboratory, inspections and process certification) Laboratories (Zutphen, the Netherlands) as representative samples of the average Dutch bovine milk. The sample collection started in May 2017 and finished in April 2018, resulting in a total of 52 samples corresponding to each week of the year. Sodium azide (0.03%) was added to the raw milk for conservation purposes. The milk samples were stored at -20°C and thawed before milk fat extraction. Milk fat extraction was done according to Tzompa-Sosa et al. (2014) in order to guarantee that only TAG were present. The milk fat was stored at -20°C and later analyzed for FA and TAG composition. The groups of FA and TAG are described in **Table 2.1**. Fat content was determined by the ISO standard 1211 (ISO, 2010) at Qlip.

2.2.2. FA analysis

Fatty acid methyl esters (**FAME**) were analyzed for the 52 weekly samples. The FAME were prepared according to the ISO Standard 15884 (ISO-IDF, 2002) and FAME composition was determined by the ISO Standard 16958 (ISO, 2015) using GC with a flame-ionization detector (**FID**; Thermo Focus, Thermo Fisher, Rodano, Italy) and an Agilent CPSil 88 FAME column (100 m × 0.25 mm i.d. × 0.2 µm film thickness). These analyses were performed by Qlip.

2.2.3. TAG composition analysis

Two methods were used to analyze the seasonal variation in TAG composition of milk fat, viz. GC-FID and MALDI-TOF-MS. The GC-FID was used as a standard method to analyze the TAG composition of the even-chain TAG groups in our milk fat samples. A TAG group can be defined as various TAG species that have the same total carbon number (**CN**) but different FA composition. The MALDI-TOF-MS was used to analyze the TAG composition for the detection and identification of even- and odd-chain TAG species in milk fat based

on their CN and number of double bonds (**DB**) (Picariello et al., 2007). In addition, with MALDI-TOF-MS TAG composition of various oils and fats, including milk fat, can be analyzed in a fast and accurate way via a quick identification of the TAG species (Schiller et al., 2004; Tzompa-Sosa et al., 2018; Yener and van Valenberg, 2019). The TAG patterns obtained by MALDI-TOF-MS and GC-FID are quite similar and comparable (Schiller et al., 2004). From these aspects, MALDI-TOF-MS provides valuable complementary information on the TAG composition that is not possible to be obtained by only using GC-FID. The technical details of these two methods are discussed below.

2.2.3.1. Weekly sample TAG analysis using GC-FID

The TAG composition was determined for the 52 weekly samples according to ISO Standard 17678/IDF 202 (ISO, 2010). The TAG were identified by carbon number (CN) on a GC-FID with a column injector port (Thermo Trace GC ultra, Thermo Scientific, Rodano, Italy) and a UltiMetal CP7532 column (5 m × 0.53 mm i.d. × 0.17 µm film thickness., Varian, Houten, The Netherlands). The TAG were analyzed by comparing their retention times to the retention time of the anhydrous milk fat TAG standard mix BCR519 (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The TAG compositions are expressed as percentages (g/100 g, %) of the normalized peak areas by dividing each peak area with the total peak area for each measurement. The response factors were close or equal to 1. The TAG identified with this method were 16 TAG groups from 24 to 54 CN. Each TAG CN refers to all TAG species with similar molecular weight, meaning the group of all TAG species with the same CN independent of degree of saturation. No odd-chain TAG can be separately identified with this method. These analyses were done at Wageningen Food Safety Research (WFSR) at Wageningen University (Wageningen, the Netherlands).

2.2.3.2. Monthly sample TAG analysis using MALDI-TOF-MS

Weekly samples were mixed in equal volumes according to their corresponding month to obtain monthly samples. The TAG composition of the monthly samples were analyzed with MALDI-TOF-MS (UltrafleXtreme, Bruker Corporation, Bremen, Germany). The identification of TAG were based on CN:DB. The analysis was based on the method by Picariello et al. (2007) as modified by Tzompa-Sosa et al. (2014). Milk fat samples were dissolved in chloroform (10 mg of milk fat/ml) in a glass tube with Teflon cap. The sample solution was mixed with 1 M NaCl at a ratio of 1:1 (v/v) for 1 min and then centrifuged for 3 minutes at 3000 rpm (Heraeus Multifuge X3R, Thermo Fisher Scientific, Langenselbold, Germany). The upper aqueous layer was discarded and the lower organic layer was used for MALDI-TOF-MS analysis. Matrix solution was prepared by dissolving 10 mg of 2,4,6-tri-hydroxy-aceto-phenone (Sigma-Aldrich Chemie GmbH) in methanol:water (1:1, v/v) containing 0.05% trifluoroacetic acid. A 0.1 *M* NaCl solution was used as cationization agent. The solutions were spotted directly on a stainless steel MALDI target plate in a ratio

1:1:0.5 µL of matrix/ fat solution/ cationization agent (v/v/v). Two technical replicates were prepared per sample. Each technical replicate was platted 5 times subjected to 1000 laser pulses taken randomly from 20 different points. The analysis was performed using the auto-execute mode, to allow the random acquisition of masses. Peak areas with signal-to-noise ratio >6, present in at least 4 out of 5 repetitions, were defined as identified TAG. The MALDI-TOF-MS mass spectra were analyzed with "MALDIquant" package by using R programming language (Gibb and Strimmer, 2012). The results were presented in relative intensities after normalization based on total ion chromatogram for each measurement, meaning that each peak intensity was divided by the total sum and expressed in percentage. The TAG species were then tentatively identified in CN:DB by using LIPID MAPS Online Tools (https://www.lipidmaps.org/resources/tools/bulk-structure-search/create?database=LMSD) using a mass range of 0.1 Da (Fahy et al., 2005). Using this method a qualitative TAG profile of milk fat can be obtained with the information of the odd- and even-chain TAG species that vary in degree of saturation (Yener and van Valenberg, 2019).

2.2.4. Statistical analysis

The yearly mean, standard deviation and coefficient of variation (**CV**) for the milk fat content, the FA and the TAG concentrations determined by GC-FID were calculated considering the 52 measured weeks. The minimum and maximum values of each trait were presented based on months. Statistical analysis were performed on monthly means, obtained from their corresponding weeks, to compare each trait with one-way ANOVA using a Tukey Post Hoc test. The correlation between FA and TAG composition was analyzed according to Pearson's comparison test. Next to this, the monthly relative TAG intensities measured by the MALDI-TOF-MS were grouped according to the corresponding seasons and one-way ANOVA with a Tukey Post Hoc test was performed to identify the differences in TAG profiles between seasons. The cut-off value considered for significant difference was $P<0.05$. All statistical analyses were executed with IBM SPSS Statistics 25.0 software (2017; UBM Corp.), and R version 3.6.1 (2019; https://www.r-project.org/).

Table 2.1. Definitions of fatty acids (FA) and triacylglycerols (TAG) groups analyzed by GC-FID.

Item[1]	Formula[2]
FA groups	
SFA	C4:0, C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0iso, C14:0, C15:0 *iso*, C15:0*anteiso*, C15:0, C16:0*iso*, Pristanic-acid, C16:0, C17:0*iso*, C17:0*an*teiso, Phytanic-acid, C17:0, C18:0, C19:0, C20:0, C22:0, C24:0
UFA	C10:1*cis*9, C12:1*cis*9, C14:1*cis*9, C16:1*trans*9, C16:1*cis*9, C17:1*cis*9; C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*cis*9, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:1*cis*14, C18:1*cis*15, C18:2*cis*9,12 (LA), C18:3*cis*6,9,12 (GLA), C18:3*cis*9,12,15 (ALA), C18:2*cis*9,*trans*11 (CLA), C20:3*cis*8,11,14 (DGLA), C20:4*cis*5,8,11,14 (AA), C20:4*cis*8,11,14,17 (ETA), C20:5*cis*5,8,11,14,17 (EPA), C22:5*cis*7,10,13,16,19 (DPA)
MUFA	C10:1*cis*9, C12:1*cis*9, C14:1*cis*9, C16:1*trans*9, C16:1*cis*9, C17:1*cis*9; C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*cis*9, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:1*cis*14, C18:1*cis*15
PUFA	C18:2*cis*9,12 (LA), C18:3*cis*6,9,12 (GLA), C18:3*cis*9,12,15 (ALA), C18:2*cis*9,*trans*11 (CLA), C20:3*cis*8,11,14 (DGLA), C20:4*cis*5,8,11,14 (AA), C20:4*cis*8,11,14,17 (ETA), C20:5*cis*5,8,11,14,17 (EPA), C22:5*cis*7,10,13,16,19 (DPA)
SCFA	C4:0, C5:0, C6:0, C 7:0, C8:0, C9:0, C10:0, C10:1*cis*9, C11:0
MCFA	C12:0, C12:1*cis*9, C13:0, C14:0iso, C14:0, C14:1*cis*9, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, Pristanic acid, C16:0, C16:1*trans*9, C16:1*cis*9, C17:0*iso*, C17:0*anteiso*, Phytanic acid, C17:0, C17:1*cis*9
LCFA	C18:0, C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*cis*9, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:1*cis*14, C18:1*cis*15, C18:2*cis*9,12 (LA), C19:0, C18:3*cis*6,9,12 (GLA), C18:3*cis*9,12,15 (ALA), C18:2*cis*9,*trans*11, C20:0, C20:3*cis*8,11,14 (DGLA), C20:4*cis*5,8,11,14 (AA), C22:0, C20:4*cis*8,11,14,17 (ETA), C20:5*cis*5,8,11,14,17 (EPA), C24:0, C22:5*cis*7,10,13,16,19 (DPA)
OBCFA	C5:0, C7:0, C9:0, C11:0, C13:0, C14:0*iso*, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, C17:0*iso*, C17:0*anteiso*, C17:0, C17:1*cis*9, C19:0
TAG groups	
LMW	CN26, CN28, CN30, CN32, CN34, CN36
MMW	CN38, CN40, CN42, CN44, CN46, CN48
HMW	CN50, CN52, CN54

[1] SCFA: short-chain fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; OBCFA: odd- and branched-chain fatty acids; LMW: low molecular weight; MMW: medium molecular weight; HMW: high molecular weight.

[2] LA: linoleic acid; GLA: γ-linolenic acid; ALA: α-linolenic acid; DGLA: dihomo-γ-linolenic acid; AA: arachidonic acid; ETA: eico-satetraenoic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid.

2.3. Results and Discussion

2.3.1. Seasonal variation in fat Content

Milk fat content significantly differed (P<0.05) between months, where the maximum content was observed in March (4.60%) and the minimum in June (4.08%; **Table 2.2**). These results are consistent with the study of Heck et al. (2009) in the Netherlands and are also consistent with seasonal patterns in Danish (Larsen et al., 2014), Polish (Frelich et al., 2012) and Greek (Govari et al., 2019) bovine milk. The major feed ingredients in Dutch dairy cattle rations include (DM basis) grass herbage (14%), grass silage (37%), corn silage (19%), wet byproducts (4%) and concentrate (25%; van Bruggen, 2018). During the grazing season, some 70% of cows consume grass herbage, mainly at the expense of grass silage and some concentrate. Lower milk fat content in summer months may be explained by increased concentrations of ruminal biohydrogenation intermediates (including various CLA isomers; Sterk et al., 2011; Leskinen et al., 2019). These intermediates are related to inhibition of *de novo* milk fat synthesis (Chouinard et al., 1999; Baumgard et al., 2000). In grass herbage, C18:3*cis*9,12,15 is the most abundant FA, and high levels of this FA is related with the production of different intermediate CLA isomers that occur during the biohydrogenation process in the rumen (Jenkins et al., 2008). In contrast, extensive lipolysis and biohydrogenation of FA in grass during wilting and ensiling leads to lower C18:3*cis*9,12,15 contents of grass silage than grass herbage (Dewhurst et al., 2006). Next to a seasonal effect of diet, increases in temperature and humidity (temperature-humidity index; THI) are associated with reduced milk fat contents under otherwise unchanged dietary and other managerial conditions (Bernabucci et al., 2015). Photoperiod is another factor that may affect milk production. During lactation, day length had a negative effect on milk fat concentration (Aharoni et al., 2002) The significant changes in milk fat content observed in the present study may thus be explained by changes in diet, heat-humidity, and photoperiod.

Supplemental Figure S2.1 shows the Pearson correlation between milk fat content, FA and TAG composition in all seasons. According to Pearson correlation analysis, milk fat content was positively correlated with C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1*cis*9, C15:0, C16:0, C16:1*cis*9, phytanic acid and with the TAG species CN30-CN36 and CN42-CN46, whereas it was negatively correlated with C15:0*iso*, C15:0*anteiso*, C16:0*iso*, C16:1*trans*9, all C17 variants, C18:0, C18:1 *trans* FA, C18:1*cis* FA (except C18:1*cis*12), C18:2*cis*9,*trans*11 and TAG species CN50-CN54. Considering the correlations between the FA composition and milk fat content, these results are in line with Baumgard et al. (2000) where milk fat content and the positively correlated FA (except phytanic acid) decreased in summer. Furthermore, the TAG species that were positively correlated to milk fat content are the ones that mainly contain SCFA (*de novo* synthesized FA) and MCFA (C14:0, C14:1*cis*9, C15:0, C16:0, C16:1*cis*9) in their structure (Gresti et al.,

1993; Liu et al., 2020). Moreover the negatively correlated FA were also identified by Baumgard et al. (2000) to have a negative effect on milk fat content and *de novo* FA synthesis. The TAG species CN50, CN52 and CN54 are mainly composed of LCFA and UFA (Gresti et al., 1993; Liu et al., 2020), and hence were also foreseen to be negatively correlated with milk fat content.

2.3.1.1. Seasonal variation in FA composition

The seasonal variation in FA composition of the bovine milk fat from May 2017 to April 2018 is shown in **Table 2.2.** Our results showed that most FA were significantly different (P<0.05) between months, except C4:0, C14:0*iso*, C18:1*trans*9, C18:3*cis*6,9,12 (GLA), C19:0, C20:3*cis*8,11,14 (DGLA), C20:5*cis*5,8,11,14,17 (EPA) and C22:5*cis*7,10,13,16,19 (DPA). We observed similar seasonal trends as previously described by Heck et al. (2009). Blood derived FA increased in summer and *de novo* synthesized FA increased in winter. Spring and autumn were considered as transition seasons where the FA increased or decreased until they reached their minimum/maximum value in winter/summer. Several studies identified similar trends for the aforementioned FA when the seasonal changes in FA composition were studied (Elgersma et al., 2006; Heck et al., 2009; Larsen et al., 2014). In particular, when comparing our FA profile in milk fat from Dutch bovine milk with the FA profile of 2005 reported by Heck et al (2009), similar seasonal trends were found. This suggests that the FA composition within seasons in the Netherlands has the same behavior every year. The proportion of SFA in milk fat from representative samples in 2017/2018 was 70.77% and similar to that in 2005 (70.63%; Heck et al., 2009).

The variation in proportion of C18:2*cis*9,12 in milk fat was comparable to that of C18:3*cis*9,12,15 (CV being 4.46% and 4.85%, respectively). However, there appeared to be no consistent pattern in variation in C18:2*cis*9,12 throughout the season, whereas C18:3*cis*9,12,15 markedly increased from winter to summer period (**Figure 2.1a**). The absence of a clear seasonal trend in C18:2*cis*9,12 in milk fat indicates that the intake of corn silage and concentrates (main sources of C18:2*cis*9,12) in the diet is most likely relatively constant throughout the year, whereas the increased concentration of C18:3*cis*9,12,15 in summer suggest a higher intake of fresh grass (main source of C18:3*cis*9,12,15) in summer months compared with grass silage (lower levels of C18:3*cis*9,12,15) in winter. These findings were consistent with large changes in C18:3*cis*9,12,15 but only moderate changes in C18:2*cis*9,12 proportions in milk fat upon replacing corn silage with grass herbage or grass silage (Couvreur et al., 2006a; van Gastelen et al., 2015) and with seasonal changes observed in previous studies (Heck et al., 2009; Van Valenberg et al., 2013; Hanus et al., 2018).

Table 2.2. Descriptive statistics [mean (% wt/wt), standard deviation (SD), coefficient of variation (CV; %), minimum and maximum value] of seasonal variation of milk fat fatty acid (FA) composition from May 2017 until April 2018 as analyzed by gas chromatography (GC) – flame ionization detector (FID).[1]

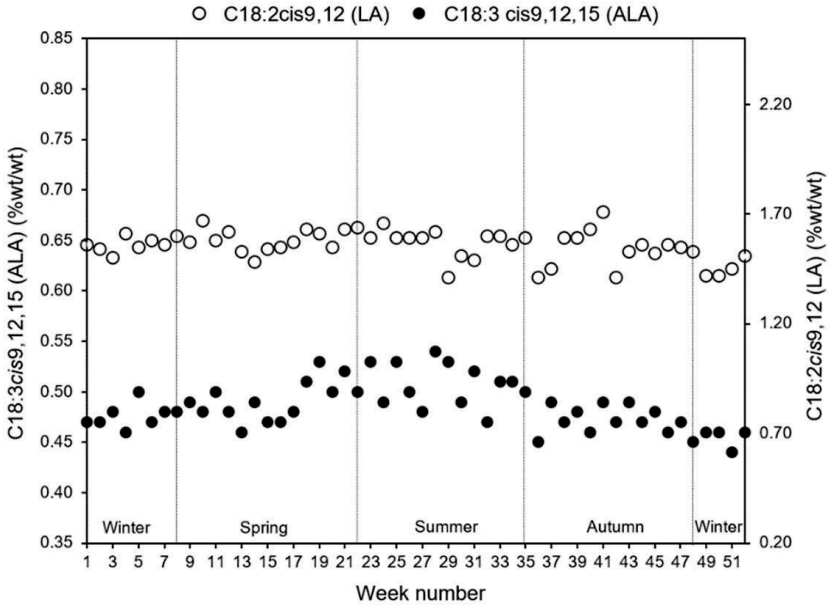
	Year			Minimum		Maximum		*P* value
Item	Mean	SD	CV (%)	Month	Mean	Month	Mean	Month effect
Fat content	4.34	0.148	3.41	July	4.08	March	4.60	<0.001
C4:0	3.61	0.132	3.65	October	3.14	January	3.98	0.220
C6:0	2.34	0.077	3.28	August	2.19	January	2.59	<0.001
C8:0	1.32	0.047	3.55	August	1.22	January	1.42	<0.001
C10:0	3.04	0.151	4.96	August	2.74	January	3.28	<0.001
C10:1*cis*9	0.32	0.016	5.09	June	0.30	November	0.36	<0.001
C11:0	0.06	0.007	11.56	July	0.04	December	0.07	<0.001
C12:0	3.99	0.181	4.53	August	3.60	January	4.27	<0.001
C12:1*cis*9	0.09	0.018	19.24	February	0.06	May	0.12	0.012
C13:0	0.09	0.008	8.64	August	0.07	November	0.10	<0.001
C14:0*iso*	0.08	0.006	7.44	March	0.07	July	0.10	0.140
C14:0	11.54	0.318	2.75	August	10.99	February	12.01	<0.001
C14:1*cis*9	1.11	0.060	5.45	June	0.97	November	1.22	<0.001
C15:0*iso*	0.23	0.012	5.18	March	0.21	July	0.26	<0.001
C15:0*anteiso*	0.45	0.020	4.57	March	0.41	July	0.49	<0.001
C15:0	1.07	0.038	3.56	June	0.99	December	1.13	<0.001
C16:0*iso*	0.19	0.008	4.27	January	0.18	July	0.21	<0.001
C16:0	31.32	1.053	3.36	June	29.04	December	32.83	<0.001
C16:1*trans*9	0.18	0.016	8.68	February	0.15	August	0.20	<0.001
C16:1*cis*9	1.60	0.057	3.54	June	1.50	October	1.72	<0.001
C17:0*iso*	0.36	0.021	5.78	March	0.33	July	0.39	<0.001
C17:0*anteiso*	0.39	0.016	4.20	February	0.37	June	0.43	<0.001
Phytanic Acid	0.18	0.016	9.35	July	0.15	January	0.22	0.002
C17:0	0.52	0.013	2.50	January	0.50	July	0.55	0.050
C17:1*cis*9	0.20	0.010	5.07	February	0.19	June	0.22	<0.001
C18:0	9.61	0.524	5.46	December	8.68	June	10.98	<0.001
C18:1*trans*6	0.23	0.021	9.40	February	0.18	June	0.27	0.003
C18:1*trans*9	0.16	0.026	16.14	October	0.00	January	0.20	0.277
C18:1*trans*10	0.26	0.027	10.55	February	0.20	August	0.32	<0.001
C18:1*trans*11	0.99	0.149	15.07	December	0.78	August	1.31	<0.001
C18:1*cis*9	18.43	0.918	4.98	December	17.04	June	20.01	<0.001
C18:1*cis*11	0.74	0.035	4.78	December	0.68	June	0.85	<0.001
C18:1*cis*12	0.23	0.017	7.25	September	0.19	March	0.27	0.031
C18:1*cis*13	0.13	0.011	8.05	March	0.12	August	0.15	<0.001
C18:1*cis*14	0.40	0.014	3.47	December	0.37	August	0.43	0.023
C18:1*cis*15	0.32	0.016	5.13	April	0.28	August	0.35	<0.001
C18:2*cis*9,12 (LA)	1.56	0.069	4.46	December	1.45	May	1.61	0.015
C18:2*cis*9,*trans*11 (CLA)	0.45	0.063	13.98	February	0.38	August	0.55	<0.001
C18:3*cis*6,9,12 (GLA)	0.06	0.010	17.93	October	0.05	May	0.06	0.343
C18:3*cis*9,12,15 (ALA)	0.49	0.024	4.85	December	0.46	June	0.51	<0.001
C19:0	0.14	0.006	4.13	October	0.13	December	0.15	0.830
C20:0	0.12	0.009	7.53	February	0.11	June	0.14	<0.001
C20:3*cis*8,11,14 (DGLA)	0.06	0.005	7.64	February	0.06	June	0.07	0.114
C20:4*cis*5,8,11,14 (AA)	0.08	0.006	6.66	February	0.07	October	0.10	<0.001
C20:5*cis*5,8,11,14,17 (EPA)	0.03	0.011	37.12	February	0.05	November	0.07	0.090
C22:5*cis*7,10,13,16,19 (DPA)	0.06	0.006	10.56	August	0.06	November	0.08	0.189
FA groups								
SFA	70.77	1.233	1.74	August	69.01	December	72.24	<0.001
UFA	28.24	1.190	4.21	December	26.69	August	29.91	<0.001
MUFA	25.19	1.093	4.34	December	23.53	August	27.04	<0.001
PUFA	2.85	0.116	4.07	December	2.64	August	3.06	<0.001
SCFA	10.73	0.347	3.24	August	9.97	January	11.64	<0.001
MCFA	53.59	1.483	2.77	June	50.43	December	55.86	<0.001
LCFA	34.66	1.669	4.82	December	32.44	June	37.46	<0.001
OBCFA	3.82	0.074	1.94	February	3.67	July	3.96	0.008

[1] LA: linoleic acid; GLA: γ-linolenic acid; ALA: α-linolenic acid; DGLA: dihomo-γ-linolenic acid; ArA: arachidonic acid; ETA: eico-satetraenoic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic

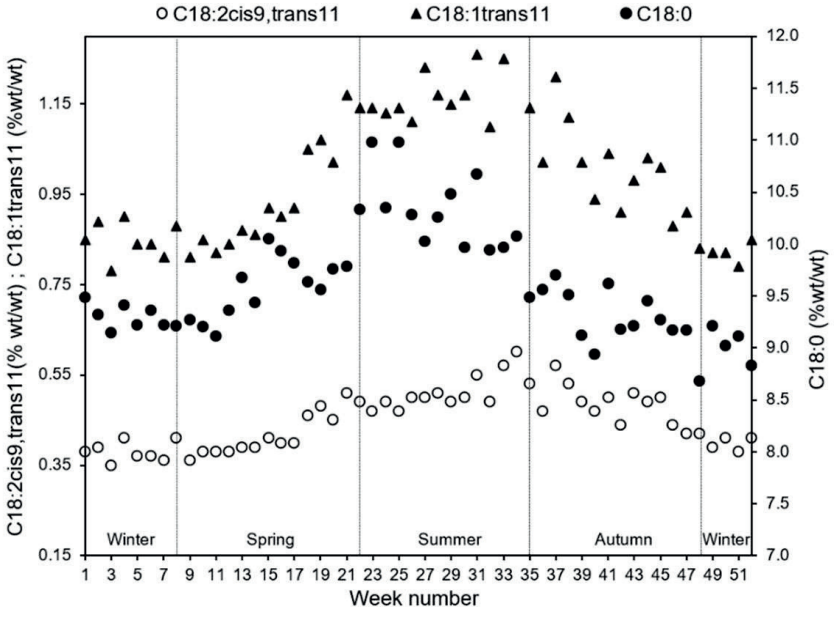
acid; SCFA: short-chain FA; MCFA: medium-chain FA; LCFA: long-chain FA; OBCFA: odd- and branched-chain FA. The FA included for the calculation of each FA group are described in Table 2.1. The FA C5:0, C7:0, C9:0, pristanic acid, C22:0, C20:4*cis*8,11,14,17 (ETA), and C24:0 are not included in the table due to their high CV (above 20%). The total sum of the FA composition is 99%; 1% of the total FA composition is not included in the table due to low concentrations (<0.05%) in the GC-FID analysis. The yearly mean, SD, and CV were calculated based on the 52 weekly samples (n=52). The cut-off value for significant difference between months is $P<0.05$.

The presence of C18:3*cis*9,12,15 and C18:2*cis*9,12 in the diet of dairy cattle leads to the formation of several biohydrogenation products including C18:2*cis*9,*trans*11, C18:1*trans*11 and ultimately C18:0 (Jensen, 2002; Cozma et al., 2013). For all these FA, an increase in their relative concentration was observed during summer months [C18:1*trans*11 (August, 1.31%); C18:2*cis*9,*trans*11 (August, 0.55%) and C18:0 (June, 10.98%); **Figure 2.1b**]. In the mammary gland, C18:1*trans*11 may be desaturated by the Δ9 desaturase enzyme (stearoyl-coenzyme A desaturase; **SCD**) forming C18:2*cis*9,*trans*11, which further explains the increase or decrease of C18:2*cis*9,*trans*11 and C18:1*trans*11 in the same month (Peterson et al., 2002). The increase of C18:1*trans*10 and of C18:2*cis*9,*trans*11 and other CLA isomers is associated with decreased formation of *de novo* FA as well as C16:0 that partly originates from dietary FA, thus leading to lower concentrations of these FA in summer months (Table 2.1; Baumgard et al., 2000; Van Valenberg et al., 2013). Furthermore, levels of C18:0 were greater during summer months, which can be explained by the biohydrogenation pathway of C18:3*cis*9,12,15 forming C18:0 as end product (Palmquist, 2006; Chilliard et al., 2007). The FA C18:1*cis*15 and C18:1*cis*14 are characteristic intermediate biohydrogenation products of C18:3*cis*9,12,15 and increased in summer (Table 2.1) (Benchaar et al., 2012). This may suggest that the increased concentrations of C18:0 are mainly due to the biohydrogenation of C18:3*cis*9,12,15 rather than that of C18:2*cis*9,12.

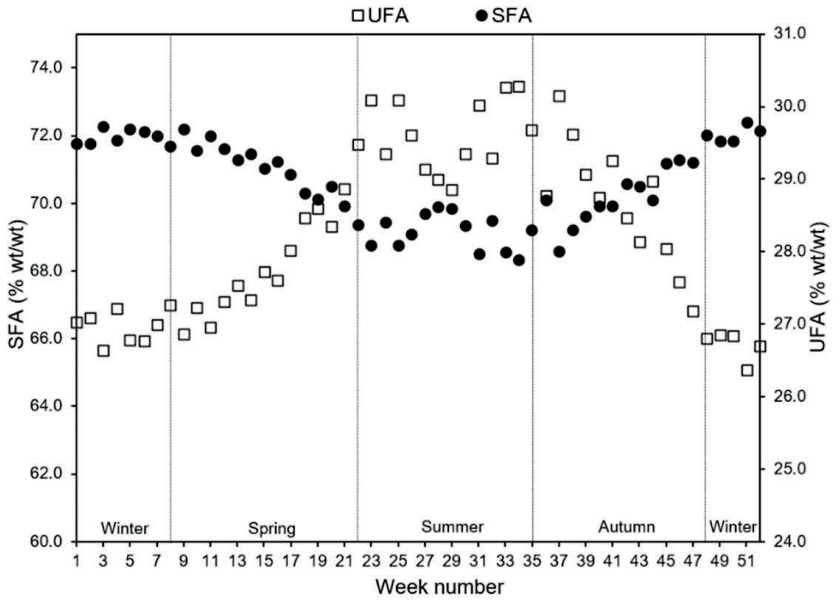
Milk UFA and SFA reached their maximum and minimum relative concentration (29.91% and 69.01%, respectively) in the same month (August), with a drop in UFA content and an increase in SFA content starting in week 29 (July; **Figure 2.1c**). Similar variations in C18:3*cis*9,12,15 were reported by Heck et al. (2009) close to week 30. As suggested by Heck et al. (2009), the yearly fluctuations of C18:3*cis*9,12,15 is most likely related to the quality and supply of fresh grass during the grazing period in the Netherlands.


C18:2cis9,12 (LA)
C18:3 cis9,12,15 (ALA)
C18:3cis9,12,15 (ALA) (%wt/wt)
C18:2cis9,12 (LA) (%wt/wt)
Winter
Spring
Summer
Autumn
Winter
Week number

a.


C18:2cis9,trans11
C18:1trans11
C18:0
C18:2cis9,trans11(% wt/wt) ; C18:1trans11 (%wt/wt)
C18:0 (%wt/wt)
Winter
Spring
Summer
Autumn
Winter
Week number

b.



c.

Figure 2.1. Weekly variation of milk fat fatty acids from May 2017 until April 2018 analyzed by gas chromatography (GC) – flame ionization detector (FID): a. C18:3*cis*9,12,15 (ALA) and C18:2*cis*9,12 (LA), b. C18:2*cis*9,*trans*11 (CLA), C18:1*trans*11 and C18:0 and c. Unsaturated fatty acids (UFA) and saturated fatty acids (SFA).

The odd- and branched-chain FA (**OBCFA**) differed significantly between months (P<0.05), but the variation was relatively small when compared to variation in other groups of FA (CV of 1.94% for OBCFA; CV of 2.77% to 4.82% for SCFA, MCFA and LCFA). The *iso* and *anteiso* branched-chain FA (**BCFA**) increased in summer, and the linear odd-chain FA C11:0, C13:0 and C15:0 increased in winter. In contrast to other odd-chain FA and in line with Heck et al. (2009), C17:0 increased in summer. In their review, Vlaeminck et al. (2006) suggested an increase in forage:concentrate ratio to be associated with altered rumen bacterial composition and generally increased milk OBCFA levels. Upon replacing corn silage with grass herbage, Couvreur et al. (2006) observed greater proportions of C15:0*iso*, C15:0*anteiso*, C17:0*iso* and C17:0*anteiso* in milk fat, but no significant effect on C14:0*anteiso* and C16:0*iso*. Upon increasing forage:concentrate ratio from 35:65 to 65:35 (DM basis), OBCFA proportion in milk fat increased (Sterk et al., 2011). All in all, these findings suggest that the high intake of fresh grass likely increased the BCFA content of milk fat in summer. Furthermore, the increased concentrations of linear odd-chain FA such as C11:0, C13:0 and C15:0 are likely related to a lower concentration of C18:3*cis*9,12,15 and associated biohydrogenation intermediates related to decreased suppression of *de*

novo FA synthesis (discussed previously) in winter. The maximum concentrations of C17:0 in milk fat in summer can be explained by the fact that C17:0 is both *de novo* synthesized and blood derived (Vlaeminck et al., 2006; Heck et al., 2012). Similarly, C17:1*cis*9 also increased in summer as a product from the desaturation of C17:0 by Δ9 desaturase enzyme activity.

Chlorophyll is the precursor for the formation of phytol and ultimately of phytanic acid, a multi-branched FA (Vlaeminck et al., 2006; Hellgren, 2010). Phytanic acid had its maximum relative concentration in winter (0.22%). This was unexpected considering the generally greater dietary chlorophyll content from grass herbage in the diet in summer/spring months. Lv et al. (2017) reported that phytol of Italian ryegrass was well preserved (87-140% of pre-ensiling levels) during ensiling at several nitrogen fertilization treatments and harvesting stages. In view of the greater total phytol content than the total chlorophyll content in silage, Lv et al. (2017) suggested that phytol may be derived from other substances besides chlorophyll, including phytol esters of FA, which may contribute towards greater phytanic acid contents in milk fat in winter than in summer.

2.3.2. Seasonal variation in TAG composition

Table 2.3 and **Figure 2.2** present the seasonal variation in TAG composition. Triacylglycerols were divided into low molecular weight TAG (**LMW**; CN26-CN36), medium molecular weight TAG (**MMW**; CN38-CN48) and high molecular weight TAG (**HMW**; CN50-CN54). The grouping was defined according to the FA esterified in these TAG and their CN (Gresti et al., 1993; Liu et al., 2020). All TAG groups significantly differed (P<0.05) between months throughout the year (Table 2.3). The seasonal variations of the LMW, MMW and HMW TAG groups are presented in **Figure 2.2a**. The LMW TAG had their maximum in autumn and winter, and minimum in summer. The HMW TAG had their maximum in summer and minimum in winter. These variations indicate that LMW and HMW groups seem to be homogeneous groups where all TAG within each group behave in a similar way (Table 2.3). However, this was not the case for MMW TAG. We have observed different patterns and smaller variations for the TAG species in the MMW group. Among them, CN38 was highest in autumn, CN42-CN46 were highest in winter, CN40 was highest in summer and CN48 was highest in spring (Table 2.3, Figure 2.2b). The variation in HMW TAG (CV=6.10%) was much higher compared to MMW (CV=1.47%) and LMW (CV=2.84%) TAG. Our results based on representative samples of the average Dutch bovine milk are in line with previous studies on seasonal variation in milk fat TAG composition based on a limited number of cows or farms (Capuano et al., 2014; Liu et al., 2017; Tzompa-Sosa et al., 2018).

Table 2.3. Descriptive statistics [mean (% wt/wt), standard deviation (SD), coefficient of variation (CV; %), minimum and maximum value] of the seasonal variation of milk fat triacylglycerol (TAG) composition from May 2017 until April 2018 as analyzed by gas chromatography (GC) – flame ionization detector (FID).[1]

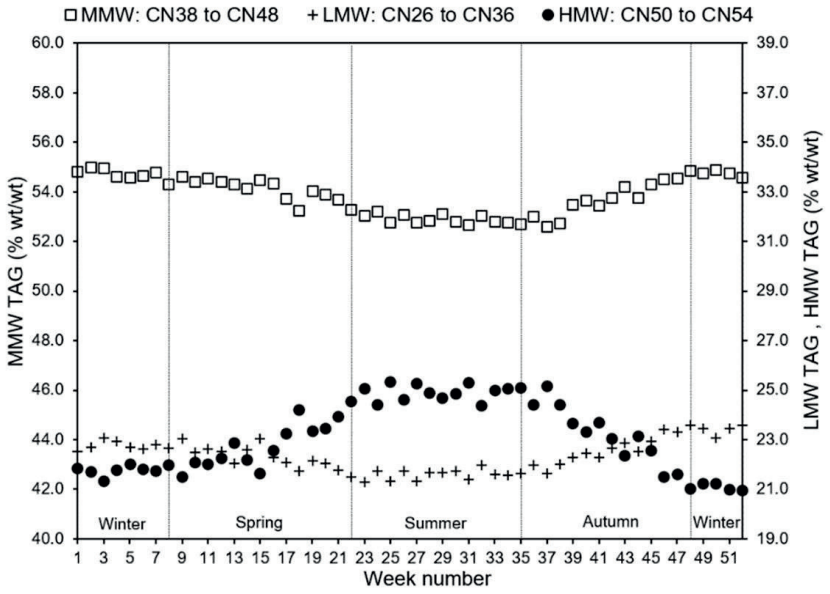
	Year			Minimum		Maximum		*P* value
Item	Mean	SD	CV (%)	Month	Mean	Month	Mean	Month effect
CN26	0.27	0.011	4.09	July	0.26	December	0.28	<0.001
CN28	0.61	0.021	3.44	July	0.58	November	0.64	<0.001
CN30	1.21	0.047	3.89	July	1.14	December	1.27	<0.001
CN32	2.67	0.103	3.85	July	2.54	December	2.83	<0.001
CN34	6.17	0.212	3.44	June	5.90	November	6.43	<0.001
CN36	11.02	0.256	2.33	June	10.68	November	11.35	<0.001
CN38	12.42	0.086	0.69	March	12.28	November	12.49	<0.001
CN40	9.69	0.107	1.10	September	9.56	June	9.84	<0.001
CN42	7.32	0.246	3.36	August	6.99	January	7.61	<0.001
CN44	7.10	0.299	4.21	August	6.72	January	7.46	<0.001
CN46	7.85	0.249	3.17	August	7.56	January	8.14	<0.001
CN48	9.42	0.143	1.51	June	9.28	March	9.54	0.005
CN50	10.86	0.302	2.78	December	10.49	July	11.27	<0.001
CN52	8.70	0.672	7.73	January	7.96	August	9.54	<0.001
CN54	3.61	0.465	12.86	January	3.13	June	4.21	<0.001
TAG groups								
LMW	22.33	0.634	2.84	June	21.29	November	23.57	<0.001
MMW	53.80	0.793	1.47	September	52.60	January	54.97	<0.001
HMW	23.18	1.413	6.10	January	21.65	August	24.95	<0.001

[1] CN: carbon number; LMW: low molecular weight (CN26 to CN36); MMW: medium molecular weight (CN38 to CN48); HMW: high molecular weight (CN50 to CN54). The TAG species included in each TAG group is described in Table S2.1. CN24 was included in the calculations but not shown in the table due to high CV (>20%). Cholesterol was also included in the calculations, but due to low reliability of its results with the ISO Standard 17678/IDF 202 (ISO, 2010) it was not reported in the table. The yearly mean, SD and CV were calculated based on the 52 weekly samples (n=52). The cut-off value for significant difference between months is $P<0.05$.

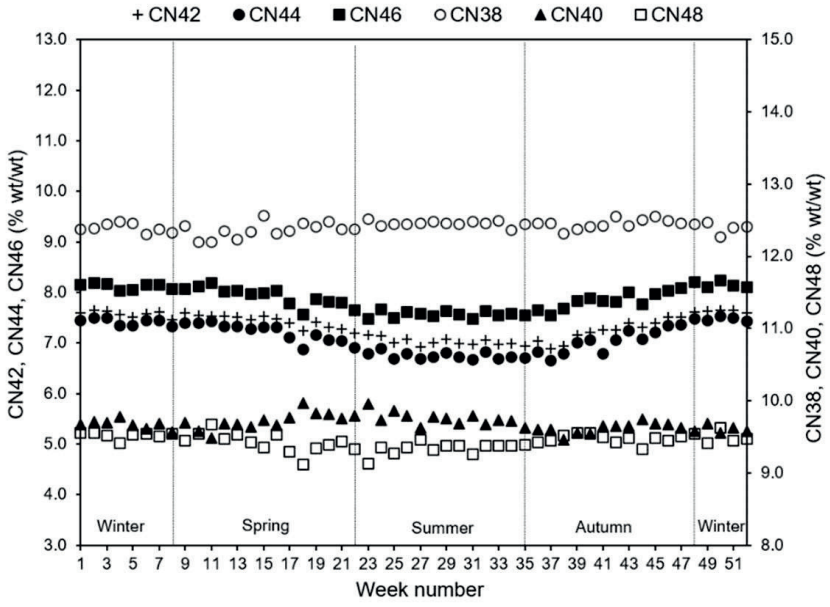
Supplemental Figure S2.1 presents the results of the Pearson correlation analysis that identifies the linear relationships between FA and TAG structures. In contrast with other milk FA, C4:0 was not significantly related to any of the TAG species. Unlike all other FA, C4:0 is esterified almost exclusively at the *sn*-3 position of the TAG (Jensen, 2002). Production of C4:0 does not require acetate because it can be produced directly from β-hydroxybutyrate derived from the blood, and furthermore changes in C4:0 concentration in milk fat upon dietary changes have been observed to differ from other FA shorter than 16 C atoms (Bargo et al., 2006). Such unique features of C4:0 relative to other milk FA might be related to the absence of significant relationships with various TAG species. The FA that were highly correlated with LMW, MMW and HMW TAG were identified in the structures of these TAG by previous studies (Gresti et al., 1993; Laakso and Manninen, 1997; Liu et al., 2020). In this study, high positive correlations ($r>0.6$, $P<0.05$) were observed between HMW

TAG species and many LCFA, PUFA and MUFA; notable exceptions include C14:1*cis*9, C16:1*cis*9, C18:1*cis*12, and DPA. The positively correlated FA in general had increased concentrations in summer, hence the increased concentrations of HMW TAG in summer milk fat. High positive correlations ($r>0.6$, $P<0.05$) were found between LMW TAG and SCFA, C12:0, C14:0, C14:1*cis*9 and C16:0. It can thus be suggested that the increased concentrations of LMW TAG happened in response to the high availability of these FA in winter milk fat. Among the MMW TAG group, TAG from CN42 to CN46 had positive correlations ($r>0.6$, $P<0.05$) with SCFA, C12:0, C14:0, C14:1*cis*9 and C16:0, which is in line with the increased concentrations of these FA in winter milk fat (Table 2.1). The TAG CN40 had high positive correlations ($r>0.6$, $P<0.05$) with C18:0, C18:1*cis*11, C18:1*cis*14 and C18:3*cis*9,12,15; therefore the higher concentrations of TAG 40 in summer months can be explained by the higher abundance of these FA in this season. We considered the correlations between the FA composition and TAG CN38 and CN48 inconclusive due to their low correlation coefficients ($-0.6<r<0.6$; $P<0.05$). This can be attributed to the rather stable concentrations of these TAG in a year and/or the variety of the FA that are esterified in these TAG species.

The low seasonal variation of the MMW group, and the different seasonal trends (the months when the highest and lowest concentrations were reached) observed for the TAG within the MMW group drew our attention. Therefore, the seasonal changes in the TAG species from CN38 to CN48 are presented separately in **Figure 2.2b** in detail. The TAG CN38 and CN48 showed a relatively stable pattern with small variations throughout the year, suggesting that these TAG are hardly affected by the FA seasonal variation in milk fat. The TAG CN40 had a slightly increased concentration from late spring to summer, and TAG from CN42 to CN46 showed similar variations between seasons with slightly decreased concentrations in summer compared to the other seasons. The small year-round variations observed in MMW TAG may be explained by the wide range of individual TAG species that have been identified within this group with large differences in FA composition. Bovine milk fat contains a large number of different TAG. Recently, Liu et al. (2020) identified 3454 TAG species characterized by CN:DB in bovine milk fat. They reported between 130 to 200 different TAG isomers (same CN:DB but different FA composition) for each TAG specie from CN38 to CN48. On the other hand, the number of isomers that were identified for each LMW TAG species from CN26 to CN36 was maximum 20 and for each HMW TAG species from CN50 to CN54 was maximum 30 (Liu et al., 2020).



a.



b.

Figure 2.2. Weekly variation of milk fat triacylglycerols (TAG) from May 2017 until April 2018 as analyzed by gas chromatography (GC) – flame ionization detector (FID). a. Low molecular weight (LMW; CN26 to CN36), medium molecular weight (MMW; CN38 to CN48) and high molecular weight (HMW; CN50 to CN54) TAG. b. MMW TAG groups CN38, CN40, CN42, CN44, CN46, CN48.

In addition to the TAG composition analyzed by GC-FID, the seasonal variation in TAG species was determined by MALDI-TOF-MS. With MALDI-TOF-MS we could determine not just the even-chain TAG species, but also the odd-chain TAG species as well as the saturation degree of the TAG species. **Table 2.4** presents the tentatively identified TAG species detected by MALDI-TOF-MS that were significantly different between seasons (P<0.05); the complete TAG profile with the 108 TAG species identified with MALDI-TOF-MS is presented in **Supplemental Table S2.1**. To be able to make a direct comparison to GC-FID, the TAG species identified with MALDI-TOF-MS were also divided into LMW TAG (CN26:0-CN36:0), MMW TAG (CN37:2-CN49:0) and HMW TAG (CN50:6-CN55:1). The seasonal trends in abundances of the LMW and HMW TAG groups identified with MALDI-TOF-MS were similar to those identified with GC-FID (Table 2.2). The LMW group had their maximum abundances in winter, where the TAG species with the highest abundance were CN34:0 and CN36:0. In contrast with the LMW group, the HMW group had their maximum abundance in summer, where the TAG species with the highest abundance were CN52:2 and CN52:1. Regarding the MMW group, its maximum concentration was identified in summer, nonetheless the difference between seasons was not significant. The TAG species with the highest abundance within the MMW were CN38:0 and CN48:1 with high concentrations in winter and CN38:1 with high concentrations in summer. Overall, the seasonal trend of the LMW, MMW and HMW are in accordance with the seasonal trends of the milk fat FA composition (Table 2.1, Supplemental Figure S2.1) and their identified presence in the different individual TAG species isomers that compose each TAG group (Liu et al., 2020) as discussed in a previous section. The increased abundances of mono- and polyunsaturated TAG species in summer are in accordance with the increased content of MUFA and PUFA in milk fat in summer (Table 2.1). Within the mono- and polyunsaturated TAG species, the HMW group had the highest abundance, due to the large number of unsaturated TAG species known to be esterified in their individual TAG species structures (Liu et al., 2020). The saturated TAG species abundance was highest in winter. Within the saturated TAG species, saturated TAG MMW group was the most abundant, in particular TAG species CN36:0 and CN38:0. These results are in accordance with the high concentrations of SFA in winter (Table 2.1). Finally, the odd-chain TAG species had maximum abundance in summer. In general, the most abundant odd-chain TAG species in summer were the MMW TAG species including CN37:1, CN39:1, and CN41:1. These results are also in accordance with the increased concentrations of the odd-chain FA in bovine milk fat in summer (Table 2.1).

Table 2.4. Descriptive statistics [mean (relative intensity (%)), standard deviation (SD), coefficient of variation (CV; %), minimum and maximum value] of the seasonal variation of milk fat triacylglycerol (TAG) composition from May 2017 until April 2018 as analyzed by MALDI-TOF-MS.[1]

		Year			Minimum		Maximum		*P* value
TAG Annotation	Mass to charge ratio (*m/z*)	Mean	SD	CV (%)	Season	Mean	Season	Mean	Season Effect
CN30:0	577.46	1.06	0.097	9.17	Spring	1.03	Summer	1.10	0.027
CN32:1	603.47	0.46	0.066	14.20	Winter	0.44	Summer	0.50	0.003
CN33:2	615.40	0.10	0.041	41.43	Winter	0.08	Summer	0.12	<0.001
CN33:1	617.52	0.17	0.029	17.67	Winter	0.15	Spring	0.17	0.008
CN34:0	633.53	3.52	0.255	7.24	Summer	3.43	Winter	3.65	0.001
CN35:1	645.52	0.17	0.031	18.94	Winter	0.16	Autumn	0.18	0.015
CN35:0	647.54	0.59	0.056	9.43	Summer	0.58	Winter	0.61	0.040
CN36:3	649.51	0.24	0.079	32.43	Summer	0.21	Winter	0.28	0.001
CN36:0	661.56	5.42	0.342	6.31	Summer	5.26	Winter	5.58	0.002
CN37:2	671.51	0.07	0.030	40.98	Winter	0.06	Autumn	0.08	0.014
CN37:1	673.55	0.33	0.047	14.21	Winter	0.31	Summer	0.35	0.013
CN38:6	677.54	0.36	0.108	29.82	Summer	0.31	Winter	0.43	<0.001
CN38:5	679.53	0.07	0.027	41.53	Summer	0.05	Winter	0.07	0.047
CN38:1	687.57	3.27	0.248	7.59	Winter	3.19	Summer	3.35	0.026
CN38:0	689.59	4.01	0.209	5.21	Autumn	3.94	Winter	4.08	0.026
CN39:2	699.55	0.11	0.024	22.02	Winter	0.10	Summer	0.12	0.003
CN39:1	701.58	0.37	0.042	11.45	Winter	0.35	Summer	0.39	0.001
CN40:6	705.57	0.32	0.083	25.71	Summer	0.28	Winter	0.37	<0.001
CN40:4	709.55	0.08	0.020	25.26	Winter	0.07	Autumn	0.08	0.026
CN40:2	713.59	0.86	0.094	10.96	Winter	0.79	Summer	0.93	<0.001
CN41:2	727.57	0.12	0.029	24.11	Winter	0.11	Summer	0.13	0.004
CN41:1	729.60	0.29	0.042	14.42	Spring	0.27	Summer	0.31	0.004
CN42:6	733.60	0.25	0.051	20.84	Spring	0.23	Winter	0.27	0.004
CN42:5	735.58	0.08	0.025	30.07	Spring	0.07	Summer	0.09	0.047
CN42:2	741.62	0.47	0.052	10.94	Winter	0.45	Summer	0.49	0.001
CN43:1	757.63	0.26	0.034	13.23	Spring	0.24	Autumn	0.27	0.003
CN44:1	771.06	0.10	0.097	95.55	Summer	0.06	Winter	0.14	0.008
CN44:0	773.68	1.12	0.109	9.69	Autumn	1.08	Spring	1.15	0.022
CN47:2	811.66	0.12	0.028	23.47	Winter	0.11	Summer	0.13	0.006
CN48:1	827.73	1.62	0.123	7.62	Summer	1.58	Winter	1.66	0.026
CN50:6	845.56	0.11	0.074	69.67	Summer	0.07	Winter	0.15	<0.001
CN52:2	881.77	1.29	0.142	11.03	Winter	1.19	Summer	1.40	<0.001
CN52:1	883.78	1.06	0.131	12.43	Winter	1.00	Summer	1.13	<0.001
CN52:0	885.78	0.31	0.057	18.66	Winter	0.28	Summer	0.33	0.014
CN54:4	905.76	0.15	0.029	19.88	Winter	0.14	Summer	0.16	0.011
CN54:3	907.78	0.37	0.061	16.28	Winter	0.33	Summer	0.41	<0.001
CN54:2	909.79	0.42	0.072	17.09	Winter	0.37	Summer	0.48	<0.001
CN54:1	911.80	0.26	0.044	16.58	Winter	0.24	Summer	0.31	<0.001
TAG groups									
LMW		31.99	1.602	5.01	Summer	31.29	Winter	32.56	0.011
MMW		51.31	0.884	1.72	Spring	51.19	Summer	51.41	0.814
HMW		16.71	1.071	6.41	Winter	16.11	Summer	17.31	<0.001
Saturated		28.61	0.878	3.07	Autumn	28.30	Winter	29.11	<0.001
Monounsaturated		19.72	0.631	3.20	Winter	19.46	Summer	20.02	0.001
Polyunsaturated		13.24	0.718	5.42	Winter	12.93	Summer	13.48	0.002
Odd-chain		9.47	0.554	5.85	Spring	9.28	Summer	9.63	0.013

[1]Only tentatively identified TAG species that differ significantly between seasons are presented. Winter: December, January, February; spring: March, April, May; summer: June, July, August; autumn: September, October, November. MALDI-TOF-MS: Matrix-assisted laser desorption/ionization - time of flight- mass spectrometry, CN: carbon number; LMW: low molecular weight; MMW: medium molecular weight; HMW: high molecular weight. The LMW (CN26:0–CN36:0), MMW (CN37:2–CN49:0), and HMW (CN50:6–CN55:1) were calculated based on the sum of all TAG species, and included identified and nonidentified TAG species. The saturated, monounsaturated, polyunsaturated, and odd-chain fatty acid groups were calculated based on the sum of the TAG species tentatively identified, presented in Supplemental Table S2.1. The yearly mean, SD, and CV were calculated based on the monthly samples (n=12).

The identified TAG compositional changes between seasons, especially between summer and winter, are known to influence the physical properties of milk fat (Mohan et al., 2020). One physical property that varies by changing the milk fat TAG composition is the solid fat content (**SFC**). High concentrations of unsaturated HMW TAG species in summer lead to a decrease in milk fat SFC (Couvreur et al., 2006b; Larsen et al., 2014), whereas high concentrations of saturated LMW and MMW in winter are expected to increase the milk fat SFC. The milk fat SFC at a specific temperature affects milk fat crystallization behavior and TAG polymorphism, which are important factors to consider for quality control of high fat containing products (Mohan et al., 2020). For example, a decrease in SFC at a specific temperature implies less fat will crystalize, thus softer milk fat texture may be expected. Moreover, depending on the TAG species present in milk fat (unsaturated or saturated) different TAG polymorphs will be formed (*e.g.* α, β' and β) that also define milk fat structure characteristics (*e.g.* smooth or "sand like" mouthfeel). Moreover, to control the crystal formation in milk fat and customize milk fat's texture characteristics (*e.g.* spreadability and hardness) for a specific product application the dairy industry applies tempering and ripening processes to milk fat. The conditions for cream ripening temperature are adjusted based on the unsaturation degree of bovine milk fat (Bylund, 2015; Khosrova, 2016; Mohan et al., 2020). Therefore the seasonal variations in TAG composition and their (un)saturation degree can greatly influence these processes. For this reason, analyzing the seasonal variations not only in the FA but also the TAG composition of milk fat will provide important insights on how to improve and adjust the parameters required for milk fat industrial processing for food product design and development.

2.4. Conclusions

Our study demonstrated a large seasonal variation in milk FA and TAG composition in Dutch bovine milk. In spring and summer, *de novo* synthesized FA decreased; biohydrogenation products and CLA isomers increased. The proportion of C18:2*cis*9,12 did not vary seasonally, suggesting rather a constant corn silage and concentrate intake throughout seasons. Maximum proportion of C18:3*cis*9,12,15 in late spring and summer reflected increased fresh herbage intake in these periods. In winter high availability of SCFA and C16:0 increased the formation of LMW and MMW (CN42-CN46) TAG, whereas in summer high concentrations of LCFA and UFA increased the formation HMW TAG. Overall, changes in FA composition throughout the year affected the FA availability for TAG synthesis resulting in seasonal changes in milk fat TAG composition. The implications of the seasonal variations in triacylglycerol composition on milk fat technological properties is of great interest to the food industry, yet it requires more in-depth study.

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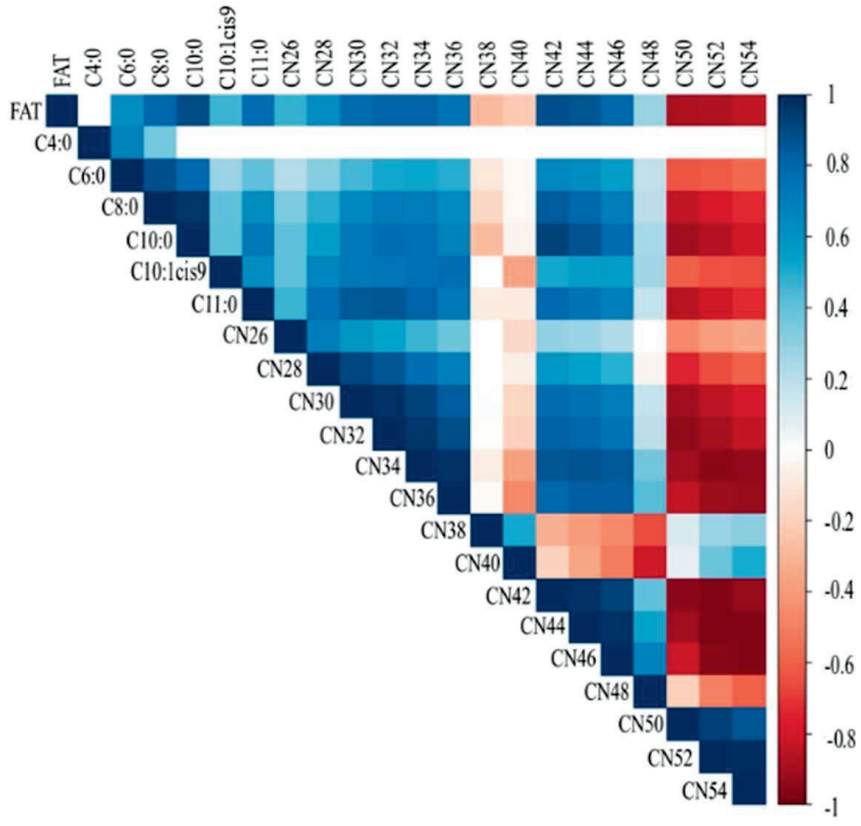
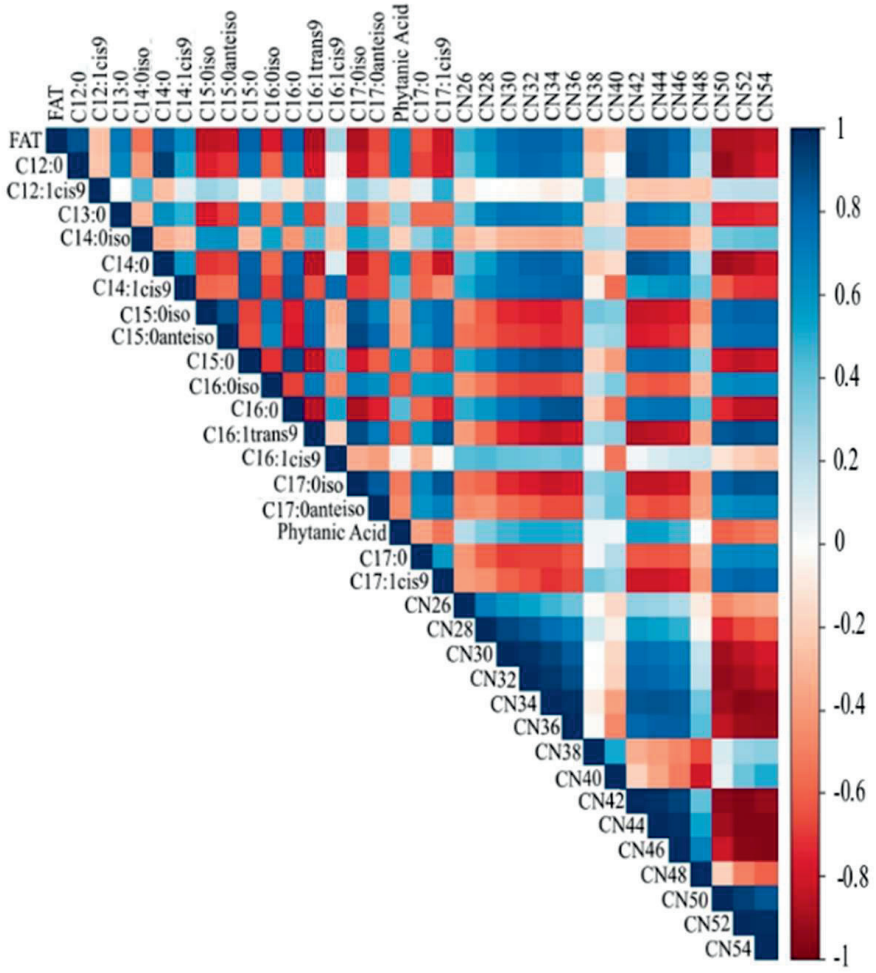
Supplementary Information



a.


FAT
C12:0
C12:1cis9
C13:0
C14:0iso
C14:0
C14:1cis9
C15:0iso
C15:0anteiso
C15:0
C16:0iso
C16:0
C16:1trans9
C16:1cis9
C17:0iso
C17:0anteiso
Phytanic Acid
C17:0
C17:1cis9
CN26
CN28
CN30
CN32
CN34
CN36
CN38
CN40
CN42
CN44
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1
0.8
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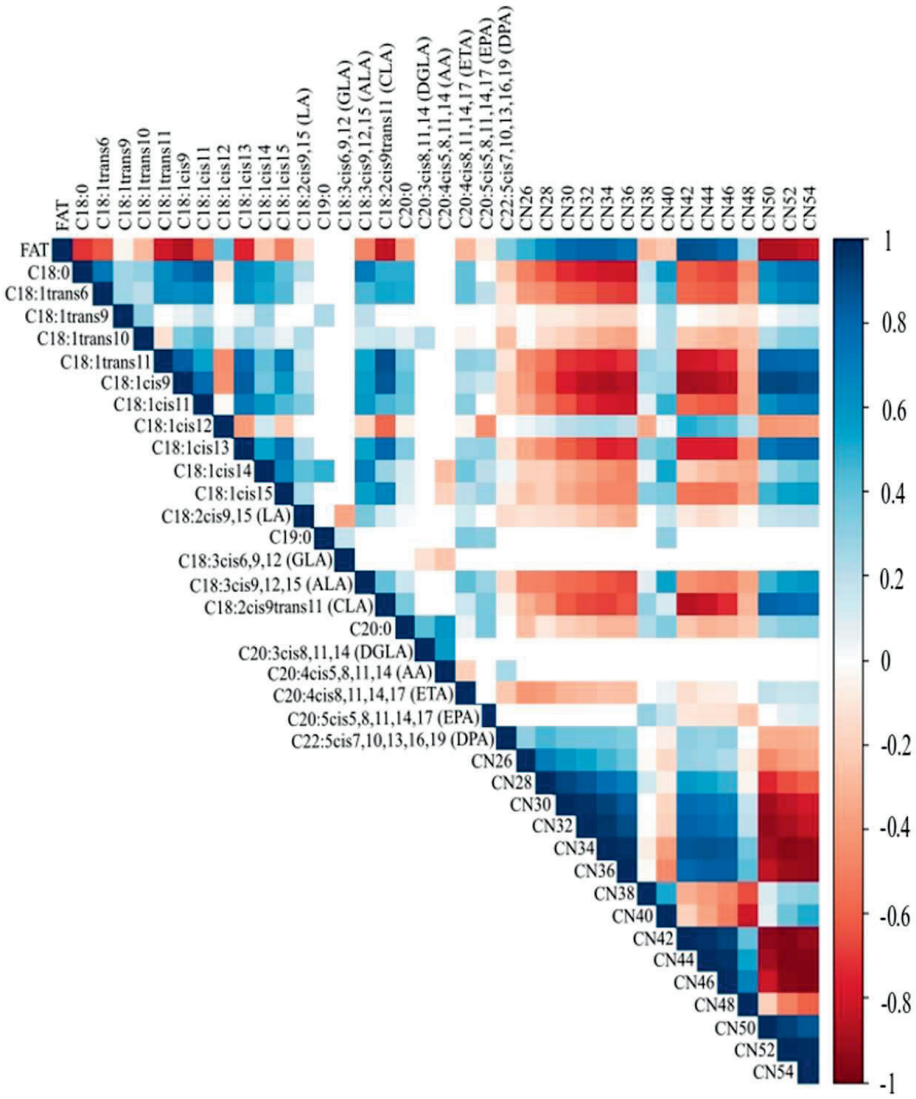
b.



c.

Figure S2.1. Pearson correlation analysis between triacylglycerol (TAG) species and fatty acids (FA) analyzed by gas chromatography (GC) – flame ionization detector (FID). The Pearson correlation analysis was divided in: a. Short-chain FA (SCFA) and all TAG species, b. Medium-chain FA (MCFA) and all TAG species, c. Long-chain FA (LCFA) and all TAG species.

Table S2.1. Descriptive statistics [mean (relative intensity (%)), standard deviation (SD), coefficient of variation (CV; %), minimum and maximum value] of the seasonal variation of milk fat triacylglycerol (TAG) composition from May 2017 until April 2018 as analyzed by MALDI-TOF-MS. Only the tentatively identified TAG species by MALDI-TOF-MS are presented.[1]

			Year			Minimum		Maximum		*P* value
TAG Annotation	Mass to charge ratio (*m/z*)	TAG Group	Mean	SD	CV (%)	Season	Mean	Season	Mean	Season Effect
CN26:0	521.39	LMW	0.31	0.052	16.86	Winter	0.30	Autumn	0.32	0.416
CN28:1	547.41	LMW	0.12	0.026	22.27	Winter	0.11	Summer	0.13	0.247
CN28:0	549.43	LMW	0.64	0.075	11.80	Winter	0.63	Summer	0.65	0.301
CN30:1	575.44	LMW	0.24	0.033	13.76	Winter	0.24	Summer	0.25	0.490
CN30:0	577.46	LMW	1.06	0.097	9.17	Spring	1.03	Summer	1.10	0.027
CN32:2	601.46	LMW	0.07	0.022	28.80	Winter	0.07	Summer	0.08	0.224
CN32:1	603.47	LMW	0.46	0.066	14.20	Winter	0.44	Summer	0.50	0.003
CN32:0	605.49	LMW	1.79	0.137	7.66	Autumn	1.76	Winter	1.84	0.093
CN33:2	615.40	LMW	0.10	0.041	41.43	Winter	0.08	Summer	0.12	<0.001
CN33:1	617.52	LMW	0.17	0.029	17.67	Winter	0.15	Spring	0.17	0.008
CN33:0	619.51	LMW	0.32	0.037	11.67	Autumn	0.32	Winter	0.33	0.603
CN34:2	629.49	LMW	0.11	0.031	27.12	Winter	0.11	Spring	0.12	0.588
CN34:1	631.51	LMW	0.84	0.079	9.43	Summer	0.83	Autumn	0.85	0.646
CN34:0	633.53	LMW	3.52	0.255	7.24	Summer	3.43	Winter	3.65	0.001
CN35:1	645.52	LMW	0.17	0.031	18.94	Winter	0.16	Autumn	0.18	0.015
CN35:0	647.54	LMW	0.59	0.056	9.43	Summer	0.58	Winter	0.61	0.040
CN36:3	649.51	LMW	0.24	0.079	32.43	Summer	0.21	Winter	0.28	0.001
CN36:2	657.53	LMW	0.24	0.037	15.11	Winter	0.23	Spring	0.25	0.171
CN36:1	659.54	LMW	1.79	0.151	8.43	Winter	1.75	Summer	1.81	0.396
CN36:0	661.56	LMW	5.42	0.342	6.31	Summer	5.26	Winter	5.58	0.002
CN37:2	671.51	MMW	0.07	0.030	40.98	Winter	0.06	Autumn	0.08	0.014
CN37:1	673.55	MMW	0.33	0.047	14.21	Winter	0.31	Summer	0.35	0.013
CN37:0	675.56	MMW	0.67	0.063	9.51	Spring	0.66	Summer	0.68	0.491
CN38:6	677.54	MMW	0.36	0.108	29.82	Summer	0.31	Winter	0.43	<0.001
CN38:5	679.53	MMW	0.07	0.027	41.53	Summer	0.05	Winter	0.07	0.047
CN38:3	683.54	MMW	0.12	0.026	21.20	Winter	0.12	Autumn	0.12	0.411
CN38:2	685.56	MMW	0.56	0.059	10.67	Winter	0.54	Autumn	0.57	0.062
CN38:1	687.57	MMW	3.27	0.248	7.59	Winter	3.19	Summer	3.35	0.026
CN38:0	689.59	MMW	4.01	0.209	5.21	Autumn	3.94	Winter	4.08	0.026
CN39:2	699.55	MMW	0.11	0.024	22.02	Winter	0.10	Summer	0.12	0.003
CN39:1	701.58	MMW	0.37	0.042	11.45	Winter	0.35	Summer	0.39	0.001
CN39:0	703.58	MMW	0.57	0.072	12.52	Spring	0.55	Winter	0.60	0.057
CN40:6	705.57	MMW	0.32	0.083	25.71	Summer	0.28	Winter	0.37	<0.001
CN40:5	707.55	MMW	0.07	0.023	32.77	Spring	0.06	Winter	0.08	0.084
CN40:4	709.55	MMW	0.08	0.020	25.26	Winter	0.07	Autumn	0.08	0.026
CN40:3	711.57	MMW	0.21	0.033	15.62	Winter	0.20	Spring	0.22	0.072
CN40:2	713.59	MMW	0.86	0.094	10.96	Winter	0.79	Summer	0.93	<0.001
CN40:1	715.60	MMW	2.17	0.139	6.43	Winter	2.12	Summer	2.19	0.084
CN40:0	717.62	MMW	2.31	0.125	5.42	Autumn	2.28	Winter	2.35	0.133
CN41:2	727.57	MMW	0.12	0.029	24.11	Winter	0.11	Summer	0.13	0.004
CN41:1	729.60	MMW	0.29	0.042	14.42	Spring	0.27	Summer	0.31	0.004
CN41:0	731.61	MMW	0.45	0.053	11.75	Spring	0.44	Winter	0.47	0.068
CN42:6	733.60	MMW	0.25	0.051	20.84	Spring	0.23	Winter	0.27	0.004
CN42:5	735.58	MMW	0.08	0.025	30.07	Spring	0.07	Summer	0.09	0.047
CN42:3	739.60	MMW	0.12	0.025	20.87	Summer	0.11	Spring	0.12	0.228
CN42:2	741.62	MMW	0.47	0.052	10.94	Winter	0.45	Summer	0.49	0.001

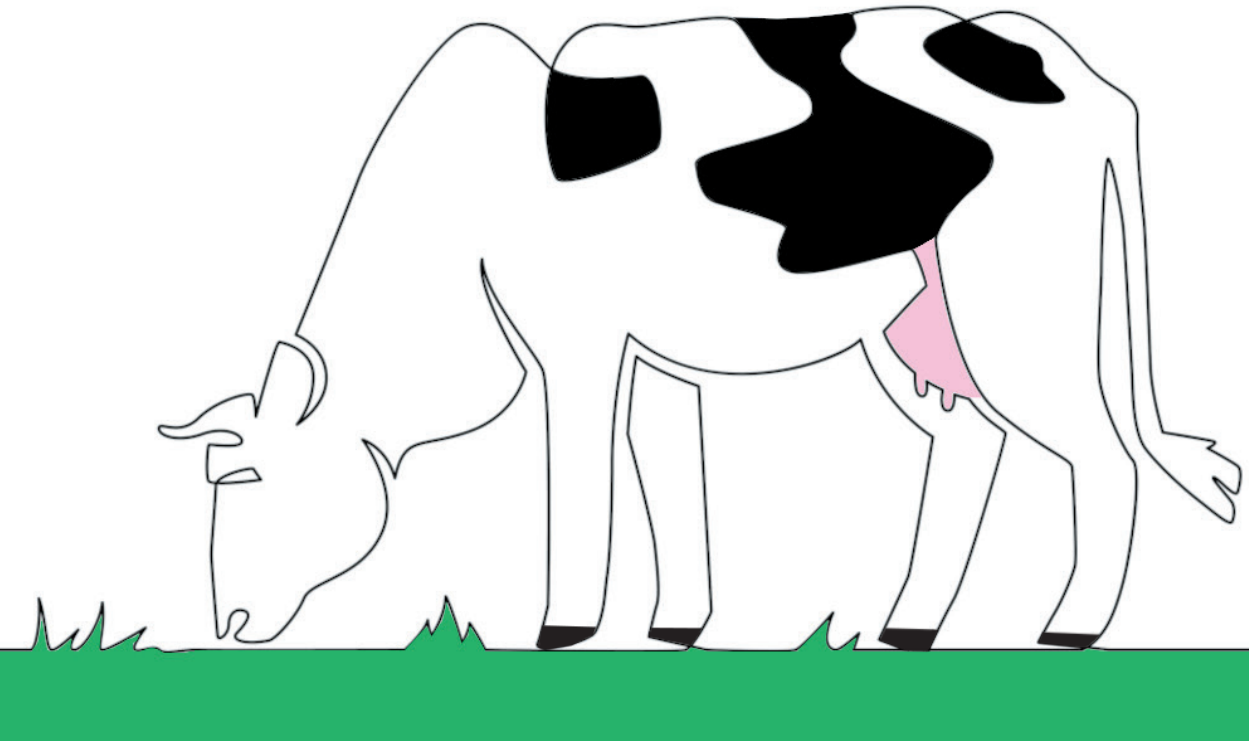
(continuation **Table S.2.1.)**

			Year			Minimum		Maximum		*P* value
TAG Annotation	Mass to charge ratio (*m/z*)	TAG Group	Mean	SD	CV (%)	Season	Mean	Season	Mean	Season Effect
CN42:1	743.63	MMW	1.38	0.120	8.70	Autumn	1.35	Winter	1.41	0.362
CN42:0	745.65	MMW	1.66	0.149	8.96	Autumn	1.61	Winter	1.70	0.094
CN43:2	755.59	MMW	0.10	0.023	22.53	Winter	0.10	Summer	0.11	0.065
CN43:1	757.63	MMW	0.26	0.034	13.23	Spring	0.24	Autumn	0.27	0.003
CN43:0	759.64	MMW	0.35	0.068	19.31	Spring	0.34	Autumn	0.36	0.396
CN44:6	761.62	MMW	0.20	0.048	23.87	Spring	0.19	Autumn	0.21	0.652
CN44:5	763.60	MMW	0.06	0.028	44.68	Spring	0.05	Autumn	0.07	0.092
CN44:3	767.63	MMW	0.10	0.022	20.90	Winter	0.10	Autumn	0.11	0.597
CN44:2	769.65	MMW	0.42	0.046	10.91	Winter	0.42	Autumn	0.43	0.384
CN44:1	771.06	MMW	0.10	0.097	95.55	Summer	0.06	Winter	0.14	0.008
CN44:0	773.68	MMW	1.12	0.109	9.69	Autumn	1.08	Spring	1.15	0.022
CN45:6	775.68	MMW	0.17	0.029	17.61	Autumn	0.16	Summer	0.17	0.387
CN45:1	785.66	MMW	0.25	0.033	13.61	Spring	0.24	Winter	0.25	0.971
CN45:0	787.66	MMW	0.30	0.048	15.92	Spring	0.29	Autumn	0.32	0.361
CN46:6	789.66	MMW	0.15	0.034	23.00	Spring	0.14	Summer	0.15	0.875
CN46:3	795.66	MMW	0.14	0.027	19.19	Summer	0.14	Spring	0.15	0.447
CN46:2	797.68	MMW	0.51	0.060	11.67	Winter	0.50	Summer	0.52	0.643
CN46:1	799.69	MMW	1.34	0.110	8.19	Summer	1.31	Winter	1.38	0.111
CN46:0	801.71	MMW	0.79	0.102	12.83	Autumn	0.76	Spring	0.81	0.183
CN47:6	803.70	MMW	0.12	0.024	19.60	Autumn	0.12	Summer	0.13	0.940
CN47:2	811.66	MMW	0.12	0.028	23.47	Winter	0.11	Summer	0.13	0.006
CN47:1	813.69	MMW	0.29	0.041	14.01	Spring	0.29	Autumn	0.30	0.844
CN47:0	815.70	MMW	0.29	0.046	16.18	Summer	0.28	Autumn	0.29	0.512
CN48:6	817.69	MMW	0.12	0.035	28.88	Spring	0.11	Autumn	0.13	0.108
CN48:3	823.69	MMW	0.16	0.029	18.05	Summer	0.16	Autumn	0.17	0.066
CN48:2	825.71	MMW	0.63	0.061	9.72	Winter	0.62	Spring	0.64	0.298
CN48:1	827.73	MMW	1.62	0.123	7.62	Summer	1.58	Winter	1.66	0.026
CN48:0	829.74	MMW	0.67	0.101	15.03	Winter	0.65	Summer	0.69	0.450
CN49:6	831.73	MMW	0.11	0.033	30.17	Summer	0.10	Spring	0.11	0.336
CN49:2	839.71	MMW	0.15	0.027	18.14	Winter	0.14	Autumn	0.16	0.254
CN49:1	841.73	MMW	0.39	0.049	12.57	Spring	0.38	Summer	0.39	0.583
CN49:0	843.73	MMW	0.29	0.046	15.60	Autumn	0.29	Winter	0.31	0.275
CN50:6	845.56	HMW	0.11	0.074	69.67	Summer	0.07	Winter	0.15	<0.001
CN50:5	847.68	HMW	0.06	0.018	30.27	Spring	0.06	Summer	0.06	0.769
CN50:4	849.70	HMW	0.09	0.030	31.68	Winter	0.09	Autumn	0.10	0.268
CN50:3	851.72	HMW	0.26	0.041	15.81	Winter	0.25	Autumn	0.27	0.440
CN50:2	853.74	HMW	0.94	0.095	10.15	Winter	0.91	Summer	0.96	0.087
CN50:1	855.76	HMW	1.81	0.155	8.58	Winter	1.79	Summer	1.83	0.628
CN50:0	857.76	HMW	0.58	0.088	15.24	Winter	0.56	Spring	0.60	0.128
CN51:6	859.75	HMW	0.09	0.028	29.06	Autumn	0.09	Summer	0.10	0.921
CN51:2	867.74	HMW	0.24	0.037	15.87	Spring	0.23	Autumn	0.25	0.152
CN51:1	869.75	HMW	0.39	0.061	15.72	Winter	0.38	Summer	0.40	0.315
CN51:0	871.75	HMW	0.29	0.067	22.61	Summer	0.28	Autumn	0.31	0.144
CN52:6	873.75	HMW	0.12	0.029	23.93	Summer	0.12	Winter	0.13	0.351
CN52:4	877.74	HMW	0.13	0.027	20.36	Autumn	0.13	Spring	0.14	0.717
CN52:3	879.75	HMW	0.38	0.052	13.72	Winter	0.37	Summer	0.39	0.463
CN52:2	881.77	HMW	1.29	0.142	11.03	Winter	1.19	Summer	1.40	<0.001
CN52:1	883.78	HMW	1.06	0.131	12.43	Winter	1.00	Summer	1.13	<0.001
CN52:0	885.78	HMW	0.31	0.057	18.66	Winter	0.28	Summer	0.33	0.014
CN53:3	893.74	HMW	0.09	0.024	27.37	Winter	0.08	Summer	0.09	0.482
CN53:2	895.76	HMW	0.20	0.033	16.30	Winter	0.19	Summer	0.21	0.123

(continuation **Table S.2.1.)**

			Year			Minimum		Maximum		*P* value
TAG Annotation	Mass to charge ratio (*m/z*)	TAG Group	Mean	SD	CV (%)	Season	Mean	Season	Mean	Season Effect
CN53:1	897.77	HMW	0.28	0.049	17.84	Winter	0.27	Summer	0.29	0.437
CN53:0	899.77	HMW	0.19	0.045	24.07	Summer	0.18	Autumn	0.20	0.230
CN54:6	901.75	HMW	0.09	0.026	29.50	Spring	0.08	Winter	0.09	0.716
CN54:5	903.75	HMW	0.10	0.024	25.25	Winter	0.09	Autumn	0.10	0.542
CN54:4	905.76	HMW	0.15	0.029	19.88	Winter	0.14	Summer	0.16	0.011
CN54:3	907.78	HMW	0.37	0.061	16.28	Winter	0.33	Summer	0.41	<0.001
CN54:2	909.79	HMW	0.42	0.072	17.09	Winter	0.37	Summer	0.48	<0.001
CN54:1	911.80	HMW	0.26	0.044	16.58	Winter	0.24	Summer	0.31	<0.001
CN54:0	913.79	HMW	0.10	0.035	33.45	Winter	0.10	Spring	0.11	0.632
CN55:2	923.77	HMW	0.10	0.024	24.14	Winter	0.09	Summer	0.11	0.082
CN55:1	925.78	HMW	0.10	0.024	24.77	Winter	0.10	Summer	0.10	0.613

[1] Only tentatively identified TAG species that differ significantly between seasons are presented. Winter: December, January, February; spring: March, April, May; summer: June, July, August; autumn: September, October, November. MALDI-TOF-MS: Matrix assisted laser desorption/ionization - time of flight - mass spectrometry, CN: Carbon number; LMW: Low molecular weight; MMW: Medium molecular weight; HMW: High molecular weight. The LMW (CN26:0–CN36:0), MMW (CN37:2–CN49:0), and HMW (CN50:6–CN55:1) were calculated based on the sum of all TAG species, and included identified and nonidentified TAG species. The yearly mean, SD, and CV were calculated based on the monthly samples (n=12).



Chapter 3

Seasonal variation in the positional distribution of fatty acids in bovine milk fat

This chapter is based on:

Yener S., S. Pacheco-Pappenheim S., J.M.L. Heck and H.J.F. van Valenberg. Seasonal variation in the positional distribution of fatty acids in bovine milk fat. Accepted for publication in the Journal of Dairy Science.

Abstract

The aim of this study was to determine the seasonal variation in the positional distribution of fatty acids (**FA**) in bovine milk fat. Bovine milk samples were collected from May 2017 to April 2018 in the Netherlands and the FA composition in the *sn*-2 position was determined by using *sn*-1(3)-selective transesterification of *Candida antarctica* lipase B. The majority of the FA showed significant variation at *sn*-2 and *sn*-1(3) positions between different seasons. The seasonal variation in *sn*-2 position was higher than the *sn*-1(3) positions. Parallel to the changes in the diet of the cows throughout a year, during summer an increase was observed in blood derived FA (*i.e.* C18:0, C18:1*cis*9) concentrations and a decrease in *de novo* synthesized FA. In winter, more saturated FA (**SFA**) were esterified in *sn*-2 position of milk fat. Highest concentrations of palmitic acid, C16:0, was observed in *sn*-2 position in winter, whereas the amount of unsaturated FA (**UFA**) at this position was highest in summer. These results showed that the FA compositions in different regiospecific positions changed due to season however the proportions of a specific FA within the three positions of the triacylglycerols in milk fat did not chance upon seasonal variation.

3.1. Introduction

Bovine milk fat has a unique chemical composition with thousands of different triacylglycerols (**TAG**) species present in various quantities (Jensen, 2002, Liu et al., 2020b). The TAG composition and structure of milk fat influences its physical properties and therefore its applicability in different food products. For example, crystallization and melting properties of butter, cheese and cream is highly dependent on milk fat TAG (Lopez, 2020, Mohan et al., 2020). Moreover, binding positions of fatty acids (**FA**) in among the different *sn*-positions (stereospecific numbering) in TAGs is specific and non-random. Short-chain FA (**SCFA**) such as C4:0 and C6:0 are preferentially esterified at the *sn*-3 position, the majority of C18:1*cis*9 is esterified at *sn*-2 position whereas about 40-45% C16:0 is found in the *sn*-2 position (Gresti et al., 1993, Jensen, 2002). Stereochemistry of TAG is known to affect lipid metabolism. The binding positions of FA in the TAG molecule may influence the hydrolysis rates of free FA (**FFA)** (Yoshinaga et al., 2015b). The authors found that docosahexaenoic acid (C22:6*cis*4,7,10,13,16,19-**DHA**) bound at the *sn*-2 position was absorbed more efficiently and reduced the TAG and cholesterol levels in the serum and liver of mice. Depending on the TAG structure, the absorption efficiency of the FA and minerals may change. Higher amounts of C16:0 at *sn*-2 position reduces the formation of insoluble calcium soaps and therefore improves calcium absorption in breast-fed infants (Innis, 2011).

The amount and composition of bovine milk fat on the other hand can be influenced by physiological factors (*i.e*. genetic, stage of lactation) and the changes in feeding regimen through supplementation of oils and fats into the cows' diet, farming practices and seasonal variation (Jensen, 2002). A large number of studies have been published on modifying the composition of milk fat via fat supplementation. However only a few studies focused on the changes in the positional distribution of FA due to the changes in feeding regimen. When cows were fed with elevated levels of linoleic acid (C18:2*cis*9,12) the distribution of FA in the high and low molecular weight TAG was affected by increased amounts of linoleic acid (Morrison and Hawke, 1977). In low molecular weight TAG, linoleic acid is preferentially esterified in the *sn*-2 position, whereas in high molecular weight TAG it is preferentially esterified the *sn*-3 position. This also affected the distribution of other FA in TAG. In another study, linoleic acid was found to be more dominant at *sn*-2 position when the feed was rumen protected (Christie and Clapperton, 1982). Reduced concentrations of C16:0 at *sn*-2 and increased concentrations of C18:1*cis*9 at *sn*-2 position were observed in milk fat of the cows fed with canola oil (rich in C18:1*cis*9) (DePeters et al., 2001).

Recent studies showed seasonal variation in milk fat FA composition mostly due to the changes in cows' diet from winter (silage-based diet and concentrate) to summer (grass-based diet; Heck et al., 2009, Liu et al., 2017, Pacheco-Pappenheim et al., 2019, Liu et al.,

2020a). In summer, higher amounts of C18 UFA were observed in milk fat whereas in winter *de novo* synthesized FA and as a consequence SFA increased. Studies that investigated the influence of seasons on the distribution of FA in bovine milk fat TAG go back to forty years ago (Parodi, 1979). The authors reported small changes in the quantities of major FA such as C16:0 and long-chain FA (**LCFA**, C18-C22) C18:0 and C18:1*cis*9 at the *sn*-2 position. The changes in the positioning of medium-chain FA (**MCFA**) were greater than the others. In their study, pancreatic lipase had been used for partial hydrolysis of TAG. Studies showed that pancreatic lipase has substrate specificity towards the SCFA containing TAG (Watanabe et al., 2009, Yoshinaga et al., 2016). As a consequence of rumen metabolism, bovine milk fat is rich in SCFA which makes it less suitable for the application of pancreatic lipase. In the recent years, an immobilized lipase from *Candida antarctica* B (**CALB**) was proposed as an alternative enzyme for the determination of positional distribution of FA in milk fat. This enzyme has been successfully applied for the characterization of various fats and oils with high contents of SCFA including milk fat (Watanabe et al., 2014, Watanabe et al., 2015, Yoshinaga et al., 2016).

The objective of this study is to determine the seasonal variation in the regiospecific composition of FA in bovine milk fat obtained in twelve consecutive months from May 2017 to April 2018 in the Netherlands.

3.2. Materials and Methods

3.2.1. Sample selection

Bovine milk samples were collected on a weekly basis from May 2017 till April 2018 by the Dutch Milk Control Institute Qlip Laboratories (Zutphen, The Netherlands) from fourteen dairy factories as described in Pacheco-Pappenheim et al., (2021). As previously described by Pacheco-Pappenheim et al., (2021), the dairy farming system in the Netherlands includes an indoor housing system in winter where the diet mainly consist of grass silage (37%), corn silage (19%), grass herbage (14%) wet by-products (4%) and concentrate (25%) on DM basis. Whereas during the grazing season, the intake of fresh pasture increases in spring and summer and the majority of the cows consume grass herbage (70%).

Milk fat was extracted from the weekly raw bulk milk samples according to Tzompa-Sosa et al., (2014) with small modifications. Shortly, the raw milk samples were centrifuged at 4°C for 15 min at 4700 rpm. Then the cream layer was separated with a spoon and transferred into clean tubes. After addition of 4M HCl to the cream, it was shaken for 30 min at 70°C in a water bath until a clear fat phase was obtained. The fat fraction was washed with MilliQ water to remove the water soluble residues. The washing step was done keeping the tubes at 70°C for 15 min in a water bath via shaking every 5 min. The impurities were removed with a glass Pasteur pipette and repeated when necessary. This method ensures

that only neutral lipids are present in the fat extract. This was confirmed with thin-layer chromatography (**TLC**) according to Yener and van Valenberg, (2019). Milk fat samples obtained from each week (n=52) were then pooled in equal ratios (v/v) in order to obtain monthly samples (n=12). The monthly samples were used for positional distribution analysis of FA.

3.2.2. Analysis of fatty acid regiospecific distribution

The composition of the FA at the 2-position of milk fat was determined according to an enzymatic transesterification method using *Candida Antarctica* lipase B (**CALB**; Novozym® 435, 10,000 PLU/g, Novozymes A/S, Frederiksberg, Denmark; AOAC, 2019). Firstly, 100 mg milk fat was dissolved in 2 ml of ethanol and 44 mg CALB was added. The mixture was first incubated at 50°C for 10 min and then up to 3 hours at 30°C by shaking them horizontally at 170 rpm. After this step, the lipase was removed from the reaction mixture by filtering through an absorbent cotton. The 2-monoacylglycerols (**2-MAG**) were then recovered from the reaction mixture and purified by using solid phase extraction. First, the reaction mixture was evaporated at 30°C by using a vacuum concentrator (RVC 2-18 CD Plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Then, the reaction mixture was dissolved in 200 µl of hexane and applied to a Sep-Pak Silica cartridge (0.69 g, Waters Co., Miliford, MA, USA), which was pre-equilibrated with hexane : diethyl ether (80:20, v/v) mixture (**Solvent 1**). Fatty acid ethyl esters (**FAEE**) were washed out with 10 ml of Solvent 1 and diacylglycerols (**DAG**) were washed out with 20 ml of the same solvent. The fraction containing 2-MAG was eluted with 10 ml of diethyl ether. This fraction was then evaporated under a gentle steam of N_2 and used for FA analyses. Enzymatic transesterification with CALB was performed in duplicate in monthly milk fat samples.

3.2.3. Fatty acid composition analyses

Fatty acids in the bovine milk fat samples and 2-MAG fractions corresponding to each month were analysed as their methyl esters by gas chromatography-flame ionization detector (**GC-FID**; Thermo Scientific Trace GC Ultra) using WCOT fused silica column (100 m × 0.25 mm i.d. X 0.2 µm f.t., Coating Select Fame, Varian, Houten, The Netherlands). Fatty acid methyl esters (**FAME**) were prepared via transesterification with methanolic KOH according to ISO standard 15884 (ISO15884, 2002). The FAME composition of *sn*-2 position of milk fat samples was analysed according to ISO standard 16958 (ISO16958, 2015) in the laboratories of Wageningen Food Safety Research (Wageningen, the Netherlands). The overall FA composition of bovine milk fat samples were performed by the Dutch Milk Control Institute Qlip (Quality, Laboratory, Inspections and Process Certification) Laboratories (Zutphen, the Netherlands) according the aforementioned ISO standard and the FA compositions were previously reported by Pacheco-Pappenheim et al., (2021). The FA compositions at the *sn*-2 and *sn*-1(3) positions

and the proportion of FA over the three positions were calculated according to Tzompa-Sosa et al., (2014) using intra- and interpositional distributions. With intrapositional distribution the FA compositions of different fats and oils can be characterized and binding positions of specific FA (*e.g. trans* FA, long-chain unsaturated FA) can be determined. It is also needed to calculate the interpositional distribution of FA. On the other hand, interpositional distribution is relevant for the nutritional and technological aspects of milk fat. It is beneficial in infant formula development since fats and oils can easily be compared to human milk to provide a better FA absorption for the infants. Besides, the TAG structure determines the types of polymorphs (α, β, β') that can be formed during crystallization which in turn affect the texture and mouthfeel of milk fat. Lastly, it can help understanding the FA binding mechanisms during TAG synthesis. The formulas used in the calculations are given in formulas 1-3. The FA compositions of intrapositional distribution were expressed in mol percentages (mol%).

Intrapositional distribution:

$$mol\%\ sn-1(3)_{\mathrm{FA}i} = \frac{3 \times \mathrm{TAG}_{\mathrm{FA}} - sn-2_{\mathrm{FA}i}}{2} \quad \text{(Equation 3.1)}$$

where *sn*-2 $_{FAi}$ is the measured molar percentage of each FA in *sn*-2 position and *sn*-1(3)$_{FAi}$ is the molar percentage of each FA at *sn*-1(3) position, TAG $_{FAi}$ is the molar percentage of a FA in TAG (before enzymatic hydrolysis) as reported by Pacheco-Pappenheim et al., (2021).

Interpositional distribution:

$$\%\ sn-2_{\mathrm{FA}i} = \frac{sn-2_{\mathrm{FA}i}}{sn-2_{\mathrm{FA}i} + (sn-1(3)_{\mathrm{FA}i} \times 2)}\ x\ 100 \quad \text{(Equation 3.2)}$$

$$\%\ sn-1(3)_{\mathrm{FA}} = \frac{sn-1(3)_{\mathrm{FA}i}}{sn-2_{\mathrm{FA}i} + (sn-1(3)_{\mathrm{FA}i} \times 2)}\ x\ 100 \quad \text{(Equation 3.3)}$$

where *sn*-2 $_{FAi}$ is the molar percentage of each FA in *sn*-2 position and *sn*-1(3)$_{FAi}$ is the molar percentage of each FA at *sn*-1(3) position.

3.2.4. Statistical analyses

The seasonal variation in the FA composition at the *sn*-2 and *sn*-1(3) positions throughout the year was assessed by using one-way ANOVA with Tukey Post Hoc comparison. The months were grouped according to the corresponding seasons (Spring: March, April, May; Summer: June, July, August; Autumn: September, October, November; Winter: December January, February). The differences between the FA compositions of the *sn*-2 and *sn*-1(3) positions in each season were tested by a two-sample T-test. The esterification preferences of FA were tested against the hypothetical values, 33.3% for *sn*-2 and 66.7% for *sn*-1(3), were also tested by a one-sample T-test. Differences were considered significant when $P<0.05$. All the statistical analyses were performed by using R programming language (Team, 2013).

3.3. Results and Discussion

Bovine milk fat has a complex composition. In the mammary gland, the FA are arranged in TAG structures over the three regiospecific positions. The variety of the FA and their binding positions within TAG molecule in turn significantly affect the physical and nutritional properties of bovine milk fat (Jensen, 2002). In this study, we looked into the FA compositions of the primary (*sn*-1(3)) and secondary (*sn*-2) positions of bovine milk fat (**Table 3.1**) and the preferential positioning of FA (**Table 3.2**) throughout a year.

3.3.1. Regiospecific distribution of fatty acids in bovine milk fat

3.3.1.1. Fatty acid compositions of *sn*-2 and *sn*-1(3) positions

The average FA compositions of Dutch bovine milk fat from May 2017 to April 2018 and the regiospecific distribution of the FA in the secondary (*sn*-2) and primary (*sn*-1(3)) positions are given in **Table 3.1**. The results shown in Table 3.1 are based on an intrapositional distribution where the FA composition of the primary and secondary positions were indicated (Blasi et al., 2008, Tzompa-Sosa et al., 2014). According to Table 3.1, the major FA at *sn*-2 position of bovine milk fat were C16:0 (35.65 mol%), C14:0 (20.16 mol%), C18:1 cis-9 (14.28 mol%), C12:0 (6.50 mol%) and C18:0 (4.19 mol%). A small proportion of SCFA such as C4:0 and C6:0 were also detected at *sn*-2 position. On the other hand, the most abundant FA in the *sn*-1(3) positions were C16:0 (22.60 mol%), C4:0 (18.45%), C18:1*cis*9 (14.01 mol%), C18:0 (8.94 mol%), C6:0 (7.71 mol%), C14:0 (6.94 mol%) and C10:0 (4.37 mol%). The values found for the compositions of these FA at primary and secondary positions of bovine milk fat are in line with literature (Jensen, 2002, Tzompa-Sosa et al., 2014, Yoshinaga et al., 2016).

The FA compositions between the *sn*-2 and *sn*-1(3) positions in each season were compared and the results are provided in **Supplemental Table S3.1**. In general, the TAG were mainly composed of SFA and the concentration of SFA were slightly higher at *sn*-2 position. The concentration of UFA at *sn*-1(3) positions was higher than that of *sn*-2 position. Within UFA, there were more PUFA located at *sn*-2 position and more MUFA at *sn*-1(3) positions (Table 3.1). In addition to the major FA, minor FA were also detected at different regiospecific positions. For example, the amount of branched-chain FA (**BCFA**) (*e.g.* C15:0*iso*, C15:0*anteiso*, C17:0*iso* and *anteiso*) at *sn*-2 and *sn*-1(3) positions were around 2.52 and 0.95 mol%, respectively. The amounts of individual BCFA located at *sn*-2 position decreased with increasing carbon chain length (*e.g.* more C15:0 than C17:0). *Anteiso* BCFA were more abundant at *sn*-2 position then their *iso* isomers. Compared to the total BCFA, C15:0*anteiso* was the most abundant FA. Similar findings were reported for regiospecific distribution of BCFA in bovine milk fat (Yan et al., 2017). In addition, around 1.15 mol% *trans* FA (**TFA**) in *sn*-2 and 1.52 mol% in *sn*-1(3) positions were detected. There were more odd-chain FA (**OCFA**) in the *sn*-2 position than *sn*-1(3) positions in bovine milk fat.

Table 3.1. Seasonal variation in the total and regiospecific distribution of fatty acids [FA; mean (SEM)] of bovine milk fat triacylglycerols according to seasons from May 2017 to April 2018 (intrapositional distribution).[1]

FA	TAG (mol %)	*sn*-2 position (mol %)						*sn-1(3)* positions (mol %)					
		Year average	Spring	Summer	Autumn	Winter	*P* value	Year average	Spring	Summer	Autumn	Winter	*P* value
C4:0	12.37 (0.05)	0.30	0.30 (0.01)	0.31 (0.02)	0.33 (0.02)	0.28 (0.01)	0.1133	18.45	18.45 (0.09)	18.45 (0.07)	18.29 (0.19)	18.63 (0.16)	0.3737
C6:0	5.36 (0.02)	0.71	0.71 (0.02)	0.72 (0.02)	0.74 (0.02)	0.69 (0.01)	0.4691	7.71	7.79 (0.05)	7.52 (0.02)	7.61 (0.03)	7.91 (0.08)	<.0001
C8:0	2.26 (0.01)	1.86	1.89 (0.03)	1.82 (0.02)	1.89 (0.04)	1.85 (0.02)	0.2091	2.47	2.51 (0.02)	2.38 (0.02)	2.43 (0.03)	2.57 (0.03)	0.0002
C10:0	4.17 (0.03)	3.80	3.90 (0.02)	3.64 (0.04)	3.81 (0.05)	3.85 (0.05)	0.0036	4.37	4.49 (0.06)	4.11 (0.03)	4.26 (0.07)	4.62 (0.03)	<.0001
C10:1*cis*9	0.44 (0.00))	0.45	0.45 (0.01)	0.42 (0.00)	0.47 (0.01)	0.47 (0.01)	0.0001	0.43	0.41 (0.01)	0.43 (0.00)	0.45 (0.01)	0.44 (0.01)	0.0195
C12:0	4.56 (0.03))	6.50	6.65 (0.02)	6.26 (0.04)	6.39 (0.08)	6.69 (0.04)	<.0001	3.61	3.68 (0.05)	3.41 (0.02)	3.56 (0.08)	3.77 (0.02)	0.0003
C12:1*cis*9	0.1 (0.00)	0.21	0.21 (0.00)	0.2 (0.00)	0.22 (0.00)	0.22 (0.00)	0.0003	0.05	0.04 (0.01)	0.06 (0.00)	0.07 (0.01)	0.04 (0.01)	0.0797
C13:0	0.09 (0.01)	0.29	0.30 (0.00)	0.27 (0.00)	0.29 (0.01)	0.31 (0.00)	<.0001	n.d.					
C14:0*iso*	0.08 (0.00)	0.17	0.16 (0.00)	0.18 (0.00)	0.17 (0.00)	0.16 (0.00)	<.0001	0.04	0.03 (0.00)	0.04 (0.00)	0.03 (0.00)	0.04 (0.00)	0.1959
C14:0	11.34 (0.04)	20.16	20.36 (0.05)	19.76 (0.13)	19.97 (0.17)	20.57 (0.05)	0.0002	6.94	7.02 (0.07)	6.73 (0.08)	6.92 (0.12)	7.11 (0.09)	0.0607
C14:1*cis*9	1.09 (0.01)	1.58	1.54 (0.01)	1.52 (0.01)	1.63 (0.01)	1.62 (0.01)	<.0001	0.85	0.82 (0.01)	0.78 (0.02)	0.9 (0.02)	0.88 (0.01)	<.0001
C15:0*iso*	0.21 (0.00)	0.34	0.33 (0.01)	0.37 (0.00)	0.34 (0.00)	0.32 (0.00)	<.0001	0.15	0.15 (0.00)	0.16 (0.00)	0.15 (0.00)	0.14 (0.00)	0.0022
C15:0*anteiso*	0.41 (0.00)	0.82	0.81 (0.01)	0.88 (0.01)	0.82 (0.01)	0.79 (0.00)	<.0001	0.20	0.2 (0.01)	0.22 (0.01)	0.2 (0.00)	0.19 (0.01)	0.08
C15:0	0.98 (0.00)	1.43	1.44 (0.01)	1.39 (0.00)	1.43 (0.01)	1.47 (0.01)	<.0001	0.75	0.75 (0.01)	0.72 (0.01)	0.76 (0.01)	0.77 (0.01)	0.003
C16:0*iso*	0.17 (0.00)	0.30	0.3 (0.00)	0.32 (0.00)	0.3 (0.00)	0.29 (0.00)	<.0001	0.10	0.1 (0.00)	0.1 (0.00)	0.1 (0.00)	0.09 (0.00)	0.0071
C16:0	26.9 (0.11)	35.65	35.63 (0.31)	34.47 (0.1)	35.85 (0.19)	36.64 (0.09)	<.0001	22.60	22.41 (0.35)	21.83 (0.2)	23.06 (0.23)	23.1 (0.2)	0.0048
C16:1*trans*9	0.15 (0.00)	0.07	0.07 (0.00)	0.09 (0.00)	0.07 (0.01)	0.06 (0.00)	0.0114	0.19	0.19 (0.01)	0.21 (0.00)	0.2 (0.01)	0.18 (0.00)	0.0101
C16:1*cis*9	1.37 (0.01)	0.17	0.15 (0.00)	0.19 (0.00)	0.17 (0.01)	0.14 (0.00)	<.0001	1.98	1.94 (0.01)	1.92 (0.02)	2.07 (0.00)	1.98 (0.02)	<.0001
C17:0*iso*	0.29 (0.00)	0.36	0.36 (0.01)	0.41 (0.00)	0.35 (0.01)	0.34 (0.00)	<.0001	0.25	0.24 (0.01)	0.26 (0.00)	0.25 (0.01)	0.23 (0.00)	0.0191
C17:0*anteiso*	0.32 (0.00)	0.52	0.52 (0.01)	0.53 (0.01)	0.51 (0.00)	0.52 (0.01)	0.0205	0.21	0.21 (0.01)	0.23 (0.01)	0.22 (0.01)	0.2 (0.01)	0.011
C17:0	0.42 (0.00)	0.46	0.46 (0.00)	0.48 (0.00)	0.44 (0.01)	0.44 (0.00)	<.0001	0.40	0.4 (0.00)	0.41 (0.00)	0.4 (0.01)	0.39 (0.00)	0.1668
C17:1*cis*9	0.17 (0.00)	0.26	0.25 (0.00)	0.27 (0.00)	0.26 (0.01)	0.24 (0.00)	<.0001	0.12	0.12 (0.00)	0.13 (0.00)	0.12 (0.00)	0.12 (0.00)	0.0146
C18:0	7.36 (0.06)	4.19	4.19 (0.07)	4.61 (0.1)	4.08 (0.08)	3.88 (0.03)	<.0001	8.94	9.01 (0.13)	9.56 (0.15)	8.62 (0.09)	8.56 (0.05)	<.0001
C18:1*trans*6	0.17 (0.0)	0.09	0.06 (0.02)	0.1 (0.06)	0.06 (0.02)	0.13 (0.03)	0.4811	0.21	0.22 (0.01)	0.23 (0.03)	0.22 (0.01)	0.18 (0.02)	0.2236
C18:1*trans*9	0.13 (0.00)	0.17	0.17 (0.01)	0.18 (0.01)	0.16 (0.01)	0.16 (0.00)	0.1569	0.10	0.11 (0.01)	0.1 (0.01)	0.09 (0.01)	0.11 (0.01)	0.5517
C18:1 *trans(*10+11)	0.75 (0.02)	0.82	0.78 (0.03)	0.98 (0.02)	0.84 (0.03)	0.68 (0.01)	<.0001	1.01	0.95 (0.06)	1.18 (0.02)	1.03 (0.04)	0.87 (0.02)	0.0001
C18:1*cis*9	14.1 (0.00)	14.28	14 (0.21)	15.42 (0.17)	14.45 (0.27)	13.26 (0.11)	<.0001	14.01	13.89 (0.22)	14.80 (0.14)	14.18 (0.27)	13.16 (0.05)	<.0001
C18:1*cis*11	0.57 (0.00)	0.26	0.25 (0.00)	0.29 (0.01)	0.26 (0.01)	0.24 (0.00)	<.0001	0.72	0.73 (0.01)	0.76 (0.01)	0.71 (0.01)	0.7 (0.01)	0.01
C18:1*cis*12	0.18 (0.00)	0.14	0.15 (0.00)	0.13 (0.00)	0.13 (0.00)	0.14 (0.00)	0.0044	0.20	0.2 (0.00)	0.19 (0.00)	0.19 (0.00)	0.21 (0.00)	0.0227
C18:1*cis*13	0.10 (0.00)	0.45	0.43 (0.03)	0.48 (0.09)	0.41 (0.04)	0.5 (0.05)	0.6226	n.d.					
C18:1*cis*14	0.31 (0.00)	0.08	0.08 (0.00)	0.08 (0.00)	0.07 (0.00)	0.07 (0.00)	0.0096	0.42	0.43 (0.01)	0.43 (0.00)	0.41 (0.00)	0.42 (0.01)	0.0727
C18:1*cis*15	0.24 (0.00)	0.06	0.05 (0.00)	0.06 (0.00)	0.06 (0.00)	0.05 (0.00)	0.0002	0.33	0.33 (0.01)	0.35 (0.00)	0.34 (0.00)	0.32 (0.00)	0.0677
C18:2*cis*9,12 (LA)	1.19 (0.00)	1.56	1.59 (0.01)	1.62 (0.01)	1.53 (0.02)	1.51 (0.01)	0.0004	1.00	1.02 (0.02)	1.01 (0.02)	1.01 (0.04)	0.98 (0.02)	0.7817
C18:3*cis*6,9,12 (GLA)	0.04 (0.00)	0.04	0.04 (0.00)	0.04 (0.00)	0.05 (0.00)	0.04 (0.00)	0.3959	0.04	0.04 (0.00)	0.04 (0.00)	0.04 (0.01)	0.04 (0.00)	0.8682
C18:3*cis*9,12,15 (ALA)	0.37 (0.00)	0.41	0.42 (0.01)	0.44 (0.00)	0.4 (0.01)	0.38 (0.01)	0.0001	0.35	0.36 (0.01)	0.37 (0.00)	0.34 (0.00)	0.34 (0.00)	0.0007
C18:2*cis*9,*trans*11	0.34 (0.00)	0.32	0.29 (0.01)	0.39 (0.01)	0.34 (0.02)	0.27 (0.00)	<.0001	0.35	0.33 (0.02)	0.4 (0.01)	0.38 (0.01)	0.31 (0.01)	<.0001
C20:0	0.09 (0.00)	0.04	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.052	0.11	0.11 (0.00)	0.11 (0.00)	0.11 (0.00)	0.11 (0.00)	0.5113

(continuation **Table 3.1.**)

FA	TAG (mol %)	*sn*-2 position (mol %)						*sn-1(3)* positions (mol %)					
		Year average	Spring	Summer	Autumn	Winter	*P* value	Year average	Spring	Summer	Autumn	Winter	*P* value
C20:3*cis*8,11,14 (DGLA)	0.04 (0.00)	0.06	0.06 (0.00)	0.06 (0.00)	0.05 (0.00)	0.06 (0.00)	0.2737	0.04	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.6785
C20:4*cis*5,8,11,14 (AA)	0.06 (0.00)	0.01	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.8635	0.08	0.08 (0.00)	0.08 (0.00)	0.09 (0.00)	0.08 (0.00)	0.312
C22:0	0.02 (0.00)	0.03	0.03 (0.00)	0.04 (0.00)	0.04 (0.00)	0.03 (0.00)	0.0001	0.02	0.02 (0.00)	0.02 (0.00)	0.01 (0.00)	0.02 (0.00)	0.0032
C20:4*cis*8,11,14,17 (ETA)	0.02 (0.00)	0.03	0.03 (0.00)	0.03 (0.00)	0.03 (0.00)	0.03 (0.00)	0.1121	0.02	0.02 (0.00)	0.02 (0.00)	0.01 (0.00)	0.02 (0.00)	0.0588
C20:5*cis*5,8,11,14,17 (EPA)	0.04 (0.00)	0.03	0.03 (0.00)	0.04 (0.00)	0.03 (0.00)	0.03 (0.00)	<.0001	0.04	0.04 (0.00)	0.04 (0.00)	0.05 (0.00)	0.04 (0.00)	0.0409
C24:0	0.01 (0.00)	0.01	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.7662	n.d.					
C22:5*cis*7,10,13,16,19 (DPA)	0.04 (0.00)	0.13	0.13 (0.00)	0.13 (0.00)	0.13 (0.00)	0.13 (0.00)	0.0602	n.d.					
FA groups													
SFA	77.82 (0.14)	78.40	78.83 (0.25)	76.9 (0.22)	78.25 (0.37)	79.63 (0.12)	<.0001	77.47	77.72 (0.33)	76.42 (0.21)	77.12 (0.29)	78.62 (0.08)	0.0466
UFA	22.22 (0.14)	21.20	20.8 (0.24)	22.65 (0.22)	21.33 (0.35)	20.02 (0.12)	<.0001	22.79	22.52 (0.33)	23.79 (0.17)	23.17 (0.3)	21.68 (0.05)	0.0466
MUFA	20.06 (0.13)	18.92	18.49 (0.23)	20.28 (0.21)	19.1 (0.33)	17.82 (0.11)	<.0001	20.86	20.60 (0.29)	21.80 (0.16)	21.22 (0.30)	19.83 (0.05)	0.0442
PUFA	2.15 (0.01)	2.60	2.6 (0.03)	2.76 (0.02)	2.57 (0.04)	2.46 (0.02)	<.0001	1.93	1.93 (0.05)	1.99 (0.03)	1.95 (0.04)	1.84 (0.01)	0.2185
SCFA	24.76 (0.08)	7.13	7.25 (0.08)	6.9 (0.1)	7.24 (0.09)	7.14 (0.08)	0.0333	20.20	33.88 (0.12)	33.09 (0.12)	33.27 (0.18)	34.4 (0.27)	0.0122
MCFA	48.77 (0.16)	69.58	69.83 (0.32)	67.86 (0.23)	69.51 (0.43)	71.13 (0.13)	<.0001	33.66	38.3 (0.45)	37.23 (0.29)	39.01 (0.41)	39.24 (0.32)	0.1434
BCFA	1.47 (0.01)	2.52	2.48 (0.05)	2.68 (0.02)	2.48 (0.02)	2.42 (0.01)	<.0001	0.95	0.93 (0.04)	1.02 (0.01)	0.95 (0.02)	0.9 (0.02)	0.0021
TFA	1.40 (0.02)	1.15	1.08 (0.06)	1.35 (0.08)	1.13 (0.07)	1.03 (0.04)	0.0003	1.52	1.46 (0.1)	1.73 (0.06)	1.55 (0.08)	1.34 (0.05)	0.0003
OCFA	1.91 (0.01)	3.19	3.2 (0.03)	3.1 (0.01)	3.21 (0.03)	3.25 (0.02)	<.0001	1.43	1.41 (0.01)	1.41 (0.02)	1.44 (0.01)	1.44 (0.02)	0.1182
Fat Content (%)		4.34	4.42 (0.11)	4.14 (0.06)	4.33 (0.09)	4.47 (0.04)	<.0001	4.34	4.42 (0.11)	4.14 (0.06)	4.33 (0.09)	4.47 (0.04)	<.0001

[1] n.d: not detected. Spring: March, April, May; Summer: June, July, August; Autumn: September, October, November; Winter: December, January, February. SFA: saturated FA, UFA: unsaturated FA, MUFA: monounsaturated FA, PUFA: polyunsaturated FA, SCFA: short-chain FA (C4-C11), MCFA: medium-chain FA (C12-C17), BCFA: branched-chain FA (C14:0*iso*, C15:0*iso*, C16:0*iso*, C17:0*iso*, C14:0*anteiso*, C15:0*anteiso*, C16:0*anteiso*, C17:0*anteiso*), OCFA: odd-chain FA, TFA: *trans* FA.

3.3.1.2. Distribution of fatty acids among the *sn*-2 and *sn*-1(3) positions

The distribution of a specific FA over the three *sn* positions (interpositional distribution) of bovine milk fat TAG are given in **Table 3.2**. From this table, the esterification preference of FA over three *sn*-positions can be estimated. The values given in Table 3.2 are calculated based on the compositions of *sn*-2 and *sn*-1(3) positions and with this approach the proportions of a FA over the three positions is considered as 100%. If a FA is preferentially esterified in one position, then its proportion should be higher than 33.3 %, considering the equal distribution of a FA within three positions of TAG molecule (Yoshinaga et al., 2015a). Since no distinction could be made between *sn*-1 and *sn*-3 positions in this study, a preference for *sn*-2 and *sn*-1(3) positions were considered when *sn*-2 >33.3% and *sn*-1(3) >66.7%, respectively. Besides, the FA esterification preferences at the *sn*-2 and *sn*-1(3) positions were compared against the hypothetical proportions of 33.3% and 66.7%, respectively and the results are given in **Supplemental Table S3.2**. Based on this, Table 3.2 and Supplemental Table 3.2 show that the majority of the SCFA (C4-C11) (>90%) and UFA (>65%) were esterified at *sn*-1 or *sn*-3 positions. There was a slight preference of SFA and MCFA (C12-C17) towards *sn*-2 and *sn*-1(3) positions, respectively. Considering the major FA in bovine milk fat, around 45% of the total C16:0 in milk fat was located in the *sn*-2 position. Oleic (C18:1*cis*9), linoleic (C18:2*cis*9,12), and α-linolenic acid (C18:3*cis*9,12,15) were favorably esterified in the *sn*-1(3) positions. These findings are line with the reported stereolocation of milk FA (Jensen, 2002). The majority of the conjugated linoleic acid (**CLA**, C18:2*cis*9,*trans*11) was also esterified in the outer positions of TAG structures. The concentrations of total TFA was less than 30% at *sn*-2 position. Similar concentrations of TFA was reported in the *sn*-2 position of Australian butter (Mansour and Sinclair, 1993). The higher proportions of BCFA in the *sn*-2 position than that of the *sn*-1(3) positions indicated a preference towards *sn*-2 position. This finding was supported by (Yan et al., 2017). Compared to human milk fat, bovine milk fat contains higher amounts of BCFA however the composition of the individual BCFA at *sn*-2 position may differ between the two. The authors stated that the BCFA are hydrolyzed at different rates than the other FA therefore their digestion and absorption in the intestine might be affected by their positioning. Considering the variety of TAG species in bovine milk fat, the non-random incorporation of FA over the glycerol backbone results in a less complex composition as it could potentially be (Jensen, 2002). Nevertheless, when the FA profile of milk fat changes, the properties of a FA (chain length, structure, melting point) might determine the positions that the FA will be esterified.

Table 3.2. Seasonal variation in the regiospecific distribution of fatty acids [FA; mean (SEM)] of bovine milk fat triacylglycerols according to seasons from May 2017 to April 2018 (interpositional distribution).[1]

FA	*sn*-2 position (mol %)					*sn-1(3)* positions (mol %)				
	Spring	Summer	Autumn	Winter	*P* value	Spring	Summer	Autumn	Winter	*P* value
C4:0	0.8 (0.03)	0.83 (0.05)	0.9 (0.05)	0.74 (0.03)	0.0708	99.1 (0.02)	99.2 (0.02)	99.17 (0.02)	99.26 (0.01)	0.0708
C6:0	4.36 (0.1)	4.55 (0.16)	4.61 (0.1)	4.21 (0.07)	0.0688	95.39 (0.05)	95.64 (0.05)	95.45 (0.08)	95.79 (0.04)	0.0688
C8:0	27.37 (0.49)	27.6 (0.38)	28.04 (0.5)	26.52 (0.29)	0.1147	71.96 (0.25)	72.63 (0.25)	72.4 (0.19)	73.48 (0.14)	0.1147
C10:0	30.26 (0.35)	30.73 (0.34)	30.93 (0.35)	29.4 (0.34)	0.0237	69.07 (0.17)	69.74 (0.17)	69.27 (0.17)	70.6 (0.17)	0.0237
C12:0	47.43 (0.38)	47.83 (0.23)	47.36 (0.49)	47.01 (0.23)	0.4466	52.64 (0.24)	52.57 (0.19)	52.17 (0.12)	52.99 (0.11)	0.4466
C12:1*cis*9	74.01 (5.52)	60.68 (1.94)	62 (2.5)	77.10 (6.88)	0.0474	38.00 (1.25)	25.99 (2.76)	39.32 (0.97)	22.9 (3.44)	0.0474
C14:0*iso*	71.13 (0.95)	69.42 (1.95)	72.34 (1.17)	68.8 (1.41)	0.3041	27.66 (0.59)	28.87 (0.47)	30.58 (0.98)	31.2 (0.71)	0.3041
C14:0	59.2 (0.23)	59.47 (0.43)	59.09 (0.47)	59.13 (0.37)	0.8925	40.91 (0.23)	40.8 (0.11)	40.53 (0.21)	40.87 (0.18)	0.8925
C14:1*cis*9	48.4 (0.32)	49.28 (0.51)	47.55 (0.36)	47.97 (0.21)	0.0207	52.45 (0.18)	51.6 (0.16)	50.72 (0.26)	52.03 (0.11)	0.0207
C15:0*iso*	52.71 (0.67)	53.48 (0.37)	53.26 (0.48)	52.55 (0.19)	0.4574	46.74 (0.24)	47.29 (0.34)	46.52 (0.19)	47.45 (0.1)	0.4574
C15:0*anteiso*	67.01 (0.65)	67.06 (0.73)	67.1 (0.47)	67.03 (0.73)	0.9997	32.9 (0.23)	32.99 (0.32)	32.94 (0.36)	32.97 (0.37)	0.9997
C15:0	49.09 (0.22)	49.03 (0.44)	48.42 (0.18)	48.78 (0.16)	0.3166	51.58 (0.09)	50.91 (0.11)	50.97 (0.22)	51.22 (0.08)	0.3166
C16:0*iso*	60.79 (0.65)	60.67 (0.47)	59.91 (0.5)	61.01 (0.5)	0.5074	40.09 (0.25)	39.21 (0.32)	39.33 (0.23)	38.99 (0.25)	0.5074
C16:0	44.29 (0.21)	44.13 (0.18)	43.74 (0.22)	44.23 (0.16)	0.2151	56.26 (0.11)	55.71 (0.1)	55.87 (0.09)	55.77 (0.08)	0.2151
C16:1*trans*9	15.77 (0.65)	17.88 (0.54)	15.11 (2.3)	14.67 (0.48)	0.3029	84.89 (1.15)	84.23 (0.33)	82.12 (0.27)	85.33 (0.24)	0.3029
C16:1*cis*9	3.81 (0.10)	4.8 (0.08)	4.03 (0.17)	3.49 (0.13)	<.0001	95.97 (0.09)	96.19 (0.05)	95.2 (0.04)	96.51 (0.06)	<.0001
C17:0*iso*	42.35 (0.29)	43.48 (0.17)	40.89 (1.55)	42.01 (0.29)	0.1893	59.11 (0.78)	57.65 (0.15)	56.52 (0.09)	57.99 (0.14)	0.1893
C17:0*anteiso*	55.85 (0.81)	53.52 (0.79)	54.01 (0.67)	56.63 (1.16)	0.0637	45.99 (0.33)	44.15 (0.4)	46.48 (0.4)	43.37 (0.58)	0.0637
C17:0	36.6 (0.45)	37.07 (0.43)	35.29 (0.56)	36.1 (0.35)	0.0647	64.71 (0.28)	63.4 (0.23)	62.93 (0.21)	63.9 (0.17)	0.0647
C17:1*cis*9	52.11 (0.69)	51.85 (0.57)	51.7 (0.64)	51.13 (0.71)	0.7559	48.3 (0.32)	47.89 (0.34)	48.15 (0.28)	48.87 (0.35)	0.7559
C18:0	18.86 (0.15)	19.42 (0.43)	19.12 (0.26)	18.47 (0.13)	0.1108	80.88 (0.13)	81.14 (0.08)	80.58 (0.21)	81.53 (0.07)	0.1108
C18:1*trans*6	11.93 (4.65)	17.91 (10.15)	12.84 (4.26)	27.04 (6.2)	0.3887	87.16 (2.13)	88.07 (2.32)	82.09 (5.08)	72.96 (3.1)	0.3887
C18:1*trans*9	44.17 (2.26)	46.6 (2.21)	48.32 (4.98)	41.36 (1.39)	0.4148	51.68 (2.49)	55.83 (1.13)	53.4 (1.1)	58.64 (0.7)	0.4148
C18:1*trans*(10+11)	29.23 (0.72)	29.21 (0.36)	28.78 (0.38)	28.08 (0.45)	0.3529	71.22 (0.19)	70.77 (0.36)	70.79 (0.18)	71.92 (0.23)	0.3529
C18:1cis9	33.5 (0.25)	34.24 (0.35)	33.77 (0.3)	33.49 (0.19)	0.2169	66.23 (0.15)	66.5 (0.12)	65.76 (0.17)	66.51 (0.09)	0.2169
C18:1*cis*11	14.8 (0.12)	15.86 (0.37)	15.46 (0.28)	14.55 (0.17)	0.0065	84.54 (0.14)	85.2 (0.06)	84.14 (0.19)	85.45 (0.09)	0.0065
C18:1*cis*12	27.84 (0.73)	25.82 (0.83)	26.4 (0.25)	25.34 (0.41)	0.0441	73.6 (0.12)	72.16 (0.36)	74.18 (0.42)	74.66 (0.21)	0.0441
C18:1*cis*14	8.4 (0.29)	8.69 (0.21)	8.21 (0.3)	7.82 (0.1)	0.1046	91.79 (0.15)	91.6 (0.15)	91.31 (0.11)	92.18 (0.05)	0.1046
C18:1*cis*15	7.2 (0.27)	8.44 (0.14)	7.9 (0.3)	6.98 (0.26)	0.0019	92.1 (0.15)	92.8 (0.13)	91.56 (0.07)	93.02 (0.13)	0.0019
C18:2*cis*9,12 (LA)	43.72 (0.54)	44.58 (0.55)	43.25 (1.24)	43.57 (0.59)	0.6710	56.75 (0.62)	56.28 (0.27)	55.42 (0.27)	56.43 (0.29)	0.671
C18:3*cis*6,9,12 (GLA)	35.12 (1.35)	34.11 (0.6)	41.11 (7.41)	34.24 (1.79)	0.5412	58.89 (3.71)	64.88 (0.67)	65.89 (0.3)	65.76 (0.89)	0.5412
C18:3*cis*9,12,15 (ALA)	37.31 (0.47)	37.33 (0.32)	36.76 (0.42)	35.85 (0.37)	0.0529	63.24 (0.21)	62.69 (0.24)	62.67 (0.16)	64.15 (0.18)	0.0529
C18:2*cis*9,*trans*11	30.59 (0.32)	32.75 (0.33)	31.08 (0.87)	30.54 (0.46)	0.0303	68.92 (0.44)	69.41 (0.16)	67.25 (0.17)	69.46 (0.23)	0.0303
C20:0	15.81 (0.42)	16.46 (1.02)	13.88 (0.71)	15.03 (1.02)	0.1823	86.12 (0.35)	84.19 (0.21)	83.54 (0.51)	84.97 (0.51)	0.1823
C20:3*cis*8,11,14 (DGLA)	43.3 (1.23)	42.65 (1.05)	42.07 (1.35)	43.55 (0.8)	0.7894	57.93 (0.68)	56.7 (0.62)	57.35 (0.52)	56.45 (0.4)	0.7894
C20:4*cis*5,8,11,14 (AA)	5.53 (0.82)	6.16 (0.94)	5.24 (0.87)	5.58 (0.95)	0.9063	94.76 (0.44)	94.47 (0.41)	93.84 (0.47)	94.42 (0.47)	0.9063
C22:0	43.89 (2.4)	49.67 (2.04)	78.18 (15.37)	38.31 (1.47)	0.009	21.82 (7.68)	56.11 (1.2)	50.33 (1.02)	61.69 (0.74)	0.009
C20:4*cis*8,11,14,17 (ETA)	42.53 (5.97)	40.74 (1.74)	67.47 (18.54)	50.82 (6.24)	0.2682	32.53 (9.27)	57.47 (2.98)	59.26 (0.87)	49.18 (3.12)	0.2682
C20:5*cis*5,8,11,14,17 (EPA)	29.51 (1.2)	34.12 (0.7)	26.89 (0.77)	28.39 (0.94)	0.0001	73.11 (0.39)	70.49 (0.6)	65.88 (0.35)	71.61 (0.47)	0.0001

(continuation **Table 3.2.**)

FA groups	*sn*-2 position (mol %)					*sn-1(3)* positions (mol %)				
	Spring	Summer	Autumn	Winter	*P* value	Spring	Summer	Autumn	Winter	*P* value
SFA	33.65 (0.06)	33.47 (0.12)	33.66 (0.1)	33.62 (0.04)	0.2392	66.35 (0.06)	66.53 (0.12)	66.34 (0.1)	66.38 (0.04)	0.2392
UFA	31.59 (0.19)	32.25 (0.35)	31.52 (0.33)	31.59 (0.16)	0.0976	68.41 (0.19)	67.75 (0.35)	68.48 (0.33)	68.41 (0.16)	0.0976
MUFA	30.98 (0.18)	31.75 (0.36)	31.04 (0.33)	31.00 (0.17)	0.0670	69.02 (0.18)	68.25 (0.36)	68.96 (0.33)	69.00 (0.17)	0.0670
PUFA	40.33 (0.44)	40.89 (0.36)	39.77 (0.85)	40.04 (0.18)	0.3123	59.67 (0.44)	59.11 (0.36)	60.23 (0.85)	59.96 (0.18)	0.3123
SCFA	9.66 (0.12)	9.44 (0.18)	9.82 (0.18)	9.41 (0.11)	0.0985	90.34 (0.12)	90.56 (0.18)	90.18 (0.18)	90.59 (0.11)	0.0985
MCFA	47.70 (0.24)	47.68 (0.27)	47.12 (0.30)	47.55 (0.23)	0.2269	52.30 (0.24)	52.32 (0.27)	52.88 (0.30)	52.45 (0.23)	0.2269
BCFA	57.25 (0.77)	56.93 (0.37)	56.61 (0.54)	57.30 (0.75)	0.684	42.75 (0.77)	43.07 (0.37)	43.39 (0.54)	42.70 (0.75)	0.684
TFA	26.94 (0.78)	28.09 (1.79)	26.84 (1.16)	27.78 (1.25)	0.715	73.06 (0.78)	71.91 (1.79)	73.16 (1.16)	72.22 (1.25)	0.715
OCFA	53.08 (0.18)	52.36 (0.32)	52.65 (0.07)	53.08 (0.25)	0.009	46.92 (0.18)	47.64 (0.32)	47.35 (0.07)	46.92 (0.25)	0.009

[1] Spring: March, April, May; Summer: June, July, August; Autumn: September, October, November; Winter: December, January, February. SFA: Saturated FA, UFA: unsaturated FA, MUFA: monounsaturated FA, PUFA: polyunsaturated FA, SCFA: short-chain FA (C4-C11), MCFA: medium-chain FA (C12-C17), BCFA: branched-chain FA (C14:0*iso*, C15:0*iso*, C16:0*iso*, C17:0*iso*, C14:0*anteiso*, C15:0*anteiso*, C16:0*anteiso*, C17:0*anteiso*), OCFA: odd-chain FA, TFA: *trans* FA.

3.3.2. Seasonal variation in the positioning of fatty acids

Significant seasonal variations in the regiospecific distribution of FA (P<0.05) throughout a year have been observed (Table 3.1). The seasonal variation in the overall FA composition of these bovine milk fat samples has been previously reported by Pacheco-Pappenheim et al. (2021). A short overview on the yearly and seasonal FA compositions (mol%) of the bovine milk fat samples are provided in **Supplemental Table S3.3**. Unlike the variations in the intrapositional distribution shown in Table 3.1, the preferential esterification of the FA over the three positions were not affected by seasonal variation (P>0.05, Table 3.2). This might indicate that the esterification preference of a FA in one specific position most likely will not change throughout the year. In other words, even if the total amount of a FA changes significantly in the *sn*-2 position, the percentage of the same FA esterified to the *sn*-2 position will not change due to seasonal variation. On the other hand, the data presented in Table 3.2 were calculated based on the FA compositions of the *sn*-2 and *sn*-1(3) positions (Table 3.1), therefore the high standard errors can be expected. For this reason the discussion of the seasonal changes will be based on the FA compositions of *sn*-2 and *sn*-1(3) positions (intrapositional distribution) as shown Table 3.1.

Considering the extent of seasonal variation, the number of FA that differed between seasons and the variation in their contents were higher for *sn*-2 position than *sn*-1(3) positions (Table 3.1 and Supplemental Table S3.1). The higher variation observed in the *sn*-2 position might indicate that the seasonal variation was more pronounced at this position than the outer positions of the TAG in milk fat. Previously, stereospecific distribution of FA in butter and the yearly variation in positioning of FA were reported by Parodi (1979). Contrary to our findings, the author reported a higher seasonal variation at the *sn*-1(3) positions than the *sn*-2 position in milk fat where seasonal variation was found to be rather small and it was noted mostly for the MCFA. However, in our study, we have identified seasonal variation not only in MCFA but for the majority of the FA including the abundant and minor FA. Besides, the number of FA identified in our study were much higher than the ones reported by Parodi (1979). The discrepancies between the study of Parodi (1979) and our results may be attributed to the differences in the conditions [*i.e.* geographical location (northern vs. southern hemisphere), breed of the cow (Holstein-Frisian vs. Jersey), milking system and therefore the feeding regimes]. The main differences are probably caused by the analytical methods used. For instance, in the study of Parodi (1979) pancreatic lipase deacylation followed by phospholipase hydrolysis was used to determine the compositions of the *sn*-2 and *sn*-1 positions, respectively. In our study, CALB has been utilized. Earlier studies showed that the pancreatic lipase hydrolyzed the SCFA and PUFA at a slower rate than MCFA and LCFA so that not all TAG species might be converted to 2-MAG at the same rate (Watanabe and Yoshinaga, 2017). In summary, the 2-MAG fraction may not be representative of the original TAG composition when pancreatic lipase is used. Besides,

the capabilities of the analytical methods used in these two studies for FA analysis also differ which in turn might have affected the number of FA detected. For example, long capillary GC-columns (100 m) provide a better separation of *cis* and *trans* C18:1 FA isomers and very long-chain FA compared to the shorter capillary GC-columns (30 m; Delmonte et al., 2009). To the best of our knowledge, no other studies have been reported on the variation in positional distribution of FA between seasons. Therefore, a direct comparison of our findings with literature might not be feasible for all the FA and observed trends within a year.

Among the *sn*-2 FA, the biggest seasonal variations were observed for the most abundant FA C16:0 (CV 2.16%), C18:1*cis*9 (CV 2.16%), C14:0 (CV 0.82%) and C18:0 (CV 0.73%) (Table 3.1). The highest seasonal variation in *sn*-1(3) positions were detected for C16:0 (CV 1.27%) and LCFA such as C18:0 (CV 1.0%) and C18:1*cis*9 (CV 1.64%); MCFA C12:0 (CV 0.36%), C10:0 (CV 0.51%) and C8:0 (CV 0.19%), and SCFA C6:0 (0.38%). There were no significant differences in C4:0 content in all the positions between seasons (P>0.05). This is in-line with the changes in C4:0 concentrations throughout the year (Pacheco-Pappenheim et al., 2021). The main observation about the seasonal trends was that the FA produced along the same pathway showed similar behavior. For instance, FA that are formed by Δ9 desaturase activity such as palmitoleic (C16:1*cis*9) and oleic acid (C18:1*cis*9) showed increased concentrations in summer and spring and a decrease in autumn and winter months. The changes observed in the positional distribution of FA were in the same direction with the changes in average milk fat composition and parallel to the feeding regimes of the cows in different seasons (Pacheco-Pappenheim et al., 2021). In summer, due to the high intake of fresh grass the concentration of blood derived FA increased in milk fat and so their abundances in the different *sn* positions of TAG. These trends were observed for the majority of UFA (*i.e*. C18:2*cis*9,12 (LA), C18:2*cis*9,12,15 (ALA), C18:1*cis*9, CLA) and C18:0 as shown in **Figure 3.1** (intrapositional distribution). These FA in the *sn*-2 and *sn*-1(3) positions followed a same trend where their highest and lowest concentrations were reached in summer and winter, respectively. The variation observed for C18:1*cis*9 was much higher compared to the other FA indicating more dynamic changes in its content throughout the year.

Due to the changes in cows' diet in winter, the proportion of *de novo* synthesized FA, especially C16:0 (partially *de novo* synthesized and blood derived) significantly increased from summer to winter in the *sn*-2 and *sn*-1(3) positions of milk fat (P<0.05). The trends in overall C16:0 content of milk fat and its concentrations in the *sn*-2 and *sn*-1(3) positions over a year are shown **Figure 3.2**. Opposite to the changes observed in Figure 3.1, Figure 3.2 shows that based on intrapositional distribution, the minimum concentration of C16:0 in the *sn*-2 and *sn*-1(3) positions were observed in summer (June - August) whereas from summer to winter its concentration increased and reached a maximum value in winter

(December - February). An explanation can be that in winter, *cis*-9 UFA remain competitive with SCFA for the *sn*-3 position, while in this season other UFA experience much more competition from long-chain saturated FA for the *sn*-1 position, because of the increased activity of glycerol-3-phosphate by acyl CoA:glycerol-sn-3-phosphate acyl transferase (**GPAT**) caused by the higher total C16:0 content of milk fat (Kinsella and Gross, 1973). This is mostly hypothetical because in this study no distinction between the *sn*-1 and *sn*-3 position could be made.

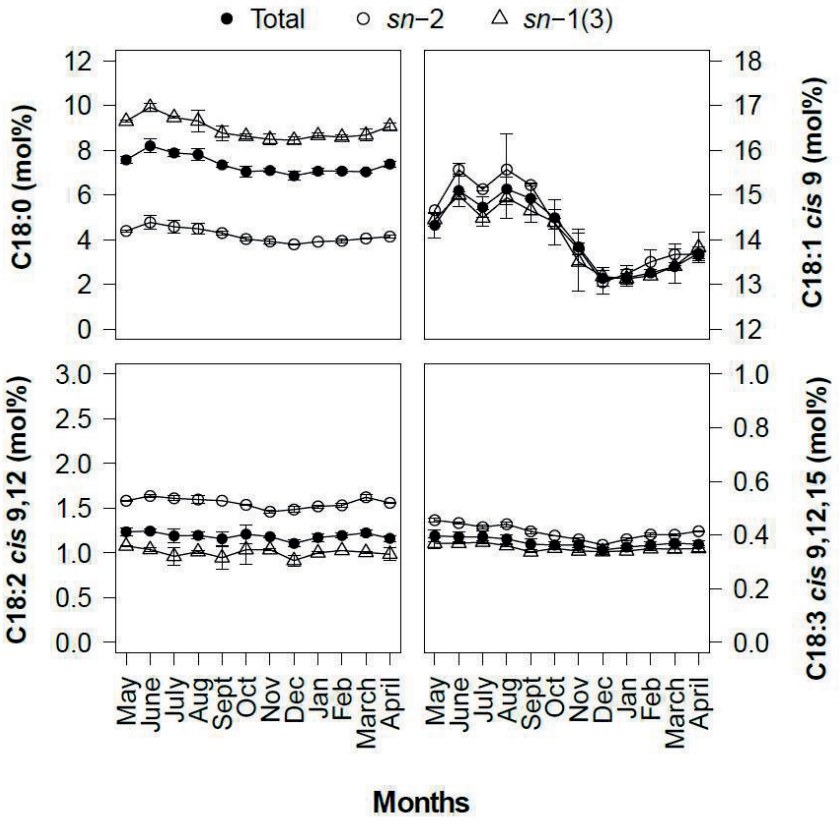


Figure 3.1. Seasonal variation in the total and intrapositional distribution of stearic (C18:0), oleic (C18:1 *cis*9), linoleic (C18:2*cis*9,12) and linolenic acid (C18:3*cis*9,12,15) contents (mol %) of bovine milk fat from May 2017 till April 2018 (● total, ○ *sn*-2 position, Δ *sn-1(3)* positions).

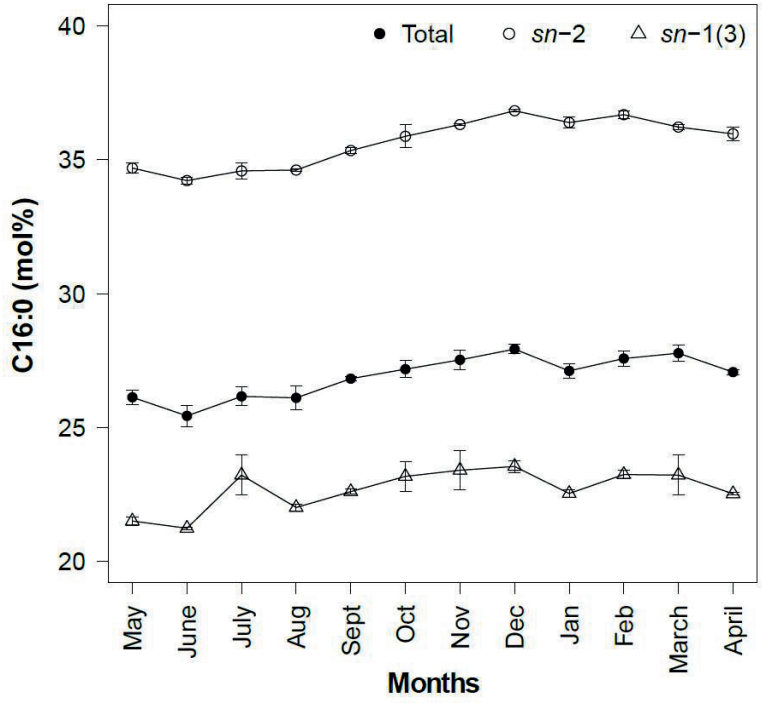


Figure 3.2. Seasonal variation in the total and intrapositional distribution of palmitic acid (C16:0) content (mol %) of bovine milk fat from May 2017 till April 2018 (● total, ○ *sn*-2 position, Δ *sn-1(3)* positions).

The changes in C16:0 concentration (intrapositional distribution, Table 3.1) indicated that in winter months more TAG that contained C16:0 in the *sn*-2 position were synthesized, yet the proportions of C16:0 in the primary and secondary positions were not influenced by this (interpositional distribution, Table 3.2). In contrast to our findings, Tzompa-Sosa et al., (2014) suggested that low concentrations of C16:0 in bovine milk fat resulted in increased proportions of C16:0 in the *sn*-2 position (interpositional distribution). The bovine milk fat samples used by Tzompa-Sosa et al., (2014) were collected in winter from individual cows that varied more in fat content (2.85 to 5.76%) and C16:0 levels (22.3-32.5 mol%) compared to our samples (4.1-4.6% fat, 25.2-28.1 mol% C16:0). Most likely the differences in C16:0 concentrations in our study were too small to have a significant effect compared to Tzompa-Sosa et al. (2014).

Overall, TAG biosynthesis in the mammary gland is a complex process. In the mammary gland, FA are arranged in the structure of TAG in a non-random order. The non-random distribution is a result of the preferences of different enzymes that are involved in TAG

biosynthesis (Palmquist, 2006). Certain FA may not only affect their own incorporation into TAG structures but also that of others. For C16:0 and C18:1 FA, this can lead to respectively a shortage against the abundance of other FA in the mammary gland. This in turn may stimulate or suppress the pathways of FA synthesis. Thus, it can be proposed that as the FA profile of milk fat changes throughout the year, the activity of the enzymes involved in TAG synthesis will be altered and consequently, FA may be positioned differently within the TAG structure.

3.4. Conclusions

Regiospecific analysis of Dutch bovine milk fat collected between May 2017 and April 2018 was performed to study the seasonal variation of the positioning of FA within the TAG structure. The strongest seasonal variation in the *sn*-2 and *sn*-1(3) FA compositions was often observed between summer and winter months (intrapositional distribution). Two distinct trends were observed for the blood derived and *de novo* synthesized FA, being the former more influenced by the changes in seasonal feeding strategies. As a consequence of fresh grass intake in summer the concentrations of UFA increased in the *sn*-2 and *sn*-1(3) positions. In winter months, the higher abundance of *de novo* synthesized FA in milk fat resulted in higher proportions of these FA in *sn*-2 and *sn*-1(3) positions. Especially, in winter months C16:0 significantly increased in *sn*-2 position. On the other hand, the changes observed in the regiospecific distribution of FA among seasons did not influence the preferential esterification of FA over the three positions (interpositional distribution). Regardless of the limited amount of information available in the literature on this topic, this study presents valuable insights and a comprehensive overview on the extent of seasonal variation in the regiospecific positioning of FA in bovine milk fat throughout a year.

3.5. Acknowledgements

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Supplementary Information

Table S3.1. Comparisons of the fatty acid (FA) concentrations (mol %) at the *sn*-2 and *sn*-1(3) positions in the triacylglycerol structures of bovine milk fat between seasons.[1]

	Spring			Summer			Autumn			Winter		
FA	*sn*-2	*sn*-1(3)	*P value*	*sn*-2	*sn*-1(3)	*P value*	*sn*-2	*sn*-1(3)	*P value*	*sn*-2	*sn*-1(3)	*P value*
C4:0	0.3 ± 0.03	18.45 ± 0.22	<0.001	0.31 ± 0.04	18.45 ± 0.18	<0.001	0.33 ± 0.04	18.29 ± 0.46	<0.001	0.28 ± 0.02	18.63 ± 0.38	<0.001
C6:0	0.71 ± 0.04	7.79 ± 0.11	<0.001	0.72 ± 0.06	7.52 ± 0.06	<0.001	0.74 ± 0.04	7.61 ± 0.07	<0.001	0.69 ± 0.03	7.91 ± 0.19	<0.001
C8:0	1.89 ± 0.08	2.51 ± 0.06	<0.001	1.82 ± 0.06	2.38 ± 0.05	<0.001	1.89 ± 0.09	2.43 ± 0.08	<0.001	1.85 ± 0.04	2.57 ± 0.06	<0.001
C10:0	3.9 ± 0.06	4.49 ± 0.14	<0.001	3.64 ± 0.1	4.11 ± 0.08	<0.001	3.81 ± 0.13	4.26 ± 0.17	<0.001	3.85 ± 0.13	4.62 ± 0.07	<0.001
C10:1*cis*9	0 ± 0	0.64 ± 0.02	<0.001	0 ± 0	0.64 ± 0.01	<0.001	0 ± 0	0.69 ± 0.03	<0.001	0 ± 0	0.67 ± 0.03	<0.001
C12:0	6.65 ± 0.05	3.68 ± 0.12	<0.001	6.26 ± 0.09	3.41 ± 0.06	<0.001	6.39 ± 0.21	3.56 ± 0.19	<0.001	6.69 ± 0.1	3.77 ± 0.04	<0.001
C12:1*cis*9	0.21 ± 0.01	0.04 ± 0.03	<0.001	0.2 ± 0.01	0.06 ± 0.01	<0.001	0.22 ± 0.01	0.07 ± 0.02	<0.001	0.22 ± 0.01	0.04 ± 0.03	<0.001
C13:0	0.3 ± 0.01	0 ± 0	<0.001	0.27 ± 0.01	0 ± 0	<0.001	0.29 ± 0.01	0 ± 0	<0.001	0.31 ± 0.01	0 ± 0	<0.001
C14:0*iso*	0.16 ± 0.01	0.03 ± 0	<0.001	0.18 ± 0.01	0.04 ± 0.01	<0.001	0.17 ± 0	0.03 ± 0	<0.001	0.16 ± 0	0.04 ± 0.01	<0.001
C14:0	20.36 ± 0.13	7.02 ± 0.18	<0.001	19.76 ± 0.32	6.73 ± 0.21	<0.001	19.97 ± 0.41	6.92 ± 0.3	<0.001	20.57 ± 0.13	7.11 ± 0.23	<0.001
C14:1*cis*9	1.54 ± 0.03	0.82 ± 0.03	<0.001	1.52 ± 0.03	0.78 ± 0.05	<0.001	1.63 ± 0.03	0.9 ± 0.04	<0.001	1.62 ± 0.02	0.88 ± 0.03	<0.001
C15:0*iso*	0.33 ± 0.01	0.15 ± 0.01	<0.001	0.37 ± 0.01	0.16 ± 0	<0.001	0.34 ± 0.01	0.15 ± 0.01	<0.001	0.32 ± 0	0.14 ± 0	<0.001
C15:0*anteiso*	0.81 ± 0.04	0.2 ± 0.02	<0.001	0.88 ± 0.02	0.22 ± 0.01	<0.001	0.82 ± 0.02	0.2 ± 0.01	<0.001	0.79 ± 0.01	0.19 ± 0.01	<0.001
C15:0	1.44 ± 0.02	0.75 ± 0.02	<0.001	1.39 ± 0.01	0.72 ± 0.03	<0.001	1.43 ± 0.03	0.76 ± 0.02	<0.001	1.47 ± 0.01	0.77 ± 0.02	<0.001
C16:0*iso*	0.3 ± 0	0.1 ± 0.01	<0.001	0.32 ± 0.01	0.1 ± 0	<0.001	0.3 ± 0.01	0.1 ± 0	<0.001	0.29 ± 0	0.09 ± 0	<0.001
C16:0	35.63 ± 0.75	22.41 ± 0.85	<0.001	34.47 ± 0.25	21.83 ± 0.48	<0.001	35.85 ± 0.48	23.06 ± 0.55	<0.001	36.64 ± 0.23	23.1 ± 0.48	<0.001
C16:1*trans*9	0.07 ± 0.01	0.19 ± 0.01	<0.001	0.09 ± 0.01	0.21 ± 0	<0.001	0.07 ± 0.03	0.2 ± 0.03	<0.001	0.06 ± 0	0.18 ± 0.01	<0.001
C16:1*cis*9	0.15 ± 0.01	1.94 ± 0.04	<0.001	0.19 ± 0.01	1.92 ± 0.04	<0.001	0.17 ± 0.02	2.07 ± 0.01	<0.001	0.14 ± 0.01	1.98 ± 0.05	<0.001
C17:0*iso*	0.36 ± 0.02	0.24 ± 0.02	<0.001	0.41 ± 0	0.26 ± 0	<0.001	0.35 ± 0.03	0.25 ± 0.02	<0.001	0.34 ± 0	0.23 ± 0.01	<0.001
C17:0*anteiso*	0.52 ± 0.02	0.21 ± 0.02	<0.001	0.53 ± 0.01	0.23 ± 0.01	<0.001	0.51 ± 0.01	0.22 ± 0.01	<0.001	0.52 ± 0.02	0.2 ± 0.02	<0.001
C17:0	0.46 ± 0.01	0.4 ± 0.01	<0.001	0.48 ± 0.01	0.41 ± 0.01	<0.001	0.44 ± 0.02	0.4 ± 0.01	<0.001	0.44 ± 0.01	0.39 ± 0.01	<0.001
C17:1*cis*9	0.25 ± 0.01	0.12 ± 0.01	<0.001	0.27 ± 0.01	0.13 ± 0	<0.001	0.26 ± 0.01	0.12 ± 0	<0.001	0.24 ± 0	0.12 ± 0.01	<0.001
C18:0	4.19 ± 0.16	9.01 ± 0.31	<0.001	4.61 ± 0.25	9.56 ± 0.37	<0.001	4.08 ± 0.19	8.62 ± 0.22	<0.001	3.88 ± 0.08	8.56 ± 0.13	<0.001
C18:1*trans*6	0.06 ± 0.06	0.22 ± 0.03	<0.001	0.1 ± 0.14	0.23 ± 0.07	0.075	0.06 ± 0.05	0.22 ± 0.03	<0.001	0.13 ± 0.08	0.18 ± 0.04	0.212
C18:1*trans*9	0.17 ± 0.02	0.11 ± 0.01	<0.001	0.18 ± 0.02	0.1 ± 0.01	<0.001	0.16 ± 0.02	0.09 ± 0.03	<0.001	0.16 ± 0.01	0.11 ± 0.02	<0.001
C18:1*trans*(10+11)	0.78 ± 0.08	0.95 ± 0.15	0.035	0.98 ± 0.05	1.18 ± 0.04	<0.001	0.84 ± 0.07	1.03 ± 0.09	<0.001	0.68 ± 0.02	0.87 ± 0.05	<0.001
C18:1*cis*9	14.00 ± 0.52	13.89 ± 0.53	0.736	15.42 ± 0.43	14.8 ± 0.34	0.020	14.45 ± 0.65	14.18 ± 0.67	0.486	13.26 ± 0.28	13.16 ± 0.11	0.452
C18:1*cis*11	0.25 ± 0.01	0.73 ± 0.02	<0.001	0.29 ± 0.01	0.76 ± 0.04	<0.001	0.26 ± 0.02	0.71 ± 0.03	<0.001	0.24 ± 0	0.7 ± 0.02	<0.001
C18:1*cis*12	0.15 ± 0.01	0.2 ± 0.01	<0.001	0.13 ± 0.01	0.19 ± 0.01	<0.001	0.13 ± 0.01	0.19 ± 0.01	<0.001	0.14 ± 0.01	0.21 ± 0.01	<0.001
C18:1*cis*13	0.43 ± 0.08	0 ± 0	<0.001	0.48 ± 0.22	0 ± 0	<0.001	0.41 ± 0.1	0 ± 0	<0.001	0.5 ± 0.11	0 ± 0	<0.001
C18:1*cis*14	0.08 ± 0.01	0.43 ± 0.01	<0.001	0.08 ± 0	0.43 ± 0.01	<0.001	0.07 ± 0.01	0.41 ± 0.01	<0.001	0.07 ± 0	0.42 ± 0.01	<0.001
C18:1*cis*15	0.05 ± 0.01	0.33 ± 0.02	<0.001	0.06 ± 0	0.35 ± 0.01	<0.001	0.06 ± 0	0.34 ± 0.01	<0.001	0.05 ± 0	0.32 ± 0.01	<0.001
C18:2*cis*9,12 (LA)	1.59 ± 0.03	1.02 ± 0.05	<0.001	1.62 ± 0.03	1.01 ± 0.06	<0.001	1.53 ± 0.06	1.01 ± 0.1	<0.001	1.51 ± 0.03	0.98 ± 0.06	<0.001
C18:3*cis*6,9,12 (GLA)	0.04 ± 0	0.04 ± 0.01	0.264	0.04 ± 0	0.04 ± 0	0.277	0.05 ± 0	0.04 ± 0.02	0.369	0.04 ± 0	0.04 ± 0.01	0.817
C18:3*cis*9,12,15 (ALA)	0.42 ± 0.03	0.36 ± 0.02	<0.001	0.44 ± 0.01	0.37 ± 0.01	<0.001	0.4 ± 0.01	0.34 ± 0.01	<0.001	0.38 ± 0.02	0.34 ± 0.01	<0.001
C18:2*cis*9,*trans*11	0.29 ± 0.04	0.33 ± 0.04	0.110	0.39 ± 0.02	0.4 ± 0.03	0.476	0.34 ± 0.04	0.38 ± 0.03	0.090	0.27 ± 0.01	0.31 ± 0.01	<0.001

(continuation **Table S3.1.**)

	Spring			Summer			Autumn			Winter		
FA	*sn*-2	*sn*-1(3)	*P value*	*sn*-2	*sn*-1(3)	*P value*	*sn*-2	*sn*-1(3)	*P value*	*sn*-2	*sn*-1(3)	*P value*
C20:0	0.04 ± 0	0.11 ± 0.01	<0.001	0.04 ± 0.01	0.11 ± 0.01	<0.001	0.04 ± 0	0.11 ± 0	<0.001	0.04 ± 0	0.11 ± 0.01	<0.001
C20:3*cis*8,11,14 (DGLA)	0.06 ± 0	0.04 ± 0	<0.001	0.06 ± 0	0.04 ± 0	<0.001	0.05 ± 0	0.04 ± 0	<0.001	0.06 ± 0	0.04 ± 0	<0.001
C20:4*cis*5,8,11,14 (AA)	0.01 ± 0	0.08 ± 0	<0.001	0.01 ± 0	0.08 ± 0.01	<0.001	0.01 ± 0	0.09 ± 0.01	<0.001	0.01 ± 0	0.08 ± 0.01	<0.001
C22:0	0.03 ± 0	0.02 ± 0	<0.001	0.04 ± 0	0.02 ± 0	<0.001	0.04 ± 0	0.01 ± 0.01	<0.001	0.03 ± 0	0.02 ± 0	<0.001
C20:4*cis*8,11,14,17 (ETA)	0.03 ± 0.01	0.02 ± 0.01	0.069	0.03 ± 0	0.02 ± 0	<0.001	0.03 ± 0	0.01 ± 0.01	0.008	0.03 ± 0	0.02 ± 0.01	<0.001
C20:5*cis*5,8,11,14,17 (EPA)	0.03 ± 0	0.04 ± 0	0.012	0.04 ± 0	0.04 ± 0	0.299	0.03 ± 0	0.05 ± 0	<0.001	0.03 ± 0	0.04 ± 0	<0.001
C24:0	0.01 ± 0	0 ± 0	<0.001	0.01 ± 0	0 ± 0	<0.001	0.01 ± 0	0 ± 0	<0.001	0.01 ± 0	0 ± 0	<0.001
C22:5*cis*7,10,13,16,19 (DPA)	0.13 ± 0.01	0 ± 0	<0.001	0.13 ± 0.01	0 ± 0	<0.001	0.13 ± 0	0 ± 0	<0.001	0.13 ± 0	0 ± 0	<0.001
FA groups												
SFA	78.83 ± 0.62	77.72 ± 0.82	0.024	76.9 ± 0.53	76.42 ± 0.52	0.144	78.25 ± 0.91	77.12 ± 0.71	0.037	79.63 ± 0.3	78.62 ± 0.19	<0.001
UFA	20.8 ± 0.59	22.52 ± 0.81	<0.001	22.65 ± 0.53	23.79 ± 0.43	<0.001	21.33 ± 0.87	23.17 ± 0.74	<0.001	20.02 ± 0.3	21.68 ± 0.11	<0.001
MUFA	18.49 ± 0.57	20.6 ± 0.7	<0.001	20.28 ± 0.52	21.8 ± 0.39	<0.001	19.1 ± 0.8	21.22 ± 0.74	<0.001	17.82 ± 0.26	19.83 ± 0.13	<0.001
PUFA	2.6 ± 0.07	1.93 ± 0.11	<0.001	2.76 ± 0.04	1.99 ± 0.07	<0.001	2.57 ± 0.11	1.95 ± 0.09	<0.001	2.46 ± 0.05	1.84 ± 0.04	<0.001
SCFA	7.25 ± 0.19	33.88 ± 0.3	<0.001	6.9 ± 0.24	33.09 ± 0.3	<0.001	7.24 ± 0.22	33.27 ± 0.43	<0.001	7.14 ± 0.19	34.4 ± 0.65	<0.001
MCFA	69.83 ± 0.77	38.3 ± 1.09	<0.001	67.86 ± 0.57	37.23 ± 0.72	<0.001	69.51 ± 1.06	39.01 ± 1	<0.001	71.13 ± 0.32	39.24 ± 0.77	<0.001
BCFA	2.48 ± 0.09	0.93 ± 0.07	<0.001	2.68 ± 0.04	1.02 ± 0.02	<0.001	2.48 ± 0.04	0.95 ± 0.04	<0.001	2.42 ± 0.02	0.9 ± 0.04	<0.001
TFA	1.08 ± 0.1	1.46 ± 0.18	<0.001	1.35 ± 0.14	1.73 ± 0.1	<0.001	1.13 ± 0.12	1.55 ± 0.13	<0.001	1.03 ± 0.07	1.34 ± 0.08	<0.001
OCFA	3.2 ± 0.05	1.41 ± 0.02	<0.001	3.1 ± 0.01	1.41 ± 0.03	<0.001	3.21 ± 0.05	1.44 ± 0.02	<0.001	3.25 ± 0.03	1.44 ± 0.03	<0.001

[1] Spring: March, April, May; Summer: June, July, August; Autumn: September, October, November; Winter: December, January, February. SFA: Saturated FA, UFA: unsaturated FA, MUFA: monounsaturated FA, PUFA: polyunsaturated FA, SCFA: short-chain FA (C4-C11), MCFA: medium-chain FA (C12-C17), BCFA: branched-chain FA (C14:0*iso*, C15:0*iso*, C16:0*iso*, C17:0*iso*, C14:0*anteiso*, C15:0*anteiso*, C16:0*anteiso*, C17:0*anteiso*), OCFA: odd-chain FA, TFA: *trans* FA.

Table S3.2. Statistical analysis on the stereolocation preferences between the interpositional distribution (%) and the hypothetical proportions at the *sn*-2 (33.3%) and *sn*-1(3) (66.7%) positions in the triacylglycerol structures.

Fatty Acid	*sn*-position	hypothetical proportion (%)	spring	*P value*	summer	*P value*	autumn	*P value*	winter	*P value*
C4:0	*sn*-2	33.3	0.8+-0.08	<0.001	0.83+-0.12	<0.001	0.9+-0.12	<0.001	0.74+-0.06	<0.001
C6:0	*sn*-2	33.3	4.36+-0.24	<0.001	4.55+-0.38	<0.001	4.61+-0.25	<0.001	4.21+-0.17	<0.001
C8:0	*sn*-2	33.3	27.37+-1.2	<0.001	27.6+-0.94	<0.001	28.04+-1.23	<0.001	26.52+-0.71	<0.001
C10:0	*sn*-2	33.3	30.26+-0.85	<0.001	30.73+-0.83	0.001	30.93+-0.85	0.001	29.4+-0.84	<0.001
C12:0	*sn*-2	33.3	47.43+-0.92	<0.001	47.83+-0.57	<0.001	47.36+-1.2	<0.001	47.01+-0.56	<0.001
C12:1*cis*9	*sn*-2	33.3	74.01+-13.51	0.001	60.68+-4.74	<0.001	62+-6.14	<0.001	77.1+-16.85	0.001
C14:0*iso*	*sn*-2	33.3	71.13+-2.32	<0.001	69.42+-4.78	<0.001	72.34+-2.87	<0.001	68.8+-3.46	<0.001
C14:0	*sn*-2	33.3	59.2+-0.55	<0.001	59.47+-1.05	<0.001	59.09+-1.14	<0.001	59.13+-0.9	<0.001
C14:1*cis*9	*sn*-2	33.3	48.4+-0.79	<0.001	49.28+-1.25	<0.001	47.55+-0.89	<0.001	47.97+-0.52	<0.001
C15:0*iso*	*sn*-2	33.3	52.71+-1.65	<0.001	53.48+-0.92	<0.001	53.26+-1.19	<0.001	52.55+-0.48	<0.001
C15:0*anteiso*	*sn*-2	33.3	67.01+-1.59	<0.001	67.06+-1.79	<0.001	67.1+-1.15	<0.001	67.03+-1.79	<0.001
C15:0	*sn*-2	33.3	49.09+-0.54	<0.001	49.03+-1.07	<0.001	48.42+-0.44	<0.001	48.78+-0.39	<0.001
C16:0*iso*	*sn*-2	33.3	60.79+-1.58	<0.001	60.67+-1.15	<0.001	59.91+-1.23	<0.001	61.01+-1.23	<0.001
C16:0	*sn*-2	33.3	44.29+-0.51	<0.001	44.13+-0.43	<0.001	43.74+-0.54	<0.001	44.23+-0.4	<0.001
C16:1*trans*9	*sn*-2	33.3	15.77+-1.6	<0.001	17.88+-1.32	<0.001	15.11+-5.63	0.001	14.67+-1.16	<0.001
C16:1*cis*9	*sn*-2	33.3	3.81+-0.25	<0.001	4.8+-0.21	<0.001	4.03+-0.43	<0.001	3.49+-0.31	<0.001
C17:0*iso*	*sn*-2	33.3	42.35+-0.71	<0.001	43.48+-0.42	<0.001	40.89+-3.8	0.005	42.01+-0.7	<0.001
C17:0*anteiso*	*sn*-2	33.3	55.85+-1.97	<0.001	53.52+-1.94	<0.001	54.01+-1.63	<0.001	56.63+-2.84	<0.001
C17:0	*sn*-2	33.3	36.6+-1.11	0.001	37.07+-1.05	<0.001	35.29+-1.37	0.016	36.1+-0.85	<0.001
C17:1*cis*9	*sn*-2	33.3	52.11+-1.69	<0.001	51.85+-1.39	<0.001	51.7+-1.58	<0.001	51.13+-1.73	<0.001
C18:0	*sn*-2	33.3	18.86+-0.37	<0.001	19.42+-1.04	<0.001	19.12+-0.64	<0.001	18.47+-0.33	<0.001
C18:1*trans*6	*sn*-2	33.3	11.93+-11.38	0.006	17.91+-24.87	0.190	12.84+-10.43	0.005	27.04+-15.18	0.359
C18:1*trans*9	*sn*-2	33.3	44.17+-5.53	0.005	46.6+-5.41	0.002	48.32+-12.21	0.030	41.36+-3.41	0.002
C18:1*trans*(10+11)	*sn*-2	33.3	29.23+-1.77	0.002	29.21+-0.89	<0.001	28.78+-0.94	<0.001	28.08+-1.11	<0.001
C18:1cis9	*sn*-2	33.3	33.5+-0.61	0.447	34.24+-0.85	0.041	33.77+-0.74	0.182	33.49+-0.46	0.345
C18:1*cis*11	*sn*-2	33.3	14.8+-0.3	<0.001	15.86+-0.92	<0.001	15.46+-0.7	<0.001	14.55+-0.42	<0.001
C18:1*cis*12	*sn*-2	33.3	27.84+-1.78	0.001	25.82+-2.04	<0.001	26.4+-0.61	<0.001	25.34+-1.01	<0.001
C18:1*cis*14	*sn*-2	33.3	8.4+-0.71	<0.001	8.69+-0.52	<0.001	8.21+-0.74	<0.001	7.82+-0.24	<0.001
C18:1*cis*15	*sn*-2	33.3	7.2+-0.66	<0.001	8.44+-0.34	<0.001	7.9+-0.75	<0.001	6.98+-0.63	<0.001
C18:2*cis*9,12 (LA)	*sn*-2	33.3	43.72+-1.33	<0.001	44.58+-1.34	<0.001	43.25+-3.03	<0.001	43.57+-1.44	<0.001
C18:3*cis*6,9,12 (GLA)	*sn*-2	33.3	35.12+-3.3	0.234	34.11+-1.48	0.235	41.11+-18.16	0.340	34.24+-4.38	0.622
C18:3*cis*9,12,15 (ALA)	*sn*-2	33.3	37.31+-1.16	<0.001	37.33+-0.77	<0.001	36.76+-1.04	<0.001	35.85+-0.9	0.001
C18:2*cis*9,*trans*11	*sn*-2	33.3	30.59+-0.78	<0.001	32.75+-0.81	0.160	31.08+-2.14	0.052	30.54+-1.12	0.002
C20:0	*sn*-2	33.3	15.81+-1.02	<0.001	16.46+-2.51	<0.001	13.88+-1.73	<0.001	15.03+-2.49	<0.001
C20:3*cis*8,11,14 (DGLA)	*sn*-2	33.3	43.3+-3.02	<0.001	42.65+-2.56	<0.001	42.07+-3.31	0.001	43.55+-1.96	<0.001
C20:4*cis*5,8,11,14 (AA)	*sn*-2	33.3	5.53+-2.01	<0.001	6.16+-2.29	<0.001	5.24+-2.14	<0.001	5.58+-2.32	<0.001
C22:0	*sn*-2	33.3	43.89+-5.88	0.007	49.67+-5	<0.001	78.18+-37.64	0.033	38.31+-3.6	0.019
C20:4*cis*8,11,14,17 (ETA)	*sn*-2	33.3	42.53+-14.61	0.182	40.74+-4.25	0.008	67.47+-45.42	0.125	50.82+-15.27	0.038
C20:5cis5,8,11,14,17 (EPA)	*sn*-2	33.3	29.51+-2.95	0.026	34.12+-1.71	0.291	26.89+-1.89	<0.001	28.39+-2.3	0.003

(continuation **Table S3.2.**)

Fatty Acid	*sn*-position	hypothetical proportion (%)	spring	*P value*	summer	*P value*	autumn	*P value*	winter	*P value*
C4:0	*sn*-1(3)	66.7	49.6+-0.04	<0.001	49.59+-0.06	<0.001	49.55+-0.06	<0.001	49.63+-0.03	<0.001
C6:0	*sn*-1(3)	66.7	47.82+-0.12	<0.001	47.72+-0.19	<0.001	47.7+-0.12	<0.001	47.9+-0.09	<0.001
C8:0	*sn*-1(3)	66.7	36.31+-0.6	<0.001	36.2+-0.47	<0.001	35.98+-0.62	<0.001	36.74+-0.36	<0.001
C10:0	*sn*-1(3)	66.7	34.87+-0.43	<0.001	34.64+-0.42	<0.001	34.54+-0.42	<0.001	35.3+-0.42	<0.001
C12:0	*sn*-1(3)	66.7	26.28+-0.46	<0.001	26.09+-0.28	<0.001	26.32+-0.6	<0.001	26.49+-0.28	<0.001
C12:1*cis*9	*sn*-1(3)	66.7	12.99+-6.76	<0.001	19.66+-2.37	<0.001	19+-3.07	<0.001	11.45+-8.42	<0.001
C14:0*iso*	*sn*-1(3)	66.7	14.44+-1.16	<0.001	15.29+-2.39	<0.001	13.83+-1.43	<0.001	15.6+-1.73	<0.001
C14:0	*sn*-1(3)	66.7	20.4+-0.28	<0.001	20.26+-0.52	<0.001	20.46+-0.57	<0.001	20.43+-0.45	<0.001
C14:1*cis*9	*sn*-1(3)	66.7	25.8+-0.39	<0.001	25.36+-0.63	<0.001	26.23+-0.44	<0.001	26.02+-0.26	<0.001
C15:0*iso*	*sn*-1(3)	66.7	23.64+-0.82	<0.001	23.26+-0.46	<0.001	23.37+-0.59	<0.001	23.73+-0.24	<0.001
C15:0*anteiso*	*sn*-1(3)	66.7	16.49+-0.8	<0.001	16.47+-0.89	<0.001	16.45+-0.57	<0.001	16.48+-0.9	<0.001
C15:0	*sn*-1(3)	66.7	25.45+-0.27	<0.001	25.48+-0.53	<0.001	25.79+-0.22	<0.001	25.61+-0.19	<0.001
C16:0*iso*	*sn*-1(3)	66.7	19.6+-0.79	<0.001	19.67+-0.57	<0.001	20.04+-0.61	<0.001	19.49+-0.61	<0.001
C16:0	*sn*-1(3)	66.7	27.85+-0.25	<0.001	27.94+-0.22	<0.001	28.13+-0.27	<0.001	27.89+-0.2	<0.001
C16:1*trans*9	*sn*-1(3)	66.7	42.12+-0.8	<0.001	41.06+-0.66	<0.001	42.44+-2.82	<0.001	42.66+-0.58	<0.001
C16:1*cis*9	*sn*-1(3)	66.7	48.09+-0.12	<0.001	47.6+-0.1	<0.001	47.98+-0.21	<0.001	48.26+-0.16	<0.001
C17:0*iso*	*sn*-1(3)	66.7	28.83+-0.36	<0.001	28.26+-0.21	<0.001	29.55+-1.9	<0.001	29+-0.35	<0.001
C17:0*anteiso*	*sn*-1(3)	66.7	22.08+-0.99	<0.001	23.24+-0.97	<0.001	23+-0.82	<0.001	21.69+-1.42	<0.001
C17:0	*sn*-1(3)	66.7	31.7+-0.55	<0.001	31.46+-0.53	<0.001	32.35+-0.69	<0.001	31.95+-0.42	<0.001
C17:1*cis*9	*sn*-1(3)	66.7	23.95+-0.84	<0.001	24.07+-0.7	<0.001	24.15+-0.79	<0.001	24.43+-0.86	<0.001
C18:0	*sn*-1(3)	66.7	40.57+-0.19	<0.001	40.29+-0.52	<0.001	40.44+-0.32	<0.001	40.77+-0.16	<0.001
C18:1*trans*6	*sn*-1(3)	66.7	44.03+-5.69	<0.001	41.04+-12.43	0.004	43.58+-5.22	<0.001	36.48+-7.59	<0.001
C18:1*trans*9	*sn*-1(3)	66.7	27.91+-2.76	<0.001	26.7+-2.7	<0.001	25.84+-6.1	<0.001	29.32+-1.71	<0.001
C18:1*trans*(10+11)	*sn*-1(3)	66.7	35.39+-0.89	<0.001	35.4+-0.44	<0.001	35.61+-0.47	<0.001	35.96+-0.56	<0.001
C18:1cis9	*sn*-1(3)	66.7	33.25+-0.3	<0.001	32.88+-0.42	<0.001	33.12+-0.37	<0.001	33.25+-0.23	<0.001
C18:1*cis*11	*sn*-1(3)	66.7	42.6+-0.15	<0.001	42.07+-0.46	<0.001	42.27+-0.35	<0.001	42.72+-0.21	<0.001
C18:1*cis*12	*sn*-1(3)	66.7	36.08+-0.89	<0.001	37.09+-1.02	<0.001	36.8+-0.3	<0.001	37.33+-0.5	<0.001
C18:1*cis*14	*sn*-1(3)	66.7	45.8+-0.36	<0.001	45.65+-0.26	<0.001	45.89+-0.37	<0.001	46.09+-0.12	<0.001
C18:1*cis*15	*sn*-1(3)	66.7	46.4+-0.33	<0.001	45.78+-0.17	<0.001	46.05+-0.37	<0.001	46.51+-0.31	<0.001
C18:2*cis*9,12 (LA)	*sn*-1(3)	66.7	28.14+-0.66	<0.001	27.71+-0.67	<0.001	28.37+-1.52	<0.001	28.21+-0.72	<0.001
C18:3*cis*6,9,12 (GLA)	*sn*-1(3)	66.7	32.44+-1.65	<0.001	32.94+-0.74	<0.001	29.45+-9.08	<0.001	32.88+-2.19	<0.001
C18:3 *cis*-9,12,15 (ALA)	*sn*-1(3)	66.7	31.35+-0.58	<0.001	31.33+-0.39	<0.001	31.62+-0.52	<0.001	32.08+-0.45	<0.001
C18:2*cis*9,*trans*11	*sn*-1(3)	66.7	34.71+-0.39	<0.001	33.62+-0.41	<0.001	34.46+-1.07	<0.001	34.73+-0.56	<0.001
C20:0	*sn*-1(3)	66.7	42.1+-0.51	<0.001	41.77+-1.25	<0.001	43.06+-0.87	<0.001	42.49+-1.25	<0.001
C20:3*cis*8,11,14 (DGLA)	*sn*-1(3)	66.7	28.35+-1.51	<0.001	28.67+-1.28	<0.001	28.96+-1.65	<0.001	28.22+-0.98	<0.001
C20:4*cis*5,8,11,14 (AA)	*sn*-1(3)	66.7	47.24+-1.01	<0.001	46.92+-1.15	<0.001	47.38+-1.07	<0.001	47.21+-1.16	<0.001
C22:0	*sn*-1(3)	66.7	28.06+-2.94	<0.001	25.16+-2.5	<0.001	10.91+-18.82	0.001	30.84+-1.8	<0.001
C20:4*cis*8,11,14,17 (ETA)	*sn*-1(3)	66.7	28.73+-7.31	<0.001	29.63+-2.13	<0.001	16.27+-22.71	0.003	24.59+-7.64	<0.001
C20:5cis5,8,11,14,17 (EPA)	*sn*-1(3)	66.7	35.24+-1.47	<0.001	32.94+-0.85	<0.001	36.56+-0.95	<0.001	35.8+-1.15	<0.001

(continuation **Table S3.2.**)

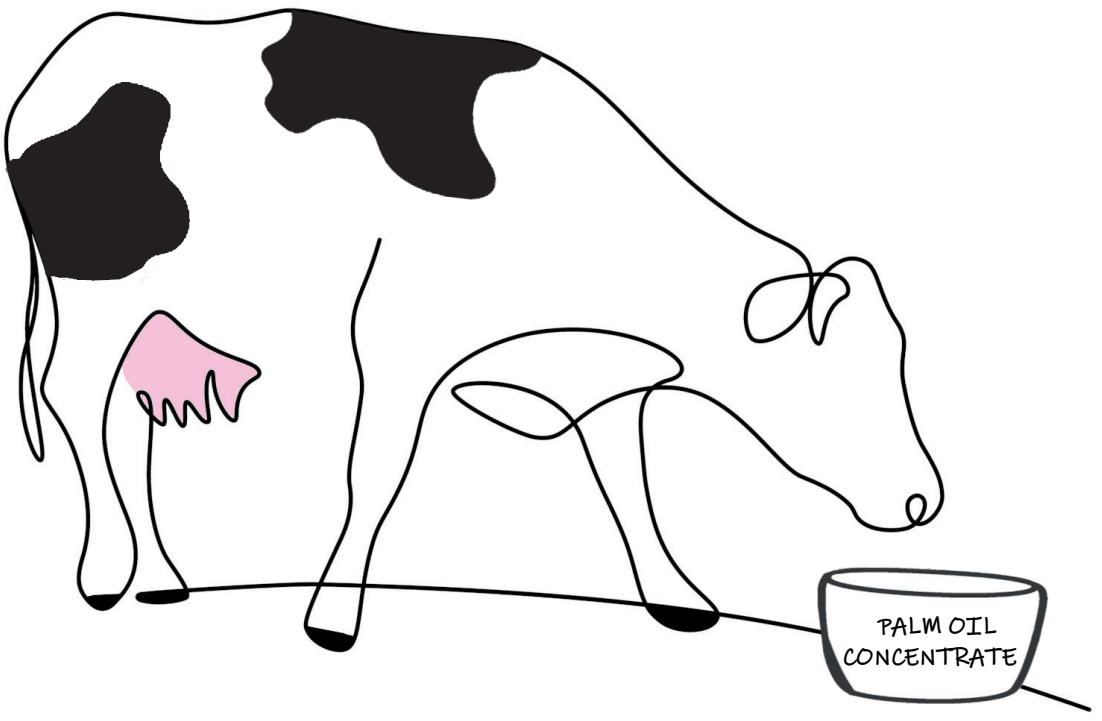
Fatty group	*sn*-position	Hypothetical proportion (%)	spring	*P value*	summer	*P value*	autumn	*P value*	winter	*P value*
SFA	*sn*-2	33.3	33.65+-0.12	0.001	33.47+-0.24	0.139	33.66+-0.19	0.006	33.62+-0.08	<0.001
UFA	*sn*-2	33.3	31.59+-0.39	<0.001	32.25+-0.7	0.014	31.52+-0.65	0.001	31.59+-0.33	<0.001
MUFA	*sn*-2	33.3	30.98+-0.36	<0.001	31.75+-0.72	0.003	31.04+-0.65	<0.001	31+-0.34	<0.001
PUFA	*sn*-2	33.3	40.33+-0.89	<0.001	40.89+-0.72	<0.001	39.77+-1.69	<0.001	40.04+-0.36	<0.001
SCFA	*sn*-2	33.3	9.66+-0.24	<0.001	9.44+-0.37	<0.001	9.82+-0.37	<0.001	9.41+-0.22	<0.001
MCFA	*sn*-2	33.3	47.7+-0.48	<0.001	47.68+-0.55	<0.001	47.12+-0.61	<0.001	47.55+-0.46	<0.001
BCFA	*sn*-2	33.3	57.25+-1.34	<0.001	56.93+-0.63	<0.001	56.61+-0.94	<0.001	57.3+-1.31	<0.001
TFA	*sn*-2	33.3	26.94+-1.36	<0.001	28.09+-3.11	0.009	26.84+-2.02	0.001	27.78+-2.16	0.002
OCFA	*sn*-2	33.3	53.08+-0.32	<0.001	52.36+-0.55	<0.001	52.65+-0.12	<0.001	53.08+-0.43	<0.001
SFA	*sn*-1(3)	66.7	66.35+-0.12	0.001	66.53+-0.24	0.139	66.34+-0.19	0.006	66.38+-0.08	<0.001
UFA	*sn*-1(3)	66.7	68.41+-0.39	<0.001	67.75+-0.7	0.014	68.48+-0.65	0.001	68.41+-0.33	<0.001
MUFA	*sn*-1(3)	66.7	69.02+-0.36	<0.001	68.25+-0.72	0.003	68.96+-0.65	<0.001	69+-0.34	<0.001
PUFA	*sn*-1(3)	66.7	59.67+-0.89	<0.001	59.11+-0.72	<0.001	60.23+-1.69	<0.001	59.96+-0.36	<0.001
SCFA	*sn*-1(3)	66.7	90.34+-0.24	<0.001	90.56+-0.37	<0.001	90.18+-0.37	<0.001	90.59+-0.22	<0.001
MCFA	*sn*-1(3)	66.7	52.3+-0.48	<0.001	52.32+-0.55	<0.001	52.88+-0.61	<0.001	52.45+-0.46	<0.001
BCFA	*sn*-1(3)	66.7	42.75+-1.34	<0.001	43.07+-0.63	<0.001	43.39+-0.94	<0.001	42.7+-1.31	<0.001
TFA	*sn*-1(3)	66.7	73.06+-1.36	<0.001	71.91+-3.11	0.009	73.16+-2.02	0.001	72.22+-2.16	0.002
OCFA	*sn*-1(3)	66.7	46.92+-0.32	<0.001	47.64+-0.55	<0.001	47.35+-0.12	<0.001	46.92+-0.43	<0.001

Table S3.3. Fatty acid (FA) composition (mol %) and fat content of bovine milk fat.

		Seasons								
Item	Year average	Winter	Autumn	Spring	Summer	min	Season	max	Season	*P* value
Fat content	4.34 (0.15)	4.47 (0.04)	4.33 (0.09)	4.42 (0.11)	4.14 (0.06)	4.08	Summer	4.60	Spring	<0.001
C4:0	12.37 (0.39)	12.5 (0.42)	12.31 (0.56)	12.33 (0.29)	12.37 (0.19)	12.31	Autumn	12.50	Winter	0.645
C5:0	0.02 (0.03)	0.02 (0.03)	0.01 (0.02)	0.01 (0.02)	0.03 (0.03)	0.01	Autumn	0.03	Summer	0.475
C6:0	5.36 (0.14)	5.5 (0.16)	5.29 (0.11)	5.41 (0.08)	5.25 (0.06)	5.25	Summer	5.50	Winter	<0.001
C7:0	0.02 (0.02)	0.02 (0.03)	0.01 (0.02)	0.02 (0.02)	0.04 (0.01)	0.01	Autumn	0.04	Summer	0.043
C8:0	2.26 (0.07)	2.33 (0.04)	2.23 (0.06)	2.30 (0.03)	2.20 (0.04)	2.20	Summer	2.33	Winter	<0.001
C9:0	0.04 (0.02)	0.05 (0.01)	0.03 (0.02)	0.04 (0.01)	0.04 (0.01)	0.03	Autumn	0.05	Winter	0.001
C10:0	4.17 (0.19)	4.36 (0.06)	4.06 (0.15)	4.30 (0.08)	3.97 (0.07)	3.97	Summer	4.36	Winter	<0.001
C10:1	0.44 (0.02)	0.45 (0.02)	0.45 (0.02)	0.43 (0.01)	0.42 (0.01)	0.42	Summer	0.45	Winter	<0.001
C11:0	0.07 (0.01)	0.08 (0.01)	0.07 (0.01)	0.07 (0.00)	0.06 (0.00)	0.06	Summer	0.08	Winter	<0.001
C12:0	4.56 (0.18)	4.75 (0.05)	4.45 (0.17)	4.68 (0.07)	4.37 (0.07)	4.37	Summer	4.75	Winter	<0.001
C12:1	0.1 (0.02)	0.1 (0.02)	0.12 (0.02)	0.09 (0.02)	0.11 (0.01)	0.09	Spring	0.12	Autumn	0.003
C13:0	0.09 (0.01)	0.1 (0.01)	0.09 (0.01)	0.10 (0.01)	0.09 (0.00)	0.09	Summer	0.10	Winter	0.001
C14:0*iso*	0.08 (0.01)	0.08 (0.01)	0.08 (0.01)	0.08 (0.01)	0.08 (0.01)	0.08	Spring	0.08	Summer	0.005
C14:0	11.34 (0.27)	11.58 (0.14)	11.2 (0.24)	11.49 (0.14)	11.07 (0.14)	11.07	Summer	11.58	Winter	<0.001
C14:1*cis*9	1.09 (0.06)	1.12 (0.03)	1.13 (0.04)	1.07 (0.03)	1.02 (0.03)	1.02	Summer	1.13	Autumn	<0.001
C15:0*iso*	0.21 (0.01)	0.2 (0.00)	0.21 (0.01)	0.21 (0.01)	0.23 (0.01)	0.20	Winter	0.23	Summer	<0.001
C15:0*anteiso*	0.41 (0.02)	0.39 (0.01)	0.41 (0.01)	0.40 (0.02)	0.44 (0.01)	0.39	Winter	0.44	Summer	<0.001
C15:0	0.98 (0.03)	1.00 (0.02)	0.98 (0.02)	0.98 (0.02)	0.94 (0.02)	0.94	Summer	1.00	Winter	<0.001
C16:0*iso*	0.17 (0.01)	0.16 (0.00)	0.17 (0.01)	0.16 (0.00)	0.18 (0.01)	0.16	Winter	0.18	Summer	<0.001
C16:0	26.9 (0.81)	27.54 (0.41)	27.09 (0.57)	26.98 (0.76)	25.94 (0.51)	25.94	Summer	27.54	Winter	<0.001
C16:1*trans*9	0.15 (0.01)	0.14 (0.00)	0.16 (0.01)	0.15 (0.01)	0.17 (0.00)	0.14	Winter	0.17	Summer	<0.001
C16:1*cis*9	1.37 (0.05)	1.36 (0.03)	1.43 (0.03)	1.35 (0.03)	1.34 (0.02)	1.34	Summer	1.43	Autumn	<0.001
C17:0*iso*	0.29 (0.02)	0.27 (0.01)	0.29 (0.01)	0.28 (0.01)	0.31 (0.00)	0.27	Winter	0.31	Summer	<0.001
C17:0*anteiso*	0.32 (0.01)	0.31 (0.01)	0.32 (0.01)	0.31 (0.01)	0.33 (0.01)	0.31	Winter	0.33	Summer	<0.001
C17:0	0.42 (0.01)	0.41 (0.01)	0.42 (0.01)	0.42 (0.01)	0.43 (0.01)	0.41	Winter	0.43	Summer	<0.001
C17:1*cis*9	0.17 (0.01)	0.16 (0.00)	0.17 (0.01)	0.16 (0.01)	0.18 (0.00)	0.16	Winter	0.18	Summer	<0.001
C18:0	7.36 (0.43)	6.99 (0.17)	7.19 (0.25)	7.32 (0.26)	7.96 (0.29)	6.99	Winter	7.96	Summer	<0.001
C18:1*trans*6	0.17 (0.02)	0.16 (0.01)	0.17 (0.02)	0.17 (0.01)	0.19 (0.01)	0.16	Winter	0.19	Summer	<0.001
C18:1*trans*9	0.13 (0.02)	0.13 (0.01)	0.12 (0.04)	0.13 (0.01)	0.13 (0.01)	0.12	Autumn	0.13	Spring	0.908
C18:1*trans*10	0.20 (0.04)	0.18 (0.01)	0.20 (0.02)	0.21 (0.06)	0.21 (0.03)	0.18	Winter	0.21	Summer	0.286
C18:1*trans*11	0.75 (0.13)	0.64 (0.03)	0.79 (0.1)	0.68 (0.13)	0.90 (0.04)	0.64	Winter	0.90	Summer	<0.001
C18:1*cis*9	14.1 (0.78)	13.17 (0.15)	14.43 (0.59)	13.81 (0.45)	14.97 (0.32)	13.17	Winter	14.97	Summer	<0.001

(continuation **Table S3.3.**)

		Seasons								
FA	Year average	Winter	Autumn	Spring	Summer	min	Season	max	Season	*P* value
C18:1*cis*11	0.57 (0.03)	0.55 (0.02)	0.57 (0.02)	0.57 (0.02)	0.60 (0.03)	0.55	Winter	0.60	Summer	<0.001
C18:1*cis*12	0.18 (0.01)	0.18 (0.01)	0.17 (0.01)	0.18 (0.01)	0.18 (0.01)	0.17	Autumn	0.18	Winter	0.003
C18:1*cis*13	0.10 (0.01)	0.09 (0.00)	0.10 (0.01)	0.10 (0.01)	0.11 (0.01)	0.09	Winter	0.11	Summer	<0.001
C18:1*cis*14	0.31 (0.01)	0.30 (0.01)	0.30 (0.01)	0.31 (0.01)	0.31 (0.01)	0.30	Winter	0.31	Summer	0.057
C18:1*cis*15	0.24 (0.01)	0.23 (0.01)	0.25 (0.01)	0.24 (0.02)	0.25 (0.01)	0.23	Winter	0.25	Summer	0.002
C18:2*cis*9,12 (LA)	1.19 (0.06)	1.16 (0.05)	1.18 (0.06)	1.21 (0.05)	1.21 (0.05)	1.16	Winter	1.21	Spring	0.055
C19:0	0.10 (0.00)	0.10 (0.00)	0.10 (0.01)	0.10 (0.00)	0.11 (0.00)	0.10	Autumn	0.11	Summer	0.060
C18:3*cis*6,9,12 (GLA)	0.04 (0.01)	0.04 (0.01)	0.04 (0.01)	0.04 (0.00)	0.04 (0.00)	0.04	Autumn	0.04	Summer	0.764
C18:3*cis*9,12,15 (ALA)	0.37 (0.02)	0.35 (0.01)	0.37 (0.01)	0.38 (0.02)	0.39 (0.02)	0.35	Winter	0.39	Summer	<0.001
C18:2*cis*9,*trans*11	0.34 (0.05)	0.29 (0.02)	0.38 (0.04)	0.31 (0.03)	0.39 (0.02)	0.29	Winter	0.39	Summer	<0.001
C20:0	0.09 (0.01)	0.08 (0.01)	0.09 (0.01)	0.08 (0.01)	0.09 (0.01)	0.08	Spring	0.09	Summer	0.001
C20:3*cis*8,11,14 (DGLA)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04	Winter	0.04	Summer	0.331
C20:4*cis*5,8,11,14 (AA)	0.06 (0.00)	0.06 (0.00)	0.06 (0.00)	0.06 (0.00)	0.06 (0.00)	0.06	Spring	0.06	Autumn	0.022
C22:0	0.02 (0.01)	0.03 (0.00)	0.02 (0.01)	0.02 (0.00)	0.03 (0.00)	0.02	Autumn	0.03	Winter	0.003
C20:4*cis*8,11,14,17 (ETA)	0.02 (0.01)	0.02 (0.00)	0.02 (0.01)	0.02 (0.00)	0.03 (0.00)	0.02	Autumn	0.03	Summer	0.005
C20:5*cis*5,8,11,14,17 (EPA)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	0.04	Spring	0.04	Autumn	0.040
C24:0	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.01 (0.00)	0.01 (0.01)	0.01	Autumn	0.01	Summer	0.037
C22:5*cis*7,10,13,16,19 (DPA)	0.04 (0.00)	0.04 (0.00)	0.05 (0.01)	0.04 (0.00)	0.04 (0.00)	0.04	Summer	0.05	Autumn	0.084
Pristanic acid	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.01	Autumn	0.01	Summer	0.842
Phytanic Acid	0.12 (0.01)	0.13 (0.01)	0.12 (0.01)	0.12 (0.01)	0.11 (0.01)	0.11	Summer	0.13	Winter	<0.001
FA groups										
SFA	77.78 (2.72)	78.98 (0.22)	77.25 (0.76)	78.23 (0.66)	76.68 (0.4)	76.68	Summer	78.98	Winter	<0.001
UFA	22.22 (1.39)	21.02 (0.22)	22.75 (0.76)	21.77 (0.66)	23.32 (0.4)	21.02	Winter	23.32	Summer	<0.001
MUFA	20.06 (1.23)	18.97 (0.19)	20.57 (0.71)	19.63 (0.59)	21.08 (0.39)	18.97	Winter	21.08	Summer	<0.001
PUFA	2.15 (0.16)	2.05 (0.05)	2.18 (0.09)	2.14 (0.09)	2.24 (0.06)	2.05	Winter	2.24	Summer	<0.001
SCFA	24.76 (0.88)	25.3 (0.6)	24.47 (0.62)	24.91 (0.33)	24.38 (0.3)	24.38	Summer	25.30	Winter	<0.001
MCFA	48.77 (1.56)	49.8 (0.56)	48.85 (0.94)	49.03 (0.89)	47.34 (0.65)	47.34	Summer	49.80	Winter	<0.001
BCFA	1.47 (0.01)	1.40 (0.00)	1.48 (0.01)	1.43 (0.01)	1.57 (0.00)	1.40	Winter	1.57	Summer	<0.001
TFA	1.40 (0.02)	1.25 (0.01)	1.44 (0.02)	1.33 (0.02)	1.60 (0.01)	1.25	Winter	1.60	Summer	<0.001
OCFA	1.91 (0.01)	1.94 (0.01)	1.89 (0.01)	1.90 (0.01)	1.91 (0.01)	1.89	Autumn	1.94	Winter	0.332


PALM OIL
CONCENTRATE

Chapter 4

Feeding hydrogenated palm fatty acids to lactating Holstein-Friesian dairy cows modifies milk fat triacylglycerol composition and structure, and solid fat content

This chapter is based on:

Pacheco-Pappenheim S., S. Yener., K. Nichols, J. Dijkstra, K. Hettinga, and H.J.F. van Valenberg. Feeding hydrogenated palm fatty acids to lactating Holstein-Friesian dairy cows modifies milk fat triacylglycerol composition and structure, and solid fat content. Submitted for publication to the Journal of Dairy Science.

Abstract

The aim of this study was to analyze the effect of fat and protein supplementation to dairy cattle rations on milk fat triacylglycerol (**TAG**) composition, fatty acid (**FA**) positional distribution in the TAG structure, and milk solid fat content (**SFC**). Fifty-six lactating Holstein-Friesian cows were blocked into 14 groups of 4 cows and randomly assigned 1 of 4 dietary treatments: 1) low protein, low fat, 2) high protein, low fat, 3) low protein, high fat, and 4) high protein, high fat. The high protein and high fat diets were obtained by isoenergetically supplementing the basal ration (low protein, low fat) with rumen-protected soybean meal and rapeseed meal, and hydrogenated palm fatty acids (mainly C16:0 and C18:0 FA), respectively. Fat supplementation modified milk fat TAG composition more extensively compared to protein supplementation. Fat supplementation resulted in decreased concentrations of the low molecular weight TAG CN26 to CN34 and medium molecular weight TAG CN40, CN44 and CN46, and increased concentrations of CN38 and the high molecular weight (**HMW**) TAG CN50 and CN52. Increased contents of C16:0, C18:0, and C18:1*cis*9 in the TAG in response to fat supplementation were related to increases in the relative concentrations of C16:0 and C18:0 at the *sn*-2 position and of C18:0 and C18:1*cis*9 at the *sn*-1(3) positions of the TAG structure. High concentrations of HMW TAG species CN50 and CN52 in response to fat supplementation increased milk SFC at 20, 25, and 30°C. Our study shows that important alterations in milk fat TAG composition and structure occur when feeding hydrogenated palm fatty acids to lactating dairy cattle, and that these alterations result in an increased SFC of milk fat. These changes may improve absorption of both fat and minerals in infants and may impact processing of milk fat.

4.1. Introduction

Bovine milk fat is mainly composed of triacylglycerols (**TAG**; 98%), which are formed by the esterification of 3 fatty acids (**FA**) to a glycerol backbone. Milk FA composition can be modified by various nutritional interventions to dairy cattle rations to serve a functional purpose, *e.g.* to improve the processing and manufacturing of milk and dairy products, or alter the nutritional value of milk to benefit human health (Jenkins and McGuire, 2006; Mohan et al., 2020). The supplementation of saturated long chain FA (**SLCFA**) into dairy cattle rations is a nutritional strategy commonly used to increase the energy density of the diet in support of milk production (Ashes et al., 1997; Nichols et al., 2018). In contrast to unsaturated FA (**UFA**) that are susceptible to biohydrogenation in the rumen (Baumgard et al., 2000), SLCFA are largely rumen-inert, and commonly do not result in negative impacts on fiber digestibility or milk fat depression at supplementation levels up to 3% of dietary DM (Ashes et al., 1997; NRC 2001; Nichols et al., 2018). Milk fat content and FA composition will vary depending on the type and amount of supplemented fat and its relative inertness in the rumen (Jacobs et al., 2011; Rabiee et al., 2012).

Variations in milk FA composition result in changes in milk fat TAG composition (Jensen, 2002; Pacheco-Pappenheim et al., 2019). Previous studies have reported that oil supplementation to dairy cattle rations (*e.g.* palm oil, canola oil, olive oil, soya oil, and linseed oil) decreased the milk concentration of TAG species with 30 to 36 and 42 to 46 carbon atoms, and increased the concentration of the TAG species with 50 to 54 carbon atoms, as a result of the high concentrations of long chain FA (**LCFA**) in the supplemented oils (Banks et al., 1989; DePeters et al., 2001). Seasonal variation in feeding regimes also affects milk fat TAG composition, with increased concentrations of high molecular weight (**HMW**) TAG and decreased concentrations of low molecular weight (**LMW**) and medium molecular weight (**MMW**) TAG upon intake of fresh grass in spring and summer (Pacheco-Pappenheim et al., 2021). Variations in milk TAG composition may in turn affect the solid fat content (**SFC**) of milk fat (Precht and Frede, 1994; Ashes et al., 1997). The SFC at a certain temperature is responsible for the mouthfeel and texture characteristics of a food product (*e.g.* softness, hardness, spreadability, and room temperature stability of butter; NorAini et al., 1995; Mohan et al., 2020). Therefore, milk SFC is considered to be one of the key parameters that defines the suitability of a fat source for a specific food product application. Earlier studies have suggested that high proportions of UFA in dairy cow diets increased UFA esterification in HMW unsaturated TAG species, resulting in lower milk fat SFC (Smet et al., 2010; Larsen et al., 2014). Most of the studies on this topic assessed the effect of unsaturated fats (rumen-protected and non-protected sources) on milk SFC, whilst scarce information can be found on the effect on milk SFC when feeding saturated fats (*e.g.* supplementation of SLCFA) to dairy cattle (Ashes et al., 1997).

The structure of TAG species change according to the non-random positional distribution of FA on the glycerol backbone (Jensen, 2002). The stereospecific numbering (***sn***) identifies the position of the FA in the TAG structure, where *sn*-1(3) refers to the primary positions and *sn*-2 to the secondary position in the TAG structure. There is great interest in the composition of the FA in the secondary position in the TAG structure, particularly in relation to C16:0. For example, a higher content of C16:0 at the *sn*-2 position in infant formula has been shown to increase free FA and calcium absorption in infants (Innis, 2011; Yaron et al., 2013). Tzompa-Sosa et al. (2014) showed that differences in the total FA composition in milk fat were related to the FA positional distribution in the TAG structure, where high concentrations of C16:0 in milk fat were associated with decreased proportions of C14:0, C16:0 and SLCFA and increased proportion of C18:1*cis*9 at the *sn*-2 position in the TAG structures. Increased concentrations of UFA from canola oil (containing mainly MUFA) in the cow's diet reduced the concentrations of C16:0 and increased the concentrations of C18:1*cis*9 at the *sn*-2 position (DePeters et al., 2001). To our knowledge, no information has been reported on the effect of saturated fat supplementation to dairy cattle on the esterification preference of C16:0 at the *sn*-2 position in the TAG structure of milk fat.

In the current study, we used milk samples collected from the study of Nichols et al. (2018) to investigate the effect of rumen-inert SLCFA from hydrogenated palm fatty acids (mainly C16:0 and C18:0) supplemented to dairy cattle on TAG composition, FA positional distribution in the TAG structure, and SFC of milk fat. In the interest of food processing and dairy product design, increased concentrations of SLCFA in dairy cattle rations may support the formation of TAG species with C16:0 at the *sn*-2 position, which is beneficial for development of infant formula. On the other hand, rations rich in C16:0 might increase the level of saturated TAG species in milk fat resulting in an increased SFC. This consequence may negatively affect the mouthfeel and texture characteristics of dairy products and thus require adjustments in the processing conditions of products made from this milk fat.

4.2. Materials and Methods

4.2.1. Milk sample collection and preparation

Milk samples were collected from 56 Holstein-Friesian cows (167±87 DIM; 2.8±1.9 lactations; mean±SD) enrolled in a feeding study that has been previously described by Nichols et al., (2018). Briefly, the experiment consisted of 2 successive periods (control and experimental), each consisting of 21 days of diet adaptation and 7 days of measurement. A basal TMR consisting of 34% grass silage, 33% corn silage, 5% grass hay, and 28% concentrate (DM basis) was fed during the control period. Cows were blocked (4 cows per block) based on parity, DIM, and DMI during the final 7 days of the control period, and were randomly assigned within block to 1 of 4 treatments for the experimental period: 1) low

protein, low fat (**LP/LF**), 2) high protein, low fat (**HP/LF**), 3) low protein, high fat (**LP/HF**), and 4) high protein, high fat (**HP/HF**). The HP and HF diets were obtained by restricting the intake of the basal TMR of individual cows by 5% of their ad libitum intake during the control period and supplementing 2.0 kg of rumen-protected soybean meal and rapeseed meal (DM basis; 50:50 mixture of SoyPass + RaPass; Borregaard LignoTech, Sarpsborg, Norway) and 0.68 kg of rumen-inert hydrogenated palm FA (DM basis; 50% C16:0 and 47% C18:0; Hidropalm; Norel, Madrid, Spain), respectively. Cows were milked twice daily at 530 and 1630 h.

Milk samples from individual cows were collected at 2 subsequent morning and afternoon milkings in the final 7 days of each period and stored at -20°C pending milk fat extraction. Frozen milk samples were thawed in a water bath (37°C for approximately 30 min), and 50 mL of each sample was pooled into a composite sample for milk fat extraction. The cream portion was separated by centrifugation (5,000 rpm for 5 min at 4°C). Fat was extracted by treating the cream with 4M HCl, heating (70°C for 30 min under gentle shaking), and centrifuging (5,000 rpm for 5 min at 4°C) to remove protein traces. A final heating was performed (70°C for 15 min under gentle shaking) to remove traces of water. Milk fat samples were stored at -20°C until further analysis.

4.2.2. Triacylglycerol composition analysis

Triacylglycerol composition was analyzed by gas chromatography (**GC**) - flame ionization detector (**FID**) and MALDI-TOF-MS. The GC-FID was used to determine the TAG composition of even-chain TAG groups. A TAG group is composed of TAG species with same number of total carbon atoms [*i.e.,* the same carbon number (**CN**)]. The molecular weight of each individual TAG species was determined using MALDI-TOF-MS, which determines the TAG profile (even- and odd-chain TAG species) based on their CN and number of double bonds (**DB**). Both methods complement each other resulting in a complete overview of milk fat TAG composition (Schiller et al., 2004; Pacheco-Pappenheim et al., 2021).

4.2.2.1. GC-FID

Triacylglycerol composition of milk fat samples from the control (n=56) and experimental (n=56) periods (total n=112) were determined according to the ISO Standard 17678 (2010) for GC-FID using a column injector port (Thermo Trace GC ultra; Thermo Scientific, Rodano, Italy) and a UltiMetal CP7532 column (5 m × 0.53 mm i.d. × 0.17 µm film thickness; Varian, Houten, The Netherlands). Triacylglycerols were classified according to their CN. Anhydrous milk fat TAG standard (BCR519; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was used as a reference for the identification of all TAG groups. Triacylglycerol

composition was expressed as the relative concentration in milk fat (g/100 g, %). The TAG groups that can be identified with this method are even-chain TAG from CN24 to CN54.

4.2.2.2. MALDI-TOF-MS

Triacylglycerol composition with MALDI-TOF-MS (UltrafleXtreme, Bruker Corporation, Germany) was determined in milk fat samples from the experimental period (n=56) according to the methods described by Tzompa-Sosa et al. (2018) and Yener and van Valenberg (2019). Each sample was plated 5 times and subjected to 1000 laser pulses taken randomly (automatic mode) from 20 different points to allow the random acquisition of masses. The obtained mass spectra were analyzed according to Yener and van Valenberg (2019) with the MALDIquant package using R (Gibb and Strimmer, 2012). Selection of the mass peaks was done using a signal-noise ratio >6. The relative intensities were calculated by dividing the intensity of each mass peak with the sum of the intensity of all mass peaks and expressed as a percentage. This was performed for each of the 5 repetitions, and the mean values were used as the relative intensity for each TAG species. For identification of TAG species, the molecular weights of TAG species measured with MALDI-TOF-MS were matched in LIPID MAPS® Online Tools library to the molecular weight of TAG specie with a known CN:DB (Fahy et al., 2005).

4.2.3. Regiospecific distribution analysis of fatty acids

The composition of FA at the *sn*-2 position of the TAG structure was analyzed in milk fat samples from cows receiving the LP/LF and LP/HF treatments in the experimental period (n=28) to investigate the effect of dietary palm fatty acids supplementation. The *sn*-2 positional analysis was performed via enzymatic transesterification using *Candida antarctica* lipase (fraction B; 10,000 PLU/g, Novozym 435; Novozymes A/S, Frederiksberg, Denmark) according to the JOCS/AOCS Joint Method Ch3a-19 (2019). After enzymatic transesterification, the 2-monoacylglycerol fraction was isolated and analyzed for FAME composition. The FAME were prepared according to ISO Standard 15884 (2002) and analyzed according to ISO Standard 16958 (2015) with GC-FID, as described by Pacheco-Pappenheim et al., (2021). The FA profile at the *sn*-2 position was first expressed as a weight percentage (g/100 g, %) by dividing each FA peak area by the total peak area, and was then transformed to a molar percentage. Since we used a regiospecific distribution analysis, no distinctions could be made between the FA profiles at the *sn*-1 and *sn*-3 positions in the TAG structures. For this reason, the FA profile at *sn*-1(3) refers to the sum of the FA identified at the *sn*-1 and *sn*-3 positions. The positional distribution of FA in TAG structure was examined according to the intra- and interpositional distributions (Tzompa-Sosa et al., 2014).

Intrapositional distribution:
Intrapositional distribution refers to the relative concentrations or abundance of a FA at the *sn*-2 and *sn*-1(3) positions in the TAG structure.

sn-2 position:
Fatty acids profile analyzed at the *sn*-2 position expressed in molar percentage (mol %).

sn-1(3) position:
$sn\text{-}1(3)_{FAi}$ position = ($TAG_i \times 3 - sn\text{-}2_{FAi}$ position)/2 (Equation 4.1)

Where $sn\text{-}1(3)_{FAi}$ position is the molar percentage of each FA estimated to be at these positions, TAG_i is the molar percentage of each FA in milk TAG transformed from the relative concentrations in percentages (g/100 g, %) reported by Nichols et al. (2018), and $sn\text{-}2_{FAi}$ position is the molar percentage of each FA analyzed at the *sn*-2 position.

Interpositional distribution:
Interpositional distribution refers to the proportions of a FA over the 3 positions in the TAG structure. The intrapositional distribution of FA at the *sn*-2 and *sn*-1(3) positions in the TAG structure was used to calculate the FA proportions over these positions in the TAG structure. The equations to calculate the FA proportions at each position are:

$sn\text{-}2_{FAi}$ (%) = [$sn\text{-}2_{FAi}$ position/($sn\text{-}2_{FAi}$ position + ($sn\text{-}1(3)_{FAi}$ position × 2))]×100 (Equation 4.2)

$sn\text{-}1(3)_{FAi}$ (%) = ($sn\text{-}1(3)_{FAi}$ *position* × 2)/($sn\text{-}2_{FAi}$ position + ($sn\text{-}1(3)_{FAi}$ position × 2))×100 (Equation 4.3)

Where $sn\text{-}2_{FAi}$ (%) is the proportion of each FA estimated at the *sn*-2 position, $sn\text{-}1(3)_{FAi}$ (%) is the proportion of each FA estimated at the *sn*-1(3) position, $sn\text{-}1(3)_{FAi}$ position is the molar percentage of each FA (calculated from equation 4.1) and $sn\text{-}2_{FAi}$ position is the molar percentage of each FA analyzed at the *sn*-2 position.

4.2.4. Solid fat content

The SFC analysis was performed for all samples (n=112) at 6 measuring temperatures (0, 10, 20, 25, 30, and 40°C) by nuclear magnetic resonance (NMR; Bruker mq20 minispec NMR analyzer; Bruker, Mississauga, Ontario, Canada) according to the AOCS Official Method Cd 16b-93 (2009). The NMR analyzer was set at a frequency of 19.95 MHz, a dead time of 0.0073 ms, and a cell temperature of 40°C. Milk fat samples were completely melted in a water bath at 40°C, and 1 g of liquid milk fat was transferred into a NMR glass tube (diameter of 10 mm, length of 150 mm, wall thickness of 0.6 mm; Bruker Nederland B.V., Leiderdorp, Netherlands). The tubes were heated to 100°C for 15 min followed by 15 min

at 60°C. To complete the tempering, the tubes were cooled to 0°C for 60 min. Once cooled, the first measurement was done at 0°C. Samples were held for 30±1 min at each measuring temperature before the SFC analysis took place.

4.2.5. Statistical analysis

The effect of supplemented protein, fat, and their interaction on milk fat TAG composition, the FA positional distribution in TAG, and SFC were analyzed using the MIXED procedure of SAS/STAT version 9.4 (SAS Institute Inc., Cary, NC). The milk fat TAG composition analyzed by GC-FID and SFC were assessed according to model 4.1:

$$Y_{ijkl} = \mu + \beta \cdot \mu_l + block_i + Protein_j + Fat_k + (Protein \times Fat)_{jk} + \varepsilon_{ijkl}, \quad \text{(Model 4.1)}$$

where Y_{ijkl} are individual observations, μ is the overall mean, β is the control period covariate parameter, μ_l is the observed trait in the control period of cow l, $block_i$ is the random block effect (i= 1 to 14), $Protein_j$ is the fixed protein effect (j= 1 to 2), Fat_k is the fixed fat effect (k= 1 to 2), $(Protein \times Fat)_{jk}$ is the interaction between fixed Protein and Fat effects, and ε_{ijkl} is the residual random error term.

The TAG composition analyzed by MALDI-TOF-MS was assessed according to model 4.2 that excluded the control period covariate parameter:

$$Y_{ik} = \mu + block_i + Protein_j + Fat_k + (Protein \times Fat)_{jk} + \varepsilon_{ijkl}, \quad \text{(Model 4.2)}$$

where Y_{ik} are individual observations, μ is the overall mean, $block_i$ is the random block effect (i= 1 to 14), $Protein_j$ is the fixed protein effect (j= 1 to 2), Fat_k is the fixed fat effect (k=1 to 2), $(Protein \times Fat)_{jk}$ is the interaction between fixed Protein and Fat effects, and ε_{ik} is the residual random error term.

The FA positional distribution in TAG was assessed according to model 4.3 that excluded the control period covariate parameter and fixed protein effect:

$$Y_{ik} = \mu + block_i + Fat_k + \varepsilon_{ik}, \quad \text{(Model 4.3)}$$

where Y_{ik} are individual observations, μ is the overall mean, $block_i$ is the random block effect (i= 1 to 14), Fat_k is the fixed fat effect (k= 1 to 2) and ε_{ik} is the residual random error term.

When a protein × fat interaction was detected at $P \leq 0.050$, the Tukey-Kramer method was used to perform multiple comparison between treatment means for the TAG composition analyzed by GC-FID and MALDI-TOF-MS and the SFC. Differences were considered

significant at $P<0.050$. Pearson correlation analysis was performed between the FA and TAG composition results measured by GC-FID using R version 3.6.1 (2019). One sample t-test was performed to determine the FA stereolocation preference comparing the interpositional distribution (mol %) with the hypothetical proportions at the *sn*-2 (33.3%) and *sn*-1(3) (66.7%) positions in the TAG structures.

4.3. Results and Discussion

4.3.1. Variation in TAG composition

Supplementation of palm fat, a fat source mainly composed of C16:0 and C18:0 FA, is commonly used to increase the energy density of dairy cattle diets in support of milk production (Ashes et al., 1997; Nichols et al., 2018). Consumption of palm fat increased arterial plasma concentrations of LCFA (both nonesterified FA and TAG), and increased mammary gland uptake of LCFA (Nichols et al., 2019). Our results show that milk TAG composition as analyzed by GC-FID was affected independently by supplementation with protein and fat, with a protein × fat interaction affecting only TAG CN42 ($P=0.049$; **Table 4.1**), which increased in response to protein supplementation but only in the absence of fat supplementation. The relative concentrations of 11 TAG groups were affected by fat supplementation, whereas only 4 TAG groups were affected by protein supplementation. In response to fat, the relative concentrations of all LMW TAG (CN26–CN34) and the MMW TAG CN40, CN44 and CN46 decreased ($P\leq0.023$) and the concentrations of CN38 and the HMW TAG CN50 and CN52 increased ($P\leq0.011$; Table 4.1). Our results are in line with previous studies showing that palm FA supplementation, which is rich in C16:0, leads to decreased concentrations of LMW and MMW TAG and increased concentrations of HMW TAG (Banks et al., 1989).

Similar findings were observed for the LMW TAG species as analysed by both GC-FID and MALDI-TOF-MS (**Figure 4.1**; **Supplemental Table S4.1**). However, opposite to the GC-FID, the MALDI-TOF-MS results showed more significant protein × fat interactions for most MMW and HMW TAG species ($P<0.049$; Supplemental Table S4.1). Only in the absence of fat supplementation, protein supplementation increased the abundance of most MMW TAG species and decreased the abundance of most HMW TAG species (Supplemental Table S4.1). Looking closely at the differences between treatments among the LMW, MMW, and HMW TAG groups according to their TAG species saturation degree, similar protein × fat interactions effects were observed for some TAG species. In the absence of fat supplementation, protein supplementation increased the abundance of monounsaturated LMW and polyunsaturated MMW TAG species and decreased the abundance of saturated and monounsaturated HMW TAG species (Figure 4.1; Supplemental Table S4.1). These interactions that were only found by MALDI-TOF-MS may be explained by the fact that this technique differentiates between saturation degrees of TAG species.

Table 4.1. Milk fat triacylglycerol (TAG) composition (%, wt/wt) of lactating dairy cows supplemented with protein and fat as analyzed by gas chromatography (GC) - flame ionization detector (FID).

		Treatment[1]					*P* value		
Item	TAG group	LP/LF	HP/LF	LP/HF	HP/HF	SEM	Protein	Fat	Protein × Fat
Cholesterol		0.40	0.38	0.43	0.33	0.056	0.200	0.901	0.396
TAG									
CN26	LMW	0.36	0.37	0.34	0.32	0.017	0.610	0.023	0.272
CN28	LMW	0.65	0.70	0.55	0.55	0.024	0.280	<0.001	0.397
CN30	LMW	1.21	1.30	0.98	1.02	0.036	0.069	<0.001	0.452
CN32	LMW	2.41	2.59	1.90	2.02	0.066	0.029	<0.001	0.633
CN34	LMW	5.68	5.77	4.78	5.05	0.119	0.077	<0.001	0.382
CN36	LMW	11.11	10.95	10.56	10.90	0.193	0.546	0.055	0.110
CN38	MMW	12.80	12.62	13.01	13.20	0.159	0.975	0.011	0.221
CN40	MMW	10.06	10.31	9.75	9.83	0.113	0.148	0.002	0.452
CN42	MMW	7.41[a]	7.85[b]	6.73[c]	6.77[c]	0.104	0.013	<0.001	0.049
CN44	MMW	6.90	7.25	6.10	6.33	0.112	0.007	<0.001	0.540
CN46	MMW	7.50	7.53	6.81	6.95	0.118	0.484	<0.001	0.644
CN48	MMW	8.87	8.59	8.96	8.86	0.129	0.154	0.171	0.471
CN50	HMW	10.50	9.74	11.78	11.44	0.177	0.004	<0.001	0.229
CN52	HMW	8.99	8.52	11.14	10.62	0.258	0.036	<0.001	0.908
CN54	HMW	5.45	5.45	6.12	5.65	0.278	0.304	0.065	0.311
TAG groups									
LMW		21.43	21.76	19.18	19.75	0.42	0.217	<0.001	0.734
MMW		53.59	54.37	51.24	52.00	0.41	0.034	<0.001	0.971
HMW		24.76	23.78	28.94	27.72	0.64	0.050	<0.001	0.822

[1] LP/LF: low protein/low fat; HP/LF: high protein/low fat; LP/HF: low protein/high fat; HP/HF: high protein/high fat. High protein supplementation consisted of a 50:50 mixture of rumen-protected soybean meal and rapeseed meal. High fat supplementation consisted of rumen-inert hydrogenated palm fatty acids. For all treatments n=14.

[a-c] Different superscripts in the same row indicate significant protein × fat interaction difference (*P*<0.050 between treatments.

Variations in TAG composition in response to fat supplementation as shown by GC-FID and MALDI-TOF-MS are related to the changes in milk FA composition that were previously reported by Nichols et al. (2018), where fat supplementation increased the concentrations of C16:0 and C18:0, but also decreased the concentrations of *de novo* synthesized FA (C6:0 to C14:0) and polyunsaturated FA (PUFA). Gresti et al. (1993) and Liu et al. (2020) identified that LMW and MMW TAG species were mainly composed of C6:0 to C16:0 and C18:1 FA. Using Pearson correlation analysis, we identified positive correlations (r>0.60; *P*<0.050) between *de novo* FA C8:0 to C12:0, C14:0, and C15:0 and LMW TAG CN28–CN34 and MMW TAG CN42–CN46. In contrast, C16:0 and C18:1*cis*9 were negatively correlated (r<-0.28; *P*<0.050) with most of these TAG (**Supplemental Table S4.2**). Considering the results of this correlation analysis, and the fact that LMW and MMW TAG species are mainly composed of *de novo* synthesized FA, it is likely that the decreased concentrations of *de novo* FA in response to fat supplementation lowered their availability

for esterification in the TAG structures and thus decreased the formation of most LMW and MMW TAG species (Table 4.1; Figure 4.1; Supplemental Table S4.1). The HMW TAG species that increased in response to fat supplementation (Table 4.1; Figure 4.1; Supplemental Table 4.1), were previously identified to be mainly composed of C14:0, C16:0, C18:0, and C18:1 FA (Gresti et al., 1993; Liu et al., 2020). This suggests that the higher availability of particularly C16:0 and C18:0 in response to fat supplementation contributed significantly to the formation of most HMW TAG species (saturated, mono-, and polyunsaturated) in the mammary gland. This is supported by the positive correlations ($r>0.50$; $P<0.050$) between C18:0 and C18:1*cis*9, and the HMW TAG CN52 and CN54 (Supplemental Table S4.2).

Protein supplementation in this study increased the concentration of *de novo* FA and PUFA in milk (Nichols et al., 2018). These authors suggested that *de novo* FA synthesis was supported by protein supplementation through mammary cell metabolism of AA into α-ketoacids to produce acetyl-CoA, which is a precursor for the formation of *de novo* FA. The increased concentrations of *de novo* FA and PUFA in response to protein may have enhanced the formation of most monounsaturated LMW and polyunsaturated MMW TAG species, only in the absence of fat supplementation (Supplemental Table S4.1). Nichols et al. (2018) also reported that protein supplementation, in the absence of fat supplementation, tended to decrease the concentration of C16:0 which in turn could have led to decreased formation of saturated and monounsaturated HMW TAG species (Supplemental Table S4.1; Gresti et al., 1993; Liu et al., 2020).

Taken together, our results show that hydrogenated palm fat supplementation into dairy cattle rations had a greater effect on milk fat TAG composition than protein supplementation. Fat supplementation reduced most saturated, mono-, and polyunsaturated LMW and MMW TAG species, and increased most saturated, mono-, and polyunsaturated HMW TAG species in milk fat. In addition, protein supplementation influenced milk fat TAG composition, but only in the absence of fat supplementation.

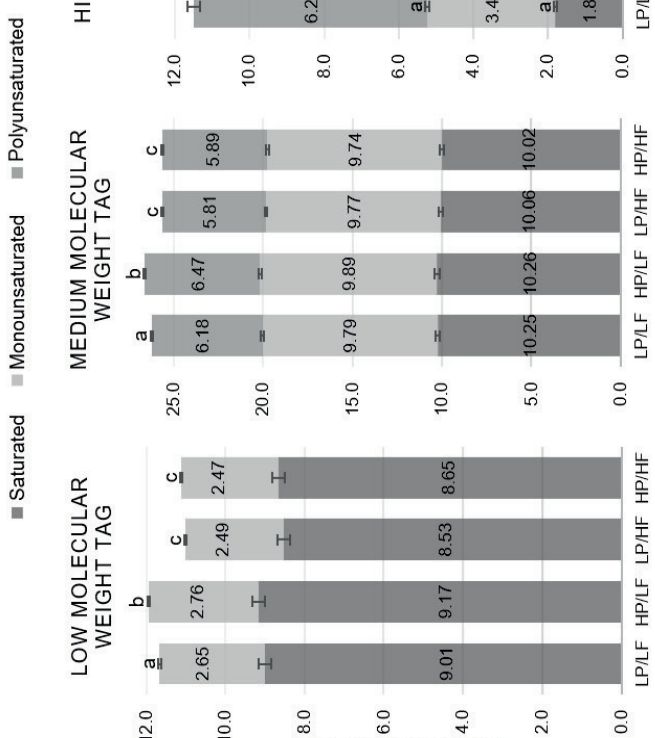


Figure 4.1. Low, medium, and high molecular weight triacylglycerol (TAG) species composition (relative intensities, %) of milk fat from lactating dairy cows supplemented with protein and fat as analyzed by MALDI-TOF-MS. Different letters (a, b, and c) in the columns indicate significant protein × fat interaction differences ($P<0.050$; Tukey-Kramer post hoc test) between treatments for the saturated, mono-, and polyunsaturated species in each TAG group. The *P* values for protein, fat and protein × fat interaction are presented in Table S4.1. LP/LF: low protein/low fat; HP/LF: high protein/low fat; LP/HF: low protein/high fat; HP/HF: high protein/high fat. High protein supplementation consisted of a 50:50 mixture of rumen-protected soybean meal and rapeseed meal. High fat supplementation consisted of rumen-inert hydrogenated palm fatty acids. For all treatments n=14.

4.3.2. Variation in positional distribution of FA in the TAG

In addition to variation in the TAG composition in response to fat and protein supplementation, changes in the TAG structure may occur. High content of C16:0 in milk fat has been reported to influence the proportions of this FA at the *sn*-2 position in TAG structures (Tzompa-Sosa et al., 2014). Therefore, we analyzed whether fat supplementation would have an effect on the concentrations of FA at the *sn*-2 or *sn*-1(3) positions in TAG structures. Since fat supplementation influenced TAG composition to a greater extent than protein supplementation in this study, positional distribution analysis was performed only for diets with and without fat supplementation, in both cases without protein supplementation. Moreover, based on the fact that the major FA in milk fat will have the largest effect on the physical properties of milk fat, the discussion will mainly focus on the variation in these FA in milk fat inter- and intrapositional distributions.

Using the interpositional distribution, the stereolocation preference of a FA over the three *sn*-positions in the TAG structure can be determined. If a FA would be positioned randomly across the *sn*-2 and *sn*-1(3) positions in the TAG structure, *sn*-2 would hold 33.3% and *sn*-1(3) (2 of the 3 positions) would hold 66.7% of that FA. Therefore, distribution of a FA above 33.3% at *sn*-2 or above 66.7% at *sn*-1(3) indicates that the enzymes responsible for the esterification of FA at these positions have a preference for that particular FA (**Supplementary Table S4.3**). Based on a one sample t-test performed between the obtained proportion for each treatment and the random hypothetical proportions, the esterification preference of a FA at both positions over the TAG structure was determined (Supplemental Table S4.3). Regardless of fat supplementation, our results show a FA esterification preference at the *sn*-1(3) positions of C4:0 to C10:0, C18:0, C18:1*cis*9, C18:1*cis*11 and C18:2*cis*9,*trans*11 (**CLA**) (P<0.050). On the other hand, the FA that were preferentially esterified at the *sn*-2 position were C12:0, C14:0, C14:1*cis*9, C15:0, C16:0, C16:1*cis*9 and C17:0 (P<0.050). These stereolocation preferences of the major FA are in line with previous studies (Jensen, 2002; Blasi et al., 2008; Tzompa-Sosa et al., 2014). Interestingly, no differences (P>0.050) were identified in the proportions of most FA (except for C17:0*anteiso*, C17:0 and ALA) over the TAG structure at both *sn*-2 and *sn*-1(3) positions when comparing the diets with and without fat supplementation (**Table 4.2**), indicating that the stereolocation of most FA did not change. Tzompa-Sosa et al. (2014) identified significant variations in the FA proportions at the *sn*-2 position of C14:0, C16:0, C18:1*cis*9 and SLCFA in the TAG structure that varied with changes in C16:0 content in milk fat. Our study may not have found such an effect due to the smaller variation in C16:0 compared to the study by Tzompa-Sosa et al. (2014).

Table 4.2. Interpositional distribution (mol %) of fatty acids (FA) in milk fat triacylglycerol structures of lactating dairy cows supplemented with fat.

	sn-2				*sn*-1(3)			
	Treatment[1]		SEM	*P* value[2]	Treatment[1]		SEM	*P* value[2]
FA	LP/LF	LP/HF			LP/LF	LP/HF		
C4:0	1.15	1.24	0.089	0.432	98.85	98.76	0.089	0.432
C6:0	4.75	5.01	0.231	0.388	95.25	94.99	0.231	0.388
C8:0	25.95	26.38	0.945	0.721	74.05	73.62	0.945	0.721
C10:0	28.71	29.61	1.113	0.561	71.29	70.39	1.113	0.561
C11:0	28.35	29.85	1.228	0.390	71.65	70.15	1.228	0.390
C12:0	44.20	46.98	1.624	0.231	55.80	53.02	1.624	0.231
C14:0*iso*	33.72	37.04	2.479	0.363	66.28	62.96	2.479	0.363
C14:0	56.99	58.18	0.851	0.268	43.01	41.82	0.851	0.268
C14:1*cis*9	44.61	46.23	1.409	0.422	55.39	53.77	1.409	0.422
C15:0*iso*	48.01	47.25	1.478	0.683	51.99	52.75	1.478	0.683
C15:0*anteiso*	59.50	58.18	1.403	0.416	40.50	41.82	1.403	0.416
C15:0	43.14	44.50	0.956	0.278	56.86	55.50	0.956	0.278
C16:0 iso	44.70	45.49	1.285	0.672	55.30	54.51	1.285	0.672
C16:0	43.15	44.26	0.727	0.302	56.85	55.74	0.727	0.302
C16:1*trans*9	5.92	5.74	0.313	0.697	94.08	94.26	0.313	0.697
C17:0*anteiso*	27.86	25.54	0.584	0.013	72.14	74.46	0.584	0.013
C16:1*cis*9	53.84	54.78	0.566	0.177	46.17	45.22	0.566	0.177
C17:0	35.97	38.38	0.685	0.029	64.03	61.62	0.685	0.029
C17:1*cis*9	57.44	54.96	2.105	0.372	42.56	45.04	2.105	0.372
C18:0	19.84	18.93	0.747	0.382	80.16	81.07	0.747	0.382
C18:1*cis*9	31.61	30.27	0.962	0.300	68.39	69.73	0.962	0.300
C18:1*cis*11	11.12	10.20	0.562	0.270	88.88	89.80	0.562	0.270
C18:2*cis*9,12 (LA)	34.07	33.98	1.088	0.957	65.93	66.02	1.088	0.957
C18:2*cis*9,*trans*11 (CLA)	14.90	12.85	1.067	0.200	85.10	87.15	1.067	0.200
C18:3*cis*6,9,12 (GLA)	6.88	7.08	0.485	0.749	93.12	92.92	0.485	0.749
C18:3*cis*9,12,15 (ALA)	34.03	36.15	0.620	0.017	65.97	63.85	0.620	0.017
C20:0	36.06	40.88	2.987	0.276	63.94	59.12	2.987	0.276
C20:3*cis*8,11,14 (DGLA)	36.55	34.17	2.268	0.433	63.45	65.83	2.268	0.433
C20:4*cis*5,8,11,14 (AA)	35.69	31.74	2.039	0.197	64.31	68.26	2.039	0.197
C22:0	18.39	16.74	0.757	0.149	81.61	83.26	0.757	0.149
C24:0	13.67	13.13	1.005	0.683	86.33	86.87	1.005	0.683
C20:5*cis*5,8,11,14,17 (EPA)	19.28	20.76	1.539	0.403	80.72	79.24	1.539	0.403

[1] The treatment refers to the two diets assigned to the lactating dairy cows in the experimental period: low protein/low fat (LP/LF) and low protein/high fat (LP/HF). Fourteen cows were considered for each treatment.

[2] The *P* values correspond to the effect of fat on the FA positional distribution at the *sn*-2 and *sn*-1(3) positions.

Moving on to the intrapositional distribution results, regardless of fat supplementation, C14:0, C16:0 and C18:1*cis*9 had the highest relative concentrations at the *sn*-2 position in the TAG structure, and C4:0, C16:0 and C18:1*cis*9 had the highest concentrations at the *sn*-1(3) positions (**Table 4.3**). These results are in accordance with previous studies (Blasi et al., 2008; Tzompa-Sosa et al., 2014; Watanabe et al., 2015). At the *sn*-2 position, most FA were affected in response to fat supplementation with the exceptions of C4:0, C6:0, C14:1*cis*9, C16:1*cis*9, C18:1*cis*9, C18:1*cis*11 and some PUFA (Table 4.3). Among the FA

at the *sn*-2 position that were affected by this, only C16:0 and C18:0 increased (P<0.003 and P<0.012, respectively) whereas all the other FA decreased in response to fat supplementation. Moreover, most FA at the *sn*-1(3) positions also showed changes upon fat supplementation with the exceptions of a few FA, including the major FA C4:0, C14:0, C16:0, C18:1*cis*9, C18:2*cis*9,12 (LA) and CLA (Table 4.3). Among the FA that changed at the *sn*-1(3) positions, only C18:0 and C18:1*cis*9 increased (P≤0.002), whereas the other FA all decreased in response to fat supplementation (Table 4.3). It appears that the increased concentrations of C16:0, C18:0, and C18:1*cis*9 in the TAG, likely as a result of their increased concentration in the diet and subsequent uptake by the mammary gland, increased the abundance of C16:0 only at *sn*-2, C18:1*cis*9 only at *sn*-1(3), and C18:0 at both the *sn*-2 and *sn*-1(3) positions of the TAG structures.

As being the most abundant FA in milk fat, the stereolocation of C16:0 at the *sn*-2 position in the TAG structure is important for fat digestion and absorption in infants (Innis, 2011; Yaron et al., 2013). Greater abundance of C16:0 at the *sn*-2 position in TAG improves absorption of free FA and calcium in infants (Innis, 2011). In contrast, hydrolysis of TAG that contain high concentrations of SLCFA, such as C16:0 and C18:0, at the *sn*-1(3) positions results in the release of free FA that bind with calcium and other dietary minerals, resulting in indigestible calcium complexes (Small, 1991; Ramírez et al., 2001; Mu and Høy, 2004). Formation of these complexes decreases fat and mineral absorption and causes hard stool and constipation in infants (Kennedy et al., 1999; Mu and Høy, 2004; Yaron et al., 2013). In addition, several studies on fat digestion in infants have reported increased absorption of fat and minerals when most of the C18:1*cis*9 was esterified at the *sn*-1(3) positions (Mu and Høy, 2004; Innis, 2011). Our results show that supplementing dairy cattle diets with hydrogenated palm fatty acids results in milk TAG structures richer in C16:0 at *sn*-2, C18:1*cis*9 at *sn*-1(3), and C18:0 at *sn*-2 and *sn*-1(3) positions. Triacylglycerol structures in milk produced by cattle consuming high levels of palm fatty acids may thus enhance the absorption of FA and minerals, due to the increased abundance of C16:0 at the *sn*-2 and C18:1*cis*9 at the *sn*-1(3) positions. However, the high concentrations of C18:0 at the *sn*-1(3) may instead decrease the absorption of FA and minerals, hindering the beneficial effect caused by C16:0 at the *sn*-2 and C18:1*cis*9 at the *sn*-1(3) positions. All in all, these findings may be of interest for developers of infant formula or other specialty dairy products, considering that by changing the concentration of specific FA in the cows' diet, the concentrations of these FA at the *sn*-2 and *sn*-1(3) positions in the TAG structure can be changed.

Table 4.3. Intrapositional distribution (mol %) of fatty acids (FA) in milk fat triacylglycerol structures of lactating dairy cows supplemented with fat.

	sn-2				*sn*-1(3)			
	Treatment[1]		SEM	*P* value[2]	Treatment[1]		SEM	*P* value[2]
FA	LP/LF	LP/HF			LP/LF	LP/HF		
C4:0	0.36	0.39	0.027	0.315	15.29	15.57	0.220	0.371
C6:0	0.71	0.70	0.033	0.850	7.05	6.61	0.095	0.004
C8:0	1.79	1.59	0.062	0.011	2.55	2.23	0.058	0.002
C10:0	3.60	3.02	0.132	0.005	4.45	3.62	0.132	0.001
C11:0	0.44	0.37	0.018	0.024	0.55	0.45	0.022	0.006
C12:0	5.41	4.54	0.167	0.003	3.41	2.61	0.130	<0.001
C14:0*iso*	0.20	0.16	0.007	0.002	0.21	0.15	0.014	0.010
C14:0	20.29	17.71	0.414	<0.001	7.65	6.39	0.197	<0.001
C14:1*cis*9	1.53	1.38	0.073	0.162	0.96	0.84	0.063	0.199
C15:0*iso*	0.38	0.31	0.010	<0.001	0.21	0.18	0.010	0.054
C15:0*anteiso*	0.78	0.63	0.020	<0.001	0.26	0.23	0.013	0.092
C15:0	1.36	1.14	0.033	0.001	0.90	0.71	0.024	<0.001
C16:0*iso*	0.33	0.28	0.010	0.001	0.21	0.17	0.010	0.031
C16:0	36.22	40.29	0.980	0.003	23.92	25.33	0.663	0.156
C16:1*trans*9	0.07	0.06	0.003	0.043	0.55	0.47	0.021	0.012
C17:0*anteiso*	0.46	0.40	0.011	0.001	0.60	0.58	0.015	0.292
C16:1*cis*9	2.19	2.40	0.079	0.075	0.94	0.99	0.039	0.249
C17:0	0.48	0.43	0.015	0.001	0.43	0.34	0.010	<0.001
C17:1*cis*9	0.31	0.26	0.013	0.001	0.11	0.11	0.007	0.652
C18:0	4.38	4.96	0.219	0.012	8.85	10.70	0.340	0.002
C18:1*cis*9	14.04	14.89	0.680	0.330	15.13	16.98	0.371	<0.001
C18:1*cis*11	0.16	0.14	0.012	0.165	0.63	0.61	0.033	0.667
C18:2*cis*9,12 (LA)	1.66	1.47	0.059	0.035	1.60	1.46	0.063	0.122
C18:2*cis*9,*trans*11 (CLA)	0.28	0.21	0.015	0.003	0.84	0.74	0.057	0.219
C18:3*cis*6,9,12 (GLA)	0.04	0.04	0.002	0.821	0.24	0.24	0.008	0.891
C18:3*cis*9,12,15 (ALA)	0.39	0.33	0.012	0.005	0.38	0.29	0.012	<0.001
C20:0	0.030	0.027	0.001	0.006	0.029	0.022	0.003	0.109
C20:3*cis*8,11,14 (DGLA)	0.05	0.05	0.004	0.947	0.05	0.06	0.007	0.472
C20:4*cis*5,8,11,14 (AA)	0.03	0.02	0.001	0.061	0.02	0.02	0.002	0.583
C20:5*cis*5,8,11,14,17 (EPA)	0.020	0.019	0.001	0.555	0.042	0.038	0.002	0.025
C22:0	0.029	0.022	0.001	<0.001	0.064	0.054	0.002	0.004
C24:0	0.01	0.01	0.001	0.016	0.03	0.02	0.001	0.005

[1] LP/LF: low protein/low fat; LP/HF: low protein/high fat. High fat supplementation consisted of rumen inert hydrogenated palm fatty acids. Fourteen cows were considered for each treatment.

[2] The *P* values correspond to the effect of fat on the FA positional distribution at the *sn*-2 and *sn*-1(3) positions.

4.3.3. Variation in SFC

Variations in TAG composition are known to influence milk SFC. Hence, due to the large variations in TAG composition, for example in response to fat supplementation in this study, alterations in milk SFC were expected. Milk SFC increased at 20, 25, and 30°C in response to fat supplementation ($P<0.001$), whereas only the milk SFC measured at 30°C decreased in response to protein supplementation ($P<0.041$). No protein × fat interactions on the SFC were observed at any of the temperatures (**Table 4.4**). These effects on SFC may be

explained by the changes in TAG composition observed in response to fat and protein supplementation. As shown by the TAG composition, fat supplementation led to an increased formation of the HMW TAG CN50 and CN52. The concentrations of HMW TAG CN50 correlated positively with C16:0 content (r=0.59) and the concentrations of HMW TAG CN52 correlated positively with C18:0 content (r=0.61) (Supplemental Table S4.2). These FA both have higher melting points than the shorter FA, thus leading to higher melting points of the HMW TAG CN50 and CN52 (Knothe and Dunn, 2009). Therefore, the higher concentration of HMW TAG, specially saturated HMW TAG species, may explain the increased SFC at 20, 25, and 30°C in response to fat supplementation. This is supported by Smiddy et al. (2012) who showed that concentrations of HMW CN50, CN52, and CN54 determined the variations in the melting profiles of milk fat of several animal species, including cow's milk.

Table 4.4. Milk solid fat content (%) at 0, 10, 20, 25, 30, and 40°C of lactating dairy cows supplemented with protein and fat.

	Treatment[1]					*P* value		
Temperature (°C)	LP/LF	HP/LF	LP/HF	HP/HF	SEM	Protein	Fat	Protein × Fat
0	61.2	60.0	62.2	60.7	1.170	0.249	0.478	0.900
10	50.3	48.5	52.7	51.0	1.285	0.193	0.068	0.942
20	21.2	20.0	26.0	24.1	0.950	0.101	<0.001	0.733
25	12.3	11.3	14.8	13.9	0.568	0.099	<0.001	0.950
30	6.6	5.8	8.8	8.1	0.356	0.041	<0.001	0.903
40	1.3	1.3	1.2	1.3	0.054	0.528	0.485	0.760

[1] LP/LF: low protein/low fat; HP/LF: high protein/low fat; LP/HF: low protein/high fat; HP/HF: high protein/high fat. High protein supplementation consisted of a 50:50 mixture of rumen-protected soybean meal and rapeseed meal. High fat supplementation consisted of rumen-inert hydrogenated palm fatty acids. For all treatments n=14.

Regarding the effect of protein supplementation on the changes in milk SFC, we hypothesize that the decreased milk SFC at 30°C (P<0.041) is related to the increased concentrations of PUFA in response to protein supplementation (Nichols et al., 2018). The PUFA have a lower melting point due to the large number of double bonds in their structures, thus their esterification in TAG species will lead to a decrease in the resulting TAG's melting points. As discussed in the previous sections, protein supplementation enhanced the formation of total diunsaturated TAG species and MMW polyunsaturated TAG species (Figure 4.1; Supplemental Table S4.1), which may have contributed to the decreased milk SFC identified at 30°C. Similar to our study, previous studies that assessed the effect of feeding UFA to dairy cows on milk fat TAG composition and SFC showed that when the dietary content of PUFA increased, SFC measured at 4 to 30°C decreased (Precht and Frede, 1994; Couvreur et al., 2006; Smet et al., 2010).

Increased milk SFC at 20, 25, and 30°C may have negative effects on milk fat processing and its application as an ingredient in dairy based food products. For example, there may be implications for the processing conditions required for milk fat fractionation to obtain stearin (SFC ≥55%, melting temperature ≥20°C) and olein (SFC <55%, melting temperature ≤19°C), fractions used in several food applications, which may require adjustments to processing conditions (Deffense, 1993; Mohan et al., 2020). Moreover, milk SFC at 20, 25, and 30°C is known to determine texture and mouthfeel properties of milk fat-containing food products, as well as their table-top quality properties (*e.g.* butter and margarine structure stability and resistance to oil exudation; Deffense, 1993; Mohan et al., 2020). Based on these results, the impact of feeding hydrogenated palm fatty acids to dairy cows on milk fat properties and how this affects quality and consumer-acceptability of high-fat dairy products should be considered by dairy nutritionists, processors, and product manufacturers.

4.4. Conclusions

This study investigated the effect of supplementing the diet of dairy cattle with protein from rumen protected sources and fat from hydrogenated palm fatty acids on milk fat TAG composition and structure, and milk SFC. Fat supplementation modified these parameters more than protein supplementation. In response to fat supplementation, the concentrations of LMW and MMW TAG decreased, and the concentrations of HMW TAG increased. Fat supplementation also increased the formation of TAG with greater proportions of C16:0 and C18:0 at the *sn*-2 position and C18:0 and C18:1*cis*9 at the *sn*-1(3) positions in their structures. Fat supplementation increased milk SFC at 20, 25, and 30°C, correlating with the increased abundance of HMW TAG species probably containing more C16:0 and C18:0. These changes in TAG composition and structure, and milk SFC may impact nutritional and physiochemical quality of dairy products originating from the milk fat produced by cattle consuming rations supplemented with hydrogenated palm fatty acids.

4.5. Acknowledgements

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Supplementary Information

Table S4.1. Milk fat triacylglycerol (TAG) species composition (relative intensities, %) from lactating dairy cows supplemented with protein and fat as analyzed by MALDI-TOF-MS.

TAG Annotation[2]	Mass-to-charge ratio (*m/z*)[3]	TAG Group	Treatment[1]				SEM	*P* value		
			LP/LF	HP/LF	LP/HF	HP/HF		Protein	Fat	Protein × Fat
CN26:0	521.45	LMW	0.440[a]	0.487[b]	0.394[c]	0.407[c]	0.013	<0.001	<0.001	0.035
CN28:1	547.46	LMW	0.235	0.256	0.208	0.211	0.006	0.011	<0.001	0.054
CN28:0	549.48	LMW	0.701	0.764	0.620	0.647	0.020	<0.001	<0.001	0.088
CN29:1	561.51	LMW	0.133[c]	0.140	0.126	0.131	0.005	0.072	0.013	0.735
CN29:0	563.51	LMW	0.312	0.322	0.276	0.296	0.011	0.008	<0.001	0.327
CN30:0	577.51	LMW	0.960	1.030[b]	0.872	0.930	0.032	<0.001	<0.001	0.600
CN32:0	605.53	LMW	1.234	1.295	1.1171	1.137[c]	0.028	<0.001	<0.001	0.075
CN33:0	619.55	LMW	0.428	0.436	0.371	0.388	0.013	0.010	<0.001	0.384
CN34:1	631.55	LMW	0.765[a]	0.814[b]	0.700[c]	0.693[c]	0.011	0.005	<0.001	<0.001
CN34:0	633.56	LMW	1.884	1.875	1.794	1.800	0.027	0.928	<0.001	0.602
CN35:1	645.57	LMW	0.266	0.282	0.239	0.250	0.007	<0.001	<0.001	0.470
CN35:0	647.58	LMW	0.637[a]	0.624[a]	0.562[b]	0.582[b]	0.012	0.622	<0.001	0.009
CN36:1	659.58	LMW	1.250[a]	1.273[a]	1.217[b]	1.188[c]	0.012	0.709	<0.001	0.003
CN36:0	661.60	LMW	2.413	2.328	2.523	2.457	0.036	0.006	<0.001	0.722
CN37:1	673.59	MMW	0.427	0.427	0.379	0.379	0.005	0.966	<0.001	0.992
CN37:0	675.60	MMW	0.688	0.670	0.621	0.627	0.011	0.418	<0.001	0.077
CN38:3	683.58	MMW	0.214[c]	0.219	0.199	0.206[c]	0.003	0.053	<0.001	0.698
CN38:2	685.59	MMW	0.594	0.622	0.590	0.598	0.009	0.006	0.029	0.108
CN38:1	687.61	MMW	1.798	1.775	1.947	1.883	0.020	0.003	<0.001	0.157
CN38:0	689.63	MMW	1.984	1.949	2.123	2.043	0.028	0.006	<0.001	0.261
CN39:2	699.59	MMW	0.191[a]	0.199[a]	0.168[b]	0.163[b]	0.006	0.620	<0.001	0.046
CN39:1	701.62	MMW	0.450	0.450	0.405	0.408	0.006	0.728	<0.001	0.721
CN39:0	703.62	MMW	0.628[a]	0.607[a]	0.563[b]	0.577[b]	0.013	0.688	<0.001	0.031
CN40:3	711.61	MMW	0.302	0.324	0.289	0.295	0.008	0.005	<0.001	0.106
CN40:2	713.63	MMW	0.782[a]	0.806[b]	0.800[ab]	0.780[a]	0.015	0.814	0.700	0.021
CN40:1	715.64	MMW	1.374[a]	1.397[a]	1.441[b]	1.413[b]	0.011	0.772	<0.001	0.007
CN40:0	717.66	MMW	1.414[ab]	1.434[a]	1.438[a]	1.395[b]	0.020	0.310	0.520	0.005
CN41:2	727.61	MMW	0.194	0.207	0.174	0.176	0.007	0.014	<0.001	0.080
CN41:1	729.64	MMW	0.374	0.383	0.329	0.333	0.007	0.085	<0.001	0.374
CN41:0	731.65	MMW	0.521	0.519	0.462	0.474	0.014	0.465	<0.001	0.224
CN42:5	735.58	MMW	0.134	0.130	0.116	0.124	0.007	0.599	0.007	0.191
CN42:3	739.64	MMW	0.208	0.225	0.184	0.190	0.004	0.001	<.0001	0.125
CN42:2	741.66	MMW	0.525[a]	0.565[b]	0.502[c]	0.501[c]	0.007	0.001	<0.001	0.001
CN42:1	743.67	MMW	1.025[a]	1.067[b]	0.998[c]	1.000[c]	0.013	0.009	<0.001	0.012
CN42:0	745.69	MMW	1.144[a]	1.177[b]	1.128[ac]	1.116[c]	0.022	0.260	<0.001	0.017
CN43:2	755.63	MMW	0.172[a]	0.184[b]	0.150[c]	0.149[c]	0.011	0.083	<0.001	0.046
CN43:1	757.19	MMW	0.071	0.066	0.074	0.071	0.005	0.229	0.210	0.893
CN43:0	759.68	MMW	0.423	0.433	0.370	0.380	0.013	0.050	<0.001	0.996
CN44:3	767.67	MMW	0.177[a]	0.197[b]	0.154[c]	0.161[c]	0.004	<0.001	<0.001	0.035
CN44:2	769.69	MMW	0.473[a]	0.514[b]	0.443[ac]	0.458[c]	0.009	<0.001	<0.001	0.038
CN44:1	771.71	MMW	1.009	1.060	0.979	0.995	0.021	0.001	<0.001	0.077
CN44:0	773.72	MMW	0.897[a]	0.933[b]	0.892[a]	0.884[a]	0.013	0.085	0.001	0.007
CN45:2	783.66	MMW	0.176	0.192	0.151	0.155	0.013	0.014	<0.001	0.107
CN45:1	785.70	MMW	0.320	0.338	0.279	0.284	0.009	0.004	<0.001	0.094
CN45:0	787.71	MMW	0.389	0.401	0.346	0.359	0.013	0.015	<0.001	0.962
CN46:3	795.70	MMW	0.218[a]	0.242[b]	0.200[c]	0.203[c]	0.005	<0.001	<0.001	0.004
CN46:2	797.72	MMW	0.526	0.571	0.488	0.505	0.012	<0.001	<0.001	0.058
CN46:1	799.74	MMW	0.975	0.996	0.969	0.977	0.014	0.143	0.200	0.458
CN46:0	801.75	MMW	0.735	0.751	0.732	0.726	0.009	0.629	0.118	0.219

(continuation **Table S4.1.**)

TAG Annotation[2]	Mass-to-charge ratio (*m/z*)[3]	TAG Group	Treatment[1]				SEM	*P*-value		
			LP/LF	HP/LF	LP/HF	HP/HF		Protein	Fat	Protein × Fat
CN47:2	811.71	MMW	0.188^{a}	0.204^{b}	0.167^{c}	0.169^{c}	0.009	0.007	<0.001	0.023
CN47:1	813.74	MMW	0.365	0.369	0.318	0.327	0.008	0.112	<0.001	0.504
CN47:0	815.75	MMW	0.375^{a}	0.365^{a}	0.330^{b}	0.348^{c}	0.007	0.347	<0.001	0.003
CN48:6	817.58	MMW	0.233^{a}	0.176^{b}	0.190^{bc}	0.184^{bd}	0.016	0.005	0.108	0.021
CN48:3	823.74	MMW	0.228	0.251	0.209	0.218	0.006	<0.001	<0.001	0.091
CN48:2	825.76	MMW	0.599	0.620	0.598	0.614	0.011	0.026	0.651	0.813
CN48:1	827.77	MMW	1.139	1.116	1.209	1.223	0.015	0.692	<0.001	0.088
CN48:0	829.79	MMW	0.661	0.653	0.692	0.698	0.010	0.904	<0.001	0.437
CN49:2	839.76	MMW	0.230	0.230	0.203	0.210	0.004	0.310	<0.001	0.337
CN49:1	841.78	MMW	0.458^{a}	0.446^{ab}	0.442^{b}	0.453^{ab}	0.007	0.924	0.366	0.015
CN49:0	843.78	MMW	0.392^{a}	0.372^{bc}	0.364^{b}	0.388^{c}	0.007	0.688	0.277	<0.001
CN50:6	845.62	HMW	0.303^{a}	0.211^{b}	0.254^{b}	0.256^{b}	0.025	0.008	0.893	0.005
CN50:3	851.77	HMW	0.329	0.354	0.324	0.342	0.007	<0.001	0.113	0.577
CN50:2	853.79	HMW	0.810	0.808	0.856	0.879	0.016	0.303	<0.001	0.201
CN50:1	855.81	HMW	1.238^{a}	1.175^{a}	1.452^{b}	1.445^{b}	0.022	0.006	<0.001	0.025
CN50:0	857.82	HMW	0.592	0.563	0.682	0.671	0.011	0.016	<0.001	0.280
CN51:2	867.80	HMW	0.319	0.311	0.299	0.306	0.006	0.995	0.005	0.078
CN51:1	869.81	HMW	0.457^{a}	0.429^{b}	0.459^{a}	0.458^{a}	0.008	0.001	<0.001	0.002
CN51:0	871.81	HMW	0.213^{ac}	0.164^{b}	0.186^{c}	0.185^{a}	0.017	0.404	0.002	<0.001
CN52:7	871.63	HMW	0.395^{a}	0.356^{bc}	0.383^{a}	0.411^{ac}	0.008	0.026	0.792	0.032
CN52:6	873.65	HMW	0.252^{a}	0.186^{bc}	0.219^{ac}	0.217^{a}	0.020	0.014	0.940	0.016
CN52:4	877.79	HMW	0.211	0.219	0.218	0.230	0.005	0.015	0.019	0.638
CN52:3	879.81	HMW	0.444	0.461	0.484	0.505	0.013	0.011	<0.001	0.775
CN52:2	881.83	HMW	0.979	0.958	1.189	1.173	0.028	0.196	<0.001	0.879
CN52:1	883.84	HMW	0.861	0.833	1.088	1.057	0.022	0.006	<0.001	0.889
CN52:0	885.85	HMW	0.374	0.349	0.444	0.426	0.009	<0.001	<0.001	0.546
CN53:2	895.82	HMW	0.290^{a}	0.277^{b}	0.278^{ab}	0.286^{ab}	0.007	0.601	0.776	0.016
CN53:1	897.83	HMW	0.366^{a}	0.343^{b}	0.376^{a}	0.392^{c}	0.010	0.528	<0.001	0.001
CN53:0	899.83	HMW	0.284^{a}	0.258^{b}	0.291^{a}	0.315^{c}	0.008	0.801	<0.001	<0.001
CN54:6	901.81	HMW	0.167^{c}	0.160	0.176	0.176	0.005	0.482	0.010	0.460
CN54:5	903.80	HMW	0.162	0.164	0.175	0.169	0.006	0.745	0.035	0.366
CN54:4	905.83	HMW	0.227	0.242	0.243	0.242	0.009	0.216	0.160	0.123
CN54:3	907.84	HMW	0.431	0.442	0.483	0.481	0.017	0.629	<0.001	0.475
CN54:2	909.86	HMW	0.461	0.458	0.546	0.534	0.018	0.361	<0.001	0.625
CN54:1	911.87	HMW	0.333	0.327	0.387	0.380	0.010	0.246	<0.001	0.902
CN54:0	913.86	HMW	0.159	0.154	0.172	0.171	0.004	0.427	<0.001	0.437
CN55:1	925.85	HMW	0.178	0.165	0.180	0.182	0.006	0.149	0.015	0.067
CN57:10	935.65	HMW	0.132	0.135	0.156	0.135	0.012	0.210	0.106	0.098
CN59:11	961.66	HMW	0.128	0.133	0.154	0.141	0.012	0.561	0.022	0.208
CN59:10	963.68	HMW	0.106	0.115	0.118	0.107	0.006	0.795	0.652	0.049
TAG groups[4]										
LMW			11.661	11.930	11.014	11.120	0.181	0.016	<0.001	0.281
MMW			26.392^{a}	26.826^{b}	25.795^{c}	25.818^{c}	0.220	0.022	<0.001	0.035
HMW			11.509^{a}	11.001^{b}	12.551^{c}	12.515^{c}	0.246	0.004	<0.001	0.010
Saturated			21.117	21.011	20.699	20.514	0.254	0.466	<0.001	0.790
Monounsaturated			15.892	15.935	16.252	16.134	0.103	0.890	<0.001	0.320
Diunsaturated			7.534	7.770	7.636	7.668	0.093	0.036	0.874	0.185
Polyunsaturated			5.075	5.075	4.937	5.082	0.130	0.403	0.405	0.278
LMW Saturated			9.011	9.167	8.526	8.646	0.158	0.041	<0.001	0.787
LMW Monounsaturated			2.650^{a}	2.763^{b}	2.489^{c}	2.473^{c}	0.030	0.015	<0.001	0.001
MMW Saturated			10.248	10.263	10.056	10.019	0.123	0.846	<0.001	0.628
MMW Monounsaturated			9.787	9.890	9.772	9.742	0.077	0.447	0.092	0.157
MMW Polyunsaturated			6.177^{a}	6.472^{b}	5.810^{c}	5.885^{c}	0.068	<0.001	<0.001	0.018
HMW Saturated			1.804^{a}	1.680^{b}	1.972^{c}	1.992^{c}	0.032	0.015	<0.001	0.001
HMW Monounsaturated			3.432^{a}	3.271^{b}	3.943^{c}	3.912^{c}	0.066	0.002	<0.001	0.030
HMW Polyunsaturated			6.272	6.051	6.635	6.615	0.168	0.075	<0.001	0.130

[1] LP/LF: low protein/low fat; HP/LF: high protein/low fat; LP/HF: low protein/high fat; HP/HF: high protein/high fat. High protein supplementation consisted of a 50:50 mixture of rumen-protected soybean meal and rapeseed meal. High fat supplementation consisted of rumen inert hydrogenated palm FA. For all treatments n=14.

[2] The TAG annotation refers to the TAG species identified by the LIPID MAPS Online Tools library of the MALDI-TOF-MS mass spectra. The TAG annotation was described as the ratio of the total carbon number (CN) to the number of double bonds (DB).

[3] The mass-to-charge ratio (*m/z*) refers to the mass peaks detected by MALDI-TOF-MS of the TAG species identified in milk fat of the different treatments.

[4] The sum intensities of LMW (CN26:0–CN36:0), MMW (CN37:2–CN49:0) and HMW (CN50:6–CN59:10) were calculated based on all TAG species, including the identified and non-identified TAG species. The saturated, mono-, di- and polyunsaturated TAG groups and the LMW, MMW and HMW saturated, mono and polyunsaturated TAG species groups were calculated based on the sum of the TAG species tentatively identified by the LIPID MAPS Online Tools library of the MALDI-TOF-MS mass spectra.

[a-c] Different superscripts in the same row indicate significant protein × fat interaction difference (P<0.050) between treatments.

Table S4.2. Significant Pearson correlation coefficients (P<0.050) between triacylglycerols (TAG) and fatty acids (FA) in milk from lactating dairy cows supplemented with protein and fat as analyzed by gas chromatography (GC) - flame ionization detector (FID).

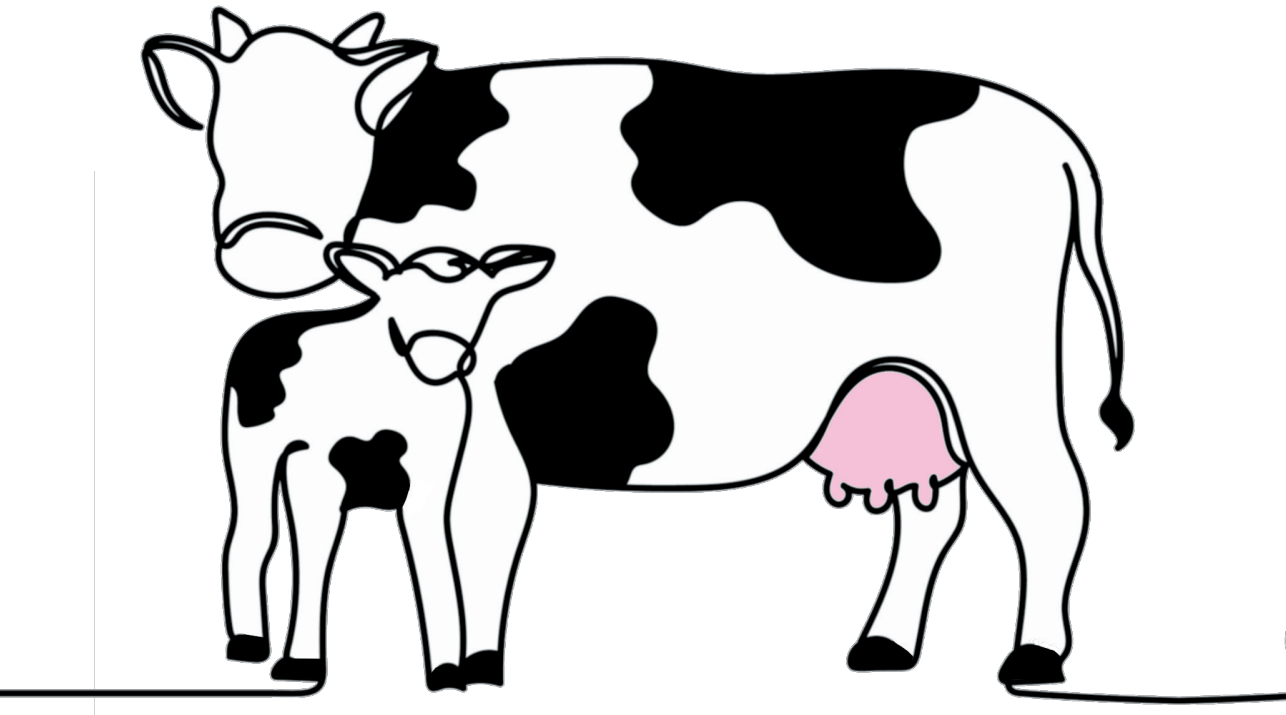
	TAG[1]														
FA	CN26	CN28	CN30	CN32	CN34	CN36	CN38	CN40	CN42	CN44	CN46	CN48	CN50	CN52	CN54
C4:0	0.35	0.32				0.65	0.85	0.50		-0.36	-0.65	-0.77	-0.31		
C6:0	0.46	0.75	0.78	0.80	0.80	0.65	0.47	0.79	0.74	0.52		-0.68	-0.90	-0.81	-0.60
C8:0	0.43	0.81	0.89	0.93	0.86	0.39		0.72	0.92	0.79	0.47	-0.47	-0.94	-0.92	-0.58
C10:0	0.36	0.74	0.85	0.91	0.83			0.58	0.96	0.90	0.64	-0.27	-0.88	-0.91	-0.56
C11:0	0.33	0.59	0.68	0.74	0.71			0.35	0.77	0.78	0.66		-0.67	-0.78	-0.58
C12:0	0.32	0.69	0.81	0.88	0.80		-0.30	0.47	0.94	0.93	0.73		-0.81	-0.89	-0.53
C14:0*iso*		0.38	0.50	0.59	0.55		-0.43		0.68	0.76	0.73		-0.51	-0.64	-0.40
C14:0	0.29	0.60	0.70	0.79	0.79		-0.29	0.36	0.86	0.87	0.74		-0.73	-0.86	-0.56
C14:1*cis*9			0.27	0.32	0.33		-0.52		0.41	0.59	0.74	0.45		-0.42	-0.34
C15:0*iso*		0.32	0.39	0.45	0.34		-0.36	0.30	0.56	0.54	0.47		-0.41	-0.46	
C15:0*ante*	0.27	0.42	0.45	0.46	0.33		-0.27	0.35	0.49	0.45	0.34		-0.41	-0.42	
C15:0		0.50	0.59	0.66	0.67	0.29		0.34	0.73	0.73	0.60		-0.61	-0.76	-0.54
C16:0*iso*				0.26			-0.28		0.43	0.44	0.42		-0.28	-0.33	
C16:0		-0.50	-0.54	-0.54	-0.28	0.41	0.35	-0.63	-0.58	-0.45		0.35	0.59	0.38	-0.27
C16:1*trans*9						-0.41	-0.51								0.27
C16:1*cis*9	-0.27	-0.39	-0.33	-0.28			-0.34	-0.44			0.38	0.62	0.31		
C17:0		0.41	0.45	0.45	0.33		-0.26	0.40	0.45	0.34			-0.44	-0.37	
C17:1*cis*9			0.29	0.33				0.42	0.39	0.32			-0.39	-0.30	
C18:0		-0.38	-0.49	-0.55	-0.65		0.36		-0.48	-0.61	-0.64		0.38	0.61	0.53
C18:1*cis*9		-0.28	-0.36	-0.44	-0.62	-0.71			-0.54	-0.55	-0.47		0.44	0.67	0.89
C18:1*cis*11		0.30	0.28			-0.29		0.42				-0.44			0.49
C18:1*cis*12	0.30	0.47	0.45	0.39				0.43				-0.44	-0.40		0.27
C18:1*cis*13		0.38	0.38	0.36				0.37				-0.32	-0.37		
C18:2*cis*9.12 (LA)		0.39	0.41	0.40	0.29			0.38	0.34			-0.32	-0.44	-0.30	
C18:2*cis*9*trans*11 (CLA)		0.37	0.42	0.44	0.33		-0.43		0.40	0.38	0.33		-0.37	-0.34	
C18:3*cis*9.12.15 (ALA)		0.43	0.50	0.50	0.36		-0.39	0.43	0.51	0.47	0.35		-0.49	-0.43	
C20:0								0.34							0.32
C20:2*cis*11.14		0.30	0.35	0.36		-0.34	-0.41	0.28	0.35	0.33	0.27		-0.32		
C22:0								0.29	0.27						
C20:3*cis*8.11.14 (DGLA)						-0.31									
C22:5cis7.10.13.16.19 (DPA)		0.39	0.42	0.37			-0.31		0.26						

[1] CN: carbon number

Table S4.3. Stereolocation preferences between the interpositional distribution (mol %) and the hypothetical proportions at the *sn*-2 and *sn*-1(3) positions in the triacylglycerol (TAG) structures.[1]

	sn-2						*sn*-1(3)					
	LP/LF			LP/HF			LP/LF			LP/HF		
FA	LP/LF	Hypothetical proportion (%)	T-Test *P* value	LP/HF	Hypothetical proportion (%)	T-Test *P* value	LP/LF	Hypothetical proportion (%)	T-Test *P* value	LP/HF	Hypothetical proportion (%)	T-Test *P* value
C4:0	1.15	33.3	<0.001	1.24	33.3	<0.001	98.85	66.7	<0.001	98.76	66.7	<0.001
C6:0	4.75	33.3	<0.001	5.01	33.3	<0.001	95.25	66.7	<0.001	94.99	66.7	<0.001
C8:0	25.95	33.3	<0.001	26.38	33.3	<0.001	74.05	66.7	<0.001	73.62	66.7	<0.001
C10:0	28.71	33.3	<0.001	29.61	33.3	0.033	71.29	66.7	<0.001	70.39	66.7	0.033
C11:0	28.35	33.3	<0.001	29.85	33.3	0.064	71.65	66.7	<0.001	70.15	66.7	0.064
C12:0	44.20	33.3	<0.001	46.98	33.3	<0.001	55.80	66.7	<0.001	53.02	66.7	<0.001
C14:0*iso*	33.72	33.3	0.842	37.04	33.3	0.217	66.28	66.7	0.842	62.96	66.7	0.217
C14:0	56.99	33.3	<0.001	58.18	33.3	<0.001	43.01	66.7	<0.001	41.82	66.7	<0.001
C14:1*cis*9	44.61	33.3	<0.001	46.23	33.3	<0.001	55.39	66.7	<0.001	53.77	66.7	<0.001
C15:0*iso*	48.01	33.3	<0.001	47.25	33.3	<0.001	51.99	66.7	<0.001	52.75	66.7	<0.001
C15:0*anteiso*	59.50	33.3	<0.001	58.18	33.3	<0.001	40.50	66.7	<0.001	41.82	66.7	<0.001
C15:0	43.14	33.3	<0.001	44.50	33.3	<0.001	56.86	66.7	<0.001	55.50	66.7	<0.001
C16:0*iso*	44.70	33.3	<0.001	45.49	33.3	<0.001	55.30	66.7	<0.001	54.51	66.7	<0.001
C16:0	43.15	33.3	<0.001	44.26	33.3	<0.001	56.85	66.7	<0.001	55.74	66.7	<0.001
C16:1*trans*9	5.92	33.3	<0.001	5.74	33.3	<0.001	94.08	66.7	<0.001	94.26	66.7	<0.001
C17:0*anteiso*	27.86	33.3	<0.001	25.54	33.3	<0.001	72.14	66.7	<0.001	74.46	66.7	<0.001
C16:1*cis*9	53.84	33.3	<0.001	54.78	33.3	<0.001	46.17	66.7	<0.001	45.22	66.7	<0.001
C17:0	35.97	33.3	<0.001	38.38	33.3	<0.001	64.03	66.7	<0.001	61.62	66.7	<0.001
C17:1*cis*9	57.44	33.3	<0.001	54.95	33.3	<0.001	42.56	66.7	<0.001	45.05	66.7	<0.001
C18:0	19.84	33.3	<0.001	18.93	33.3	<0.001	80.16	66.7	<0.001	81.07	66.7	<0.001
C18:1*cis*9	31.61	33.3	0.072	30.27	33.3	0.014	68.39	66.7	0.072	69.73	66.7	0.014
C18:1*cis*11	11.12	33.3	<0.001	10.20	33.3	<0.001	88.88	66.7	<0.001	89.80	66.7	<0.001
C18:2*cis*9,12 (LA)	34.07	33.3	0.289	33.98	33.3	0.634	65.93	66.7	0.289	66.02	66.7	0.634
C18:2*cis*9,*trans*11 (CLA)	14.90	33.3	<0.001	12.85	33.3	<0.001	85.10	66.7	<0.001	87.15	66.7	<0.001
C18:3*cis*6,9,12 (GLA)	6.88	33.3	<0.001	7.08	33.3	<0.001	93.12	66.7	<0.001	92.92	66.7	<0.001
C18:3*cis*9,12,15 (ALA)	34.03	33.3	0.231	36.15	33.3	0.001	65.97	66.7	0.231	63.85	66.7	0.001
C20:0	36.06	33.3	0.186	40.88	33.3	0.072	63.94	66.7	0.186	59.12	66.7	0.072
C20:3*cis*8,11,14 (DGLA)	36.55	33.3	0.064	34.17	33.3	0.767	63.45	66.7	0.064	65.83	66.7	0.767
C20:4*cis*5,8,11,14 (AA)	35.69	33.3	0.092	31.74	33.3	0.566	64.31	66.7	0.092	68.26	66.7	0.566
C20:5*cis*5,8,11,14,17 (EPA)	19.28	33.3	<0.001	20.76	33.3	<0.001	80.72	66.7	<0.001	79.24	66.7	<0.001
C22:0	18.39	33.3	<0.001	16.74	33.3	<0.001	81.61	66.7	<0.001	83.26	66.7	<0.001
C24:0	13.67	33.3	<0.001	13.13	33.3	<0.001	86.33	66.7	<0.001	86.87	66.7	<0.001

[1] Performed by a one sample t-test between the obtained proportion for each treatment and the random hypothetical proportions of 33.3% at the *sn*-2 position and 66.7% at the *sn*-1(3) position.



Chapter 5

Bovine triacylglycerol composition and structure differs between early and late lactation influencing milk fat solid fat content

This chapter is based on:

Pacheco-Pappenheim S.,S. Yener, R. Goselink, M.X. Quintanilla-Carvajal, H.J.F. van Valenberg and K. Hettinga. Bovine triacylglycerol composition and structure differs between early and late lactation influencing milk fat solid fat content. Submitted for publication to the International Dairy Journal.

Abstract

The aim of this study was to analyze the differences between early and late lactation in bovine milk fatty acid (**FA**) and triacylglycerol (**TAG**) composition, FA positional distribution in the TAG structure, and milk fat solid fat content (**SFC**). Two milk samples were collected from 11 cows, one in the second week of lactation (8 to 14 days in milk; **DIM**) and one in late lactation (199 to 326 DIM). The milk FA composition variation was associated with the shift from FA mobilization from body fat stores in early lactation to the increased synthesis of *de novo* FA in late lactation. Milk fat in early lactation had increased concentrations of C4:0, C18:0, C18:1*cis*9 and other monounsaturated FA, whereas late lactation milk fat had increased concentrations of *de novo* FA C10:0 to C16:0. In response to these changes in FA composition, high molecular weight (**HMW**) TAG species increased in early lactation and low and medium molecular weight (**LMW, MMW**) TAG species increased in late lactation. Except for C16:0, high FA concentrations in early and late lactation increased the FA relative concentrations at both *sn*-2 and *sn*-1(3) positions in the TAG structures. C16:0 showed opposite trends between early and late lactation, where high concentrations of C16:0 in late lactation decreased its concentrations at the *sn*-2 position and increased its concentration at the *sn*-1(3) positions. The esterification preference of FA in the TAG structures did not vary between early and late lactation. Higher concentrations of C18:0 and C18:1*cis*9 in early lactation enhanced the synthesis of mono- and polyunsaturated HMW TAG, decreasing milk fat solid fat content (**SFC**). Our study showed that lactation stage had a large effect on milk FA and TAG composition, FA distribution over the TAG structure, and SFC, driven by the variation in abundance of C16:0, C18:0, C18:1*cis*9, and *de novo* synthesized FA between lactation stages.

5.1. Introduction

Milk fat, mainly composed of triacylglycerols (**TAG**; ~98%), is one of the high value components in milk (Jensen, 2002; Mohan et al., 2020). Triacylglycerols are composed of three fatty acids (**FA**) esterified to a glycerol molecule located at the stereospecific numbering (***sn***)-positions 1, 2, or 3. Many different FA (~400) are used for TAG synthesis, therefore changes in the FA composition may influence the formation of specific TAG species in the mammary gland. Several studies analyzed the variation in the FA composition in the different lactation stages of dairy cattle (Kay et al., 2005; Garnsworthy et al., 2006; Stoop et al., 2009). The FA differences between lactation stages were related to the energy status of the cows, that triggered changes in the origin of FA used for TAG synthesis in the mammary gland (Van Knegsel et al., 2005; Stoop et al., 2009). More specifically, the major changes between lactation stages involve the strong FA release from body fat stores in early lactation (<100 days in milk; **DIM**) to the predominant *de novo* FA synthesis in middle (100–200 DIM) and late lactation (>200 DIM) (Kay et al., 2005; Stoop et al., 2009; Samková et al., 2012). Dairy cows in early lactation are usually in a negative energy balance due to the high energy requirements for milk production after calving (Kay et al., 2005; Van Knegsel et al., 2005; Stoop et al., 2009). To compensate for this energy need, the cow's body starts mobilizing FA from body fat stores, that are mainly composed of C16:0, C18:0, and C18:1*cis*9 (Christie, 1981). As a result, previous studies identified high concentrations of mobilized long-chain FA (**LCFA**) in early lactation milk fat (Kay et al., 2005; Garnsworthy et al., 2006). Moving towards mid and late lactation, the mobilization of body fat decreases and the synthesis of *de novo* FA C6:0 to C16:0 increases (Palmquist et al., 1993; Kay et al., 2005; Stoop et al., 2009).

These large differences between lactation stages in the concentrations of LCFA and *de novo* FA in milk fat are likely to influence milk fat TAG composition. High concentrations of LCFA are expected to increase the formation of high molecular weight (**HMW**) TAG species, whereas high concentrations of *de novo* FA are expected to increase the synthesis of low molecular weight (**LMW**) and medium molecular weight (**MMW**) TAG species in milk fat (Banks et al., 1989; DePeters et al., 2001). To our knowledge, no studies have reported on the effect of lactation stage on the TAG composition in bovine milk fat. In addition, no studies were found on the effect of lactation stage on the FA positional distribution in bovine milk fat TAG structures. The positioning of FA within TAG is of importance for digestion and FA absorption after milk consumption. For example, the composition and concentration of FA at the secondary position (*sn*-2) in the TAG structure are important for infant formula development, because high concentrations of C16:0 at this position were identified to increase free FA (**FFA**) and calcium absorption in infants (Innis, 2011; Yaron et al., 2013). Moreover, the TAG composition and the stereolocation of FA in the TAG structures are important for the dairy and food industry because both influence the physical properties of

milk fat (*e.g.* solid fat content (**SFC**) and crystallization behavior). Variations in milk fat physical properties, especially its SFC, are relevant for its suitability and use for food product applications (Mohan et al., 2020). In this study, we aim to analyze the effect of the early versus late lactation stage on bovine milk fat composition by providing insights into its influence on the FA and TAG composition, FA positional distribution in the TAG structure, and SFC. These findings can help the dairy industry to better understand the changes in chemical and physical properties of milk fat that are associated with the cows' lactation stage.

5.2. Materials and Methods

5.2.1. Sample collection

Raw milk samples were collected at Dairy Campus (Leeuwarden, The Netherlands) from 11 multiparous cows twice in the same lactation period: in early (8 to 14 DIM) and late lactation (199 to 326 DIM). The DIM for late lactation was chosen based on previous research, which has shown that FA composition becomes stable after 90 DIM (~12 weeks; Garnsworthy et al., 2006; Stoop et al., 2009). The cows in early lactation consumed on average ~18 kg of dry matter, including a fixed amount of 5.5 kg DM of pelleted concentrates and an unrestrictedly fed roughage-based diet. The main components of this roughage-based diet were 70% maize silage and 20% grass silage mixed with 5-6% soy bean meal, and a premix with minerals and vitamins. During late lactation, the cows consumed ~23 kg of dry matter. The amount of pelleted concentrate within total intake varied for each cow, as concentrate allowance was individually determined based on each cow's milk yield. The ranges used were determined as follows: cows with a milk yield of 17 kg of milk/d were given 1.8 kg dry matter concentrate, and cows with a milk yield of 40 kg of milk/d were given 7.2 kg dry matter concentrate. The main components of the roughage diet fed unrestrictedly to the cows in late lactation were 60% grass silage and 20% maize silage mixed with 9% soy bean meal, 10% wheat meal, and a premix with minerals and vitamins. Sampling was done during the morning milking (between 5 and 8 AM) by taking a representative 100 mL sample from the total milking of each individual cow. Samples were stored frozen (-20°C) until fat extraction. Before fat extraction, milk samples were thawed in a water bath at 37°C for approximately 30 min. The fat extraction was done based on the method described by Tzompa-Sosa et al. (2014). This method only extracts TAG from the fat samples. Milk fat samples were stored at -20°C until further analysis.

5.2.2. Fatty acid composition analysis

Fatty acid methyl esters (**FAME**) were analyzed for all milk fat samples (n=22) as described by Pacheco-Pappenheim et al., (2019). Fatty acid methyl esters were prepared according to the ISO Standard 15884 (ISO15884, 2002) and the FAME composition was determined by the ISO Standard 16958 (ISO, 2015) using gas chromatography (**GC**) with a flame-

ionization detector (**FID**; **GC-FID**; Thermo Focus, Thermo Fisher, Rodano, Italy) and an Agilent CPSil 88 FAME column (100 m × 0.25 mm i.d. × 0.2 µm film thickness).

5.2.3. Triacylglycerol composition analysis

Two methods were used to analyze the TAG composition: GC-FID and MALDI-TOF-MS. The same approach was previously described by Pacheco-Pappenheim et al. (2021), where combining the two methods provided a complete overview of the TAG composition in milk fat. On the one hand, with GC-FID the TAG composition of even chain TAG groups composed of TAG species with the same number of total carbon atoms was determined. On the other hand, to obtain information on TAG species level, MALDI-TOF-MS was used to determine the molecular weight of individual TAG species expressed in carbon number (**CN**) and number of double bonds (**DB**; **CN:DB**).

5.2.3.1. GC-FID

Triacylglycerol compositions of all milk fat samples (n=22) were analyzed according to ISO Standard 17678 (2010). Even-chain TAG groups with total CN from 24 to 54 and cholesterol were determined with GC-FID, with a column injector port (Thermo Trace GC ultra; Thermo Scientific, Rodano, Italy) and a UltiMetal CP7532 column (5 m × 0.53 mm i.d. × 0.17 µm film thickness; Varian, Houten, The Netherlands). Anhydrous milk fat standard (BCR519; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was included in the analysis as reference for the identification of the TAG groups. Triacylglycerol composition was expressed as percentages (g/100 g, %) based on the normalized peak areas of each TAG group.

5.2.3.2. MALDI-TOF-MS

Triacylglycerol composition of all milk fat samples (n=22) were also analyzed with MALDI-TOF-MS (UltrafleXtreme, Bruker Corporation, Germany) according to Tzompa-Sosa et al. (2018) and Yener and van Valenberg (2019). Each milk fat sample was measured 5 times in automatic mode, which allows acquisition of mass peaks in a random manner. The obtained mass spectra were analyzed with the MALDIquant package using R (Gibb and Strimmer, 2012; Yener and van Valenberg, 2019). Signal-to-noise ratio >6 was used for the selection of the mass peaks. Each mass peak intensity was divided by the sum of the intensities of all mass peaks to express the results as relative intensities, which were reported as percentages (%). To assign the mass peaks to TAG species, the LIPID MAPS Online Tools library was used (https://www.lipidmaps.org/resources/tools/bulk-structure-search/create?database=LMSD; Fahy et al., 2005). Triacylglycerol species were defined according to their CN:DB and reported as relative intensities of each TAG species, based on the averages of the five replicates.

5.2.4. Regiospecific distribution analysis of fatty acids

The composition of FA at the *sn*-2 position of TAG structures was analyzed for all milk fat samples (n=22) based on the JOCS/AOCS Joint Method Ch3a-19 (2019). In this method, enzymatic transesterification using *Candida antarctica Lipase* (fraction B; Novozym 435 (10,000 PLU/g), Novozymes A/S, Frederiksberg, Denmark) was performed to determine the FA composition at the *sn*-2 position in the TAG structures. After the enzymatic transesterification, the 2-monoacylglycerol fractions of the 22 samples were collected through solid-phase extraction and analyzed for FAME composition. The FAME were prepared and analyzed as described in the FA composition analysis section. The FA composition at the *sn*-2 position was calculated and expressed in molar percentages (%). The FA composition at the *sn*-1(3) (the sum of the FA at the *sn*-1 and *sn*-3 positions) was calculated based on the composition analyzed at the *sn*-2 position. As described by Tzompa-Sosa et al., (2014) intra- and interpositional distribution approaches were used to describe the FA positional distribution in the TAG structure. The intrapositional distribution assesses the FA relative concentrations (mol %) at the *sn*-2 and *sn*-1(3) positions, whereas the interpositional distribution assesses the proportions (%) of FA over the 3 positions in the TAG structures. Intra- and interpositional distributions were calculated based on Tzompa-Sosa et al., (2014).

Intrapositional distribution calculations:

sn-1(3):

$$sn\text{-}1(3)_{FAi} = (TAG_i \times 3 - sn\text{-}2_{FAi}) / 2 \quad \text{(Equation 5.1)}$$

where $sn\text{-}1(3)_{FAi}$ is the molar percentage of the FA at *sn*-1(3) position, TAG_i is the molar percentage of the FA in TAG and $sn\text{-}2_{FAi}$ is the molar percentage of the FA measured at the *sn*-2 position.

Interpositional distribution calculations:

sn-2:

$$\%\ sn\text{-}2_{FAi} = (sn\text{-}2_{FAi} / (sn\text{-}2_{FAi} + (sn\text{-}1(3)_{FAi} \times 2)) \times 100 \quad \text{(Equation 5.2)}$$

sn-1(3):

$$\%\ sn\text{-}1(3)_{FAi} = sn\text{-}1(3)_{FAi} \times 2) / (sn\text{-}2_{FAi} + (sn\text{-}1(3)_{FAi} \times 2)) \times 100 \quad \text{(Equation 5.3)}$$

Where $\%sn\text{-}2_{FAi}$ is the proportion of the FA at the *sn*-2 position, $\%\ sn\text{-}1(3)_{FAi}$ is the proportion of the FA at the *sn*-1(3) position, $sn\text{-}2_{FAi}$ is the molar percentage of the FA measured at the *sn*-2 position and $sn\text{-}1(3)_{FAi}$ is the molar percentage of the FA at *sn*-1(3) position calculated with equation 5.1.

5.2.5. Solid fat content

The SFC analysis was carried out by nuclear magnetic resonance (**NMR**; Bruker mq20 minispec NMR analyzer, Bruker, Mississauga, Ontario, Canada) according to the AOCS Official Method Cd 16b-93 (2009). The SFC was determined at 0, 10, 20, 25, 30, and 40°C. The parameters for the NMR were a frequency of 19.95 MHz, a dead time of 0.0073 ms and a cell temperature of 40°C. Milk fat samples were totally melted using a water bath at 40°C and once melted 1 g of sample was transferred into a NMR glass tube (diameter of 10 mm, length of 150 mm and a wall thickness of 0.6 mm) (Bruker Nederland B.V., Leiderdorp, Netherlands). Before analysis of the SFC, the tubes containing the milk fat samples were heated to 100°C for 15 min followed by 15 min at 60°C and then cooled to 0°C for 60 min. At 0°C the first SFC was measured and after that the samples were kept for 30±1 min at each temperature (10, 20, 25, 30, and 40°C) before subsequent SFC measurements.

5.2.6. Statistical analysis

The effect of lactation stage on milk FA composition, TAG composition, FA regiospecific distribution in the TAG structures, and SFC was tested with one-way ANOVA with repeated measures. The cut-off value defined for significant differences was $P<0.050$. All statistical analyses were performed with IBM SPSS Statistics 25.0 software (2017). Pearson correlation analysis was performed between FA, TAG, and SFC using R version 3.6.1 (2019).

5.3. Results and Discussion

5.3.1. Fatty acid composition

The average milk fat content was not different between both lactation stages (4.50%; **Table 5.1**). Most FA were significantly affected by the stage of lactation ($P<0.050$), with the exceptions of C6:0, C8:0 C16:1*cis*9, C17:0*anteiso*, C18:0:1*trans*9, C18:1*trans*10, C18:1*cis*12, C18:1*cis*14, C18:2*cis*9,12 (linoleic acid (**LA**)), C19:0, and C20:0 (Table 5.1). Increased concentrations of C4:0, LCFA ≥C17:0 and most of the unsaturated FA (**UFA**) were found in early lactation, whereas increased concentrations of medium chain FA (**MCFA**; C10:0 to C16:0), C18:1*cis*15, C18:3*cis*9,12,15 (linolenic acid (**ALA**)), C18:2*cis*9,*trans*11 (**CLA**), C20:3*cis*8,11,14 (**DGLA**), and C22:5*cis*7,10,13,16,19 (**DPA**) were found in late lactation. These results are in agreement with previous studies, where similar changes in bovine milk fat FA composition were reported between early and late lactation (Kay et al., 2005; Garnsworthy et al., 2006; Stoop et al., 2009).

As expected, cows in early lactation were in negative energy balance due to the high energy requirements after calving for milk production (**Supplemental table S5.1**; Keanthao et al., 2021; Van Knegsel et al., 2005; Garnsworthy et al., 2006; Stoop et al., 2009). In order to

fulfill their energy needs for milk production, cows start mobilizing adipose tissue. This explains the increased concentrations of C18:0 and C18:1*cis*9 in early lactation (Table 5.1). Bovine adipose tissue is mainly composed of C16:0, C18:0, and C18:1*cis*9 (Christie, 1981), so large proportions of these FA are expected to be used by the mammary gland for milk fat synthesis. Moreover, in early lactation we identified lower concentrations of *de novo* FA from C10:0 to C15:0 and C16:0 (50% *de novo* formed) (Table 5.1). This may be the result of the limited energy availability for the synthesis of *de novo* FA in the mammary gland due to the negative energy balance of cows in early lactation (Van Knegsel et al., 2005). In contrast to the *de novo* FA, C4:0 concentrations were higher in early lactation compared to late lactation. This result is in line with previous studies, which suggested that the C4:0 increase in early lactation was related to the high concentrations of β-hydroxybutyrate found in the blood after calving (Palmquist et al., 1993; Garnsworthy et al., 2006; Ward et al. 1995). High concentrations of β-hydroxybutyrate in the blood may have enhanced the formation and esterification of C4:0, which can be directly formed in the mammary epithelial cells from β-hydroxybutyrate taken up from the blood.

Our results showed a characteristic shift in the FA composition between early and late lactation (Kay et al., 2005; Garnsworthy et al., 2006; Stoop et al., 2009). However, the differences between the contents of maize and grass silage in the diets fed to the cows in early and late lactation might also be partly responsible for the variation found in FA composition. The diet fed to the cows in early lactation was relatively high in maize silage (~70%) and low in grass silage (~20%) compared to the diet in late lactation (maize ~20%, grass ~60%). Maize silage is rich in LA, whereas grass silage is rich in ALA. Based on these differences in FA composition between maize and grass silage, increased concentrations of LA were expected in early lactation and increased concentrations of ALA were expected in late lactation, assuming that feed plays a major role in milk fat FA composition. High concentrations of ALA lead to increased formation of intermediate biohydrogenation products such as CLA, C18:1*trans* FA, C18:1*cis*14, and C18:1*cis*15 (Pacheco-Pappenheim et al., 2021). Our study indicates that the high concentration of grass silage in late lactation indeed resulted in increased concentrations of ALA, CLA, and C18:1*cis*15. Overall, the major differences in the FA composition, being the increase in mobilized FA vs *de novo* FA, could be related to lactation stage, yet some small additional differences between lactation stages in the concentrations of ALA, LA, CLA, and C18:1*cis*15 suggest that feed played a role as well. Moreover, the FA composition of our study matched with the differences in FA composition between early and late lactation of previous studies that assessed this effect (Van Knegsel et al., 2005; Garnsworthy et al., 2006; Stoop et al., 2009). This confirms that the lactation stage was the main driving factor responsible for the milk FA compositional differences.

Table 5.1. Milk fatty acids (FA) composition (g/100 g, %) of cows in early and late lactation stages.[1]

	Early[1]		Late[2]		Lactation Stage Effect[3]
Item	Mean	SD	Mean	SD	*P* value
Fat content	4.51	0.37	4.49	0.52	0.917
FA[4]					
C4:0	4.14	0.45	3.39	0.23	<0.001
C6:0	2.34	0.39	2.36	0.14	0.868
C8:0	1.16	0.30	1.34	0.11	0.091
C10:0	2.42	0.85	3.23	0.41	0.014
C10:1*cis*9	0.16	0.07	0.34	0.08	0.001
C12:0	2.62	0.98	4.16	0.61	0.002
C14:0iso	0.05	0.02	0.08	0.02	0.003
C14:0	9.36	2.14	12.13	0.71	0.001
C14:1*cis*9	0.62	0.22	1.19	0.31	0.001
C15:0iso	0.18	0.04	0.25	0.04	0.005
C15:0*anteiso*	0.32	0.07	0.49	0.07	<0.001
C15:0	0.74	0.19	1.17	0.18	0.001
C16:0*iso*	0.16	0.03	0.20	0.03	0.014
C16:0	28.63	1.82	32.10	2.08	0.002
C16:1*trans*9	0.20	0.03	0.16	0.03	0.011
C16:1*cis*9	2.06	0.44	1.92	0.41	0.344
C17:0iso	0.39	0.04	0.36	0.02	0.039
C17:0anteiso	0.39	0.07	0.40	0.06	0.505
C17:0	0.61	0.09	0.52	0.06	0.007
C17:1*cis*9	0.33	0.09	0.22	0.05	0.003
C18:0	11.34	1.25	8.34	1.64	0.001
C18:1*trans*6	0.25	0.05	0.19	0.02	0.004
C18:1*trans*9	0.16	0.01	0.15	0.01	0.304
C18:1*trans*10	0.21	0.03	0.23	0.06	0.169
C18:1*trans*11	0.86	0.10	0.75	0.13	0.022
C18:1*cis*9	24.88	5.13	18.48	2.09	0.005
C18:1*cis*11	1.04	0.16	0.66	0.15	0.001
C18:1*cis*12	0.19	0.04	0.19	0.03	0.641
C18:1*cis*13	0.16	0.04	0.10	0.02	0.001
C18:1*cis*14	0.31	0.04	0.34	0.02	0.087
C18:1*cis*15	0.18	0.03	0.26	0.06	0.002
C18:2*cis*9,12 (LA)	1.73	0.20	1.69	0.16	0.561
C18:3*cis*9,12,15 (ALA)	0.34	0.05	0.41	0.06	0.032
C18:2*cis*9,*trans*11 (CLA)	0.30	0.06	0.40	0.06	0.001
C19:0	0.14	0.01	0.13	0.01	0.052
C20:0	0.11	0.02	0.11	0.03	0.812
C20:3*cis*8,11,14 (DGLA)	0.05	0.02	0.08	0.02	<0.001
C20:4*cis*5,8,11,14 (AA)	0.13	0.02	0.12	0.02	<0.001
C22:5*cis*7,10,13,16,19 (DPA)	0.06	0.01	0.08	0.02	0.008
FA groups					
SCFA[5]	10.22	1.51	10.71	0.77	0.348
MCFA[6]	46.82	4.62	55.68	3.05	0.001
LCFA[7]	42.48	5.79	32.77	3.57	0.002
SFA	65.27	5.62	71.10	2.59	0.010
UFA	34.39	5.61	28.29	2.51	0.008

[1] Collection of the early lactation milk samples was done between 8 and 14 days in milk (DIM) of the 11 cows.
[2] Collection of the late lactation milk samples was done between 199 and 326 DIM of the 11 cows.
[3] The lactation stage effect was considered significant at $P \leq 0.050$.

[4] The total sum of the FA composition is 99.6%, 0.4% of the total FA composition is not included in the table due to low concentrations (<0.05%) in the GC-FID analysis.
[5] SCFA: short chain FA. Sum of FA with chain length <12.
[6] MCFA: medium-chain FA. Sum of FA with chain length ≥12 and <18.
[7] LCFA: long-chain FA. Sum of FA with chain length ≥18.

5.3.2. Triacylglycerol composition

Triacylglycerol composition between early and late lactation changed significantly. **Table 5.2** presents the TAG composition in early and late lactation as analyzed by GC-FID. The MMW TAG CN38 and CN40 and the HMW TAG CN52 and CN54 increased (P<0.050) in early lactation, whereas in late lactation the LMW TAG CN32 and CN34 and the MMW TAG CN42 to CN48 increased (P<0.050). Similar variations in TAG composition were also identified by MALDI-TOF-MS between early and late lactation (**Supplemental Table S5.2**). These variations in the TAG composition can be explained by the changes in the FA composition in each lactation stage (Table 5.1). The LMW and MMW TAG groups are mainly composed of *de novo* FA, whereas HMW TAG group are mainly composed of LCFA (Gresti et al., 1993; Liu et al., 2020). This was supported by Pearson correlation analyses between the TAG and FA composition as analyzed by GC-FID (**Supplemental Table S5.3**), where the HMW TAG CN52 and CN54 showed high positive correlations (r>0.6; P<0.050) with C18:0, C18:1*cis*9, C18:1*cis*11, and C18:1*cis*13, and high negative correlations (r<-0.6; P<0.050) with *de novo* FA. The LMW TAG CN32 and CN34 and the MMW TAG CN42 to CN48 were positively correlated (r>0.6; P<0.050) with mainly SCFA and MCFA (C8:0 to C16:0) and negatively correlated (r<-0.6; P<0.050) with LCFA C17:0 to C18:1*cis*13 (Supplemental Table S5.3). We, therefore, suggest that the high availability of the LCFA and UFA (mainly C18:0 and C18:1*cis*9, respectively) mobilized from body fat in early lactation underlies the increased synthesis of HMW TAG species (specially CN52 and CN54) in the mammary gland. Supporting this hypothesis, MALDI-TOF-MS data showed that in early lactation higher amounts of unsaturated HMW species were formed (**Figure 5.1**, Supplementary Table S5.2). Next to the HMW TAG species, the MMW TAG species CN38 and CN40 were reported to also include C16:0, C18:0, and C18:1*cis*9, together with *de novo* short-chain FA, in their structures (Gresti et al., 1993; Liu et al., 2020). The TAG CN38 and CN40 presented high positive correlations (r>0.6; P<0.050) with C18:0, C18:1*cis*9, C18:1*cis*11 and C18:1*cis*13 similar to the correlations observed for the HMW TAG (**Supplemental Table S5.3**). For this reason, it is likely that the high availability of C18:0 and C18:1*cis*9 in early lactation also led to the increased synthesis of TAG species CN38 and CN40. In late lactation, on the other hand, the high concentrations of *de novo* FA C8:0 to C16:0 may have increased the formation of the LMW TAG species CN32 and CN34 and the MMW TAG species CN42–CN48. Similar TAG profiles in early and late lactation were identified by both GC-FID and MALDI-TOF-MS (Supplemental Table S5.2).

Table 5.2. Milk fat triacylglycerol (TAG) composition (g/100 g, %) from cows in early and late lactation stages.

		Early[1]		Late[2]		Lactation Stage Effect[3]
Item	TAG Group	Mean	SD	Mean	SD	*P* value
Cholesterol		0.26	0.03	0.33	0.04	0.001
TAG[4]						
CN24	LMW	0.03	0.01	0.03	0.01	0.910
CN26	LMW	0.24	0.07	0.26	0.04	0.515
CN28	LMW	0.53	0.17	0.60	0.08	0.218
CN30	LMW	0.98	0.32	1.24	0.17	0.059
CN32	LMW	2.03	0.67	2.76	0.36	0.023
CN34	LMW	4.96	1.22	6.36	0.67	0.019
CN36	LMW	10.78	1.23	11.15	0.87	0.465
CN38	MMW	14.67	1.23	12.23	0.57	<0.001
CN40	MMW	10.95	0.69	9.70	0.34	<0.001
CN42	MMW	6.34	1.30	7.87	0.61	0.016
CN44	MMW	5.33	1.50	7.81	0.72	0.002
CN46	MMW	5.68	1.44	8.53	0.68	0.001
CN48	MMW	7.69	1.08	9.89	0.44	<0.001
CN50	HMW	11.71	1.68	10.67	0.97	0.166
CN52	MMW	12.47	4.26	7.61	1.71	0.015
CN54	MMW	5.36	2.00	2.96	0.99	0.017
	TAG groups					
	LMW[4]	19.54	3.61	22.40	2.04	0.077
	MMW[5]	50.66	4.38	56.02	1.93	0.013
	HMW[6]	29.54	7.78	21.24	3.60	0.025

[1] Collection of the early lactation milk samples was done between 8 and 14 DIM of the 11 cows.
[2] Collection of the late lactation milk samples was done between 199 and 326 DIM of the 11 cows.
[3] The lactation stage effect was considered significant at $P \leq 0.050$.
[4] LMW: low molecular weight TAG. Sum of CN24-CN36.
[5] MMW: medium molecular weight TAG. Sum of CN38-CN48.
[6] HMW: high molecular weight TAG. Sum of CN50-CN54.

Looking more in-depth at the effect of lactation stage on the saturation degree of TAG species, **Figure 5.1** presents the variations of the saturated, mono-, and polyunsaturated TAG species between early and late lactation, assessed separately for the LMW, MMW, and HMW TAG groups. Regardless of the saturation degree, the HMW TAG species increased ($P<0.050$) in early lactation and most LMW and the MMW TAG species increased ($P<0.050$) in late lactation (Figure 5.1; Supplemental Table S5.2). Based on the FA identified in each TAG group structure, combined with the Pearson correlation analysis (Supplemental Table S5.3; Liu et al., 2020), we suggest that the high abundance of C18:0 and C18:1 FA isomers (mainly C18:1*cis*9) enhanced the formation of saturated, mono-, and polyunsaturated HMW TAG species in early lactation. On the other hand, in late lactation the high concentrations of *de novo* FA and C16:0 increased the formation of saturated and polyunsaturated LMW TAG species and all MMW TAG (saturated, mono-, and polyunsaturated).

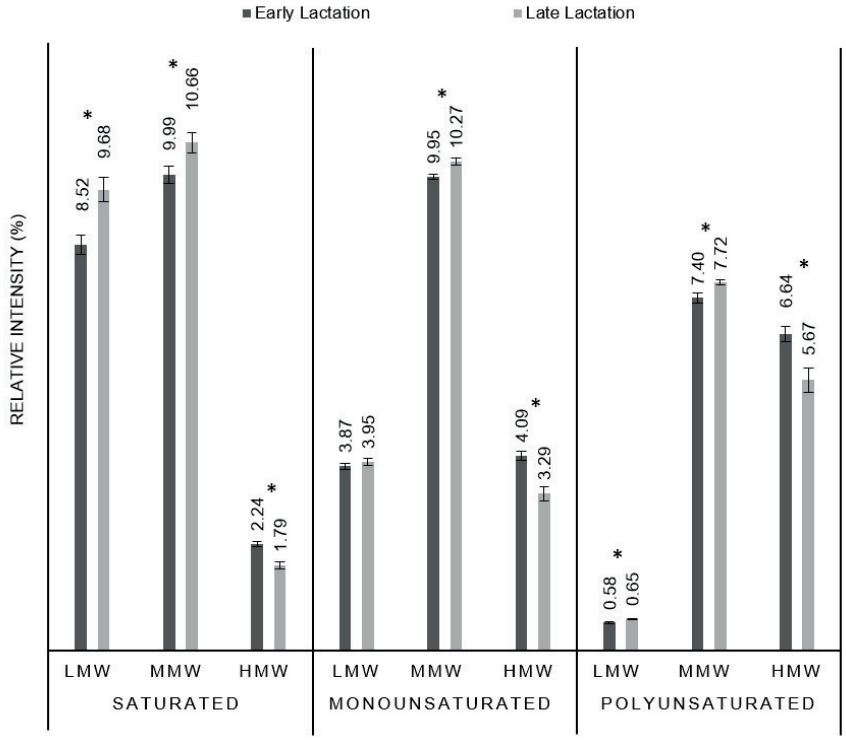


Figure 5.1. Milk fat saturated, mono-, and polyunsaturated triacylglycerol (TAG) profiles (relative intensities; %) from cows in early and late lactation stages analyzed by MALDI-TOF-MS. Differences between low, medium, and high molecular weight (LMW, MMW, and HMW) TAG species are presented separately in each TAG profile. *Significant differences (P<0.050) between early and late lactation LMW, MMW, and HMW TAG species.

5.3.3. Regiospecific positional distribution of FA in the TAG

Higher concentrations of C18:0 and C18:1*cis*9 in early lactation and higher concentrations of C16:0 in late lactation may have affected the FA positional distribution in the TAG structures. These three major FA in milk fat are esterified in most TAG species, hence changes in their total abundance, and thereby their availability for TAG synthesis in the mammary gland, can be expected to affect FA positional distribution in the TAG structures. To examine this effect, first we analyzed the FA composition at the *sn*-2 and *sn*-1(3) positions using the intrapositional distribution. The intrapositional distribution provides information on the FA relative concentrations (mol %) at the *sn*-2 and *sn*-1(3) positions in the TAG structures. Second, based on these results, the interpositional distribution was determined, where the proportions of a specific FA over the three positions (*sn*-2 and *sn*-

1(3)) in early and late lactation was calculated. With the interpositional distribution, a FA distributed over the three *sn*-positions in TAG structure represents 100%, meaning that based on equal FA distribution the proportion of each individual *sn*-position corresponds to 33.3%. Knowing the proportions of a FA at the *sn*-2 and *sn*-1(3) positions is a suitable approach to analyze the esterification preferences of FA in the TAG structures and to understand the mechanisms for TAG synthesis in the mammary gland (Blasi et al., 2008; Tzompa-Sosa et al., 2014). Combining intra- and interpositional distributions, we were able to determine the influence of lactation stage on the FA abundance variation and the FA esterification preferences at the *sn*-2 and *sn*-1(3) positions in the TAG structures.

Table 5.3 presents the results of the FA interpositional distributions in early and late lactation stages. At the *sn*-2 position, FA proportions larger than 33.3% indicate an esterification preference, whereas proportions larger than 66.7% at the *sn*-1(3) positions indicate an esterification preference for these positions. We identified that, regardless of the lactation stage, the FA that were preferentially esterified at the *sn*-2 position were the MCFA from C12:0 to C16:1*cis*9, C17:0, C17:1*cis*9, C18:1*cis*9, LA, and ALA. Moreover, the FA that were preferentially esterified at the *sn*-1(3) position were the SCFA from C4:0 to C12:0, C18:0, C18:1*cis*9, C18:1*cis*11, and C20:0. These esterification preferences of the FA are in accordance with previous studies (Blasi et al., 2008; Tzompa-Sosa et al., 2014). In addition, we also found differences in the FA interpositional distributions at both positions between lactation stages. At the *sn*-2 position, early lactation milk fat samples had higher proportions of C4:0 to C15:0, C16:0, C16:1*cis*9, C17:0, C17:1*cis*9, C18:1*cis*11, C20:0, and C18:3*cis*9,12,15 (P<0.050). Late lactation milk fat samples had higher proportions of C17:0*iso*, C16:1*trans*9, and C18:1*cis*9 at the *sn*-2 position (P<0.050). The opposite trends were observed at the *sn*-1(3) position. These shifts from *sn*-2 to *sn*-1(3) positions in the TAG structure between lactation stages may be related to the differences in the availability of C16:0, 18:0, and C18:1*cis*9 for TAG synthesis in each lactation stage. In early lactation, high concentrations of C18:0 and C18:1*cis*9 may have enhanced the proportions of most FA (mainly *de novo* FA and C16:0) at the *sn*-2 position, and resulted in decreased proportions of the same FA at the *sn*-1(3) position in the TAG structures. On the other hand, high concentrations of *de novo* FA and C16:0 in late lactation may have enhanced the proportions of most *de novo* FA at the *sn*-1(3) positions, which in turn decreased the proportions of these FA at the *sn*-2 position. It was previously suggested by Tzompa-Sosa et al., (2014) that high abundance of C16:0 may increase the activity of the glycerol-3-phospate acyltransferase (**GPAT**) enzyme towards C16:0, that is responsible for the esterification of FA at the *sn*-1 position during TAG synthesis in the mammary gland. The high availability of C16:0 in the TAG in late lactation may have increased the activity of the GPAT enzyme towards different FA, and thereby increased the proportions of mainly *de novo* FA at the *sn*-1 position and decreased the proportions of these FA at the *sn*-2 position

in the TAG structure. To balance the higher proportions of C16:0 at the *sn*-1(3) positions in late lactation, the proportions of C18:1*cis*9 were increased at the *sn*-2 position in the TAG structures. These results were similar to Tzompa-Sosa et al. (2014), who showed the same trends for interpositional distribution of C16:0 and C18:1*cis*9.

Table 5.3. Interpositional distribution of FA in milk fat triacylglycerol (TAG) structures of cows in early and late lactation stages. The interpositional distribution at the *sn*-2 and *sn*-1(3) positions are expressed in mol (%).

	sn-2					*sn*-1(3)				
	Early[1]		Late[2]		Lactation Stage Effect[3]	Early[1]		Late[2]		Lactation Stage Effect[3]
FA	Mean	SD	Mean	SD	*P* value	Mean	SD	Mean	SD	*P* value
C4:0	0.84	0.13	0.70	0.11	0.040	99.16	0.13	99.30	0.11	0.040
C6:0	3.98	0.59	3.87	0.81	0.592	96.02	0.59	96.13	0.81	0.592
C8:0	28.66	2.78	26.59	1.93	0.001	71.34	2.78	73.41	1.93	0.001
C10:0	29.15	2.21	26.34	1.57	<0.001	70.85	2.21	73.66	1.57	<0.001
C12:0	52.78	4.74	44.89	1.17	<0.001	47.22	4.74	55.11	1.17	<0.001
C14:0	65.65	4.14	56.97	2.15	<0.001	34.35	4.14	43.03	2.15	<0.001
C15:0*iso*	60.01	4.98	55.51	3.35	0.017	39.99	4.98	44.49	3.35	0.017
C15:0*anteiso*	73.10	3.63	67.05	1.98	0.001	26.90	3.63	32.95	1.98	0.001
C14:1*cis*9	55.06	4.22	49.09	1.23	0.001	44.94	4.22	50.91	1.23	0.001
C15:0	57.85	4.30	48.58	2.74	<0.001	42.15	4.30	51.42	2.74	<0.001
C16:0*iso*	64.53	8.90	61.47	5.99	0.354	35.47	8.90	38.53	5.99	0.354
C16:0	51.26	2.95	44.21	2.68	<0.001	48.74	2.95	55.79	2.68	<0.001
C17:0*iso*	40.63	3.50	44.45	3.14	0.008	59.37	3.50	55.55	3.14	0.008
C16:1*trans*9	13.55	2.48	15.74	2.28	0.020	86.45	2.48	84.26	2.28	0.020
C17:0*anteiso*	52.25	3.65	53.62	4.13	0.379	47.75	3.65	46.38	4.13	0.379
C16:1*cis*9	66.67	4.43	60.99	1.40	0.002	33.33	4.43	39.01	1.40	0.002
C17:0	43.78	3.60	38.09	3.39	0.006	56.22	3.60	61.91	3.39	0.006
C17:1*cis*9	63.94	4.43	57.54	2.98	0.002	36.06	4.43	42.46	2.98	0.002
C18:0	20.01	1.61	20.91	1.91	0.129	79.99	1.61	79.09	1.91	0.129
C18:1*trans*	13.54	3.17	15.51	5.89	0.368	86.46	3.17	84.49	5.89	0.368
C18:1*cis*9	32.94	1.61	35.53	2.35	0.002	67.06	1.61	64.47	2.35	0.002
C18:1*cis*11	13.20	1.99	10.15	1.26	0.003	86.80	1.99	89.85	1.26	0.003
C18:2*cis*9,12 (LA)	49.03	3.12	48.56	2.67	0.708	50.97	3.12	51.44	2.67	0.708
C18:3*cis*9,12,15 (ALA)	41.55	2.91	38.79	1.68	0.006	58.45	2.91	61.21	1.68	0.006
C18:2*cis*9,*trans*11(CLA)	31.35	2.56	31.46	2.01	0.883	68.65	2.56	68.54	2.01	0.883
C20:0	14.77	6.61	10.24	2.06	0.017	85.23	6.61	89.76	2.06	0.017
C20:4*cis*5,8,11,14 (AA)	27.48	3.45	26.48	2.52	0.449	72.52	3.45	73.52	2.52	0.449

[1] Collection of the early lactation milk samples was done between 8 and 14 DIM of the 11 cows.

[2] Collection of the late lactation milk samples was done between 199 and 326 DIM of the 11 cows.

[3] The lactation stage effect was considered significant at $P \leq 0.050$.

Table 5.4 presents the intrapositional distribution at the *sn*-2 and *sn*-1(3) positions in the TAG structures at each lactation stage. Regardless of the lactation stage, we identified that the most abundant FA at the *sn*-2 position were C14:0, C16:0, 18:0, and C18:1*cis*9 and at the *sn*-1(3) were FA C4:0, C16:0, C18:0, and C18:1*cis*9. These results are in agreement

with previous studies (Jensen, 2002; Blasi et al., 2008; Tzompa-Sosa et al., 2014). In early lactation, higher concentrations of C4:0, C16:0, C17:0, C17:1*cis*9, C18:0, C18:1*cis*9, and C20:0 were identified at the *sn*-2 position. At this same position in late lactation, concentrations of *de novo* FA C12:0 to C15:0, ALA and DGLA were higher. Similar to the *sn*-2 position, at the *sn*-1(3) positions increased concentrations of C4:0, C17:0*iso*, C16:1*trans*9, C17:1*cis*9, C18:0, C18:1*trans* FA, C18:1*cis*9, and C18:1*cis*11 were found in early lactation, and in late lactation increased concentrations of *de novo* FA from C8:0 to C16:0, ALA and DGLA were found. Except for C16:0, all these described changes in FA esterification at the *sn*-2 and *sn*-1(3) positions are in line with the overall FA composition of each lactation stage (Table 5.1). These results suggests that, with the exception of C16:0, increased total abundance of a FA in the TAG leads to increases at both the *sn*-2 and *sn*-1(3) positions. At the *sn*-2 position, the C16:0 concentration was higher in early lactation, whereas at the *sn*-1(3) position, the C16:0 concentration was higher in late lactation. Thus, it can be suggested that the C16:0 concentration may play an important role in the positioning of FA. It is possible that the high concentrations of C16:0 in the TAG in late lactation may have increased the GPAT activity towards C16:0 and therefore resulted in higher concentrations of C16:0 at the *sn*-1(3) positions and lower concentration of C16:0 at the *sn*-2 position (Tzompa-Sosa et al., 2014). All in all, the results indicate that the differences between lactation stages in the FA distribution in the TAG structures were due to the changes in the overall concentrations of FA in each lactation stage (Table 5.1). Alterations in the total FA concentrations in the TAG structures resulted in changes in the abundance and the proportions of the FA at the *sn*-2 and *sn*-1(3) positions in the TAG structures, yet without affecting the main esterification preferences of the FA.

Information of the FA positional distribution in the TAG structures is important for infant formula producers (Yong-Hua et al., 2010; Kloek et al., 2020). The stereolocation of C16:0 at the *sn*-2 position in the form of palmitate enhances the absorption of FFA and minerals in infants. In contrast, C16:0 at the *sn*-1 and *sn*-3 positions in the TAG structures are hydrolyzed into free C16:0. Free C16:0 can bind to dietary minerals (*e.g*. calcium and magnesium) forming indigestible complexes. These complexes are difficult to absorb and result in a lower availability of both FA and minerals in infants (Mu and Høy, 2004; Innis, 2011). In early lactation, C16:0 has a greater abundance and proportion at the *sn*-2 position than late lactation milk fat. Therefore, to increase the abundance of C16:0 at the *sn*-2 position in the TAG structures and improve FA and mineral absorption in infants, milk fat from early lactation may be suggested as a more suitable lipid source for infant formula developers.

Table 5.4. Intrapositional distribution of FA in milk fat triacylglycerol (TAG) structures of cows in their early and late lactation stages. The intrapositional distribution at the *sn*-2 and *sn*-1(3) positions are expressed in mol (%).

	sn-2					*sn*-1(3)				
	Early[1]		Late[2]		Lactation Stage Effect[3]	Early[1]		Late[2]		Lactation Stage Effect[3]
FA	Mean	SD	Mean	SD	*P* value	Mean	SD	Mean	SD	*P* value
C4:0	0.43	0.08	0.30	0.06	0.001	25.36	2.65	20.83	1.17	<0.001
C6:0	0.69	0.14	0.68	0.14	0.843	8.30	1.20	8.41	0.41	0.770
C8:0	1.74	0.37	1.89	0.18	0.265	2.20	0.57	2.61	0.19	0.036
C10:0	2.86	0.89	3.50	0.40	0.057	3.54	1.26	4.91	0.62	0.005
C12:0	4.53	1.32	6.28	0.83	0.005	2.13	0.96	3.87	0.55	0.001
C14:0iso	0.13	0.03	0.17	0.03	0.007	0.00	0.02	0.03	0.02	0.005
C14:0	17.32	2.96	19.72	0.99	0.015	4.66	1.46	7.46	0.63	<0.001
C15:0*iso*	0.29	0.05	0.36	0.05	0.002	0.10	0.04	0.15	0.04	0.010
C15:0*anteiso*	0.62	0.12	0.86	0.12	<0.001	0.12	0.04	0.21	0.03	<0.001
C14:1*cis*9	0.96	0.27	1.67	0.45	0.001	0.41	0.18	0.86	0.21	0.001
C15:0	1.13	0.24	1.50	0.16	0.002	0.42	0.14	0.80	0.15	<0.001
C16:0 iso	0.25	0.04	0.30	0.06	0.050	0.07	0.02	0.10	0.03	0.043
C16:0	36.27	2.35	35.10	2.47	0.020	17.29	1.85	22.18	1.79	<0.001
C17:0*iso*	0.36	0.04	0.37	0.03	0.433	0.27	0.04	0.23	0.02	0.016
C16:1*trans*9	0.07	0.01	0.06	0.01	0.336	0.21	0.04	0.17	0.03	0.011
C17:0*anteiso*	0.47	0.07	0.50	0.07	0.183	0.22	0.05	0.22	0.05	0.990
C16:1*cis*9	3.42	0.82	2.90	0.59	0.052	0.85	0.23	0.93	0.21	0.311
C17:0	0.63	0.13	0.46	0.08	0.005	0.40	0.06	0.37	0.05	0.128
C17:1*cis*9	0.49	0.16	0.30	0.07	0.002	0.14	0.04	0.11	0.02	0.025
C18:0	4.96	0.66	3.78	0.69	0.001	9.90	1.05	7.24	1.60	0.002
C18:1*trans*	0.43	0.11	0.45	0.18	0.871	1.39	0.16	1.22	0.18	0.023
C18:1*cis*9	17.93	3.91	14.35	1.88	0.021	18.30	4.28	13.05	1.86	0.006
C18:1*cis*11	0.31	0.08	0.15	0.05	0.001	0.99	0.15	0.65	0.15	0.002
C18:2*cis*9,12 (LA)	1.86	0.29	1.79	0.23	0.542	0.96	0.14	0.95	0.10	0.750
C18:3*cis*9,12,15 (ALA)	0.31	0.06	0.35	0.06	0.231	0.22	0.03	0.27	0.04	0.013
C18:2*cis*9,*trans*11 (CLA)	0.20	0.04	0.28	0.05	0.001	0.22	0.05	0.30	0.05	0.002
C20:0	0.03	0.01	0.02	0.00	0.017	0.09	0.02	0.10	0.03	0.729
C20:3*cis*8,11,14 (DGLA)	0.06	0.02	0.09	0.03	0.023	0.02	0.02	0.04	0.01	0.030
C20:4*cis*5,8,11,14 (AA)	0.07	0.01	0.06	0.01	0.055	0.09	0.01	0.09	0.01	0.118

[1] Collection of the early lactation milk samples was done between 8 and 14 DIM of the 11 cows.

[2] Collection of the late lactation milk samples was done between 199 and 326 DIM of the 11 cows.

[3] The lactation stage effect was considered significant at $P \leq 0.050$.

5.3.4. Solid fat content

Variations in the FA and TAG composition can influence the milk fat SFC. **Table 5.5** presents the milk fat SFC in early and late lactation stages measured at 0, 10, 20, 25, 30 and 40°C. The SFC was higher ($P<0.05$) in late lactation for all measured temperatures, except for 40°C. Because milk fat is almost completely liquid at 40°C, no difference in milk fat SFC between early and late lactation at this temperature is to be expected. The SFC differences between 0 and 30°C can be explained by the changes in milk FA and TAG composition in early and late lactation (Table 5.1; Table 5.2; Figure 5.1; Supplemental Table

S5.2). As mentioned previously, the high concentrations of C18:0 and different C18:1 FA isomers in early lactation may have increased the formation of HMW TAG species and decreased the formation of LMW and MMW TAG species (Table 5.1; Table 5.2; Supplemental Table S5.2; Supplemental Table S5.3). The TAG with the largest differences between early and late lactation were the saturated LMW and MMW TAG species (1.16% and 0.68%), and the mono- and polyunsaturated HMW TAG species (0.79% and 0.96%) (Figure 5.1; Supplemental Table S5.2). C18:1 FA isomers (especially C18:1*cis*9), the FA that are most likely present in unsaturated HMW TAG species (Figure 5.1; Supplemental Table S5.3; Liu et al., 2020), are characterized by low melting points (Knothe and Dunn, 2009). In turn, mono- and polyunsaturated HMW TAG species are thus expected to decrease milk fat SFC. This is confirmed by the Pearson correlation analysis between the FA and TAG compositions and milk fat SFC, showing negative correlations between C18:1*cis*9, C18:1*cis*11, C18:1*cis*13, CN52, CN54 and the SFC measured between 0 and 30°C ($r<-0.6$; $P<0.05$; Supplemental Table S5.4). Therefore, it can be suggested that the high availability of C18:1 isomers mobilized from body fat in early lactation enhanced the formation of unsaturated HMW TAG species in the mammary gland, resulting in a decreased milk fat SFC. Compared to early lactation, the high SFC in late lactation can be explained by the high concentrations of saturated LMW and MMW TAG species (except CN38 and CN40) and by the lower concentrations of unsaturated HMW TAG (Table 5.2; Figure 5.1; Supplemental Table S5.2). The MCFA C14:0 and C16:0, mainly found in saturated LMW and MMW TAG species, have a higher melting point compared to C18:1 FA isomers (Knothe and Dunn, 2009). This may have increased SFC in late lactation, as the Pearson correlation analysis showed high positive correlations ($r>0.6$; $P<0.05$) between *de novo* FA C8:0 to C16:0, LMW TAG CN32 and CN34, MMW TAG CN42 to CN48, and the SFC measured between 0 and 30°C (Supplemental Table S5.4). All in all, milk fat SFC differences between early and late lactation were mainly related to the concentrations of saturated LMW and MMW TAG species and unsaturated HMW TAG species. Similar to our results, previous studies reported a decrease in milk fat SFC as a result of high concentrations of unsaturated LCFA in milk fat (Precht and Frede, 1994; Couvreur et al., 2006; Smet et al., 2010), in our case mainly C18:1*cis*9.

Table 5.5. Milk fat solid fat content (%) from cows in early and late lactation stages.

	Early[1]		Late[2]		Lactation Stage Effect[3]
Temperature (°C)[4]	Mean	SD	Mean	SD	*P* value
0	52.78	7.91	62.33	2.91	0.002
10	40.50	8.04	50.35	3.55	<0.001
20	14.46	4.56	20.47	2.46	0.001
25	8.28	2.52	11.94	1.50	0.001
30	4.18	1.50	6.08	1.04	0.004
40	0.98	0.16	1.00	0.25	0.842

[1] Collection of the early lactation milk samples was done between 8 and 14 DIM of the 11 cows.

[2] Collection of the late lactation milk samples was done between 199 and 326 DIM of the 11 cows.

[3] The lactation stage effect was considered significant at $P \leq 0.050$.

[4] Measuring temperatures selected to analyze the milk fat solid fat content.

To summarize, the SFC in early lactation was lower than in late lactation, mainly due to the higher concentrations of unsaturated HMW TAG species in early lactation and higher concentration of saturated LMW and MMW TAG species in late lactation. Variations in milk SFC are important for product development of dairy and dairy-based food products because it determines products texture characteristics at refrigerator (4°C<T<10°C) and room temperatures (20 to 30°C) (*e.g.* spreadability of butter and spreads). Variations in milk fat SFC driven by the characteristic FA and TAG compositions in each lactation stage may result in unexpected changes in milk fat processing conditions and texture characteristics in such products (Mohan et al., 2020). This might be the case in seasonal milking systems where cows calve at the same time, thus a high impact of lactation stage can be especially expected on the seasonal milk fat composition and physical properties (Auldist et al., 1998; Li et al., 2019).

5.4. Conclusions

Our study showed that milk fat FA in early lactation showed higher concentrations of C18:0 and C18:1*cis*9, likely mobilized from body fat reserves. Late lactation milk fat FA showed higher concentrations of *de novo* FA, including C16:0. These FA differences between lactation stages resulted in higher concentrations of unsaturated HMW TAG in early lactation milk fat and higher concentrations of saturated LMW and MMW TAG in late lactation milk fat. Variations in the total FA concentrations in the TAG structure resulted in changes in the abundance and the proportions of the FA at the *sn*-2 and *sn*-1(3) positions in the TAG structure, yet without changing the main esterification preferences of the FA. The SFC in early lactation was lower than in late lactation, which may be the result of higher concentrations of unsaturated HMW TAG in early lactation and higher concentration of saturated LMW and MMW TAG in late lactation.

5.5. Acknowledgements

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Supplementary Information

Table S5.1. Energy balance of cows in early lactation (kJ/kg$^{0.75}$/day).[1]

	Week of lactation							
	1	2	3	4	5	6	7	8
Energy balance	-358	-399	-403	-350	-238	-181	-126	-141

[1] Information reported in Keanthao et al. accepted for publication in the Journal of Dairy Science.

Table S5.2. Milk fat triacylglycerol (TAG) species composition (relative intensities, %) from early and late lactation milk fat analyzed by MALDI-TOF-MS.

TAG Annotation[1]	Mass to charge ratio (m/z)[2]	TAG group	Early Lactation[3]		Late Lactation[4]		Lactation Stage Effect[5]
			Mean	SD	Mean	SD	*P* value
CN26:0	521.29	LMW	0.49	0.07	0.51	0.07	0.664
CN27:0	535.32	LMW	0.22	0.03	0.26	0.04	0.023
CN28:1	547.31	LMW	0.28	0.04	0.27	0.04	0.510
CN28:0	549.32	LMW	0.65	0.11	0.73	0.08	0.179
CN29:0	563.35	LMW	0.28	0.04	0.37	0.05	0.002
CN30:1	575.34	LMW	0.40	0.04	0.40	0.03	0.810
CN30:0	577.36	LMW	0.83	0.07	0.92	0.08	0.049
CN31:0	591.39	LMW	0.37	0.03	0.48	0.06	<0.001
CN32:1	603.37	LMW	0.56	0.02	0.56	0.04	0.933
CN32:0	605.38	LMW	1.03	0.17	1.24	0.09	0.009
CN33:1	617.40	LMW	0.30	0.03	0.35	0.03	0.001
CN33.0	619.39	LMW	0.39	0.04	0.49	0.03	0.001
CN34:2	629.38	LMW	0.20	0.01	0.25	0.04	0.006
CN34:1	631.39	LMW	0.74	0.08	0.82	0.06	0.074
CN34:0	633.41	LMW	1.56	0.20	1.78	0.13	0.015
CN35:1	645.40	LMW	0.29	0.02	0.32	0.03	0.016
CN35:0	647.42	LMW	0.58	0.07	0.69	0.05	0.005
CN36:2	657.40	LMW	0.38	0.04	0.40	0.02	0.107
CN36:1	659.42	LMW	1.31	0.10	1.24	0.05	0.093
CN36:0	661.44	LMW	2.12	0.14	2.20	0.19	0.258
CN37:1	673.43	MMW	0.48	0.05	0.47	0.03	0.708
CN37:0	675.44	MMW	0.70	0.05	0.71	0.04	0.602
CN38:3	683.42	MMW	0.24	0.03	0.26	0.05	0.254
CN38:2	685.43	MMW	0.71	0.09	0.64	0.04	0.018
CN38:1	687.45	MMW	1.96	0.22	1.66	0.08	0.002
CN38:0	689.46	MMW	1.92	0.14	1.81	0.16	0.064
CN39:2	699.43	MMW	0.25	0.04	0.22	0.03	0.108
CN39:1	701.45	MMW	0.52	0.07	0.48	0.02	0.022
CN39:0	703.45	MMW	0.69	0.07	0.68	0.13	0.905
CN40:3	711.45	MMW	0.40	0.05	0.37	0.05	0.130
CN40:2	713.46	MMW	1.02	0.17	0.78	0.06	0.002
CN40:1	715.48	MMW	1.47	0.10	1.31	0.06	0.001
CN40:0	717.49	MMW	1.29	0.15	1.35	0.11	0.227
CN41:1	729.47	MMW	0.43	0.04	0.42	0.02	0.467

(continuation **Table S5.2.**)

TAG Annotation[1]	Mass to charge ratio (*m/z*)[2]	TAG group	Early Lactation[3]		Late Lactation[4]		Lactation Stage Effect[5]
			Mean	SD	Mean	SD	*P* value
CN41:0	731.48	MMW	0.54	0.05	0.60	0.16	0.271
CN42:6	733.47	MMW	0.40	0.05	0.41	0.04	0.549
CN42:3	739.47	MMW	0.24	0.02	0.27	0.07	0.168
CN42:2	741.49	MMW	0.59	0.04	0.57	0.02	0.015
CN42:1	743.51	MMW	0.96	0.08	1.06	0.05	0.014
CN42:0	745.52	MMW	0.98	0.18	1.15	0.08	0.012
CN43:1	757.50	MMW	0.34	0.03	0.39	0.04	0.013
CN43:0	759.51	MMW	0.41	0.05	0.51	0.08	0.008
CN44:6	761.50	MMW	0.31	0.04	0.35	0.07	0.182
CN44:2	769.52	MMW	0.46	0.04	0.54	0.03	0.001
CN44:1	771.53	MMW	0.89	0.11	1.05	0.06	0.002
CN44:0	773.55	MMW	0.82	0.15	0.95	0.04	0.009
CN45:6	775.55	MMW	0.25	0.04	0.29	0.02	0.002
CN45:1	785.53	MMW	0.30	0.04	0.40	0.04	<0.001
CN45:0	787.53	MMW	0.37	0.04	0.45	0.04	0.003
CN46:6	789.53	MMW	0.25	0.03	0.29	0.03	0.013
CN46:3	795.52	MMW	0.22	0.03	0.29	0.04	0.002
CN46:2	797.54	MMW	0.51	0.05	0.60	0.04	0.001
CN46:1	799.56	MMW	0.87	0.12	1.06	0.06	0.001
CN46:0	801.58	MMW	0.73	0.10	0.82	0.03	0.018
CN47:6	803.57	MMW	0.23	0.03	0.25	0.02	0.190
CN47:1	813.56	MMW	0.34	0.04	0.42	0.04	0.001
CN47:0	815.56	MMW	0.37	0.04	0.43	0.03	0.004
CN48:6	817.40	MMW	0.28	0.08	0.30	0.08	0.499
CN48:3	823.56	MMW	0.22	0.02	0.30	0.03	<0.001
CN48:2	825.58	MMW	0.58	0.06	0.69	0.05	0.002
CN48:1	827.59	MMW	1.08	0.08	1.18	0.05	0.003
CN48:0	829.60	MMW	0.73	0.06	0.73	0.03	0.949
CN49:2	839.58	MMW	0.24	0.02	0.28	0.04	0.016
CN49:1	842.59	MMW	0.32	0.04	0.36	0.03	0.014
CN49:0	843.59	MMW	0.44	0.03	0.45	0.04	0.145
CN50:6	845.58	HMW	0.28	0.05	0.28	0.02	0.857
CN50:3	851.59	HMW	0.36	0.03	0.40	0.04	0.033
CN50:2	853.60	HMW	0.92	0.08	0.86	0.06	0.092
CN50:1	855.62	HMW	1.37	0.10	1.24	0.07	0.009
CN50:0	857.63	HMW	0.77	0.06	0.65	0.05	0.001
CN51:2	867.60	HMW	0.37	0.05	0.37	0.04	0.890
CN51:1	869.61	HMW	0.54	0.06	0.49	0.05	0.074
CN51:0	871.61	HMW	0.49	0.06	0.42	0.04	0.018
CN52:6	873.61	HMW	0.29	0.04	0.26	0.03	0.059
CN52:4	877.60	HMW	0.27	0.03	0.27	0.04	0.743
CN52:3	879.62	HMW	0.59	0.09	0.53	0.05	0.074
CN52:2	881.63	HMW	1.28	0.22	0.98	0.11	0.005
CN52:1	883.64	HMW	1.20	0.19	0.83	0.11	0.001
CN52:0	885.65	HMW	0.58	0.08	0.41	0.06	<0.001
CN53:2	895.62	HMW	0.38	0.09	0.31	0.04	0.058
CN53:1	897.63	HMW	0.48	0.11	0.38	0.06	0.024
CN53:0	899.63	HMW	0.40	0.08	0.31	0.06	0.012
CN54:6	901.48	HMW	0.23	0.07	0.22	0.05	0.636

(continuation **Table S5.2.**)

TAG Annotation[1]	Mass to charge ratio (*m/z*)[2]	TAG group	Early Lactation[3]		Late Lactation[4]		Early Lactatio[5]
			Mean	SD	Mean	SD	*P* value
CN54:4	905.62	HMW	0.32	0.05	0.27	0.04	0.076
CN54:3	907.64	HMW	0.65	0.15	0.45	0.09	0.005
CN54:2	909.66	HMW	0.70	0.14	0.47	0.11	0.002
CN54:1	911.66	HMW	0.50	0.09	0.35	0.07	0.001
TAG groups[6,7]							
LMW			28.54	1.52	30.44	1.24	0.021
MMW			49.40	1.32	51.31	1.12	0.006
HMW			22.06	2.76	18.24	1.63	0.006
Saturated			20.74	1.34	22.13	0.96	0.021
Monounsaturated			17.91	0.61	17.52	0.35	0.056
Polyunsaturated			14.62	0.96	14.05	0.63	0.130
LMW saturated			8.52	0.84	9.68	0.65	0.014
MMW saturated			9.99	0.71	10.66	0.58	0.023
HMW saturated			2.24	0.23	1.79	0.18	0.002
LMW Monounsaturated			3.87	0.24	3.95	0.22	0.463
MMW Monounsaturated			9.95	0.26	10.27	0.19	0.009
HMW Monounsaturated			4.09	0.51	3.29	0.33	0.003
LMW Polyunsaturated			0.58	0.04	0.65	0.05	0.014
MMW Polyunsaturated			7.40	0.19	7.72	0.36	0.023
HMW Polyunsaturated			6.64	0.88	5.67	0.53	0.016

[1] TAG annotations of the TAG species identified using LIPID MAPS Online Tools library, which corresponds to 45% of the total TAG composition. The total sum of the unidentified TAG species intensities is 46%.

[2] The mass-to-charge ratio (*m/z*) refers to the mass peaks detected by MALDI-TOF-MS of the TAG species identified in milk fat of the different treatments.

[3] Collection of the early lactation milk samples was done between 8 and 14 DIM of the 1 cows.

[4] Collection of the late lactation milk samples was done between 199 and 326 DIM of the 11 cows.

[5] The lactation stage effect was considered significant at $P<0.050$.

[6] The LMW (CN26:0–CN36:0), MMW (CN37:1–CN49:0), and HMW (CN50:6–CN54:1) refer to the sum of all TAG species including the non-identified and identified TAG species by the LIPID MAPS Online Tools library of the MALDI-TOF-MS mass spectra.

[7] The total saturated, mono-, and polyunsaturated and the LMW, MMW, and HMW saturated, mono- , and polyunsaturated TAG groups were determined based on the summation of the TAG species identified by the LIPID MAPS Online Tools library of the MALDI-TOF-MS mass spectra.

Table S5.3. Significant Pearson correlation coefficients (P<0.05) between triacylglycerols (TAG) and fatty acids (FA) in early and late lactation.

	TAG[1]															
FA	CN24	CN26	CN28	CN30	CN32	CN34	CN36	CN38	CN40	CN42	CN44	CN46	CN48	CN50	CN52	CN54
C4:0					-0.53	-0.53		0.90	0.77	-0.71	-0.81	-0.85	-0.90		0.63	0.65
C6:0	0.68	0.65	0.67	0.61	0.54	0.51	0.53			0.48				-0.61	-0.50	-0.42
C8:0	0.54	0.59	0.73	0.79	0.79	0.75	0.54	-0.47		0.80	0.73	0.66	0.51	-0.75	-0.78	-0.72
C10:0	0.43	0.48	0.66	0.77	0.80	0.76	0.44	-0.65		0.87	0.84	0.79	0.67	-0.72	-0.82	-0.77
C10:1*cis*9			0.51	0.68	0.77	0.78		-0.78	-0.73	0.75	0.85	0.89	0.85	-0.59	-0.79	-0.79
C12:0		0.43	0.61	0.76	0.82	0.79		-0.78	-0.57	0.88	0.90	0.89	0.80	-0.69	-0.84	-0.80
C14:0		0.44	0.63	0.76	0.83	0.82	0.45	-0.76	-0.57	0.87	0.89	0.88	0.80	-0.68	-0.86	-0.82
C14:1*cis*9			0.37	0.54	0.63	0.66		-0.76	-0.71	0.64	0.76	0.82	0.84	-0.45	-0.69	-0.69
C15:0*iso*			0.40	0.60	0.69	0.67		-0.80	-0.62	0.80	0.86	0.85	0.78	-0.54	-0.73	-0.72
C15:0*anteiso*			0.44	0.62	0.72	0.73		-0.83	-0.76	0.70	0.81	0.86	0.85	-0.50	-0.73	-0.71
C15:0			0.45	0.63	0.73	0.76		-0.83	-0.77	0.73	0.84	0.90	0.89	-0.52	-0.77	-0.77
C16:0*iso*					0.45			-0.72	-0.61	0.60	0.69	0.72	0.69		-0.51	-0.53
C16:0			0.31	0.47	0.57	0.63		-0.67	-0.68	0.60	0.70	0.75	0.76		-0.65	-0.66
C16:1*trans*9					-0.45	-0.47		0.58	0.63	-0.49	-0.59	-0.65	-0.67		0.52	0.53
C17:0*iso*			-0.45	-0.49	-0.51	-0.56	-0.58			-0.43	-0.45	-0.44		0.52	0.53	0.53
Phytanic.acid				0.46	0.56	0.56		-0.71	-0.64	0.65	0.75	0.79	0.75	-0.44	-0.63	-0.62
C17:0	-0.45	-0.53	-0.66	-0.76	-0.80	-0.79	-0.55	0.57		-0.81	-0.80	-0.79	-0.69	0.72	0.83	0.80
C17:1*cis*9	-0.38	-0.44	-0.60	-0.72	-0.77	-0.78	-0.56	0.55	0.42	-0.76	-0.77	-0.75	-0.63	0.71	0.79	0.76
C18:0				-0.49	-0.58	-0.60		0.79	0.76	-0.59	-0.72	-0.79	-0.85		0.63	0.63
C18:1*trans*6								0.76	0.66	-0.48	-0.59	-0.66	-0.69			
C18:1*trans*11					-0.44			0.50	0.42	-0.40	-0.46	-0.49	-0.48	0.32	0.40	0.37
C18:1*cis*9	-0.37	-0.43	-0.60	-0.74	-0.81	-0.82	-0.52	0.70	0.54	-0.84	-0.86	-0.85	-0.76	0.68	0.84	0.81
C18:1*cis*11				-0.59	-0.69	-0.69		0.78	0.72	-0.74	-0.84	-0.87	-0.81	0.55	0.73	0.72
C18:1*cis*13				-0.54	-0.63	-0.63	-0.27	0.73	0.69	-0.66	-0.73	-0.74	-0.68	0.46	0.62	0.62
C18:1*cis*14			0.44	0.54	0.59	0.62	0.53			0.57	0.59	0.57	0.47	-0.58	-0.63	-0.63
C18:1*cis*15					0.51	0.54		-0.69	-0.74	0.47	0.62	0.70	0.76		-0.53	-0.56
C19:0								0.59	0.54		-0.44	-0.49	-0.50			
C18:3*cis*9,12,15 (ALA)								-0.53	-0.57		0.45	0.52	0.54			
C18:2*cis*9,*trans*11 (CLA)					0.48	0.52		-0.73	-0.79	0.50	0.65	0.73	0.80		-0.54	-0.60
C22:5*cis*7,10,13,16,19 (DPA)			0.47	0.61	0.66	0.60		-0.59		0.70	0.70	0.67	0.56	-0.57	-0.62	-0.60

[1] CN: carbon number

Table S5.4. Significant Pearson correlation coefficients (P<0.05) between fatty acids (FA), triacylglycerols (TAG) and solid fat content (SFC) in early and late lactation.

	Temperatures (°C)[1]					
FA	0	10	20	25	30	40
C4:0	-0.64	-0.69	-0.69	-0.72	-0.66	
C6:0	0.50					
C8:0	0.80	0.74	0.66	0.60	0.48	
C10:0	0.88	0.85	0.78	0.74	0.63	
C10:1*cis*9	0.75	0.71	0.73	0.71	0.67	
C12:0	0.90	0.87	0.83	0.81	0.73	
C14:0	0.94	0.91	0.84	0.81	0.70	
C14:1*cis*9	0.64	0.62	0.64	0.62	0.61	
C15:0*iso*	0.72	0.71	0.66	0.69	0.60	
C15:0*anteiso*	0.65	0.63	0.62	0.61	0.56	
C15:0	0.76	0.73	0.73	0.72	0.69	
C16:0*iso*	0.48	0.50	0.47	0.56	0.52	
C16:0	0.80	0.78	0.85	0.82	0.83	
C16:1*trans*9	-0.62	-0.60	-0.65	-0.67	-0.65	
C16:1cis9	-0.58	-0.57	-0.52	-0.54	-0.48	
C17:0*iso*	-0.67	-0.62	-0.64	-0.65	-0.59	
Phytanic acid	0.65	0.66	0.65	0.73	0.69	
C17:0	-0.76	-0.74	-0.70	-0.66	-0.56	
C17:1*cis*9	-0.91	-0.88	-0.83	-0.82	-0.73	
C16:0	0.80	0.78	0.85	0.82	0.83	
C18:0	-0.54	-0.54	-0.56	-0.54	-0.53	
C18:1*cis*9	-0.96	-0.92	-0.89	-0.85	-0.77	
TAG						
C28	0.48					
C30	0.64	0.58	0.51	0.47		
C32	0.73	0.68	0.62	0.59	0.48	
C34	0.75	0.69	0.65	0.61	0.51	
C36	0.45					
C38	-0.72	-0.74	-0.73	-0.74	-0.68	
C40	-0.57	-0.60	-0.63	-0.64	-0.63	
C42	0.81	0.78	0.74	0.73	0.62	
C44	0.83	0.82	0.79	0.79	0.69	
C46	0.83	0.82	0.79	0.80	0.72	
C48	0.77	0.78	0.76	0.77	0.70	
C50	-0.59	-0.52	-0.48	-0.45		
C52	-0.79	-0.75	-0.70	-0.68	-0.57	
C54	-0.77	-0.74	-0.70	-0.69	-0.58	

[1] Measuring temperatures selected to analyze the milk fat solid fat content.

Chapter 6

DGAT1 K232A polymorphism and feeding modify milk fat triacylglycerol composition

This chapter is based on:

Pacheco-Pappenheim, S., S. Yener, H.J.F. van Valenberg, D.A. Tzompa-Sosa, and H. Bovenhuis. 2019. The DGAT1 K232A polymorphism and feeding modify milk fat triacylglycerol composition. J. Dairy Sci. 102:6842–6852. doi:10.3168/jds.2019-16554. Published.

Abstract

In the present study, we aimed to investigate the changes in triacylglycerol (**TAG**) composition as affected by alterations in the cows' diet due to seasonal variations and genetic factors. For this study, 50 milk fat samples in winter and 50 in summer were used from 25 cows with the DGAT1 KK genotype and 25 cows with the DGAT1 AA genotype. The samples were analyzed for milk fat content (%), fat composition, and TAG composition. We found that the content of TAG species CN54 was higher and that of CN34 and CN36 lower in summer than in winter. This seasonal variation in TAG profile was related to seasonal changes in the fatty acids C14:0, C16:0, C18:0, C18:1*cis*9, total unsaturated fatty acids, and total long-chain fatty acids, most likely resulting from dietary differences between seasons. Furthermore, we quantified the effect of DGAT1 K232A polymorphism on TAG profile and detected a significant effect on TAG species CN36, with higher values for the DGAT1 KK genotype. When adjusting for differences in fat content, we found no significant effects of the DGAT1 K232A polymorphism on TAG profile. We detected a significant interaction between DGAT1 K232A polymorphism and season for TAG species CN42 and CN52; in summer, the KK genotype was associated with higher levels for CN42 than the AA genotype, whereas in winter, the difference between the genotypes was small. For CN52, in summer the AA genotype was associated with higher levels than the KK genotype. In winter, the difference between the genotypes was also small. We show that, regardless of preference for DGAT1 genotype (AA or KK) and depending on the availability of FA according to season, UFA (C18:1*cis*9), short-chain FA (C6:0 and C10:0), and medium-chain FA might be esterified on the glycerol backbone of the TAG, keeping the structure characteristics of each TAG species. To our knowledge, this is the first report on the interaction effect of DGAT1 K232A polymorphism and season on the TAG composition in milk fat

6.1 Introduction

Milk Fat is one of the most important components of milk, influencing technological, sensorial, and nutritional properties of milk and dairy products (Cozma et al., 2013). Milk fat is composed of triacylglycerols (**TAG**; >95% of total lipids) with about 400 fatty acids (**FA**) with different chain lengths, degrees of saturation and *sn*-positional distribution (Jensen, 2002). The wide range of TAG species with different composition and structure result in a great range of melting and crystallization temperatures (from 0°C to 40°C), which define many quality specifications such as mouth feel in chocolate or ice cream, and lightness of pastry (Karupaiah and Sundram, 2007; Fredrick et al., 2011). Therefore, a better understanding of factors determining variations in TAG composition and structure is of great interest for product application purposes.

Triacylglycerol composition and structure are influenced by feeding regime and genetic factors (Capuano et al., 2014; Tzompa-Sosa et al., 2014, 2016). Feeding regime is an easy-to-implement and effective managerial strategy to modify FA composition and TAG profile. Feeding dairy cows with PUFA present in fresh grass reduces *de novo* synthesized FA and increases the long-chain FA (**LCFA**) in milk fat (Chilliard et al., 2001), probably inducing significant changes in TAG composition. Seasonal changes in TAG composition occur mainly due to alterations in cow's diet, which goes from a silage-based diet (winter) to a fresh grass-based diet (summer) irrespective of the country (Capuano et al., 2014; Liu et al., 2017; Tzompa-Sosa et al., 2018a). Capuano et al (2014) reported that milk fat of cows rich in unsaturated C18 FA (*e.g.* linolenic acid), shows relative decreases in concentrations of TAG CN34, CN36, CN42, CN44, and CN46; and relative increases in concentrations of TAG CN24, CN26, CN40, CN50, CN52, and CN54 compared with cows on a winter diet. Liu et al. (2017) found that levels of unsaturated TAG increased in summer compared with winter, indicating a positive correlation between fat content and TAG CN36:1 and CN42:1. Overall, these studies highlighted differences in seasonal milk fat TAG composition, where the summer milk fat TAG profile was found to have increased levels of high-carbon-number TAG (>50) and unsaturation levels.

Milk TAG composition can also be influenced by genetic factors (Tzompa-Sosa et al., 2016). Milk fat TAG are synthesized via the glycerol-3-phosphate pathway, where the enzyme diacylglycerol O-acyltransferase 1 (**DGAT1**) is of interest due to its role in the final step of triglyceride synthesis (Coleman and Lee, 2004; Palmquist, 2006).and because it is polymorphic in many dairy cattle populations. DGAT1 K232A polymorphism, has been shown to affect milk fat content and the FA composition, inducing changes in TAG composition. DGAT1 KK genotype has been associated with increased fat content (%), higher concentrations of saturated fat and C16:0, lower concentrations of C14:0, unsaturated C18, and CLA (Schennink et al., 2007; Tăbăran et al., 2015; Bovenhuis et al.,

2016). Few studies investigated the effect of DGAT1 K232A polymorphism on TAG composition. A recent study by Tzompa et al. (2016) reported significant effects of DGAT1 K232A polymorphism on TAG composition in winter milk fat samples. They suggested that genotype KK is associated with increased levels of the TAG species CN38 in winter milk fat. However, the combined influence of season and DGAT1 K232A polymorphism on milk fat TAG composition is unknown. Duchemin et al. (2013) reported significant DGAT1 K232A by season interaction for FA C4:0, C6:0, C8:0 C10:0, 12:0, 14:0, C16:1*cis*9, C18:1*cis*9, SFA and UFA in milk fat, but no information is available on the interaction of both factors on TAG composition.

In the present study, we aim to investigate the changes in TAG composition as affected by the changes in cow's diet due to the season, variations in the DGAT1 K232A polymorphism and the season by DGAT1 interaction.

6.2 Materials and Methods

6.2.1 Sample selection

For this study 50 milk fat samples in winter and 50 milk fat samples in summer were used from 25 cows with the DGAT1 KK genotype and 25 cows with the DGAT1 AA genotype. These cows were randomly selected from the total samples which were collected as part of the Dutch Milk Genomics Initiative project. Within this project, 1918 morning milk samples were collected from individual cows (Duchemin et al., 2013). The cows that were included were on 37 different herds and originated form 22 different sires. For the winter milk samples the cows were between 79 and 249 DIM and for the summer milk samples cows were between 163 and 332 DIM. Sodium azide (0.03%) was added to the milk to prevent microbiological growth. Milk fat was extracted from the milk samples as described by Tzompa-Sosa et al (2014). This fat extraction ensures that only non-polar lipids will be present in the extract. After extraction, the fat was stored at -80°C until further analysis.

6.2.2 FA analysis

Fatty acid methyl esters (**FAME**) were prepared from milk fat as described by ISO Standard 15884 (ISO-IDF, 2002). Methyl esters were analyzed with the method ISO Standard 16958 (ISO-IDF, 2015) by a gas chromatography (**GC**; Thermo Focus, Thermo Fisher, Italy) using an Agilent CPSil 88 FAME column (100m x 0.25mm I.D.; film 0.2µm). The standard used for identification and quantification of the FA were pure FA methyl ester (Sigma-Aldrich; Larodan). This analysis was done at the COKZ laboratory (Qlip NV, Leusden, the Netherlands).

6.2.3 TAG profiling using GC-Flame-Ionization Detection (FID)

The identification and quantification of TAG composition was carried out according to ISO Standard 17678/IDF 202 (ISO-IDF, 2010). The standard mix used for identification of the TAG was BCR 519 (anhydrous milk fat) mix (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The TAG were separated by carbon number using GC fitted with a flame-ionization detector (**FID**) and a column injector port (Thermo Trace GC ultra, Thermo Scientific, Rodano, Italy). The GC column was UltiMetal CP7532 (5 m × 0.53 mm × 0.17 µm, Varian, The Netherlands). TAG were identified by comparing their retention times to those found in the TAG standard mixture with certified reference materials BCR 632a and BCR632b (Institute for Reference Materials and Measurements, IRRM, Geel, Belgium). The results were expressed as percentage of the normalized peak areas.

6.2.4 Statistical analysis

The effect of DGAT1 K232A polymorphism and seasonal changes on the TAG and FA composition in milk fat was examined using the following linear mixed model:

$$Y_{jklm} = \mu + Season_j + DGAT1_k + Cow_l + \varepsilon_{jklm} \quad \text{(Model 6.1)}$$

where Y_{jklm} are observations for individual carbon number (TAG species from CN26 – CN54), fixed effects are the overall mean µ; Season is the effect of summer or winter, DGAT1 is the effect of AA and KK genotypes, Cow_l is the random effect accounting for repeated observations of cows in winter and summer and ε_{jklm} is the random residual error. Cow is assumed to be distributed as $N(0,M\sigma^2_{cow})$ where M is the unstructured variance-covariance matrix.

It is known that DGAT1 K232A polymorphism has a major effect on milk fat content. To investigate whether potential effects of the DGAT1 K232A polymorphism on TAG can be explained based on its effect on fat content we also performed analysis in which we adjusted for differences in fat content using the following model:

$$Y_{jklm} = \mu + Season_j + DGAT1_k + \beta Fat_{jklm} + Cow_l + \varepsilon_{jklm} \quad \text{(Model 6.2)}$$

where the effects are as described previously and β is a regression coefficient and Fat_{jklm} is a covariable accounting for differences in fat content.

Possible Season and DGAT1 interactions were analyzed using the following models:

$$Y_{jklm} = \mu + Season_j + DGAT1_k + (Season*DGAT1)_{jk} + Cow_l + \varepsilon_{jklm} \quad \text{(Model 6.3)}$$

$$Y_{jklm} = \mu + Season_j + DGAT1_k + \beta Fat_{jklm} + (Season*DGAT1)_{jk} + Cow_l + \varepsilon_{jklm} \quad \text{(Model 6.4)}$$

where the effects are as described previously and $(Season * DGAT1)_{jk}$ is the interaction between season and DGAT1 genotype.

The significance of fixed effects on the different traits (TAG and FA) was tested using the Proc Mixed method in the SAS/STAT statistic software. The Kenward and Rogers approximation (Roger et al., 1997) was used for calculating degrees of freedom.

The Bonferroni correction was used to determine the appropriate significance level while accounting for multiple testing. The number of independent traits was determined based on a principal component analysis. Five principal components were identified that represented more than 90% of the variation in the data and the corrected *P* value was calculated as $P = {}^{\alpha}/_{n} = {}^{0.05}/_{5}$ defining as cut-off significance value *P*<0.01. Finally, the correlation between TAG and FA was calculated using the Pearson correlation coefficient.

6.3 Results and Discussion

6.3.1 Effect of season and DGAT1 genotypes on fat content

Differences in fat content between seasons and DGAT1 K232A genotypes are shown in **Table 6.1** and **Table 6.2** (Model 6.1). No significant differences (Table 6.2, Model 6.1) were observed between winter and summer milk fat content (*P*=0.11). However, there is a tendency for higher milk fat content in winter (-0.14), similar as reported in previous studies (Couvreur et al., 2006; Heck et al., 2009; Duchemin et al., 2013). The intake of fresh grass in summer increases the content of C18:3*cis*9,12,15 (linolenic acid), which is related to high concentrations of long-chain PUFA in the blood that reduces milk fat content due to impaired rumen biohydrogenation. The increased concentration of PUFA (intermediate rumen biohydrogenation products) has an impact on ruminants fermentation and fibre digestibility, resulting in lower acetate production leading to lower milk fat synthesis (Harvatine et al., 2009). The DGAT1 K232A genotype KK cows had a higher fat content than the AA cows (-1.18; *P*<0.001; Table 6.2, Model 6.1). The association of KK genotype with increased fat content was previously reported (Schennink et al., 2007; Da Silva et al., 2010; Tăbăran et al., 2015), where Schennink et al (2007) reported a smaller difference between AA and KK genotypes based on a larger sample size and considering only winter milk samples.

Table 6.1. Descriptive statistics for season (winter/summer) and DGAT1 K232A genotypes (AA/KK) for fat content (%) and relative triacylglycerols (TAG) composition [mean values (% wt/wt), SD] based on milk fat samples from 50 individual cows.

	SEASON				DGAT1 K232A			
	WINTER		SUMMER		AA		KK	
Trait	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fat Content (%)	4.49	0.84	4.28	1.07	3.79	0.83	5.08	0.57
TAG								
CN26	0.28	0.03	0.29	0.04	0.28	0.04	0.29	0.04
CN28	0.86	0.13	0.87	0.14	0.87	0.15	0.86	0.12
CN30	1.51	0.23	1.49	0.25	1.50	0.26	1.50	0.22
CN32	2.91	0.43	2.80	0.48	2.86	0.49	2.84	0.42
CN34	6.51	0.68	6.16	0.89	6.31	0.84	6.36	0.78
CN36	11.91	1.31	11.23	1.53	11.16	1.49	12.04	1.28
CN38	13.16	0.55	12.87	1.10	12.77	0.86	13.29	0.83
CN40	10.27	0.64	10.64	0.74	10.43	0.67	10.48	0.77
CN42	8.22	0.74	8.18	0.83	8.12	0.78	8.29	0.78
CN44	8.00	0.73	7.69	0.85	7.81	0.85	7.88	0.75
CN46	8.52	0.56	8.28	0.82	8.40	0.74	8.40	0.68
CN48	9.59	0.63	9.47	0.81	9.63	0.74	9.42	0.70
CN50	9.95	1.08	10.18	1.36	10.29	1.33	9.81	1.06
CN52	6.57	1.41	7.41	2.48	7.52	1.95	6.39	2.03
CN54	1.64	0.93	1.92	1.55	1.75	1.22	1.81	1.37

Table 6.2. Effects of season (winter/summer) and DGAT1 K232A genotypes (AA/KK) on fat content (%) and triacylglycerols (TAG) composition (model estimates and *P* values) based on 50 individual cow samples.[1]

	SEASON				DGAT1 K232A			
	MODEL 6.1		MODEL 6.2		MODEL 6.1		MODEL 6.2	
Item	Estimate Summer	*P* value	Estimate Summer	*P* value	Estimate AA	*P* value	Estimate AA	*P* value
Fat Content (%)	-0.14	0.11	-	-	-1.18	0.00**	-	-
TAG								
CN26	0.01	0.40	0.01	0.38	-0.01	0.36	-0.01	0.58
CN28	0.00	0.90	0.00	0.99	0.01	0.73	0.00	0.96
CN30	-0.03	0.60	-0.03	0.56	0.00	0.97	0.01	0.94
CN32	-0.11	0.21	-0.13	0.16	0.02	0.89	-0.07	0.71
CN34	-0.36	0.03	-0.40	0.01*	-0.07	0.75	-0.34	0.26
CN36	-0.76	0.01*	-0.76	0.01*	-0.88	0.01*	-0.86	0.06
CN38	-0.30	0.13	-0.25	0.20	-0.53	0.02	-0.19	0.55
CN40	0.37	0.02	0.38	0.02	-0.04	0.81	0.00	1.00
CN42	-0.06	0.65	-0.07	0.63	-0.18	0.43	-0.16	0.61
CN44	-0.33	0.06	-0.36	0.04	-0.09	0.67	-0.21	0.51
CN46	-0.25	0.12	-0.28	0.08	-0.01	0.97	-0.17	0.55
CN48	-0.13	0.43	-0.17	0.32	0.22	0.24	-0.11	0.68
CN50	0.25	0.30	0.23	0.36	0.45	0.18	0.10	0.83
CN52	0.90	0.03	0.95	0.02	1.13	0.04	1.19	0.13
CN54	0.81	0.00**	0.80	0.00**	-0.37	0.26	-0.58	0.24

[1] Estimates and *P* values calculated from models 6.1 and 6.2. Results are from the solution for fixed effects where genotype KK and winter were set to zero.

* *P*<0.01, ** *P*<0.001: significantly different TAG.

6.3.2 Season

Descriptive statistics for the milk fat TAG profiles is shown in **Table 6.1**.The most abundant TAG species present in both seasons (winter and summer) were CN36, CN38, CN40, CN48 and CN50. These TAG composition profiles were similar to previous studies using milk fat from individual cows (Tzompa-Sosa et al., 2016; Liu et al., 2017) and bulk milk fat samples (Capuano et al., 2014; Larsen et al., 2014; Tzompa-Sosa et al., 2018b). The relative concentrations of each identified TAG species (CN26–CN54) were within the ranges that have been reported in the review by Jensen (2002).

The TAG species CN36 and CN54 were significantly different (P<0.01) between winter and summer when not accounting for fat content (Table 6.2, Model 6.1). Adjusting for differences in fat content CN34 was also significantly different between seasons (Table 6.2, Model 6.2). These results indicate that there is an effect of season on TAG composition in milk fat. The effect of season is most likely due to diet changes between seasons. Winter milk fat samples showed a higher relative concentration in the TAG species CN34 (-0.40; Table 6.2, Model 6.2) and CN36 (-0.76; Table 6.2, Model 6.1). The summer milk fat samples on the other hand, showed higher relative concentration in the TAG species CN40 (0.37%), CN52 (0.90%) and CN54 (0.81;Table 6.2, Model 6.1).

To further explore the differences between summer and winter TAG profiles and FA composition, we calculated Pearson correlation coefficients between TAG and FA (**Table 6.3**). Tzompa et al. (2016) showed that FA with common synthesis pathways have a similar effect on TAG composition. This trend was also observed in our results; the correlations were similar between TAG and FA for TAG species CN26-CN34 and CN48-CN52 with *de novo* FA (C6:0 to C14:0). The *de novo* FA (C6:0 to C14:0) were positively correlated with TAG species CN26 to CN34 and negatively correlated with TAG species CN48 to CN52. Most of the FA with chain length >18 carbons were positively correlated with TAG species CN50, CN52 and CN54, which indicates that most likely, these FA are in the structure of these TAG species. These findings are consistent with previous studies (Capuano et al., 2014; Tzompa-Sosa et al., 2016; Liu et al., 2017) and are associated to the fact that feeding dairy cows with PUFA present in pastures (C18:3*cis*9,12,15) reduces the *de novo* synthesized FA and increases the LCFA in milk fat (Chilliard et al., 2001); hence, inducing a significant increase in the high molecular weight (**HMW**) TAG species CN52 and CN54 in summer. Accordingly, it was reported that the HMW TAG species (CN50–CN54) are mainly composed of C18:0, C18:1*cis*9 and C16:0 (Gresti et al., 1993; Jensen, 2002). The FA composition is reported in **Supplemental Table S6.1**. These FA differed significantly (P<0.001) between seasons for both models (**Supplemental Table S6.2**).

Table 6.3. Pearson correlation coefficients between triacylglycerols (TAG) and fatty acids (FA) in milk fat.

		TAG														
FA	Fat (%)[1]	CN26	CN28	CN30[c]	CN32[c]	CN34[a,c]	CN36[a,b]	CN38[b]	CN40[a,c]	CN42[c]	CN44	CN46	CN48	CN50[b,c]	CN52[a,c]	CN54[a]
C4:0	-0.05	-0.02	-0.15	-0.14	-0.11	0.08	0.33*	0.47**	0.08	-0.32*	-0.46**	-0.51**	-0.28*	0.11	0.17	0.02
C6:0	0.32*	0.24*	0.50**	0.62**	0.66**	0.66**	0.55**	0.62**	0.40**	0.59**	0.29*	-0.11	-0.69**	-0.76**	-0.64**	-0.07
C8:0	0.22	0.26*	0.69**	0.78**	0.78**	0.58**	0.26*	0.26*	0.50**	0.80**	0.53**	0.12	-0.58**	-0.82**	-0.66**	-0.15
C10:0	0.21	0.24*	0.73**	0.83**	0.84**	0.61**	0.21	0.15	0.45**	0.89**	0.66**	0.26*	-0.50**	-0.85**	-0.71**	-0.17
C10:1	0.05	0.24*	0.43**	0.45**	0.41**	0.33*	-0.02	-0.12	0.15	0.46**	0.50**	0.40**	0.01	-0.42**	-0.44**	-0.07
C11:0	0.30**	0.13	0.61**	0.61**	0.55**	0.31*	0.07	-0.05	0.30*	0.63**	0.48**	0.25*	-0.23	-0.53**	-0.52**	-0.07
C12:0	-0.10	0.08*	0.61**	0.68**	0.79**	0.57**	-0.02	-0.13	0.20	0.73**	0.73**	0.54**	-0.14	-0.70**	-0.57**	-0.27*
C12:1	-0.13	0.09	0.35*	0.35*	0.38**	0.24*	-0.24*	-0.36*	-0.01	0.40**	0.58**	0.61**	0.24*	-0.33*	-0.32*	-0.12
C14:0 iso	0.09	0.02	-0.29*	-0.30*	-0.37*	-0.25*	-0.06	0.03	-0.11	-0.25*	-0.26*	-0.15	0.10	0.27*	0.20	0.37*
C14:0	-0.23	0.12	0.56**	0.60**	0.71**	0.62**	0.10	-0.10	0.04	0.62**	0.62**	0.49**	-0.05	-0.58**	-0.55**	-0.28*
C15:0*iso*	-0.03	-0.04	-0.16	-0.20	-0.23	-0.22	-0.08	0.04	0.06	-0.20	-0.32*	-0.33*	-0.16	0.15	0.19	0.42*
C14:1*cis*9	-0.01	0.08	0.04	0.02	0.01	0.03	-0.19	-0.32*	-0.25*	0.07	0.33*	0.52**	0.46**	0.00	-0.14	-0.06
C15:0*anteiso*	-0.30*	-0.07	-0.10	-0.24*	-0.32*	-0.36*	-0.40**	-0.34*	0.02	-0.29*	-0.23*	-0.12	0.09	0.26*	0.32*	0.55**
C15:0	0.25*	0.08	0.00	0.00	-0.08	-0.02	0.12	0.06	0.00	0.00	-0.07	-0.09	-0.02	0.04	-0.13	0.23*
C16:0*iso*	0.05	0.01	-0.18	-0.19	-0.28	-0.22	-0.09	0.05	0.04	-0.18	-0.29*	-0.26*	-0.07	0.17	0.21	0.34*
C16:0	0.42**	-0.07	-0.28*	-0.15	-0.02	0.37**	0.71**	0.43**	-0.55**	-0.05	0.13	0.22	0.21	-0.06	-0.43**	-0.23
C16:1*trans*9	-0.29*	0.00	0.01	-0.13	-0.27*	-0.41**	-0.42**	-0.34**	0.23*	-0.23*	-0.33*	-0.31*	-0.06	0.28*	0.41**	0.46**
C16:1*cis*9	0.37**	0.09	-0.35*	-0.33*	-0.34*	-0.21	0.09	-0.01	-0.35*	-0.18	0.06	0.28*	0.42**	0.20	-0.01	-0.06
C17:0*iso*	-0.42**	-0.10	-0.20	-0.34*	-0.46**	-0.52**	-0.54**	-0.40**	0.00	-0.40*	-0.36**	-0.17	0.21	0.46**	0.55**	0.49**
C17:0*anteiso*	-0.26*	0.02	-0.09	-0.19	-0.29*	-0.37*	-0.32*	-0.31*	0.02	-0.26*	-0.25*	-0.11	0.14	0.23*	0.32*	0.42**
C17:0	0.01	-0.01	-0.34*	-0.39**	-0.53**	-0.46**	-0.11	0.03	0.03	-0.43**	-0.55**	-0.50**	-0.04	0.48**	0.47**	0.29*
C17:1*cis*9	-0.03	0.00	-0.55**	-0.61**	-0.71**	-0.65**	-0.32*	-0.13	-0.11	-0.55**	-0.49**	-0.28*	0.26*	0.63**	0.63**	0.21
C18:0	0.07	-0.11	-0.21	-0.2*5	-0.30*	-0.39**	-0.21	0.14	0.37*	-0.20	-0.46**	-0.55**	-0.37**	0.21	0.41**	0.34*
C18:1*trans*6	-0.31**	-0.17	0.15	0.03	-0.10	-0.41**	-0.55**	-0.42**	0.28*	-0.15	-0.24*	-0.22	-0.02	0.21	0.45**	0.04
C18:1*trans*9	-0.39**	-0.08	0.24*	0.12	-0.03	-0.41**	-0.58**	-0.51**	0.21	-0.09	-0.06	-0.01	0.12	0.18	0.38**	0.05
C18:1*trans*10	-0.23*	-0.10	0.13	0.05	-0.07	-0.38**	-0.53**	-0.54**	0.18	-0.07	-0.08	-0.02	0.08	0.14	0.33*	-0.15
C18:1*trans*11	-0.24*	0.00	0.06	-0.07	-0.22	-0.39**	-0.39**	-0.29*	0.24*	-0.18	-0.29*	-0.33*	-0.10	0.23*	0.37**	0.45**
C18:1*cis*9	-0.40**	-0.08	-0.29*	-0.45**	-0.56**	-0.71**	-0.67**	-0.41**	0.13	-0.53**	-0.52**	-0.33*	0.19	0.63**	0.82**	0.19
C18:1*cis*11	-0.17	0.09	0.11	0.04	-0.05	-0.29*	-0.49**	-0.29*	0.23*	-0.01	-0.07	-0.09	-0.02	0.12	0.37**	0.01
C18:1*cis*12	-0.20	0.07	0.39**	0.38**	0.31*	0.00	-0.30*	-0.29*	0.17	0.22	0.22	0.17	0.00	-0.17	0.01	-0.21

(continuation **Table 6.3.**)

		TAG														
FA	Fat (%)[1]	CN26	CN28	CN30[c]	CN32[c]	CN34[a,c]	CN36[a,b]	CN38[b]	CN40[a,c]	CN42[c]	CN44	CN46	CN48	CN50[b,c]	CN52[a,c]	CN54[a]
C18:1*cis*13	-0.29*	0.09	0.08	-0.01	-0.13	-0.39**	-0.60**	-0.45**	0.24*	-0.08	-0.10	-0.08	0.06	0.23	0.41*	0.28*
C19:0	-0.12	0.00	-0.15	-0.16	-0.24	-0.20	-0.11	0.02	0.12	-0.18	-0.29*	-0.33*	-0.12	0.21	0.26*	0.10
C18:2*cis*9,12	-0.37**	0.14	0.33*	0.26*	0.17	-0.15	-0.40*	-0.33*	0.20	0.12	0.08	0.05	0.02	-0.01	0.20	-0.22
C18:3*cis*9,12,15	-0.25*	0.02	-0.12	-0.23*	-0.34*	-0.42**	-0.48**	-0.21	0.17	-0.27*	-0.35*	-0.31*	-0.04	0.32*	0.46**	0.35*
FA groups																
SFA[2]	0.40**	0.04	0.17	0.34*	0.49**	0.72**	0.75**	0.54**	-0.16	0.44**	0.40**	0.23*	-0.20	-0.55**	-0.74**	-0.27*
UFA[3]	-0.40**	-0.04	-0.19	-0.36*	-0.51**	-0.72**	-0.74**	-0.52**	0.15	-0.45**	-0.43**	-0.24*	0.22	0.58**	0.76**	0.25*
SCFA[4]	0.12	0.21	0.73**	0.84**	0.91**	0.74**	0.31*	0.29*	0.44**	0.82**	0.60**	0.21	-0.55**	-0.89**	-0.72**	-0.24
MCFA[5]	0.36**	-0.01	-0.16	-0.03	0.11	0.47**	0.67**	0.33*	-0.56**	0.09	0.30*	0.40*	0.28*	-0.17	-0.55**	-0.26*
LCFA[6]	-0.35**	-0.08	-0.21	-0.37**	-0.51**	-0.73**	-0.69**	-0.37**	0.28*	-0.45**	-0.55**	-0.46**	0.00	0.55**	0.81**	0.34*
OBCFA[7]	-0.09	0.03	-0.21	-0.31*	-0.44**	-0.45**	-0.29*	-0.20	0.06	-0.32*	-0.39**	-0.28*	0.06	0.37*	0.37*	0.52**

[a] FA and TAG significantly different (P<0.05) between seasons (winter/summer); [b] FAs and TAG significantly different (P<0.05) between DGAT1 genotypes AA/KK; [c] FAs and TAG with significant difference (P<0.05) for the interaction effect DGAT1 genotypes and season; * Correlation with significant difference (P<0.05); ** Correlation with significant difference (P<0.001); [1] Fat (%): milk fat content; [2] SFA: saturated FA, includes C4:0, C5:0, C6:0, C8.0, C10:0, C12:0, C14:0, C15:0, C16:0, C18:0, C19:0, C20:0, C22:0; [3] UFA: unsaturated FA, includes C16:1*trans*9, C16:1*cis*9, C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*trans*12, C18:1*cis*9, C18:1*trans*15, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:2*cis*9,12, C18:3*cis*9,12,15, C18:2*cis*9,*trans*11, C20:1*cis*11, C20:3*cis*8,11,14, and other unsaturated FA with lower relative concentrations not included in the table; [4] SCFA: short-chain FA, include FA from C4:0 to C12:0; [5] MCFA: medium-chain FA, includes FA from C12:0 to C17:1*cis*9; [6] LCFA: long-chain FA, includes FA from C18:0 to DHA (not reported in the table); [7] OBCFA: odd- and branched-chain FA, includes C14:0*iso*, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, C17:0*iso*, C17:0*anteiso*, C17:0 and C17:0*cis*9 (Vlaeminck et al., 2006).

The *cis*-9 FAs, resulting from the Δ9 desaturase activity in the mammary gland, had similar significant correlation trends between TAG species CN28 to CN46 and CN48 to CN52. The FA C17:1*cis*9 and C18:1*cis*9 were negatively correlated with TAG species CN28 to CN38 and CN42 to CN46 and positively correlated with CN48 to CN52. The FA C16:1*cis*9 was negatively correlated with CN28 to CN32 and along with C14:1*cis*9 was negatively correlated to CN40 and positively correlated with CN44 and CN48. The FA C18:1*cis*9 was found to differ significantly among seasons (P<0.001), with increased concentrations in summer. According to previous studies (Jensen, 2002), C18:1*cis*9 is incorporated in the structure of TAG species CN48 and HMW TAG CN50 to CN52. The increase of CN48 and HMW TAG (CN50–CN54) in summer can be explained by the increased levels of C18:1*cis*9, which can be attributed to the intake of fresh grass. It is known that C18:1*cis*9 is mainly formed from the biohydrogenation of PUFA and the subsequent desaturation of C18:0. Finally, C14:1*cis*9, which is mainly *de novo* synthesized, showed significantly (P<0.001) higher levels in winter as compared to summer. It was negatively correlated with low molecular weight (**LMW**) TAG CN28 to CN32 and positively correlated with medium molecular weight (**MMW**) TAG CN44 to CN48. These findings suggest that there is a preference for C14:1*cis*9 to be included in the structure of these TAG species (CN44 to CN48). In the literature reviewed for this study, we found evidence only for C18:1*cis*9 (Tzompa-Sosa et al., 2014) and its relations with TAG composition and structure. The samples used for Tzompa Sosa et al (2014) were as well collected and selected from the Dutch Milk Genomics project.

The relative concentration of long-chain odd- and branched-chain FA (OBCFA; >C14), specifically C15:0*anteiso*, C17:0*iso* and C17:0*anteiso*, were significantly higher (P<0.001) in summer than in winter in both models (**Supplemental Table S6.2**). These findings are consistent with that of Heck et al (2009). Furthermore, this group of FA was found positively correlated with HMW TAG species CN50 (P<0.05), CN52 (P<0.05) and CN54 (P<0.001), which might be explained by the increase in the forage:concentrate ratio in the feed that leads to higher relative concentrations of *iso* FA and lower concentrations of *anteiso* and linear odd-chain FA (Fievez et al., 2012) available for TAG synthesis. There is limited information on the relation between OBCFA and TAG composition in milk fat. Duncan et al (1978) reported the differences in proportions of branched-chain FA in subcutaneous TAG of barley-fed ruminants but no studies were found on the relation between OBCFA and TAG composition in milk fat. Therefore, this is important to consider when relating TAG composition to crystallization behavior in milk fat because of their low melting points relative to chain length. It is known that *anteiso* FA have particularly low melting points (-14°C to 7°C; Knothe and Dunn, 2009) and are used by some bacteria to maintain their membrane fluidity when exposed to low temperatures (Annous et al., 1997; Vlaeminck et al., 2006).

In winter, as expected, more MMW TAG species (CN34 to CN38 and CN44 to CN48) are present that incorporate mainly short- and medium-chain FA (Capuano et al., 2014). According to previous studies (Gresti et al., 1993; Jensen, 2002; Tzompa-Sosa et al., 2014), the FA composition suggested for these TAG species are SCFA, C14:0, C16:0, C18:0, and C18:1*cis*9. In our study, all FA besides SCFA were found to be different (P<0.010) between seasons for both models (Supplemental Table S6.2); C14:0 was no longer significantly different between seasons when differences in fat content where not accounted for in the model.

In general, it can be concluded that the main changes between winter and summer milk fat TAG composition were the higher levels of HMW TAG species CN54 in summer and higher levels of MMW TAG (CN34 and CN36) species in winter. These differences can be explained by the changes in FA composition (C14:0, C16:0, C18:0 C18:1*cis*9, UFA, and LCFA), which are most likely due to differences in diet between seasons.

6.3.3 DGAT1 K232A polymorphism

The effects of DGAT1 genotype AA and KK on milk fat TAG composition are presented in **Table 6.2.** Milk fat samples from the genotype AA had higher relative concentrations of TAG species CN28 (0.01), CN32 (0.02), CN48 (0.22), CN50 (0.45), and CN52 (1.13) (**Table 6.2**, Model 6.1). The TAG species CN34 (-0.07), CN36 (-0.88), CN38 (-0.53) , CN42 (-0.18), CN44 (-0.09), and CN54 (-0.37) were higher for genotype KK (Table 6.2, Model 6.1). No TAG species were significantly different (P<0.01) between genotypes when accounting for differences in fat content (Model 6.2). If differences in fat content were not considered in the model (Table 6.2, Model 6.1) significant difference (P<0.01) were observed for TAG species CN36. Therefore, the DGAT1 K232A genotype did affect the TAG composition in milk fat but these effects can be explained by the effects of the DGAT1 K232A polymorphism on fat content (Table 6.2, Model 6.2). The TAG species CN36 is positively correlated with short- and medium-chain FA (mainly SFA). Schennink et al. (2008) showed that the DGAT1 K232A KK genotypes is associated with higher fat content and higher levels of *de novo* synthesized short and medium-chain FA, explaining the higher levels for CN36. Tzompa et al. (2016) reported significant difference for TAG species CN38 between AA and KK genotypes in winter samples, which positively associated with the effect of genotype KK on the TAG composition in milk fat; this difference was not found in our study but it was in the same direction (P=0.02). Tzompa et al. (2016) selected cows with similar fat content in their study and therefore differences in fat content were relatively small. In the current study, differences in fat content between cows are taken into account using model 6.2. Hence, a model that does not account for differences in fat content shows an effect of DGAT1 K232A genotype on TAG composition (in our study, on TAG CN36). Model 6.2 shows that DGAT1 K232A genotype has no effect on TAG composition after accounting for differences in fat content in milk fat. These results should not be influenced by the different lactation stages

(DIM) in summer and in winter of the cows selected for this study. Bovenhuis et al. (2015) showed that the effect of DGAT1 is not constant but changes during lactation. However these changes occur rarely during early lactation and therefore are not expected to affect the results reported.

6.3.4 Interaction of DGAT1 K232A polymorphism and season

The significance of the interaction of DGAT1 genotype (AA/KK) and season (winter/summer) on milk fat TAG composition is shown in **Table 6.4**. The TAG species CN32 (Model 6.3), CN42, and CN52 (Model 6.4) were found to be significant (P<0.01).

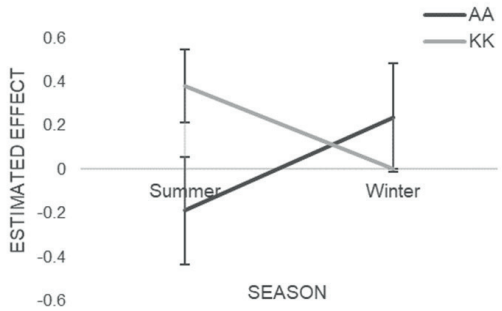
Table 6.4. Interaction seasonxDGAT1 K232A genotypes effect on Fat content (%) and TAG composition (P value) based on 25 individual cow samples for each genotype.

	MODEL 6.3	MODEL 6.4
Item	*P* value	*P* value
Fat Content (%)	0.63	
TAG	*P* value	*P* value
CN26	0.23	0.24
CN28	0.16	0.13
CN30	0.03	0.03
CN32	0.02	0.01*
CN34	0.03	0.02
CN36	0.14	0.13
CN38	0.16	0.19
CN40	0.05	0.05
CN42	0.00**	0.00**
CN44	0.24	0.18
CN46	0.98	0.93
CN48	0.08	0.11
CN50	0.0*	0.03
CN52	0.01*	0.01*
CN54	0.16	0.15

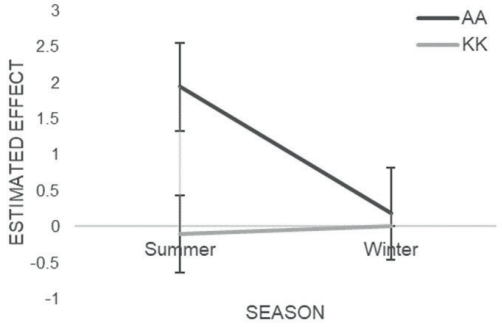
*P<0.05, **P<0.001: significantly different TAG; TAG species found significantly different for both models 6.3 and 6.4 show that regardless of whether differences in fat content are considered, there is an effect of the interaction of season × DGAT1 K232A genotypes on that TAG species.

Figure 6.1 shows the estimated effects between AA and KK genotypes and season for TAG species CN42 and CN52, which showed a significant (P<0.01) DGAT1 × season interaction but were not significantly affected by DGAT1 or Season (Table 6.2). The estimated effects were obtained from model 6.3 without the main effects (Season and DGAT1). Effects of DGAT1 genotypes AA and KK on TAG species CN42 are reversed in winter and summer: in summer, KK cows had higher levels of TAG CN42 than AA cows, whereas in winter, AA cows tended to have higher levels than KK cows, although this difference was not significant. In contrast, DGAT1 AA cows had higher levels of CN52 than KK cows in both seasons but in winter the difference was not significant. In summer, UFA and LCFA were higher in AA cows than in KK cows and SFA were higher in KK cows than in AA cows

(**Figure 6.2**). This is in agreement with previous studies (Duchemin et al., 2013; Tăbăran et al., 2015). The SCFA showed a different trend where the KK genotype had higher relative concentrations in summer and lower relative concentrations in winter than the AA genotype (Figure 6.2d).

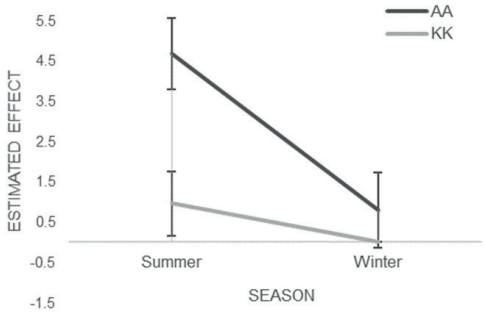


a. Estimated effect of DGAT1 K232A AA KK in winter and summer for TAG species CN42.

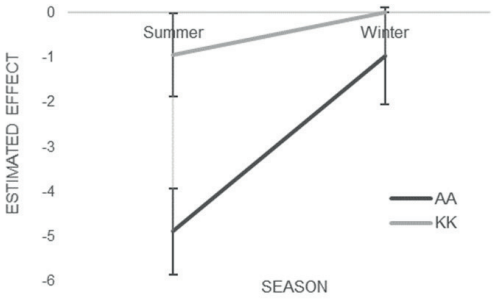


b. Estimated effect of DGAT1 K232A AA KK in winter and summer for TAG species CN52.

Figure 6.1. Estimated effects of DGAT1 K232A AA/KK in winter and summer based on 25 individual cow samples for TAG species: CN42 (a) and 52 (b). Both TAG species were significantly different with and without accounting for differences in fat content for the interaction effect season x DGAT1 K232A AA versus KK. Genotype KK was set to zero in winter for both TAG species. The error bars indicate the standard error (SE) of the solution of the model for fixed effects.

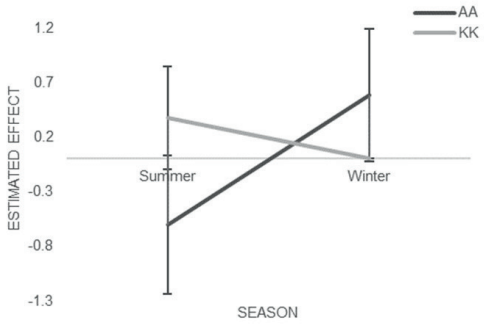


a. Estimated effect of DGAT1 K232A AA KK in winter and summer on the UFA group.



b. Estimated effect of DGAT1 K232A AA KK in winter and summer on the SFA.

c. Estimated effect of DGAT1 K232A AA KK in winter and summer on LCFA.



d. Estimated effect of DGAT1 K232A AA KK in winter and summer on SCFA.

Figure 6.2. Estimated effect of DGAT1 K232A AA/KK in winter and summer for FA groups UFA (a), SFA (b), LCFA (c) and SCFA (d) based on 25 individual cow samples. Genotype KK was set to zero in winter for all FA groups. The unsaturated fatty acids (UFA) include includes C16:1*trans*9, C16:1cis9, C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*trans*12, C18:1*cis*9, C18:1*trans*15, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:2*cis*9,12, C18:3*cis* 9,12,15, C18:2*cis*9,*trans*11, C20:1*cis*11, C20:3*cis*8,11,14, and other unsaturated FA with lower relative concentrations. The saturated fatty acids (SFA) include C4:0, C5:0, C6:0, C8.0, C10:0, C12:0, C14:0, C15:0, C16:0, C18:0, C19:0, C20:0, C22:0; long-chain fatty acids (LCFA) include FA from C18:0 to docosahexaenoic acid (DHA); short-chain fatty acids (SCFA) include from C4:0 to C12:0. The error bars indicate the standard error (SE) of the solution of the model for fixed effects.

Our findings suggest that dietary FA variations due to seasonal changes have affected the FA esterified by the different DGAT1 genotypes. According to previous studies, the structure of the TAG species CN42 and CN52 have the following FAs in the *sn*-3 position: CN42: C10:0, C12:0, C8:0, C6:0, C16:1, C14:1*cis*9 and CN52: C16:0, C16:1*cis*9 and C18:1*cis*9 (Gresti et al., 1993; Jensen, 2002). Consequently, in our study significant differences in both models (P<0.01) were found in FA C6:0, C10:0 and C18:1*cis*9 levels under the interaction effect of DGAT1 genotype and seasons (**Supplemental Table S6.3**). Considering the role of the DGAT1 enzyme as catalyzing agent in the last step of TAG synthesis (Lehner and Kuksis, 1996; Yen et al., 2008) and the association of KK genotype with increased preference for esterifying SCFA and C16:0 in the *sn*-3 position of a diacylglycerol (Tăbăran et al., 2015; Tzompa-Sosa et al., 2016), we speculate that these FA are most likely to be found at the *sn*-3 position and preferred by DGAT1. In addition, we suggest that according to the pool of FA available in each season, the preferences of the genotypes for a specific type of FA might change during TAG synthesis, always keeping the structure characteristics of each TAG species. This could be a mechanism regulated by the cow to lower the melting point of TAG during milk fat synthesis, as previously suggested (Tzompa-Sosa et al., 2016). To achieve this, the mammary gland increases the degree of

unsaturation of the LCFA during lactation in a seasonal system, increases the proportion of SCFA and MCFA and uses asymmetric positioning of SCFA on the glycerol backbone of the TAG (Dils, 1986). Hence, considering the FA in the *sn*-3 position, in summer when the available pool of FA is mainly UFA and LCFA, for TAG species CN42 the KK genotype will most likely esterify UFA as well as SFA. In winter, when the pool of FA is mainly SCFA, MCFA and SFA the AA genotype will favor esterification of SCFA at first (Figure 6.3d) in these TAG structures; therefore, the preference of the genotype will be altered. In summer, the AA genotype will favor the esterification of UFA and LCFA in TAG CN52; whereas the KK genotype will favor esterification of SFA. This is probably because of the positioning of C18:1*cis*9 in CN52 (Gresti et al., 1993; Jensen, 2002). In winter, the AA genotype will likely favor SFA (C16:0) and UFA (C18:1*cis*9) for esterification of CN52. Tzompa-Sosa et al (2016) identified an increase in CN38 in winter milk fat TAG composition; however, in our study, this TAG was not significantly different when the interaction effect of DGAT1 genotype and season was taken into account. Nevertheless, our results show the same trend in AA and KK genotypes on the esterification of MMW TAG species (*i.e*. CN42) in winter.

6.4 Conclusion

The main goal of the current study was to investigate the changes in TAG composition as affected by the changes in cow's diet due to the seasonal variations and genetic traits. The main changes between winter and summer in milk fat TAG composition, with and without considering the differences in fat content, were the increase of TAG species CN54 in summer and the increase of TAG species CN34 and CN36 in winter. Our study suggests that diet changes among seasons have a great effect on milk fat TAG composition. We showed that FA C14:0, C16:0, C18:0, C18:1*cis*9, UFA, and LCFA are the main FA involved in the changes in milk fat TAG composition. The DGAT1 K232A polymorphism has an individual effect on TAG composition when differences in fat content are not accounted for, such that TAG species CN36 showed a significant increase with the KK genotype. Furthermore, the interaction effect of DGAT1 K232A genotype and diet according to seasonal variation was found to be significant with and without accounting for differences in fat content for TAG species CN42 and CN52. Thus, there is an interaction effect of DGAT1 and season on the TAG composition in milk fat. Regardless of the preference of the genotype (AA/KK) and depending on the availability of FA according to season, the mammary gland will most likely esterify UFA (C18:1*cis*9), SCFA (C6:0 and C10:0), and MCFA to the *sn*-3 position on the glycerol backbone of the TAG, keeping the structural characteristics of each TAG. We propose that this is a mechanism of the mammary gland to maintain the melting point of TAG during milk fat synthesis. Further compositional and structural analyses should be done to confirm our proposed mechanism of the DGAT1

K232A polymorphism during milk fat TAG synthesis. To our knowledge, this is the first report where the combined effect of DGAT1 K232A polymorphism was analyzed in milk fat.

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Supplementary Information

Table S6.1. Descriptive statistics for season (winter/summer) and DGAT1 K232A genotypes (AA/KK) for FA composition [mean values (mol %), SD] based on 50 individual cow samples.

	SEASON				DGAT1 K232A			
	Winter		Summer		AA		KK	
FA	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C4:0	9.40	0.57	9.35	0.67	9.39	0.67	9.36	0.57
C5:0	0.05	0.04	0.03	0.04	0.03	0.03	0.05	0.04
C6:0	4.48	0.26	4.38	0.38	4.33	0.34	4.54	0.27
C7:0	0.04	0.03	0.03	0.03	0.03	0.03	0.05	0.03
C8:0	2.18	0.23	2.12	0.28	2.11	0.27	2.19	0.23
C9:0	0.06	0.03	0.04	0.04	0.04	0.03	0.06	0.03
C10:0	3.96	0.62	3.85	0.63	3.85	0.66	3.97	0.58
C10:1	0.52	0.09	0.50	0.10	0.51	0.10	0.52	0.08
C12:0	4.61	0.92	4.54	0.86	4.69	1.00	4.44	0.72
C12:1	0.14	0.04	0.14	0.04	0.14	0.05	0.13	0.02
C13:0	0.11	0.03	0.10	0.03	0.10	0.03	0.11	0.03
C14:0iso	0.09	0.02	0.10	0.03	0.09	0.02	0.10	0.03
C14:0	11.50	0.97	11.22	1.10	11.64	0.99	11.03	1.00
C15:0iso	0.01	0.05	0.21	0.05	0.12	0.11	0.11	0.12
C14:1*cis*9	1.41	0.25	1.26	0.31	1.34	0.32	1.33	0.27
C15:0anteiso	0.46	0.06	0.53	0.11	0.49	0.09	0.49	0.11
C15:0	1.07	0.15	1.03	0.16	0.99	0.14	1.12	0.15
C16:0iso	0.19	0.04	0.20	0.04	0.19	0.04	0.19	0.04
C16:0	29.08	2.78	26.33	3.50	26.48	3.23	29.08	3.17
C16:1*cis*9	1.32	0.40	1.30	0.27	1.18	0.28	1.47	0.34
C17:0iso	0.26	0.03	0.30	0.05	0.29	0.05	0.27	0.04
C17:0anteiso	0.40	0.11	0.52	0.08	0.45	0.13	0.46	0.09
C17:0	0.38	0.05	0.39	0.06	0.38	0.06	0.38	0.05
C17:1*cis*9	0.15	0.03	0.17	0.04	0.16	0.04	0.16	0.03
C18:0	6.76	0.95	7.43	1.14	7.04	1.11	7.16	1.10
C18:1*trans*6	0.17	0.04	0.19	0.06	0.20	0.05	0.16	0.04
C18:1*trans*9	0.12	0.03	0.13	0.03	0.13	0.03	0.11	0.02
C18.1*trans*11	0.66	0.20	0.99	0.55	0.88	0.51	0.77	0.35
C18:1*cis*9	14.47	1.70	16.16	2.91	16.23	2.51	14.29	2.12
C18:1*trans*15	0.17	0.04	0.19	0.09	0.19	0.05	0.18	0.09
C18:1*cis*11	0.33	0.07	0.36	0.11	0.37	0.10	0.31	0.07
C18:1*cis*12	0.16	0.05	0.15	0.05	0.17	0.05	0.14	0.04
C18:1*cis*13	0.07	0.01	0.07	0.03	0.08	0.02	0.06	0.02
C18:2*cis*9,12	0.99	0.25	0.93	0.22	1.05	0.23	0.85	0.21
C18:3*cis*9,12,15	0.35	0.08	0.42	0.12	0.39	0.10	0.38	0.11
C20:0	0.09	0.02	0.08	0.02	0.08	0.02	0.09	0.02
C18:2*cis*9,*trans*11	0.34	0.12	0.48	0.25	0.45	0.23	0.36	0.17
FA groups								
SFA[1]	75.37	2.36	73.00	4.37	73.02	3.50	75.49	3.53
UFA[2]	21.79	2.10	24.13	3.80	24.06	3.25	21.73	2.89
SCFA[3]	25.38	1.73	24.92	1.96	25.04	2.07	25.27	1.59
MCFA[4]	46.59	2.71	43.86	3.75	44.09	3.24	46.49	3.47
LCFA[5]	25.19	2.43	28.36	4.16	27.94	3.72	25.47	3.38
OBCFA[6]	2.99	0.33	3.44	0.43	3.16	0.45	3.29	0.42

[1] SFA: saturated FA, includes C4:0, C5:0, C6:0, C8.0, C10:0, C12:0, C14:0, C15:0, C16:0, C18:0, C19:0, C20:0, C22:0.

[2] UFA: unsaturated FA, includes C16:1*trans*9, C16:1*cis*9, C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*trans*12, C18:1*cis*9, C18:1*trans*15, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:2*cis*9,12, C18:3*cis*9,12,15, C18:2*cis*9,*trans*11, C20:1*cis*11, C20:3*cis*8,11,14, and other unsaturated FA with lower relative concentrations not included in the table.

[3] SCFA: short-chain FA, include FA from C4:0 to C12:0.

[4] MCFA: medium-chain FA, includes FA from C12:0 to C17:1*cis*9.

[5] LCFA: long-chain FA, includes FA from C18:0 to DHA (not reported in the table).
[6] OBCFA: odd- and branched-chain FA, includes C14:0*iso*, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, C17:0*iso*, C17:0*anteiso*, C17:0 and C17:0*cis*9 (Vlaeminck et al., 2006).

Table S6.2. Effects of season (winter/summer) and DGAT1 genotypes (AA/KK) on milk FA composition (model estimates, and *P* value) based on 50 individual cow samples.

	SEASON				DGAT1 K232A			
	MODEL 6.1		MODEL 6.2		MODEL 6.1		MODEL 6.2	
FA	Estimate[1]	*P* value[1]	Estimate[2]	*P* value[2]	Estimate[1]	*P* value[1]	Estimate[2]	*P* value[2]
C4:0	-0.04	0.80	-0.05	0.73	0.02	0.89	-0.04	0.86
C5:0	-0.01	0.29	-0.01	0.24	-0.01	0.03*	-0.01	0.27
C6:0	-0.10	0.08	-0.07	0.21	-0.22	0.02*	0.03	0.80
C7:0	-0.00	0.58	-0.00	0.67	-0.01	0.04	-0.01	0.38
C8:0	-0.06	0.12	-0.06	0.18	-0.08	0.30	-0.01	0.96
C9:0	-0.00	0.69	-0.00	0.72	-0.02	0.02*	-0.00	0.63
C10:0	-0.13	0.17	-0.12	0.25	-0.13	0.48	0.07	0.76
C10:1	-0.02	0.24	-0.02	0.20	-0.01	0.62	-0.03	0.40
C12:0	-0.09	0.57	-0.13	0.43	0.24	0.34	-0.01	0.97
C12:1	0.00	0.99	-0.00	0.88	0.00	0.57	-0.00	0.77
C13:0	-0.01	0.25	-0.01	0.17	-0.02	0.06	-0.02	0.09
C14:0*iso*	0.01	0.03*	0.012	0.03	-0.01	0.22	-0.01	0.42
C14:0	-0.34	0.03*	-0.39	0.01*	0.60	0.04*	0.22	0.58
C15:0*iso*	0.01	0.79	0.01	0.68	-0.01	0.50	-0.03	0.30
C14:1*cis*9	-0.16	0.00**	-0.16	0.00**	0.00	0.97	-0.04	0.73
C15:0*anteiso*	0.07	0.00**	0.06	0.00**	-0.00	0.96	-0.08	0.01*
C15:0	-0.04	0.19	-0.05	0.09	-0.13	0.00**	-0.19	0.00**
C16:0iso	0.01	0.10	0.01	0.12	-0.00	0.90	-0.00	0.86
C16:0	-2.72	0.00**	-2.62	0.00**	-2.71	0.00**	-1.27	0.24
C16:1*cis*9	-0.02	0.73	-0.01	0.83	-0.30	0.00**	-0.26	0.04*
C17:0*iso*	0.04	0.00**	0.04	0.00**	0.02	0.05	-0.01	0.56
C17:0*anteiso*	0.12	0.00**	0.10	0.00**	0.00	0.85	-0.07	0.03*
C17:0	0.01	0.41	0.01	0.41	-0.01	0.67	-0.01	0.70
C17:1*cis*9	0.01	0.05	0.02	0.06	-0.00	0.86	-0.01	0.49
C18:0	0.68	0.00**	0.69	0.00**	-0.07	0.81	0.08	0.85
C18:1*trans*6	0.02	0.06	0.02	0.07	0.04	0.01*	0.02	0.20
C18:1*trans*9	0.01	0.12	0.01	0.13	0.02	0.01*	0.01	0.47
C18:1*cis*9	1.80	0.00**	1.79	0.00**	1.94	0.00**	1.34	0.12
C18:1*trans*15	0.01	0.36	0.01	0.34	0.02	0.17	0.01	0.53
C18:1*cis*11	0.04	0.07	0.04	0.07	0.06	0.01*	0.07	0.03
C18:1*cis*12	-0.01	0.29	-0.01	0.28	0.03	0.01*	0.03	0.08
C18:1*cis*13	0.01	0.01	0.01	0.00**	0.01	0.00**	0.01	0.01*
C18:2*cis*9,12	-0.06	0.20	-0.06	0.15	0.19	0.00**	0.11	0.22
C18:3*cis*9,12,15	0.06	0.01	0.06	0.01*	0.01	0.62	-0.04	0.27
C20:0	-0.01	0.00**	-0.01	0.00**	-0.01	0.11	-0.01	0.36
C18:2*cis*9,*trans*11	0.14	0.00**	0.13	0.00**	0.09	0.08	-0.01	0.88
FA groups								
SFA[3]	-2.51	0.00**	-2.48	0.00**	-2.49	0.01*	-1.45	0.25
UFA[4]	2.49	0.00**	2.49	0.00**	2.29	0.00**	1.45	0.20
SCFA[5]	-0.49	0.15	-0.49	0.16	-0.23	0.66	0.08	0.92
MCFA[6]	-2.63	0.00**	-2.61	0.00**	-2.57	0.00**	-1.69	0.13
LCFA[7]	3.21	0.00**	3.25	0.00**	2.53	0.00**	2.15	0.08
OBCFA[8]	0.45	0.00**	0.42	0.00**	-0.14	0.18	-0.37	0.01*

* Significantly different FA (*P*<0.050).

** Significantly different FA (*P*<0.001).

[1] Estimates and *P* values calculated from model 6.1. Results from the solution for fixed effects where genotype KK and winter were set to zero.

[2] Estimates and *P* values calculated from model 6.2. Results from the solution for fixed effects where genotype KK and winter were set to zero.

[3] SFA: saturated FA, includes C4:0, C5:0, C6:0, C8.0, C10:0, C12:0, C14:0, C15:0, C16:0, C18:0, C19:0, C20:0, C22:0.

[4] UFA: unsaturated FA, includes C16:1*trans*9, C16:1*cis*9, C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*trans*12, C18:1*cis*9, C18:1*trans*15, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:2*cis*9,12, C18:3*cis*9,12,15, C18:2*cis* 9,*trans*11, C20:1*cis*11, C20:3*cis*8,11,14, and other unsaturated FA with lower relative concentrations not included in the table.

[5] SCFA: short-chain FA, include FA from C4:0 to C12:0.

[6] MCFA: medium-chain FA, includes FA from C12:0 to C17:1*cis*9.

[7] LCFA: long-chain FA, includes FA from C18:0 to DHA (not reported in the table).

[8] OBCFA: odd- and branched-chain FA, includes C14:0iso, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, C17:0*iso*, C17:0*anteiso*, C17:0 and C17:0*cis*9 (Vlaeminck et al., 2006).

Table S6.3. Interaction DGAT1 AA/KK and summer/winter effect on milk FA composition (*P* values) based on 25 individual cow samples for each genotype.

	MODEL 6.3	MODEL 6.4
FA	*P* value	*P* value
C4:0	0.66	0.61
C5:0	0.52	0.78
C6:0	0.00**	0.01*
C7:0	0.83	0.92
C8:0	0.03	0.03
C9:0	0.33	0.24
C10:0	0.01*	0.01*
C10:1	0.06	0.05
C12:0	0.26	0.19
C12:1	0.45	0.42
C14:0 *iso*	0.70	0.77
C14:0	0.27	0.17
C14:1*cis*9	0.87	0.85
C15:0*anteiso*	0.87	0.94
C15:0	0.87	0.98
C16:0iso	0.47	0.47
C16:0	0.50	0.44
C16:1*cis*9	0.07	0.06
C17:0*iso*	0.12	0.13
C17:0*anteiso*	0.31	0.36
C17:0	0.20	0.19
C17:1*cis*9	0.00**	0.00**
C18:0	0.90	0.86
C18:1*trans*6	0.51	0.49
C18:1*trans*9	0.36	0.31
C18:1*cis*9	0.01*	0.01*
C18:1*cis*11	0.02	0.02
C18:1*cis*12	0.37	0.35
C18:1*cis*13	0.03	0.01*
C18:2*cis*9,12	0.60	0.63
C18:3*cis*9,12,15	0.48	0.46
FA groups		
SFA[1]	0.03	0.02
UFA[2]	0.01*	0.01*
SCFA[3]	0.02*	0.01*
MCFA[4]	0.60	0.53
LCFA[5]	0.05	0.04
OBCFA[6]	0.32	0.37

* Significantly different FA (*P*<0.010).

** Significantly different FA (*P*<0.001).

[1] SFA: Saturated FA, includes C4:0, C5:0, C6:0, C8.0, C10:0, C12:0, C14:0, C15:0, C16:0, C18:0, C19:0, C20:0, C22:0.

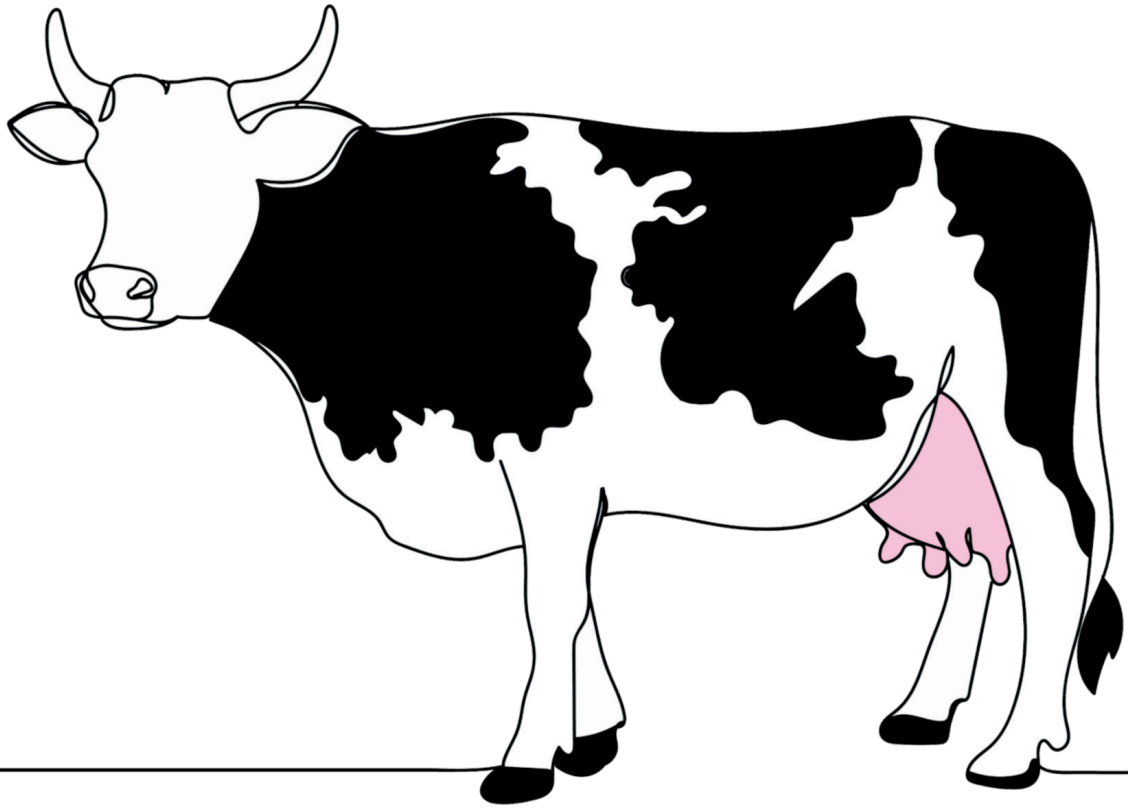
[2] UFA: unsaturated FA, includes C16:1*trans*9, C16:1*cis*9, C18:1*trans*6, C18:1*trans*9, C18:1*trans*10, C18:1*trans*11, C18:1*trans*12, C18:*cis*9, C18:1*trans*15, C18:1*cis*11, C18:1*cis*12, C18:1*cis*13, C18:2*cis*9,12, C18:3*cis*9,12,15, C18:2*cis*9,*trans*11, C20:1*cis*11, C20:3*cis*8,11,14, and other unsaturated FA with lower relative concentrations not included in the table.

[3] SCFA: short-chain FA, include FA from C4:0 to C12:0.

[4] MCFA: medium-chain FA, includes FA from C12:0 to C17:1*cis*9.

[5] LCFA: long-chain FA, includes FA from C18:0 to DHA (not reported in the table).

[6] OBCFA: odd- and branched-chain FA, includes C14:0*iso*, C15:0*iso*, C15:0*anteiso*, C15:0, C16:0*iso*, C17:0*iso*, C17:0*anteiso*, C17:0 and C17:0*cis*9 (Vlaeminck et al., 2006).



Chapter 7

General discussion

Feed and animal related factors are known to affect milk fatty acid (**FA**) and triacylglycerol (**TAG**) composition. Feed related factors refer to changes in the feeding regime, whereas animal related factors include, amongst others, lactation stage, breed and genetics. Until now, most studies available on the effect of animal and feed related factors on milk lipids have assessed the effect of these two factors only on the FA composition. This is important considering three FA and a glycerol molecule are the building blocks that compose the TAG structure. However, further analysis is required on the TAG species that can be formed from a specific FA composition. Depending on the TAG composition, milk fat varies in its technological, sensorial, and nutritional properties. Hence, variations in milk fat TAG composition determine its suitability for dairy and non-dairy food product applications. Therefore, the aim of this research was to assess the effect of feed and animal related factors on the variation in milk fat TAG composition and structure to provide insights on the variation in the TAG composition and structure that can be expected for each factor. Furthermore, this study determines the influence of the variation in TAG composition on the solid fat content (**SFC**), one of the key factors that define the use of a fat for specific food product applications. This chapter compares and discusses the main findings of **chapters 2 to 6**, which are summarized in **Table 7.1**. **Chapters 2 to 4** address feed related factors (seasonal feed variations, and hydrogenated palm FA and protein supplementation), and **chapters 5 and 6** address animal related factors (cow's lactation stage and genetics). The comparison between different chapters will be divided into three parts: 1) the variations in FA and TAG (Sections 7.2 and 7.3), 2) the changes observed in the FA distribution within the TAG structure (Section 7.4), and 3) the relation between the variations in the TAG composition and alterations in milk fat SFC (Section 7.5). Finally, recommendations for future research based on the main conclusions of this thesis will be addressed. The results of this thesis offer insights into the FA and TAG compositional changes of milk fat produced under varying feed and animal related factors. These compositional changes, and their resulting physical changes, are especially relevant for the food industry.

Table 7.1. Summary of the main effects of feed and animal related factors on milk fatty acid (FA) and triacylglycerol (TAG) composition, FA regiospecific distribution, and milk solid fat content (SFC).[1]

	CHAPTERS 2 AND 3 Seasonal variation	CHAPTER 4 Hydrogenated palm FA and protein supplementation	CHAPTER 5 Early/Late Lactation	CHAPTER 6 Genetic variation: DGAT1 A232K / Season
FA composition	• High concentrations of PUFA (especially C18:3*cis*9,12,15 (ALA)) in summer decreased the concentrations of *de novo* synthesized FA and increased the concentrations of biohydrogenation products and CLA isomer FA in spring and summer. • Winter milk fat contained increased concentrations of *de novo* synthesized FA compared to summer.	• Supplementation of hydrogenated palm oil rich in C16:0 and C18:0 decreased the concentrations of *de novo* synthesized FA and increased the concentrations of LCFA in milk fat (Nichols et al., 2018). • Supplementation of protein increased the concentration of *de novo* synthesized FA in milk. (Nichols et al., 2018).	• High concentrations of C18:0 and C18:1*cis*9 mobilized from cows' body fat stores increased the concentrations of these FA in milk fat and decreased the concentrations of *de novo* synthesized FA in early lactation. • Late lactation milk fat presented higher concentrations of *de novo* synthesized FA C8:0 to C16:0 compared to early lactation.	• Summer milk fat presented increased concentrations of C18:0, C18:1*cis*9, C18:3*cis*9,12,15 (ALA), CLA, and OBCFA (>C14). • Winter milk fat presented increased concentrations of C14:0, C14:1*cis*9 and C16:0. • No major FA differed between DGAT1 AA and KK genotypes. • Compared to the DGAT1 AA genotype, SCFA showed higher relative concentrations in summer for the DGAT1 KK genotype, whereas in winter its relative concentrations decreased.
TAG composition	• High concentrations of LCFA and UFA in summer increased the concentrations of HMW TAG groups. • High availability of SCFA and C16:0 in winter increased the formation of LMW and MMW TAG (CN42 to CN46).	• Supplementation of hydrogenated palm FA decreased the concentrations of LMW (CN28 to CN34) and MMW (CN40 to CN46) TAG and increased the concentrations of HMW TAG (CN50 to CN52). • High concentrations of *de novo* synthesized FA and PUFA in response to protein enhanced the formation of most monounsaturated LMW and polyunsaturated MMW TAG species, but only in the absence of palm oil supplementation.	• Higher concentrations of C16:0, C18:0, C18:1*cis*9 and other C18:1 FA in early lactation increased the concentrations of mono- and polyunsaturated HMW, and TAG groups CN38 and CN40. • Higher concentrations of *de novo* synthesized FA in late lactation increased the synthesis of MMW TAG.	• Summer milk fat presented increased concentrations of TAG species CN54, whereas winter showed increased concentrations of TAG groups CN34 and CN36. • In summer, the DGAT1 KK genotype was associated with higher levels for TAG species CN42 than the DGAT1 AA genotype whereas in winter the difference between the genotypes was small. • DGAT1 AA genotype was associated with higher levels of CN52 in summer compared to the DGAT1 KK genotype.
FA Positional Distribution	• Neither summer nor winter feeding regimes influenced the esterification preference of FA over the three positions in the TAG structure. • High concentrations of LCFA and UFA in summer increased the concentrations of these FA at both *sn*-2 and *sn*-1(3) positions in the TAG structure. • High concentrations of *de novo* synthesized FA in winter increased the concentration of these FA at both *sn*-2 and *sn*-1(3) positions in the TAG structure.	• The FA esterification preferences over the three positions in the TAG structure was not influenced by hydrogenated palm FA supplementation. • Hydrogenated palm FA supplemented diets increased the relative concentrations of C16:0 and C18:0 at the *sn*-2 and C18:0 and C18:1*cis*9 at the *sn*-1(3) positions of the TAG structure.	• Neither early nor late lactation FA composition variations affected the esterification preference of the FA over the three positions in the TAG structure. • Except for C16:0, regardless of the lactation stage, high concentrations of FA increased the FA concentrations at all positions over the TAG structure. • Higher concentrations of C16:0 in late lactation compared to early lactation increased C16:0 concentrations at the *sn*-1(3) positions and decreased C16:0 concentrations at the *sn*-2 position in the TAG structure.	
Solid Fat content		• High concentrations of saturated HMW TAG in milk fat of cows supplemented with hydrogenated palm FA increased the SFC at 20, 25, and 30°C. • High concentrations of PUFA in response to protein supplementation decreased milk SFC at 30°C.	• High concentrations of mono- and polyunsaturated HMW TAG in early lactation milk fat decreased the SFC at 0, 10, 20, 25, and 30°C. • High concentrations of saturated LMW and MMW TAG in late lactation milk fat increased the SFC at 0, 10, 20, 25, and 30°C.	

[1] FA: fatty acid; PUFA: polyunsaturated FA; CLA: conjugated linoleic acid; SCFA: short-chain FA; LCFA: long-chain FA; UFA: unsaturated FA; OBCFA: odd-branched chain FA; TAG: Triacylglycerol; LMW TAG: low molecular weight TAG; MMW: medium molecular weight TAG; HMW: high molecular weight TAG; SFC: solid fat content.

7.1. Methodological considerations for milk fat TAG composition analysis: GC-FID vs MALDI-TOF-MS

In this thesis, two methods were implemented to assess the TAG composition: gas chromatography with flame ionization detector (**GC-FID**) and matrix-assisted laser desorption/ionization - time of flight - mass spectrometry (**MALDI-TOF-MS**). The GC-FID is one of the most common methods used for milk fat TAG composition analysis and for quality control of bovine milk fat, whereas MALDI-TOF-MS has only recently come into use for milk fat TAG fingerprinting (Picariello et al., 2007; Tzompa-Sosa et al., 2018; Yener and van Valenberg, 2019). This thesis demonstrates that combining the two methods provides a more detailed milk fat TAG profile with information on both TAG molecular weight (GC-FID) and TAG saturation degree (MALDI-TOF-MS). More specifically, the TAG composition analysis by GC-FID offers information on the TAG groups with even carbon numbers from 24 to 54, where each TAG group is composed of TAG species with similar molecular weight, independent of their saturation degree. On the other hand, the MALDI-TOF-MS analyses TAG profiles with information about odd- and even-chain TAG species as well as about the number of double bonds in each TAG species' structure. The analysis of the TAG composition with MALDI-TOF-MS (**chapters 2, 4, and 5**) rendered similar TAG profiles compared to those obtained with GC-FID when summing up the relative intensities of the TAG species identified by MALDI-TOF-MS into even-chain TAG groups, similar to GC-FID. As an example, **Figure 7.1** presents the TAG profiles obtained by GC-FID and MALDI-TOF-MS from the same bovine milk fat sample, in this case, late lactation milk fat.

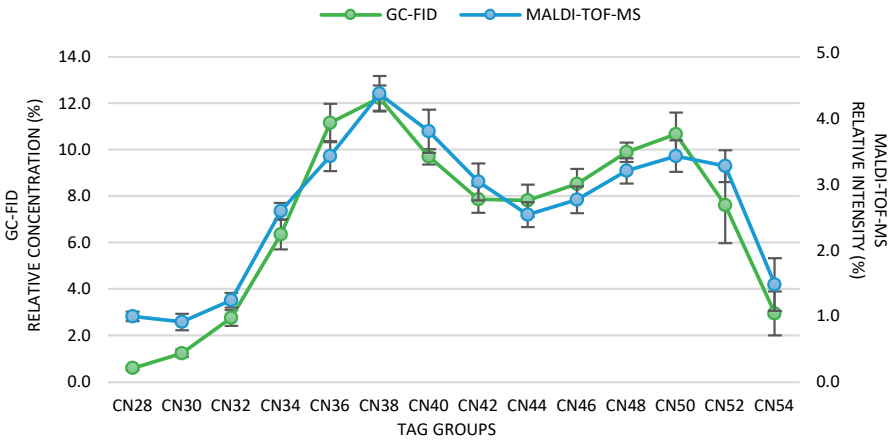


Figure 7.1. Triacylglycerol (TAG) profiles obtained by gas chromatography - flame ionization detector (**GC-FID**) and matrix-assisted laser desorption/ionization - time of flight - mass spectrometry (**MALDI-TOF-MS**) of late lactation milk fat.

The larger differences in Figure 7.1 between GC-FID and MALDI-TOF-MS analysis correspond to the content of the LMW TAG group CN28. This may be explained by the lower concentrations of this TAG group compared to the other TAG groups in milk fat. Lower concentrations may lead to larger variations in the detection of the TAG species by either method.

When considering the individual MALDI-TOF-MS TAG profiles, it is possible to obtain a more detailed perspective than GC-FID due to the ability of MALDI-TOF-MS to discriminate many more TAG species. The resulting inclusion of the saturation degree in the TAG profiles allows the differences in the total abundances of saturated, mono- and polyunsaturated TAG species within the low molecular weight (**LMW**), the medium molecular weight (**MMW**), and the high molecular weight (**HMW**) TAG groups in milk fat to be highlighted better. In general, the combination of both techniques as presented in this thesis provided more information on the TAG composition variation in milk fat and helped us to better understand the effect of feed and animal related factors on the TAG composition variations in bovine milk fat.

7.2. Influence of FA availability for TAG synthesis in the mammary gland

To better understand the role of the availability of different types of FA for TAG synthesis, the combined results of **chapters 2, 4, 5, and 6** were analysed. **Figure 7.2** presents the relation between the *de novo* synthesized and blood derived FA concentrations and the LMW, MMW, and HMW TAG groups. The data included in Figure 7.2 are the averages of the total *de novo* synthesized FA, the total blood derived FA, and the total sum of each TAG group (LMW, MMW, and HMW) analysed by GC-FID.

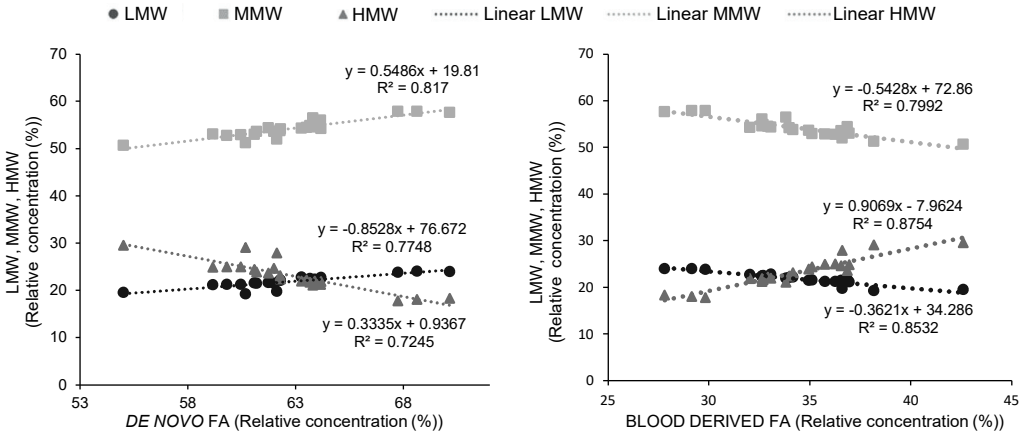


Figure 7.2. The relations between *de novo* synthesized and blood derived fatty acids (FA) with low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight (HMW) triacylglycerol (TAG) groups. This figure is based on the combined FA and TAG results analysed by gas chromatography - flame ionization detector (**GC-FID**) of chapters 2, 4, 5, and 6.

During milk fat biosynthesis in the mammary epithelial cell (**MEC**), TAG are synthesized in the smooth endoplasmic reticulum membrane (**SER**). Two sources of FA are available for TAG synthesis in the SER: *de novo* FA synthesized in the MEC itself and FA absorbed from blood (Lu et al., 2014; Osorio et al., 2016). The *de novo* synthesized FA are FA with 16 and less carbon atoms, whereas blood derived FA are FA with 16 and more carbon atoms (Palmquist, 2006). C16:0 is known to be approx. 50% *de novo* synthesized and 50% blood derived; thus, it is considered as both *de novo* synthesized and blood derived. These two sources of FA varied greatly in response to changes in the feed and animal related factors as studied in this thesis. Among the feed and animal related factors, regardless of the specific factor, all the samples with high concentrations of *de novo* synthesized FA showed increased concentrations of LMW and most MMW, as well as decreased concentrations of HMW TAG species. An opposite trend was identified when high concentrations of blood derived FA were present. In this case, LMW and most MMW TAG species decreased, and HMW TAG species increased (**Figure 7.2**). These results were according to our expectations, considering the types of FA in the structures of LMW, MMW, and HMW TAG groups (Gresti et al., 1993; Liu et al., 2020). The differentiation between the LMW, MMW, and HMW groups was based on the FA that mainly compose the TAG structures of each group (Gresti et al., 1993; Liu et al., 2020). The LMW TAG group (CN26 to CN36) is characterized to be mainly composed of *de novo* synthesized short-chain FA (**SCFA**), the MMW TAG group (CN38 to CN48) is characterized to be composed of mainly *de novo* synthesized SCFA and medium-chain FA (**MCFA**), and the HMW TAG group (CN50 to CN54) is characterized to be mainly composed of C16:0 and blood derived FA (long-chain FA (≥C18); **LCFA**; Table 1.1). These findings show that regardless of the specific feed or animal related factor being studied, high concentrations of *de novo* synthesized FA will always enhance the formation of the LMW and most MMW TAG groups, whereas high concentrations of blood derived FA will always enhance the formation of the HMW TAG group. Thus, this indicates that it is possible to change the TAG composition of milk fat by interventions that change the abundance of *de novo* synthesized or blood derived FA in the mammary gland through feed or animal related factors.

In **chapters 2, 4, 5, and 6**, different trends were observed among the MMW TAG group, especially for TAG species with 38 and 40 carbon atoms (CN38 and CN40). These differences were identified to be related to the wider range of FA that may be esterified in this TAG group compared to the LMW and HMW TAG. The MMW TAG are mainly composed of *de novo* MCFA, but several MMW TAG species (*e.g.* CN38 and CN40; Liu et al., 2020) in milk fat were identified to contain also LCFA such as C18:0 and C18:1 FA. This might explain the similar trends between the MMW TAG CN38 and CN40 and the HMW TAG groups, both of which positively correlated with the abundance of C18:0 and C18:1 FA in **chapters 2 and 5**. Therefore, considering the whole MMW TAG group, it may be

suggested that changes in any FA type can influence the formation of MMW TAG in the MEC. Therefore, the large variety of FA that can be esterified in this TAG group results in smaller differences in MMW TAG composition of milk fat, such as in the case of the seasonal variation effect on the MMW TAG composition in **chapter 2** (CV=1.47%; max (winter): 54.56 g/100 g; min (summer): 52.59 g/100 g). Similar variations were observed for this TAG group in all chapters. In general, the variations in the MMW TAG group were the smallest, whereas the largest variations were identified for the HMW TAG group. Considering that the TAG composition is responsible for the physical properties of milk fat (*e.g.* melting point, SFC, crystallization behaviour; Mohan et al., 2020), the influence of the TAG composition on milk fat physical properties might be mainly related to the larger changes in HMW TAG species. Section 7.5 will present a detailed discussion on the effects of TAG composition variation on the SFC.

In addition, by analysing the TAG composition with GC-FID and MALDI-TOF-MS in **chapters 2, 4, and 5**, the variation between saturated, monounsaturated and polyunsaturated TAG species in milk fat could be assessed more comprehensively. In general, regardless of the specific factor that was studied, high concentrations of *de novo* synthesized FA increased the concentration of saturated TAG species, whereas high concentrations of blood derived FA increased the concentrations of mono- and polyunsaturated TAG species in milk fat. Together with the GC-FID results, it seems that by increasing the abundance of *de novo* synthesized FA, the LMW and MMW TAG can be increased in milk fat mainly in the form of saturated TAG species. On the other hand, high concentrations of blood derived FA increase the abundance of HMW TAG species, mainly in the form of unsaturated (mono- and polyunsaturated) TAG species (**chapter 2, 4, and 5**). Moreover, looking closer to the changes in TAG saturation degree among the different TAG groups (LMW, MMW, and HMW), **chapters 2, 4, and 5** show a similarity in the trends of the saturated, mono-, and polyunsaturated species among the LMW, MMW, and HMW TAG. In all chapters, high concentrations of C16:0 (50% blood derived) and other blood derived FA increased the formation of saturated, mono-, and polyunsaturated HMW TAG species. Opposite findings were obtained when *de novo* synthesized FA increased, as this resulted in the increased formation of saturated, mono-, and polyunsaturated LMW and MMW TAG species. It can thus be suggested that, regardless of the specific factor, the FA that are mainly esterified in each TAG group determine the abundance of saturated, mono-, and polyunsaturated TAG species. These relations between FA availability and the TAG synthesized in the MEC are presented in **Figure 7.3**, as a summary of the common TAG synthesis trends obtained by MALDI-TOF-MS in **chapters 2, 4, and 5**.

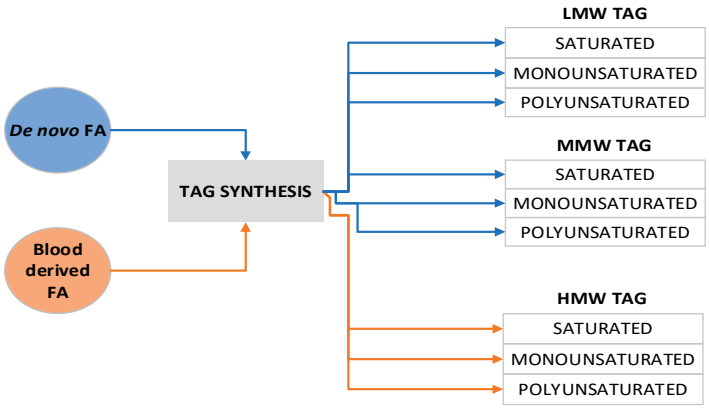


Figure 7.3. The relation between *de novo* synthesized and blood derived fatty acids (FA) availability and triacylglycerols (TAG) species with different saturation degrees. The blue arrow represents the TAG species formed at high abundance of *de novo* FA. The orange arrow represents the TAG species formed at high abundance of blood derived FA. LMW TAG: low molecular weight TAG, MMW TAG: medium molecular weight TAG; HMW TAG: high molecular weight TAG.

In summary, it seems that, regardless of the specific factor, the pool of FA available for TAG synthesis, *i.e.*, *de novo* or blood derived FA, determine which TAG species in the mammary gland are synthesized. A high availability of *de novo* synthesized FA will always result in enhanced formation of LMW and MMW TAG species and high concentrations of blood derived FA in enhanced formation of HMW TAG species. These changes in the availability of FA determines also the saturation degree of the TAG species formed. On the one hand, a larger abundance of *de novo* synthesized FA will most likely increase the concentrations of saturated TAG species due to the larger concentrations of saturated LMW and MMW TAG. On the other hand, a larger abundance of blood derived FA will most likely increase the concentration of unsaturated TAG species due to the larger concentrations of HMW mono- and polyunsaturated TAG.

7.3. The effect of feed and animal related factors on the FA and TAG composition: Similarities and differences

In this thesis, **chapters 2, 4, 5, and 6** explain the variations in FA and TAG composition depending on the feed and animal related factors. To distinguish the effects of the different feed and animal related factors on the FA and TAG composition, a principal component analysis (**PCA**) was conducted on the combined data from these chapters. **Figure 7.4** presents the PCA biplot based on the FA and TAG compositions of all factors, as analyzed by GC-FID. Together, principal component 1 (**PC1**) and principal component 2 (**PC2**) explained 67.6% of the total variance, where the loadings of the different FA and TAG show that PC1 accounted for the differences between factors in *de novo* FA, LMW and MMW TAG groups and PC2 accounted for the differences between factors in blood derived FA and HMW TAG groups.

Accounting for differences in *de novo* FA, LMW, and MMW TAG groups, two clusters of samples can be identified: one including the effect of the variations in the DGAT1 K232A genotype and seasonal feeding regimes (**chapter 6**; cluster 1) and a second one that includes the effect of the seasonal variation (**chapter 2**) and late lactation (**chapter 5**) (Cluster 2; Figure 7.4). The first cluster, “DGAT1 K232A x Season”, showed positive relations with *de novo* FA (except C16:0), LMW TAG groups (except CN26) and most MMW TAG groups (except CN38 and CN40) and negative relations with blood derived FA and HMW TAG groups. These relations may be explained by the fact that, compared to the other analyzed factors, the “DGAT1 K232A x Season” samples had the highest concentrations of *de novo* FA and lowest concentration of blood derived FA. In turn, these high concentrations of *de novo* FA greatly enhanced the formation of most LMW and MMW TAG groups and reduced the formation of HMW TAG groups in milk fat, as described in section 7.2. In general, the overall similarities in the FA and TAG composition in cluster 1 can be related to the greater genetic influence of DGAT1 K232A polymorphism on the synthesis of *de novo* FA in the MEC (Schennink et al., 2007), and in turn on the LMW and MMW TAG groups, compared to the other factors. Moreover, the high positive relation of de *novo* FA and most LMW and MMW TAG within cluster 1 are in line with the effects of the DGAT1 K232A polymorphism on the FA and TAG composition in bovine milk fat (Schennink et al., 2007; Duchemin et al., 2013). Schennink et al. (2007) suggested that the DGAT1 K232A KK genotype was associated with higher concentrations of saturated FA in milk fat and lower concentrations of C18 unsaturated FA. In addition, **chapter 6** presents the interaction found in this thesis between DGAT1 K232 AA/KK and the seasonal feeding effect on the formation of TAG CN42 in milk fat, where higher concentrations of CN42 were associated with summer feeding regimes. These findings explain the identified relations of the saturated *de novo* FA with both summer and winter DGAT1 KK genotype samples and

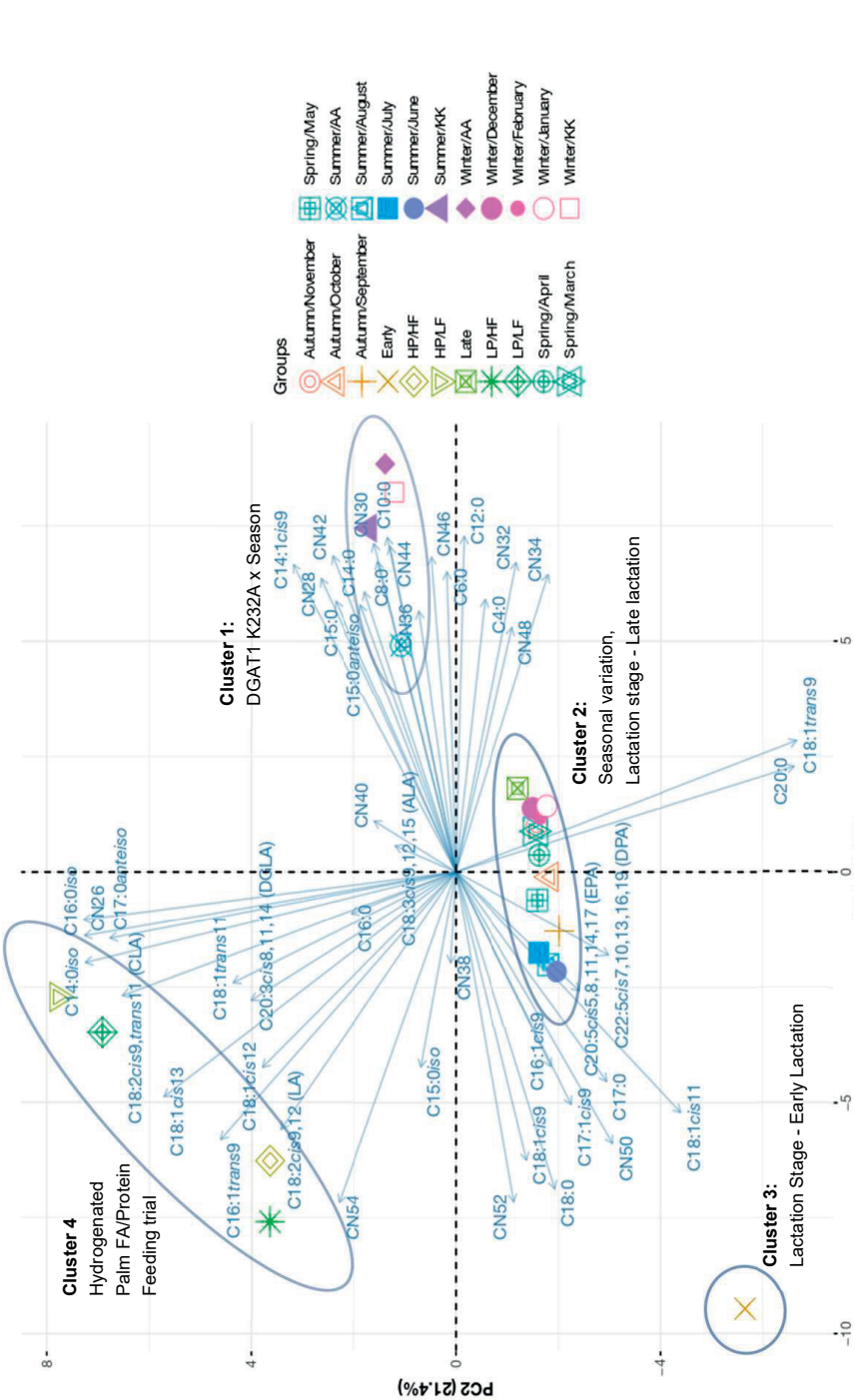


Figure 7.4. Biplot of principal component analysis (PCA) of feed and animal related factors on the fatty acids (FA) and triacylglycerol (TAG) groups composition of bovine milk fat based on the results of chapters 2,4, 5 and 6. PC1: principal component 1; PC2: principal component 2; CN: carbon number; Cluster 1: DGAT1 K232A x Season; Cluster 2: seasonal variation, lactation stage - late lactation; Custer 3. lactation stage - early lactation; Cluster 4: hydrogenated palm FA/protein feeding trial.

CN42 with summer/KK samples in cluster 1. Moreover, summer and winter DGAT1 KK genotype samples and the winter DGAT1 AA genotype samples presented similar concentrations of de *novo* FA and most of the LMW and MMW TAG. This can be explained by the relation between the DGAT1 K232A KK genotype and the saturated *de novo* FA and the high concentrations of *de novo* FA in winter milk fat in **chapter 6**. In both cases, the increased concentration of *de novo* FA, as a result of either the DGAT1 KK genotype or winter seasonal feeding regime, led to higher concentrations of LMW and MMW TAG groups.

Within the second cluster, the samples from winter and late lactation are close together in the PCA plot, showing that these samples had similar FA and TAG composition, with higher amounts of *de novo* FA, LMW, and most MMW TAG groups (**chapters 2 and 5**). However, the concentrations of these FA and TAG were lower than that of the "DGAT1 K232A x Season" samples (cluster 1) which may explain the difference between these clusters on PC1. Between the winter and summer milk fat samples in cluster 2, the transition seasons autumn and spring can be found, as can be expected based on their intermediate FA and TAG profiles (**chapter 2**). The summer FA and TAG profiles, characterized by higher concentrations of blood derived UFA compared to winter, were positively related with the EPA and DPA, some rumen biohydrogenation products (C18:0 and C18:1cis11), C17:0, C18:1*cis*9, HMW CN50, and HMW CN52. These relations were explained in **chapter 2**, where the increased intake of C18:3*cis*9,12,15 (Linolenic acid (**ALA**); the main FA of fresh grass) in summer resulted in increased formation of HMW mono- and polyunsaturated TAG species and decreased formation of LMW and MMW TAG species. This also explains the negative relations in Figure 7.4 of *de novo* FA and LMW and most MMW with the summer seasonal samples in cluster 2.

Accounting for the differences between the factors in blood derived FA and in HMW TAG groups, two clusters were identified in Figure 7.4: the cluster of the early lactation samples (**chapter 5**; cluster 3) and the cluster of the hydrogenated palm FA/protein supplementation feeding trial samples (**chapter 4**, cluster 4). Early lactation samples showed positive relations with mainly C18:0, C18:1*cis*9, C18:1*cis*11, C17:0, HMW CN50, and HMW CN52. This trend is in line with the FA and TAG compositional variations described in **chapter 5** for early lactation. Due to a negative energy balance, cows in early lactation mobilize LCFA from body fat stores (mainly C16:0, C18:0, and C18:1*cis*9) to support milk production. High concentrations of LCFA from body fat stores result in increased formation of HMW TAG and decreased concentration of LMW and MMW TAG species in early lactation. It seems from Figure 7.4 that the relations between C18:0, C18:1*cis*9, CN50, and CN52 are especially high in early lactation (cluster 3). Therefore, it may be suggested that, compared to the other analyzed factors, early lactation results

in greater concentrations of C18:0 and C18:1*cis*9 that enhance the formation of HMW TAG groups CN50 and CN52 the most.

Cluster 4, “hydrogenated palm FA/protein supplementation feeding trial”, was related to higher concentrations of C16:0 and most MUFA and PUFA in milk fat as a result of cow’s feed supplemented with hydrogenated palm FA and protein (**chapter 4**; Nichols et al., 2018). Only the HMW TAG CN54 showed a strong relation with the high fat diets, whereas the other HMW TAG groups CN50 and CN52 showed weaker relations. This can be explained by the fact that early lactation showed the largest concentrations of C18:0 and C18:1*cis*9, which resulted in larger concentrations of CN50 and CN52 compared to the effect of hydrogenated palm FA supplementation. Hence, the loadings of these FA and HWM TAG clustered together with the scores of the early lactation samples. Lastly, C16:0 showed the strongest relations with the hydrogenated palm FA/protein supplementation feeding trial (cluster 4). It seems that, compared to the other factors, the high content of C16:0 in the hydrogenated palm FA supplemented diets increased the concentrations of C16:0 in milk fat the most. However, C16:0 concentrations did not seem to vary much between factors, due to the small loading of C16:0 towards cluster 4. This can be explained by the fact that C16:0, the most abundant FA in milk fat, is present in high concentrations in all milk fat samples. Hence, only small differences were identified between the different factors studied.

To summarize, the biggest differences identified between factors were in their effect on the abundance of de *novo* synthesized and blood derived FA. Changes in the abundance of these FA directly affected the synthesis of TAG in the MEC, as described in section 7.2 and Figure 7.2. The factor that most significantly increased the abundance of *de novo* FA was the genetic effect studied in this thesis, the DGAT1 K232A polymorphism. In late lactation and winter seasonal feeding regime, the abundance of *de novo* FA and most LMW and MMW TAG species also increased but to a lower degree compared to the effect of the DGAT1 K232A polymorphism. In contrast, the factors that increased the blood derived FA in milk, and in turn, the synthesis of HMW TAG, were early lactation, summer seasonal feeding regime, and the hydrogenated palm FA supplementation. Early lactation presented the highest concentrations of C18:0 and C18:1*cis*9, resulting in the highest concentrations of HMW TAG CN50 and CN52. The summer feeding regime resulted in similar FA and TAG profiles to that of early lactation, but to a lesser extent. Supplementing lactating cows with hydrogenated palm FA presented also high concentration of HMW TAG, especially of CN54. However, a different HMW and a different FA profile were obtained compared to early lactation and seasonal feeding regime due to the specific composition of the hydrogenated palm FA supplementation.

7.4. The effect of feed and animal related factors on the positioning of FA in the TAG structure

The effect of the different feed and animal related factors on the positioning of FA in the TAG structures was assessed in **chapters 3, 4, 5, and 6**. The assessment of the feed and animal related factors provided examples of different abundances of *de novo* and blood derived FA available for TAG synthesis in the MEC (Section 7.3). As described in sections 7.2 and 7.3, changes in the concentrations of these two FA sources for TAG synthesis in the MEC determined the formation of LMW, MMW, and HMW TAG species in milk fat. These changes in the FA composition may not only influence the type of TAG species formed (LMW, MMW, and HMW), but also the structure of the TAG species. This hypothesis is based on the fact that during TAG synthesis in the SER, the FA at the *sn*-1, *sn*-2, and *sn*-3 positions in the glycerol molecule are determined by three different enzymes: glycerol-3-phosphate acyltransferase (**GPAT**), 1-acylglycerol-3-phosphate O-acyltransferase (**AGPAT**), and diacylglycerol O-acyltransferase (**DGAT**), respectively (Lu et al., 2014; Osorio et al., 2016). Each enzyme has its specific FA preferences, causing the esterification of the FA in the TAG structure to be non-random (Jensen, 2002; Blasi et al., 2008; Tzompa-Sosa et al., 2014). Moreover, Tzompa-Sosa et al., (2014) identified that the preferences of the enzymes involved in the TAG synthesis in the MEC could be influenced by changing the availability of C16:0. It was suggested in their study that by increasing the availability of C16:0, the GPAT enzyme may esterify C16:0 in the TAG structures to a larger extend, leading to a higher proportions of C16:0 at *sn*-1 position and decreasing the proportions of C14:0, C16:0 as well as of other saturated LCFA at the *sn*-2 position. Therefore, major changes in the abundance of C16:0 in the MEC, as influenced by feed or animal related factors, may affect the esterification of FA in the TAG structure.

An enzymatic regiospecific method was used in this thesis to examine the effect of the different feed and animal related factors on the FA positional distribution in the TAG structure. This means that we were able to determine the composition of the FA at the *sn*-2 position (inner position) and the composition at the *sn*-1(3) positions (outer positions). With a regiospecific method, it is not possible to differentiate the FA compositions of the *sn*-1 and the *sn*-3 positions in the TAG structure, thus the FA composition determined at the *sn*-1(3) is the sum of the FA concentrations at these positions. The positional distribution of FA was expressed by means of two approaches: intrapositional and interpositional. The intrapositional distribution shows the FA relative concentrations at each analysed position, *sn*-2 and *sn*-1(3), whereas the interpositional distribution indicates the FA proportions over the *sn*-2 and *sn*-1(3) positions in TAG structures (**chapters 3, 4, and 5**). The intrapositional distribution is relevant for the identification of total changes in the FA abundance at the *sn*-2 and *sn*-1(3) position in the TAG structure, whereas the interpositional distribution is relevant when analysing the mechanisms of the enzymes involved in TAG synthesis in the

MEC and to determine differences between animal species in the TAG structures (Blasi et al., 2008; Tzompa-Sosa et al., 2014).

Based on the interpositional results of **chapters 3, 4, and 5,** neither feed nor animal related factors had a significant effect on the proportions (or stereolocations) of most FA at either *sn*-2 and *sn*-1(3) positions over the TAG structure. As discussed in these chapters, the FA stereolocations were determined based on the premise that the FA at *sn*-2 and *sn*-1(3) positions make up 100% of the TAG structure, so the corresponding proportion at each *sn* position is 33.3% based on equal distribution. Hence, proportions greater than 33.3% at the *sn*-2 position and proportions greater than 66.7% at the *sn*-1(3) positions indicate a stereolocation preference for the *sn*-2 and the *sn*-1(3) positions, respectively. Therefore, regardless of the specific factor, the FA stereolocation over the TAG structure of the major FA will most likely be: C12:0, C14:0 and C16:0 at the *sn*-2 position, and C4:0 to C10:0 and C18:0 at the *sn*-1(3) positions. The stereolocation of C18:1*cis*9, C18:1*cis*9,12 (**LA**) and C18:1*cis*9,12,15 (**ALA**) were almost identical for all positions in the TAG structure, suggesting that these FA may be stereolocate at both the *sn*-2 and the *sn*-1(3) positions.

Moving on to the intrapositional distribution results, **chapters 3, 4, and 5** showed that, regardless of the specific factors, increasing the concentrations of any FA increased the concentrations of that FA at both the *sn*-2 and the *sn*-1(3) positions in the TAG structures. One exception was observed in **chapter 5**, where high concentrations of C16:0 in late lactation decreased its concentrations at the *sn*-2 position. This was explained by the possibly enhanced activity of the GPAT enzyme that, due to its high preference for C16:0, may have increased the concentrations of C16:0 at the *sn*-1(3) positions in the TAG structures at the expense of its esterification at the *sn*-2 position. The effect of lactation stage on the FA intrapositional distribution in the TAG structures presented the largest differences of C16:0 and C18:1*cis*9 between samples (**chapter 5**) compared to **chapters 3 and 4**. Larger differences in C16:0 levels between early and late lactation may have enhanced the activity of the GPAT enzyme compared to the other studied factors, resulting in decreased concentrations of C16:0 at the *sn*-2 position. Therefore, it may be concluded that lactation stage is the factor with the largest influence on the FA intrapositional distribution.

The changes in the positional distributions of FA may be relevant findings for food product development, particularly for the design of infant formula. One of the major goals of infant formula design is to replicate the human milk TAG structure as much as possible to improve milk fat digestion and absorption in infants (Kloek et al., 2020; Viriato et al., 2020). Triacylglycerols in human milk contain greater concentrations of C16:0 (52-62%) at the *sn*-2 position in the TAG structures compared to bovine milk (~45%) (Haddad et al., 2012; Blasi et al., 2013; Chen et al., 2020). During fat digestion, the FA at the *sn*-1 and *sn*-3 positions are hydrolyzed by pancreatic lipase resulting in two free FA (**FFA**) and a *sn*-2

monoacylglycerol. The presence of C16:0 at the *sn*-2 position thereby leads to formation of 2-monopalmitin during fat digestion, which is easily absorbed and also improves the absorption of FFA and minerals in infants. On the contrary, C16:0 at the *sn*-1 and *sn*-3 positions in the TAG structures are hydrolyzed into free C16:0. Free C16:0 can bind to dietary minerals forming non digestible complexes. These complexes are hard to absorb and result in decreased bioavailability of both FA and minerals in infants (Mu and Høy, 2004; Karupaiah and Sundram, 2007; Innis, 2011). Therefore, to improve milk fat digestion and absorption in infants, we may suggest that it would be possible to indirectly increase the concentrations of C16:0 at the *sn*-2 position in the TAG structure by changing the availability of FA through changes in the cows feed (**chapters 4 and 3**).

This study also examined the individual and combined effect of DGAT1 K232A AA/KK polymorphism (**chapter 6**) as well as the summer and winter seasonal feeding regimes on the variations in milk fat TAG composition. The assessment of the combined effect of DGAT1 K232A AA/KK polymorphism and the seasonal feeding regimes showed the larger influence of the FA abundance in the MEC over the preferences of the DGAT1 AA/KK polymorphism. **Figure 7.5** presents the mechanism proposed in **chapter 6**, explaining the relation between the FA availability influenced directly by the seasonal feeding regimes and the preferences of the analysed DGAT1 AA/KK genotypes.

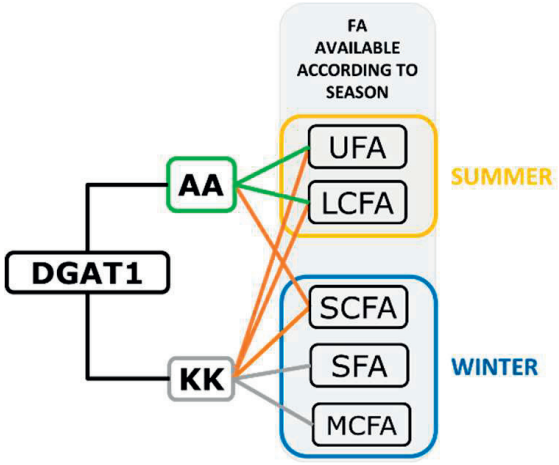


Figure 7.5. Proposed mechanism of the interaction between fatty acid (FA) availability in the mammary epithelial cell (MEC) influenced directly by the seasonal feeding regimes of summer and winter and the preferences of the analysed DGAT1 AA/KK genotypes (chapter 6). UFA: unsaturated FA; LCFA: long-chain FA; SCFA: short-chain FA; SFA: saturated FA; MCFA medium-chain FA. The green line connectors refer to the FA preferably esterified by the DGAT1 AA genotype, and the grey line connectors refer to the FA preferably esterified by the DGAT1 KK genotype. The orange line connectors refer to the changes in the FA esterification preference of the DGAT1 AA/KK genotypes influenced by the summer and winter seasonal feeding regimes.

The seasonal feeding regimes in summer and in winter determined the FA availability for TAG synthesis in the MEC, where, as expected, the summer diet increased the concentrations of UFA and LCFA (blood derived FA), and winter diet increased the concentration of *de novo* SCFA and MCFA and in SFA in milk fat (yellow and blue box Figure 7.5). Depending on the FA availability in each season, the DGAT1 genotypes AA/KK showed different FA preferences, keeping the characteristics of the synthesized TAG species (LMW, MMW, and HMW). Two TAG groups, CN42 and CN52, showed a significant interaction effect between the DGAT1 K232A genotype and season (chapter 6). The DGAT1 AA genotype was characterized to preferably esterify blood derived LCFA and UFA, but it also showed a preference for SCFA in winter. While the DGAT1 KK genotype was characterized to preferably esterify *de novo* SCFA and MCFA, it also showed a preference for LCFA and UFA in summer (Figure 7.5; green, orange and grey line connectors). This change of preference of the DGAT1 AA/KK polymorphs was suggested in **chapter 6** to be a mechanism to regulate the melting point of TAG and in turn keep the melting point of milk fat droplets below the cow's body temperature (~39°C) (Jensen, 2002). For this purpose, the MEC may increase the proportions of SCFA and MCFA and asymmetrically esterify the SCFA in the glycerol molecule (Dils, 1986), as explained in **chapter 6**.

The intra- and interpositional distribution analysis of FA, together with the combined effect of the DGAT1 K232A polymorphism and seasonal feeding regimes on the variations in the TAG composition, show that changes in the FA availability for TAG synthesis in the MEC is the major driver influencing the FA abundance at all positions in the TAG structures. Large variations in the FA availability for TAG synthesis may change the enzymes' preferences and vary the FA abundance at the *sn*-2 and *sn*-1(3) positions in the TAG structures, yet without influencing the stereolocation of the FA in the TAG structures.

7.5. Influence of TAG composition variations on the SFC in milk fat

The studies in **chapters 4 and 5** assessed the effect of the TAG composition variation on the SFC. **Chapter 4** focused on the effect of hydrogenated palm FA supplementation, whereas **chapter 5** focused on the effect of early and late lactation stage. These two factors led to different FA and TAG compositions resulting in opposite effects on the milk fat SFC. **Figure 7.6** summarizes and compares these opposite effects on the SFC.

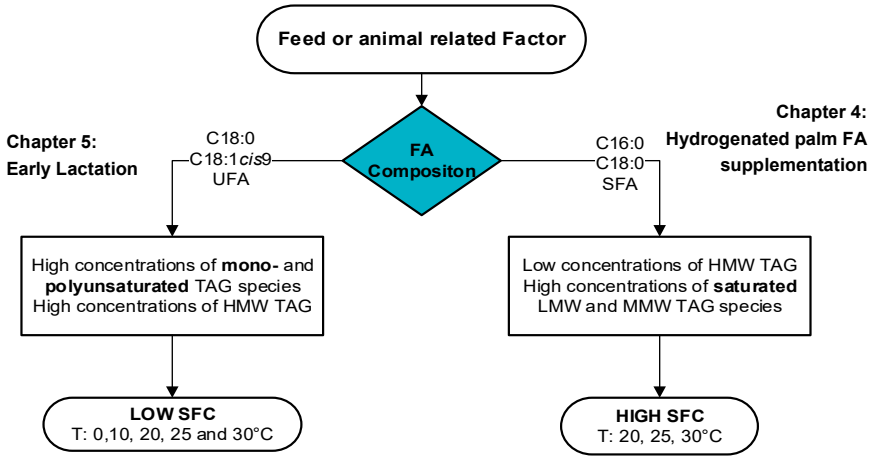


Figure 7.6. Influence of the fatty acid (FA) and triacylglycerol (TAG) compositions variations on the solid fat content (SFC) in bovine milk fat measured at 0, 10, 20, 25 and 30°C. SFA: saturated FA; UFA: unsaturated FA; LMW: low molecular weight TAG; MMW: medium molecular weight TAG; HMW: high molecular weight TAG; SFC: solid fat content; T: temperature (°C).

In both cases, early lactation and hydrogenated palm FA supplementation, all the HMW TAG species (saturated, mono-, and polyunsaturated) increased due to high concentrations of blood derived FA (Section 7.2). Comparing the MALDI-TOF-MS TAG species profiles in both cases, early lactation milk fat samples presented greater concentrations of mono- and polyunsaturated TAG species as well as a higher concentration of all HMW TAG in milk fat, whereas the hydrogenated palm FA supplemented milk fat samples presented lower concentrations of these TAG species and increased concentrations of saturated LMW and MMW TAG species (Figure 7.6). As a result of these differences in TAG species' saturation degree, two opposite trends in the resulting SFC were identified. On the one hand, a high content of saturated FA led to increased formation of LMW and MMW saturated TAG species, resulting in a high SFC between 20 and 30°C. On the other hand, high concentrations of C18:1*cis*9 and UFA, which, in turn, led to increased formation of unsaturated TAG species, resulted in a low SFC between 0 and 30°C (Figure 7.6). The influence of the TAG species' saturation degree on the SFC was explained in **chapters 4**

and 5 by the differences in the melting points of the FA available for TAG synthesis, which further determined the melting points of the formed TAG species. In general, the length of the carbon chain and the number of double bonds in the TAG structure determines its melting point (Knothe and Dunn, 2009). Therefore, milk fat with a higher content of unsaturated TAG species, as is the case for the early lactation, is expected to have lower melting points, leading to a lower SFC at most temperatures (**chapter 5**). In contrast, milk fat with a higher content of saturated TAG species, as is the case for the hydrogenated palm FA supplementation, leads to higher melting points and thus a higher SFC (**chapter 4**; Figure 7.6).

Previous studies have suggested that the SFC is mainly determined by changes in the composition of the HMW TAG species in milk fat (Precht and Frede, 1994; Smiddy et al., 2012). This hypothesis was supported by the results described in **chapters 4 and 5**, where changes in the HMW TAG species were indeed strongly associated with the SFC measured between 0 and 30°C. However, as explained in chapter 4, the increase in the SFC was not only related to the decreased concentration of unsaturated HMW TAG species, but also to the high content of saturated LMW and MMW TAG species. Moreover, in chapter 5 a high abundance of unsaturated HMW TAG species and a low abundance of saturated LMW and MMW TAG species were associated with decreased milk fat SFC. Therefore, it may be suggested that it is actually the shift from unsaturated HMW TAG species to the saturated LMW and MMW TAG species that leads to increased milk fat SFC. Changes in the saturation degree of HMW TAG, and also in general of the composition of all TAG, can be influenced by different factors as presented in this thesis. Hence, it may be possible to control the milk fat SFC by influencing variations in the TAG species' saturation degree by means of feed and animal related factors.

Variations in milk fat SFC at a specific temperature are important in the food industry because they determine its suitability for a certain food product application. Milk fat with a high SFC at a specific temperature results in a harder texture, whereas a low milk fat SFC results in a softer texture. The range of temperatures at which SFC was assessed in this thesis (0 to 40°C) provided insights into the effect of the feed and animal related factors on milk fat texture and mouthfeel characteristics. In general, temperatures from 0 to 10°C define refrigeration texture characteristics, whereas room temperatures from 20 to 30°C define table top product characteristic and product stability. Mouthfeel product characteristics are mainly determined by the SFC of milk fat from 30 to 40°C. Milk fat is almost liquid at 40°C (SFC: 1–2%; **chapter 4 and 5**), so regardless of the analysed factor, no differences in the SFC at 40°C were found. In both chapters, the major changes in the SFC were observed between 10 and 30°C, which is in line with the melting profile of bovine milk fat analysed in previous studies (Ten Grotenhuis et al., 1999; Smiddy et al., 2012). Changes in the SFC at these temperatures may influence processing conditions of milk fat

(*e.g.* milk fat ripening and fractionation processes), resulting in further processing challenges for the food industry. Therefore, the insights obtained in this thesis on the effect of feed and animal related factors on the SFC can help the food industry to better understand the processing challenges of milk fat associated with these factors.

To summarize, two major effects of the feed and animal related factors were identified in the SFC due to the shift between the contents of unsaturated HMW TAG species and saturated LMW and MMW TAG species in milk fat. A lower milk fat SFC was associated with increased content of unsaturated HMW TAG species (**chapter 5**) and with lower content of saturated LMW and MMW TAG species, whereas a higher SFC was associated with increased contents of saturated LMW and MMW and decreasing content of unsaturated HMW TAG species (**chapter 4**). Therefore, through feed and animal related factors, it may be possible to change the formation of saturated LMW and MMW and unsaturated HMW TAG species, and in turn, obtain different SFC at specific temperatures. All in all, information on the TAG composition variations and its saturation degree are important for a complete understanding of the influence of variations in the TAG composition on milk fat SFC.

7.6. Main conclusions

This thesis investigated the effect of several feed and animal related factors on the variation in milk FA and TAG composition and structure and the effect of these variations on milk fat SFC. Based on the assessment of the FA and TAG composition in regard to all analysed factors, it was possible to conclude that variations in the synthesis of the total LMW, MMW, and HMW TAG groups were determined by the abundances of *de novo* synthesized FA and blood derived FA available for TAG synthesis in the MEC. Regardless of the specific factor studied, a high abundance of *de novo* FA will always lead to increased synthesis of LMW and MMW TAG species. Moreover, high abundance of blood derived FA will always lead to increased synthesis of HMW TAG species.

Changes in the availability of FA for TAG synthesis did also cause structural changes in the TAG species. Regardless of the specific factors, changes in the abundance of either *de novo* or blood derived FA modified the concentrations of the these FA at the *sn*-2 and the *sn*-1(3) positions in the TAG structures. Larger differences in the abundance of C16:0 between milk fat samples led to increased concentrations of C16:0 at the *sn*-1(3) position and decreased concentrations of C16:0 at the *sn*-2 position, probably due to increased esterification of this FA by GPAT. However, regardless of the changes in FA availability for TAG synthesis, the proportions of the FA over the *sn*-2 and *sn*-1(3) positions in the TAG structures did not change.

The assessment of the effect of hydrogenated palm FA supplementation and early lactation on the TAG composition and its influence on milk fat SFC highlighted the importance of the saturation degree of TAG. Changes in milk fat SFC were mainly associated with the shift in the content of unsaturated HMW TAG species to saturated LMW and MMW TAG species. A low milk fat SFC was obtained as a result of high concentrations of unsaturated HMW TAG species and lower content of saturated LMW and MMW TAG species, whereas a higher SFC was associated with opposite changes in these TAG species.

To conclude, the different FA compositions (*de novo* synthesized FA and blood derived FA) of all analysed feed and animal related factors and their influence on the formation of LMW, MMW, and HMW TAG species, may be used as a strategy for food product design and development. Firstly, increasing the concentrations of C16:0 at the *sn*-2 position in the TAG structure by means of feed and animal related factors may be of interest for infant formula development. Secondly, the effect of feed and animal related factors on the TAG composition and structure and its influence on the SFC shown in this thesis may be relevant for dairy nutritionists and food product developers.

7.7. Recommendations for future work

This thesis highlights the effect of different feed and animal related factors on the FA and TAG composition and structure variations and their consequences for milk fat SFC. The following are suggestions for future research to further enhance our knowledge on the composition and properties of milk fat:

- Further work to determine a more detailed TAG profile with information on the specific FA present in the structures of each TAG species could be carried out. This would require more advanced and sophisticated analytical methods (*e.g.* LC-MS and LC-MS/MS; Liu et al., 2020). Deeper insights in the FA composition of the individual TAG species formed in the MEC, as influenced by the feed and animal related factors, may provide a better understanding of the effect of the differences in the availability of *de novo* synthesized and blood derived FA in the TAG structures. This information may provide insights into the influence of changes in TAG composition and structure on the SFC.

- The assessment of the FA positional distribution in the TAG structures may be enhanced by using a stereospecific approach, instead of a regiospecific approach, where the FA composition at each position (*sn*-1, *sn*-2, and *sn*-3) is identified by the former. The stereospecific distribution of the TAG structures was analysed previously following the phospholipase A_2 method proposed by Christie (2003), as further explained in the study of Blasi et al. (2008). This method starts from *sn*-1,2(2,3)-diacylglycerols, obtained from a partial chemical degradation using ethyl magnesium bromide coupled with thin-layer chromatography (**TLC**). Subsequently, s*n*-1,2(2,3)-phosphatidylcholines are chemically synthesized, isolated by TLC, and further mixed with phospholipase A_2 to form a *sn*-1-lysophosphatidylcholines (***sn*-1-LPC**) and free FA. The *sn*-1-LPC and FFA components are separated by TLC and analysed for FA composition, representing the compositions of the FA at the *sn*-1 and *sn*-2 positions in the TAG structures, respectively (Blasi et al., 2008). The FA composition at the *sn*-3 position is calculated based on the FA compositions analysed at the *sn*-1 and *sn*-2 positions in the TAG structures. Determining the composition of the FA at the *sn*-1, *sn*-2 and *sn*-3 positions in the TAG structure may provide a better understanding of the preferences of the enzymes involved during TAG synthesis in the MEC. This information may also lead to further knowledge on the mechanisms during TAG synthesis in the MEC. Therefore, this can be proposed as a methodological approach to further study and confirm the suggested changes in the preference of the enzymes GPAT and DGAT1 during TAG synthesis in the MEC. A deeper insight of these mechanisms will contribute to an improved understanding of the formation of TAG species in the MEC, which may in turn better explain the influence of all feed and animal related factors on the TAG composition and structure.

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Summary

Summary

Triacylglycerols (**TAG**) composition and structure in milk fat determine the physical, nutritional and functional characteristics in milk fat. Research on the variation in milk fat TAG composition and structure is of great relevance for the use of milk fat as an ingredient in food product design and development. TAG are composed of three fatty acids (**FA**) bound to a glycerol molecule. Therefore, changes in milk FA composition are expected to influence milk fat TAG composition and structure. Feed and animal related factors are known to affect milk FA composition. Feed related factors refer to alteration in the dairy cows' feeding regimes, whereas animal related factors refer to factors such as lactation stage, breed, and genetics. Little information is available on the effect of these factors on milk fat TAG composition and structure, as most of the studies have only assessed their effect on FA composition. Therefore, this thesis aimed to assess the effect of feed and animal related factors on the variation in FA and TAG composition and structure and to determine its influence on the milk fat solid fat content (**SFC**). Milk fat SFC is one of the physical properties that define the suitability of a fat for a specific food product application. SFC was thus selected as the physical property of study in this thesis to highlight the effect of milk FA and TAG variations. The feed related factors analyzed in this thesis were the seasonal feeding regimes (**chapters 2 and 3**) as well as hydrogenated palm FA and protein supplementation to the feed of lactating dairy cows (**chapter 4**). The animal related factors analyzed in this thesis were the cows' lactation stage (**chapter 5**) and polymorphisms of the diacylglycerol O-acyltransferase 1(DGAT1) K232A gene (**chapter 6**).

In **chapters 2 and 3**, the effect of the seasonal feeding regimes on the FA and TAG composition and structure were assessed in a non-seasonal milking system. Both chapters were based on weekly-pooled milk samples collected from fourteen dairy factories in the Netherlands, during one year, from May 2017 to April 2018. Changes in the seasonal feeding regimes are associated with the grazing period of the cows. In the Netherlands, the grazing period begins in April/May and ends in October. Hence, spring and summer feeding regimes include a larger amount of fresh pastures. In contrast, in autumn and winter, when cows are kept in stables, their diet shifts to a higher content of concentrate and silage. The most abundant FA in fresh pastures is C18:3*cis*9,12,15 (linolenic acid, **ALA**), whereas the most abundant FA in concentrate and corn silage is C18:2*cis*9,12 (linoleic acid, **LA**). Seasonal FA and TAG composition variation were presented in **chapter 2**, where the largest FA variations were found between summer and winter, while spring and autumn were considered transition periods. The FA variations included a smaller proportion of *de novo* synthesized FA and an increased proportion of biohydrogenation products and conjugated linoleic acid (**CLA**) isomer FA in spring and summer. The proportion of LA in milk fat did not have a clear seasonal variation, suggesting that the intake of LA from corn silage and concentrate is rather constant throughout seasons. Moreover, the proportion of

ALA in milk fat was greatest during late spring and summer, reflecting the increased intake of fresh pasture in these periods. Seasonal FA variations led to changes in the TAG composition of milk fat. The high availability of short-chain FA (**SCFA**) and C16:0 in winter increased the formation of the low molecular weight (**LMW**) TAG group and the medium molecular weight (**MMW**) TAG group from CN42 to CN46, whereas the high concentrations of long-chain FA (**LCFA**) and unsaturated FA (UFA) in summer increased the formation of CN40 and the HMW TAG group (CN50–CN54). In **chapter 3**, the influence of the FA seasonal variation on the TAG structure was assessed. The FA regiospecific distribution analysis showed that high concentration of FA in summer and winter increased the FA concentrations at both the *sn*-2 and the *sn*-1(3) positions in the TAG structure, yet without affecting the FA stereolocation preference over the TAG structures. Between summer and winter, the FA with the larger variation at the *sn*-2 and the *sn*-1(3) positions were C16:0 and C18:1*cis*9. At both *sn*-2 and *sn*-1(3) positions, C16:0 showed the highest concentrations in winter, whereas C18:1*cis*9 showed the highest concentration on these positions in summer.

In **chapter 4**, the influence of hydrogenated palm FA (mainly 16:0 and C18:0) and protein supplementation in cows' feed on the TAG composition and structure, as well as the SFC of bovine milk fat were assessed. In this study, milk fat samples from 56 Holstein-Friesian cows were blocked into 14 groups of 4 cows and randomly given 1 of 4 dietary treatments. The assigned treatments were: 1) low protein/low fat, 2) high protein/low fat, 3) low protein/high fat, and 4) high protein/high fat. The high protein and high fat diets were attained by supplementing the basal ration (low protein/low fat) with rumen-protected soybean meal and rapeseed meal, and hydrogenated palm oil FA, respectively. It was found that the effect of palm FA supplementation on the TAG composition was larger than the effect of protein supplementation. The supplementation of palm FA led to decreased concentrations of LMW TAG CN26 to CN34 and MMW TAG CN40 to CN46 and increased concentrations of HMW TAG CN50 and CN52. Similar to **chapter 3**, these changes did not affect the FA stereolocation preferences over the TAG structures. Higher concentrations of C16:0, C18:0, and C18:1*cis*9 in milk fat in response to palm FA supplementation were associated with increased concentrations of C16:0 and C18:0 at the *sn*-2 position and C18:0 and C18:1*cis*9 at the *sn*-1(3) position of the TAG structures. Higher concentration of saturated HMW TAG CN50 and CN52 in response to palm FA supplementation increased milk fat SFC measured at 20, 25, and 30°C, probably due to the high abundance of HMW TAG species with C16:0 and C18:0 in their structures.

In **chapter 5**, the effect of early and late lactation stage on the FA and TAG composition and structure, and the SFC of bovine milk fat was analyzed. The samples used in this study were two milk fat samples from 11 cows collected in their early (8 to 14 DIM) and late (199 to 326 DIIM) lactation stages. The results showed a characteristic change in FA composition between early and late lactation. In early lactation, the FA mobilization from the cows' body

stores resulted in increased concentrations of C4:0, C18:0, and C18:1*cis*9, and other monounsaturated FA. In contrast, in late lactation higher concentrations of *de novo* FA C10:0 to C16:0 were observed. This shift in FA composition between early and late lactation led to increased formation of unsaturated HMW TAG species in early lactation and increased formation of saturated LMW and MMW TAG species in late lactation. Similar to the findings of **chapters 3 and 4**, changes in milk FA concentration did not affect the stereolocation preference of the FA over the TAG structures. Except for C16:0, regardless of the lactation stage, high FA concentrations in milk increased the respective FA concentrations at the *sn*-2 and *sn*-1(3) positions in the TAG structures. In late lactation, high concentrations of C16:0 in milk fat were associated with a decreased concentration of C16:0 at the *sn*-2 position and an increased concentration of C16:0 at the *sn*-1(3) positions in the TAG structures. This was explained by possible changes in the FA esterification preferences of the glycerol-3-phospate acyltransferase (**GPAT**) enzyme during TAG synthesis in the mammary epithelial cell (**MEC**). This enzyme is responsible for the esterification of FA at the *sn*-1 position in the TAG structure. An increase in the availability of C16:0 for esterification in the TAG structure may have increased the GPAT esterification preference for C16:0, subsequently leading to higher concentrations of C16:0 at the *sn*-1(3) positions and relatively lower concentration of C16:0 at the *sn*-2 position. The increased concentration of C18:0 and C18:1*cis*9 that enhanced the formation of mono- and polyunsaturated HMW TAG in early lactation milk fat also led to a lower milk fat SFC compared to late lactation.

In **chapter 6**, the effect of the DGAT1 K232A polymorphism and the summer and winter seasonal feeding regimes on the TAG composition was assessed. The study of the DGAT1 K232A polymorphism was selected in this thesis due to its known influence on the milk fat content and its FA composition. The samples analyzed in this study were 50 winter and 50 summer milk fat samples from 25 cows with the DGAT1 KK and 25 cows with the DGAT1 AA genotype. Statistical mixed models with and without fat content as covariable were used to test for the individual and combined effect of DGAT1 AA/KK genotypes and seasonal feeding regimes on milk fat TAG composition. Considering the individual effect of the seasonal feeding regimes on the TAG composition, summer milk fat samples showed a higher concentration of the HMW TAG CN54 and a lower concentration of the LMW TAG CN34 and CN36 compared to winter milk fat samples. Similar to **chapter 2**, these changes in the TAG composition were associated with the FA composition of summer and winter milk fat, mainly related to the seasonal variations of C14:0, C16:0, C18:0, total UFA (especially C18:1*cis*9), and total LCFA. Regarding the effect of the DGAT1 K232A polymorphism, the KK genotype showed higher concentrations of CN36 without adjusting for fat content. After adjusting for fat content, no effect of the DGAT1 K232A polymorphism on milk fat TAG composition was observed. These findings suggest that the increase of

CN36 for the DGAT1 K232A KK genotype can be explained by the differences in fat content between AA and KK genotypes. An interaction effect of the DGAT1 K232A polymorphism and seasonal feeding regimes was identified with and without accounting for differences in fat content for the TAG species CN42 and CN52. It was, therefore, suggested that depending on the FA availability in each season (summer/winter), UFA (mainly C18:1*cis*9), SCFA (C6:0 and C10:0), and medium-chain FA may be esterified to the TAG structure regardless of the DGAT1 K232A genotype (AA/KK) FA preferences.

In **chapter 7,** all the feed and animal related factors were compared and discussed based on their different effects on the variations in the TAG composition and structure as well as milk fat SFC. In general, for all the feed and animal related factors, the changes in FA composition were strongly related to the type of TAG species formed in the MEC. Higher availability of *de novo* synthesized FA (FA≤C16:0) in the MEC enhanced the formation of LMW TAG and most MMW TAG species, whereas blood derived FA (FA≥C18:0) enhanced the formation of HMW TAG species. Regardless of the specific factor, higher milk FA concentrations increased the FA concentrations at both the *sn*-2 and *sn*-1(3) position in the TAG structure; however, this did not affect the stereolocation preferences of the FA over the TAG structure. Only for the effect of lactation stage, in late lactation higher C16:0 concentrations seem to have influenced the esterification preference of the GPAT enzyme, thus increasing the concentration of C16:0 at the *sn*-1(3) position. These findings may be relevant for infant formula developers to enhance the concentrations of C16:0 at the *sn*-2 position in the TAG structure to improve fat and mineral absorption in infants. Lastly, changes in milk fat SFC were mainly associated with the shift in the content of unsaturated HMW TAG species to saturated LMW and MMW TAG species. Therefore, changing the content of these TAG in milk fat by means of feed or animal related factors may be a strategy for food product design and development to customize milk fat SFC for a specific food product application.

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About the Author

Author Biography

Sara Liliana Pacheco Pappenheim was born on February 24, 1984 in Bogotá, Colombia. She obtained her bachelor degree in Agroindustrial Process Engineering at La Sabana University, Chia (Colombia) in 2009. After finishing her bachelor she started a master in Food Product Design and Quality Management at Wageningen University and Research that she finished in 2012. In her second master's year she did her internship at Fonterra in Palmerston North (New Zealand), working in the development of dairy products with high protein content. Once obtained her master degree she returned to Bogotá, where she soon started working as Junior product developer at the dairy company Alpina, being responsible for supporting the research and development area, improving existing products and creating new ones to expand the company's portfolio. In 2016 she was granted a scholarship for her PhD studies by COLCIENCIAS, the Colombian National Administrative Department of Science, Technology and Innovation. In February 2017 she arrived in the Netherlands to begin her PhD project on milk fat composition at Food Quality and Design (FQD) Department at Wageningen University and Research, supervised by Hein van Valenberg and Vincenzo Fogliano. She presented her PhD research results and defended her thesis in October 2021.

List of Publications

This thesis:

Pacheco-Pappenheim, S., S. Yener, H.J.F. van Valenberg, D.A. Tzompa-Sosa, and H. Bovenhuis. 2019. The DGAT1 K232A polymorphism and feeding modify milk fat triacylglycerol composition. J. Dairy Sci. 102:6842–6852. doi:10.3168/jds.2019-16554.

Pacheco-Pappenheim, S., S. Yener, J.M.L Heck., J. Dijkstra, and H.J.F. van Valenberg. 2021. Seasonal variation in fatty acid and triacylglycerol composition of bovine milk fat. J. Dairy Sci. 104:14. doi:10.3168/jds.2020-19856.

Yener S., S. Pacheco-Pappenheim S., J.M.L. Heck and H.J.F. van Valenberg. Seasonal variation in the positional distribution of fatty acids in bovine milk fat. In press.

Pacheco-Pappenheim S., S. Yener., K. Nichols, J. Dijkstra, K. Hettinga, and H.J.F. van Valenberg. Feeding hydrogenated palm fatty acids to lactating Holstein-Friesian dairy cows modifies milk fat triacylglycerol composition and structure, and solid fat content. Submitted for publication to the Journal of Dairy Science.

Pacheco-Pappenheim S.,S. Yener, R. Goselink, M.X. Quintanilla-Carvajal, H.J.F. van Valenberg and K. Hettinga. Bovine triacylglycerol composition and structure differs between early and late lactation influencing milk fat solid fat content. Submitted for publication to the International Dairy Journal.

Other publications:

Nichols K., A. Bannink, S. Pacheco, H.J. van Valenberg, J. Dijkstra, and H. van Laar. 2018. Feed and nitrogen efficiency are affected differently but milk lactose production is stimulated equally when isoenergetic protein and fat is supplemented in lactating dairy cow diets. J. Dairy Sci. 101:7857–7870. doi:10.3168/jds.2017-14276.

Nichols K, J. Dijkstra, H. van Laar, S. Pacheco, H.J. van Valenberg, A. Bannink. 2019. Energy and nitrogen partitioning in dairy cows at low or high metabolizable protein levels is affected differently by postrumen glucogenic and lipogenic substrates. J. Dairy Sci. 102:395–412. doi.org/10.3168/jds.2018-15249.

Overview of complete training activities

Discipline specific activities

Courses:

Rheology: The do's and don'ts, VLAG, Wageningen, The Netherlands, 2019.
Healthy and sustainable diets: synergies and trade-offs, VLAG, Wageningen, The Netherlands, 2019.
Masterclass Dairy Protein Biochemistry, VLAG, Wageningen, The Netherlands, 2018.
Masterclass Applied Biocatalysis, VLAG, Wageningen, The Netherlands, 2019.
Advanced Food analysis, VLAG, Wageningen, The Netherlands, 2019.

Symposia and conferences:

16th Euro Fed Lipid Congress and Expo, Gesellschaft Deutscher Chemiker e.V., Belfast, Ireland, 2018.
17th Euro Fed Lipid Congress and Expo, Gesellschaft Deutscher Chemiker e.V., Seville, Spain, 2019.
Rheology Symposium, VLAG, Wageningen, The Netherlands, 2019.
American Oil Chemistry Society (AOCS) 2020, AOCS, virtual conference, 2020.
19th Euro Fed Lipid Congress and Expo, Gesellschaft Deutscher Chemiker e.V., virtual conference, 2021.

General courses:

VLAG PhD week, VLAG, Baarlo, The Netherlands, 2017.
Scientific Writing, VLAG, Wageningen, The Netherlands, 2017.
Applied Statistics, VLAG, Wageningen, Wageningen, The Netherlands, 2018.
Chemometrics (Multivariate Statistics), VLAG, Wageningen, Wageningen, The Netherlands, 2018.
Mixed Linear Models, PE&RC, Wageningen, The Netherlands, 2018.
Career assessment, WGS, Wageningen, The Netherlands, 2018.
Introduction to R, VLAG, Wageningen, The Netherlands, 2018.
Reviewing Scientific Papers, WGS, Wageningen, The Netherlands, 2018.
Scientific Publishing, WGS, Wageningen, The Netherlands, 2018.
Brain Training, WGS, Wageningen, The Netherlands, 2018.

Optional courses and activities

Scientific meetings, seminar, colloquia, FQD, Wageningen, The Netherlands, 2017-2021.
Organizing PhD trip - PhD committee, FQD, Melbourne/WagaWaga/Sydney, Australia, 2018.
DST Meeting, FQD, Wageningen, The Netherlands, 2017-2021.
Preparation of research proposal, FQD, Wageningen, The Netherlands, 2017-2018.

Teaching obligations

Supervision of BSc and MSc students, FQD, Wageningen, The Netherlands, 2017-2020.
Practical Dairy Chemistry and Physics, FQD, Wageningen, The Netherlands, 2017-2020.

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