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Effects of Dietary Supplements of DHA-enriched Micro Algae Diet on Physical and Technological Properties of Dairy Cow Milk Fat

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

This study examined the effect of dietary supplementation of DHA-enriched micro algae diet on physical and technological properties of dairy milk fat in terms of the dynamic crystallization and melting behaviour. Two dairy cows were subjected to feeding regime of DHA-enriched micro algae diet and control diet. The experiment was carried out during 21-d to determine the normal (control) and DHA-enriched micro algae diet modified milk fat that were taken for further analysis. The melting and crystallization behaviour of the milk fat from the cows fed control and DHA-enriched micro algae diets was studied using differential scanning calorimetry (DSC), Q1000 (TA Instruments, New Castle, DE, United States). DHA-enriched micro algae supplementation strongly affected the melting and crystallization properties of milk fat. Generally, the onset temperature (°C) of milk fat crystallization was significantly lower in DHA-enriched milk fat as compared to the control. The quantity of heat released by fat crystallization expressed as J/g (peak area) was significantly lower in enriched milk fat. DHA-enriched milk fat also had a lower peak maximum temperature as compared to control in all samples investigated. All melting curves displayed two

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peaks (lower melting and higher melting peaks) and for melting peaks, DHA-enriched milk fat melted at significantly lower temperature as compared to the control indicating an increase in the degree of unsaturation of milk fat. Melting offset temperature was significantly lower for DHA-enriched milk fat as compared to the control. It can be concluded that from the results of this study, micro algae supplementation significantly altered the milk fat composition and positively affected melting and crystallization behaviour of milk fat.

Keywords: Docosahexaenoic acid; algae diet; milk fat; crystallization; melting.

1. INTRODUCTION

"The fatty acid composition of milk fat has a number of effects on milk quality, including aspects such as its physical properties (e.g. melting point and hardness of butter, crystallisation and fractionation of milk fat) as well as its nutritional properties (e.g. potential effects of specific FA on human health. Cow milk fat is the most complex fat found in the nature, with more than 400 different fatty acids (FAs). About 70% of milk fat corresponds to saturated FAs and 20% to monounsaturated FAs mainly oleic acid (C18:1c9), with variations as a function of season and diet. This diversity of FAs and TAGs confers to milk fat specific physical properties, in particular the presence of a solid TAG phase over a wide range of temperatures" [1]. "The TAG composition induces a complex crystallization behavior and a wide melting range with multiple melting points. This is why modification of FAs of cow milk fat through feeding is inevitable. The FA and TAG composition of milk can be tailored for technological, nutritional, and health reasons (e.g., increase in unsaturated FA and decrease in palmitic acid). In this respect, numerous techniques have been applied including physical, chemical, and dietary manipulation by means of feeding dairy animals. Technological treatments are often applied to have desired functionalities (e.g., improved cold spreadability of butter) and expend the use of milk fat in the food industry" [1]. "Milk fat composition can be modified readily by changing the feeding regime. Attempted dietary feeding regimes include: forages, oilseeds, fish oil, and fat supplements incorporated in either protected or unprotected form" [2,3]. For instance, Rego et al. [4] reported "an increase in concentrations of CLA, TVA and n-3 PUFA by inclusion of fish oil in the diet of grazing dairy cows, which represents a positive impact upon the dietetic quality of milk and consequently results in a promotion of the image of this product by the consumer", and this was supported by Kairenius et al. [5] and Toth et al. [6].

"It is therefore expected that a diet with vegetable oils rich in unsaturated fatty acids and secondary fatty components could not only improve the nutritional properties (fatty acid profile) but could also improve the physical properties of milk" [7]. "The most significant changes in milk fat quality relate to rheological (melting) properties, which influence numerous quality aspects of manufactured dairy products. An interesting side-benefit of increasing the content of unsaturated fatty acids in milk fat is the improvement in cold spreadability of butter" [8]. "Because of its great importance, studies have been carried out on the effect of feed supplementation on the physical properties of milk fat properties such as solid fat content (SFC). For instance, a decrease in the SFC of milk derived from cow fed full fat soybeans and rape seed" was reported [9,10] and was associated with a reduction in saturated fatty acid content and an increase in mono-unsaturated fatty acid content. Shazly et al. [11] observed that "the physical properties of anhydrous milk fat (AMF) such as sold fat content (SFC) of cows and buffaloes supplemented with flaxseed oil (FO), soybean oil (SO), or their mixture (FSO) were affected not only by its content of USFA but also by its contents of lipid-minor components such as cholesterol and vitamin E". Antonacci et al. [7] reported that "the soybean-linseed oil blend at 50% resulted in the highest number of favorable nutritional changes in ewe's milk including the decrease in the hypercholesterolemic fraction of milk, the simultaneous increase in vaccenic, rumenic and linolenic acids, the n-6/n-3 ratio lower than 4 and a low atherogenic index". In another study, Lin et al. [12] reported "a decrease in the hardness and solid fat index (SFI) of milk fat derived from the cows supplemented with calcium salts of oleic acid and this was also attributed to an increase in mono-unsaturated fatty acids and a decrease in saturated fatty acids". Similarly, Singh et al. [8] reported "an increase in milk fat firmness with a slightly lower SFC at 5°C. The crystallization behaviour of milk fat was also significantly affected".

"The most important consideration concerning milk fat for manufacturing, other than total fat concentration, is the melting point of the fat. Milk fat does not melt at a defined temperature, but over a range, because of its diversity of FA and low, medium, and high melting glycerides" [13]. Likewise Van Aken et al. [14] elucidated that "triglycerides are composed of a large number of different fatty acids leading to a heterogeneous composition of triglycerides and a very broad melting range, which varies between approximately −40 and 35°C. The major FA contributing to variation in melting that can be manipulated are palmitic acid (C16:0) and Oleic acid (C18:1). Because of the relatively specific action of stearoyl CoA desaturase, increasing the content of cis FA increases the content of C18:1 and the de novo synthesis of C16:0 tends to decrease. On the other hand, crystallization of milk fat largely determines the physical stability of the fat globule and the consistency of high-fat dairy products, but crystal behaviour is also complicated by the wide range of different triglycerides. Like all triglycerides, milk fat may crystallize into several crystal polymorphs, which are classified into the main forms γ, α, β' and β". Due to the highly asymmetric molecular structure of a large part of the triglyceride molecules in milk fat, the β form is rarely observed [15]. The stability of the polymorphic crystal forms increases in the order γ < α <β'. This is reflected by a higher clear-point of the more stable polymorphic form and a slow transformation of crystals into a more stable polymorph, finally leading to a complete transformation into the β'-form. However, the γ form is the least stable and is rarely observed in slowly cooled fat. Van Aken and Visser [16] reported "a slow formation of β'-crystals after 20 to 40 min when milk fat was cooled to a temperature below the α-clear-point preceded by α-crystallization, which was complete within a few minutes (usually 2 min). Triglycerides that were initially crystallized in the α-form dissolved into the liquid fat and subsequently recrystallized on the growing β′-crystals". In a study to assess the effect of algae supplementation on the physical properties of the milk fat, Singh et al. [8] reported "a significant alteration of milk fat crystallization behaviour. The enriched milk fat showed a more rapid crystallization process and shorter induction time for nucleation in the temperature range (20-27 °C)". "Micro algae and its extracted oil provide an important source of EPA and DHA and a major advantage of its use has been reported that the technology exists to produce industrial quantities under controlled and environmentally safe conditions to modify milk fat technological properties such as melting and crystallization" [17]. The crystallization properties of milk fat can be affected by many parameters including the changes in the FA and TAG composition. Besides solid fat content, the functionality of a fat is also affected by the crystal structure formed by TAGs [1]. Understanding the crystallization and melting behaviors of milk TAGs as well as the polymorphism of milk TAGs is of great importance from a scientific point of view and with regard to the economic impact of milk fat, especially in fat-rich products.

The objective of this study therefore was to assess the effect of dietary supplementation of DHA-enriched micro algae diet on physical and technological properties of dairy milk fat in terms of the dynamic crystallization and melting behaviour.

2. METHODOLOGY

2.1 Experimental Animals

Two bearing heifers were selected to make part of the trial, in which one was from Black Holstein breed (Cow 1) while other belonged to the Red Holstein breed (Cow 2). Selection was based on the expected calving date, uniformity and origin. Cows were fed twice daily at milking time (07.30 and 19.30 h) for 21-d, where weeks 1 and 2 were used to adjust diets (adaptation weeks) and a third week for data collection (experimental week).

2.2 Experimental Diet

Animals were fed a total mixed ration based on corn and grass silage (corn:grass, 50:50, DM basis) and supplemented with proteins to meet the nutrient requirements of dairy cows producing 25 kg of milk/d. Forage was added to the ration following the type of ration. The control diet (C) and algae diet (A) ration received the standard forage. Additionally, the algae diet (A) ration was supplemented with DHA-gold (*Schizochytrium* sp., 19 % DHA). The ingredient composition of the experimental diets is shown in Table 1.

The algae were supplemented twice a day by the rumen fistula 1h after feeding. The algae addition increased gradually to maximally 4 % of the ration uptake of the previous day on DM basis. This corresponded with a maximum daily addition of 2 % on fresh matter base (35.8 kg). The amount of fresh product weighted for one feeding turn is shown in Table 2.

Table 1. Formulated experimental diets (kg DM)

Diet	Corn silage	Grass silage	Soybean meal	Standard forage
Control Diet (C)	6	6	0.5	5.5
Algae Diet (A)	6	6	1.0	4.4

Table 2. The amount of ingredients in the formulated experimental diet

Diet	Description
C (Control diet)	7,4 kg grass silage; 7,8 kg corn silage; 3 kg standard forage; 250 g soybean meal
A (Algae diet)	7,4 kg grass silage; 7,8 kg corn silage; 2,2 kg standard forage; 500 g soybean meal

2.3 Differential Scanning Calorimetry (DSC) analysis to study crystallization of milk fat

The melting and crystallization behaviour of the milk fat from the cows fed control and algae diets was studied using differential scanning calorimetry (DSC), Q1000 (TA Instruments, New Castle, DE, United States). The DSC works by comparing the difference in either the temperature or heat input between a sample contained in a DSC pan and an empty reference pan [18]. The difference is used to characterise changes associated with crystal formation or melting. Milk samples were processed at 'Melkcontrolecentrum' (MCC) into butter and anhydrous milk fat to allow long term storage. To obtain anhydrous milk fat, butter was frozen overnight and then melted and centrifuged at 400 x g at 50°C for 15 min. The upper phase was passed through filters (type 595, Schleicher & Schuell, Dassel, Germany) filled with anhydrous Na_2SO_4 as a desiccant at 60°C. The extracted fat was stored at -20°C. Frozen milk fat samples were melted in the oven at about 65 °C for 1 hour. Pasteur pipettes were washed with acetone, placed in a glass beaker and dried in the oven at about 105 °C. Approximately 5–15 mg of molten milk fat samples were introduced into hermitic DSC aluminium pans using the warmed Pasteur pipettes and then hermetically sealed using the sample encapsulating press. The pans were cleaned with acetone to remove possible traces of milk fat and accurately weighed on the analytical balance to obtain the actual sample weight. An empty hermetically sealed DSC aluminium pan was used as reference.

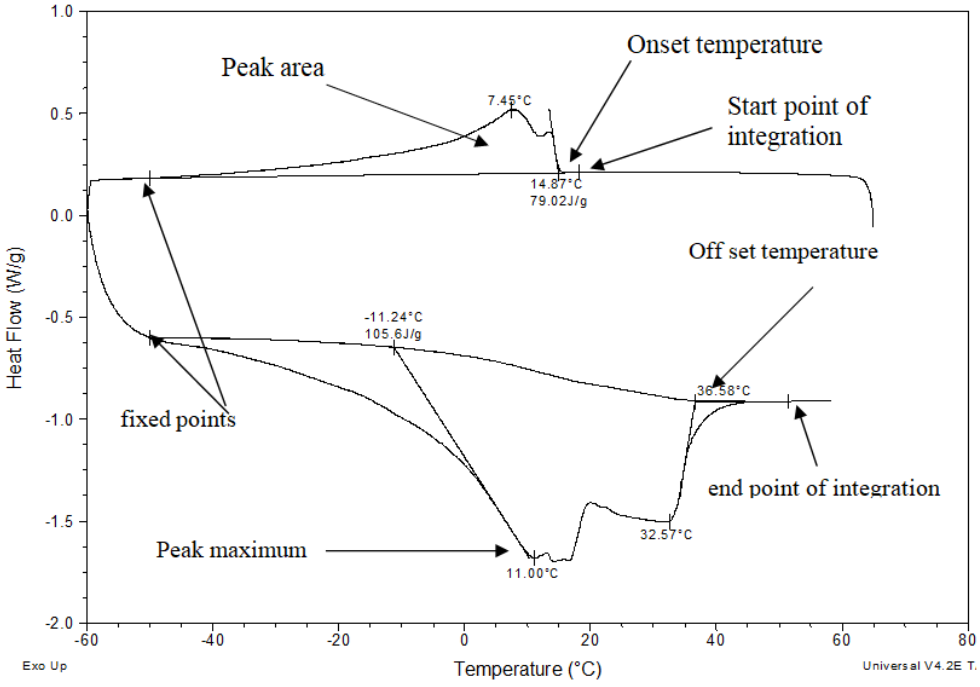

Fig. 1. Typical extractable data from endothermal and exothermal DSC curves

The following time-temperature program was used for examining the non-isothermal crystallization of milk fat in a conventional DSC: Equilibration at 65°C, Isothermal for 10 min at 65°C so that all the existing crystals and nuclei are melted, Ramp 5 °C/min until -60°C, Isothermal for 1 min at -60 °C, and the ramp 20°C/min until 65 °C. This heating rate was chosen because it is fast enough to prevent polymorphic transitions as much as possible during heating and it is slow enough to eliminate the effect of thermal lag. From the resulting endotherms and exotherms, the following parameters were obtained after integration of the curves: The onset temperature of the crystallization peak and the offset temperature of the melting peak (°C), determines the start and the end of crystallization and melting, respectively (Fig. 1). The peak maximum (°C), the temperature at which the speed of melting is maximal and the total peak area (J/g), i.e. the area enclosed by the baseline and the exothermal or endothermal peak, corresponding to the heat released by crystallization and the heat needed for melting.

The curves were integrated using TA Universal analyzer (Universal Analysis for Windows 2000/XP, version 4.2, 1998-2005 TA Instruments-waters, LLC). The resulting melting profiles give an idea about the amount of crystallized fat (peak area) in the sample. The total peak area is calculated by integration with the starting point and the end point of crystallization being known and taken as integration limits. However, when determined visually, these limits are very operator dependent. To avoid this, Foubert, et al. [19] developed an objective calculation algorithm to determine these limits automatically. The integration limits (starting point and the end point of crystallization) for the DSC curves in this experiment therefore, were determined using the objective calculation based on the algorithm developed by Foubert [19]. Temperature of -50°C was used as fixed points for establishment of DSC curves (Fig.1).

2.4 Statistical Analysis

Statistical analyses for melting and crystallization data were performed using SPSS 12.0 (SPSS software for Windows, release 16.0, SPSS, Inc., USA). Standard Two-Sample t-Test was applied, and the effects with $P < 0.05$ were considered significant.

3. RESULTS AND DISCUSSION

3.1 Effect of DHA-enriched Micro Algae diet on Melting and Crystallization of Milk Fat

The average milk fat melting and crystallization parameters as determined by DSC are shown in Table 3. DHA-enriched micro algae supplementation strongly affected the melting and crystallization properties of milk fat (Table 3 and Fig. 2). Generally, the onset temperature (°C) of milk fat crystallization was significantly lower in DHA-enriched milk fat as compared to the control. The quantity of heat released by fat crystallization expressed as J/g (peak area) was significantly lower in enriched milk fat, except for Cow3-Day 5. DHA-enriched milk fat also had a lower peak maximum temperature as compared to control in all samples investigated (Table 3 and Fig. 2). All melting curves (Fig. 2) displayed two peaks (lower melting and higher melting peaks). For both lower and higher melting peaks, DHA-enriched milk fat melted at significantly lower temperature as compared to the control indicating an increase in the degree of unsaturation of milk fat. Melting offset temperature was significantly lower for DHA-enriched milk fat as compared to the control. Thus in all cases, melting of DHA-enriched milk fat ended at a relatively lower temperature than the control (Table 3). The quantity of heat required to melt the milk fat crystals formed was lower for DHA-enriched milk fat compared to the control.

In current study, melting and crystallization of milk fat were used to assess the change in physical and technological properties of DHA-enriched milk fat. A significant change in melting and crystallization behaviour of milk fat was noted as a result of algae supplementation (Fig. 2). At this stage, it must be kept in mind that melting and crystallization of natural fats are complicated phenomena and this is certainly true for milk fat with its very wide range of different triglycerides. Milk fat crystallisation is of great practical importance, as it largely determines the melting behaviour, the physical stability of the fat globule, the consistency of high-fat products [1], the compatibility with other fats and the aeration properties [20]. “The FA and TAGs (triacylglyceride) composition of milk can be tailored for technological, nutritional, and health reasons (e.g., increase in unsaturated FA and decrease in palmitic acid). In this respect,

numerous techniques have been applied including physical, chemical, and dietary manipulation by means of feeding dairy animals" [1]. Technological treatments are often applied to have desired functionalities such as improved cold butter spreadability and expand the use of milk fat in the food industry. Boeckaert et al. [21] reported that "dietary supply of DHA-enriched micro algae resulted in an altered milk fatty acid composition toward increased concentrations of C18:2 t11c15, CLA c9t11, CLA t9c11, C18:1 *trans*, and DHA and decreased concentrations of SFA, in particular C18:0. A modified milk fatty acid composition upon algae feeding was associated with decreased milk fat content when algae were supplemented with the diet".

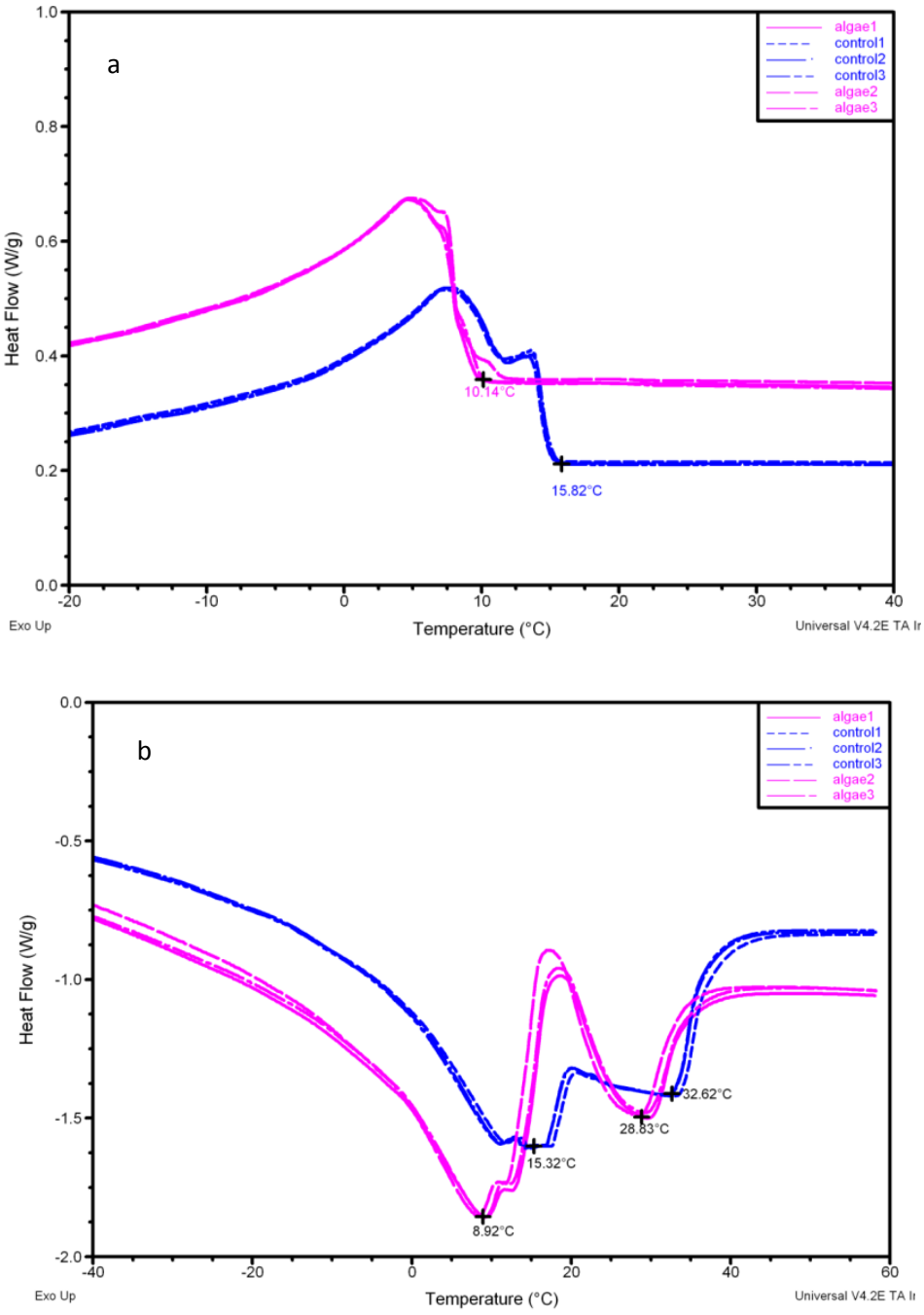

Fig. 2. Differential scanning calorimetry thermographs for the melting and crystallization of control and DHA enriched milk fat, immediately upon crystallization

Table 3. Crystallization and melting parameters for milk fat from algae and control diet fed cows

Sample		Crystallization			Melting			
		Onset T[4] °C	Peak area KJ/g	Peak Max °C	Offset T[5] °C	Peak area KJ/g	Peak[1]max °C	Peak[2] max °C
Cow1-Day3	Control	15.6	76.2	7.70	38.5	104	12.9	34.2
	Algae	10.4	72.9	5.30	34.9	91.6	9.40	29.7
	Signif[1]	***	0.122	**	**	*	**	***
	SEM[2]	*0.138*	*1.703*	*0.242*	*0.600*	*3.170*	*0.465*	*0.518*
Cow1-Day5	Control	14.8	78.2	7.36	36.9	106	11.3	33
	Algae	8.70	70.8	4.61	35.0	94.6	8.62	29.5
	Signif[1]	***	***	***	*	**	**	***
	SEM[2]	*0.140*	*0.468*	*0.100*	*0.669*	*1.999*	*0.398*	*0.438*
Cow2-Day1	Control	13.8	75.8	9.61	37.2	101	13.3	32
	Algae	11.85	63.8	8	36.5	81.3	11.6	29
	Signif[1]	***	**	0.113[3]	0.555	**	0.065	0.232
	SEM[2]	*0.149*	*1.878*	*0.637*	*1.305*	*3.565*	*0.679*	*2.135*
Cow2-Day3	Control	14.4	77.8	10.14	38.1	110	14.4	33.5
	Algae	9.9	66.3	7.13	31.3	77.5	10.2	24.8
	Signif[1]	0.175[3]	**	***	**	***	**	***
	SEM[2]	*0.940*	*1.825*	*0.298*	*1.010*	*2.066*	*0.632*	*0.942*
Cow2-Day5	Control	13.6	73.6	9.55	37.9	105	14.1	32.8
	Algae	12.2	73.6	8.45	33.6	93.7	12.3	28.2
	Signif[1]	**	0.968	0.155[3]	*	***	**	**
	SEM[2]	*0.212*	*1.084*	*0.505*	*0.961*	*0.755*	*0.301*	*0.720*

[1]Signif = the level of significance difference between the mean values for control and algae (or p-value)

[2]SEM = the standard error of mean

[3]Welch Modified Two-Sample t-Test was used (since the equality of variance assumption was not fulfilled). In rest of the samples, the equality of variance assumption was fulfilled, and a simple Two Sample t-Test was used

[4]The temperature at which crystallization of milk fat starts

[5]The temperature at which melting of milk fat ends

*Data presented are simple/modified two sample t-Test estimated means, n = 3 for control and algae diet in all parameters. *** for P < 0.001, ** for P < 0.01 and * for P < 0.05;and P>0.05 are not significantly different and their respective p-values are shown*

The variations in fatty acid composition as a result of diet alteration affect the crystallization and melting properties of milk fats. Melting behaviour is important to many applications of milk fat in food products. The melting behaviour of milk fat can be characterised by its final melting point (defined as the temperature at which milk fat becomes visually clear and free of crystals), melting range, also called thermal profile, and solid fat content profile [22,23]. In the current study, all melting curves (Fig. 2) displayed two peaks (lower melting and higher melting peaks) as observed by DSC. For both lower and higher melting peaks, DHA-enriched milk fat melted at significantly lower temperature as compared to the control indicating an increase in the degree of unsaturation of milk fat. The melting behaviour of milk is affected by chain length, degree of saturation, branching, position of the double bound and *cis* or *trans* configuration of fatty acids [1]. The melting point of untreated milk fat varies between approximately 32 to 38 °C. Anhydrous milk fat (AMF), which is the fat isolated from original milk butter, has a broad melting range from –40°C to +40°C and no sharp melting temperature such as pure compounds (Fig. 1). AMF is not completely solid until it reaches a temperature below –40°C and must be warmed at least to +40 °C to ensure complete melting of TAGs.

In current study, DSC recordings show two successive isotherms that have been attributed to the successive crystallization of milk fat (Fig. 2.b) and an exotherm in the DSC thermogram confirms a polymorphic transition (α→β') occurring on heating of milk fat (Fig. 2.a). This is in accordance with [24,25,26] who studied crystallization and melting behaviour of milk fat by differential scanning calorimetry (DSC) and confirmed that it crystallizes and melts in several steps. The rate of the polymorphic transition (α→β') and formation of β'-crystals from the melt is dependent on the chemical composition and is slower in the LMF (low melting fraction) than in the HMF (high melting fraction) [14]. Polymorphic transitions depend largely on the heating rates during DSC-analysis of not tempered samples. These authors identified three endothermic peaks for milk fat, the first endothermic peak corresponding to the melting of α-polymorphs of low melting fraction (LMF) and middle melting fraction (MMF). The second endothermic peak, which had a maximum at 14 °C, was a result of further melting of triglycerides that are in the α-form as the short spacings of the α-form disappear at 17°C to 20°C [15]. The third and broad shoulder peak in the DSC curves is probably caused by the melting of mixed β'-crystals of high melting fraction (HMF) and middle melting triglycerides. However, in the present study, only two melting peaks were identified by DSC (Fig. 2). The disappearance of the third polymorphic transition peak could be a result of the higher heating rate (20°C/min) used during the experiment. The peak maximum for the first and second peak of DHA-enriched milk fat was on average between 9 and 28°C, respectively, whereas for control milk fat the first and second peak had their maximum on average at 15 and 33°C, respectively. In the present study, the melting point of DHA-enriched milk fat ranged between 9.4 and 12.3°C for the low melting fraction and between 24.8 and 29.7°C for the high melting fraction. The lower peak maximum temperature for melting and crystallization, lower onset temperature for crystallization, lower offset temperature for melting and the lower quantity of heat energy required for complete melting of fat crystals formed upon crystallization observed in DHA-enriched milk fat compared to the control, can be explained by the increase in the degree of unsaturation of DHA-enriched milk (Fig. 2 and Table 3).

Indeed, the degree of unsaturation increased in DHA-enriched milk fat as it was represented by an increase in total unsaturated fatty acid and decrease in total saturated fatty acids in DHA-enriched milk fat as it was reported in previous study [21]. The increase in the degree of unsaturation of milk fat represents a positive impact upon dietetic quality of milk and consequently results in a promotion of the image of this product by the consumer. However, increase in degree of unsaturation may be associated with increased risk of oxidative deterioration of enriched dairy products. When considering the technological properties of DHA enriched milk, it is therefore important to take into account this fact.

4. CONCLUSION

Algae supplementation positively affected melting and crystallization behaviour of milk fat. The onset temperature for crystallization, offset temperature for melting, quantity of heat energy required for crystallization and melting of milk fat, and the peak maximum temperature for both melting and crystallization were decreased by algae supplementation. From these findings, it is concluded that micro algae supplementation

significantly alter the milk fat composition and physical properties of milk fat by lowering melting temperature as compared to the control indicating an increase in the degree of unsaturation of milk fat which improves its human nutrition and health image by increasing CLA and DHA levels and lowering SFA.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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