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RESEARCH ARTICLE

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A two-step feeding of calcium salts of fish oil improves reproductive performance in Holstein cows

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Abstract

Aim of study: To examine the effects of a biphasic schedule of feeding *n-3* fatty acids on dairy cows.

Area of study: Isfahan, Iran.

Material and methods: 140 lactating Holstein cows were allotted at calving into two groups of 70 animals and received one of two dietary treatments: 1) saturated fatty acids (SFA, containing 80% palmitic acid) or 2) calcium salt of fish oil (CSFO, containing 16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)), with an *n-6*/*n-3* FA ratio of approximately 7 for SFA and 5 for CSFO treatments. The dietary supplements were fed to the respective groups at 240 g/head.day from 0 to 21 days in milk, and 120 g/head.day from 22 to 150 days in milk. Milk yield was recorded biweekly and milk composition was evaluated monthly. The concentration of FA in the milk and blood was determined on d-90 of the experiment in 10 cows randomly selected from each group. Reproductive indices were recorded until d-150.

Main results: The CSFO supplementation did not affect average milk yield, milk composition or milk somatic cell count (SCC); however, in some weeks it decreased milk SCC ($p<0.05$). Plasma concentrations of palmitic acid and *n-3* FA as well as milk fat concentration of EPA and DHA increased in the CSFO-fed cows ($p<0.05$). Feeding the CSFO decreased open days (100 *vs* 119 days, $p<0.05$), service per conception and all service conception rates ($p<0.05$).

Research highlights: The implementation of a two-stage feeding program of *n-3* FA improved reproductive variables and reduced milk SCC in dairy cows.

Additional key words: conception rate; *n-6*/*n-3* ratio; postpartum; udder health

Abbreviations used: ADF (acid detergent fiber); CLA (conjugated linoleic acid); COX2 (cyclooxygenase 2); CSFO (calcium soap of fish oil); DHA (decosahexaenoic acid); DIM (days in milk); DM (dry matter); EPA (eicosapentaenoic acid); FA (fatty adcid); LOX (lipoxygenase); NDF (neutral detergent fiber); PUFA (poly-unsaturaed fatty acids); SCC (somatic cell count); SFA (saturated fatty acids); TMR (total mixed ration).

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Introduction

Reis *et al.* (2012) demonstrated that feeding cows with moderate quantities of poly-unsaturated fatty acids (PUFA) improves production performance as compared to feeding saturated FA (SFA). More recently, McFadden (2020) explored the role of saturated and unsaturated FA as bioactive signaling molecules in modulating insulin secretion and sensitivity. Progresses in this field has demonstrated that the ratio of two important subclasses of FA, namely *n-6* and *n-3* (or alternatively omega-FA), plays a prominent role in reproduction success, embryonic survival, service per conception (Ballard & Byrd, 2018), and open days (Dirandeh *et al.*, 2013). However, different subclasses of omega PUFA differ in terms of their impact on reproduction. Silvestre *et al.* (2011) reported that the reproductive performance of dairy cows was improved in a two-step feeding program through which the *n-6* FA supply was increased after calving, followed by an increase

in *n-3* FA supply during the service period. This strategy deprives fresh cows of taking the advantages of *n-3* FA in suppressing the inflammatory reactions that have been observed, for instance, in endothelial cells of periparturient cows (Contreras *et al.*, 2012). Therefore, a strategic feeding of both subclasses of omega FA should be explored in such a way that the benefits of *n-3* FA in enhancing reproduction and health coincided with the potential of *n-6* FA in cyclicity resumption (Silvestre *et al.*, 2011). The latter strategy constructs the underlying hypothesis of the present study; therefore, the objectives of this farm-scale trial, were to investigate the impacts of feeding a combination of *n-6* and *n-3* FA in a biphasic feeding schedule from calving to 150 days in milk (DIM) on reproduction and performance of dairy cows.

Material and methods

Study design and data collection

The study was undertaken in a large commercial dairy facility (FKA Agri-Animal Husbandry Co., Isfahan, Iran). All experimental procedures were approved by the Animal Welfare Committee of the Department of Animal Science, University of Tehran, Iran. A total of 140 multiparous Holstein cows were randomly allotted into two groups of 70 animals each, blocked for similar calving date, and received one of two dietary treatments immediately postpartum: 1) saturated fatty acids (SFA, containing 80% palmitic acid, Energizer RP10©, IFFCO, Malaysia) or 2) calcium salt of fish oil (CSFO, containing a minimum of 16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA), StrataG113, Virtus Nutrition LLC, Corcoran, CA, USA). The dietary supplements were fed to the respective groups at 240 g/head per d from 0 to 21 days of lactation, and 120 g/head per d from 22 to 150 days in milk. According to the manufacturer's indications, the CSFO contained also 33% palmitic acid, 15% stearic acid, 25% oleic acid, 5% linoleic acid and 2% linolenic acid. The decrease in the CSFO from 240 g to 120 g/head per day in the CSFO diet was accompanied the removal of extruded soybean from d-21 to d-150 in both treatments in such a manner that the ratio of *n-6*/*n-3* FA in the SFA and CSFO groups was retained at 7.6 and 4.5 from d-1 to d-21, and at 7.0 and 5.0 from d-22 to d-150, respectively. As shown in Table 1, the diets had a

Table 1. Ingredients of the experimental diets fed from parturition until 150 days in milk (DIM)

Ingredients, % DM	1-21 DIM		22-150 DIM	
	SFA[1]	CSFO[1]	SFA	CSFO
Alfalfa hay	15.5	15.5	17	17
Corn silage	27.6	27.6	17.7	17.7
Ground barley grain	10.4	10.4	18.3	18.3
Ground corn grain	18.6	18.6	18.3	18.3
Whole cottonseed	5	5	3.75	3.75
Soybean meal	8.5	8.5	13.5	13.5
Canola meal	4.6	4.6	3.3	3.3
Poultry meat + feather meal	1.1	1.1	2.5	2.5
Extruded soybean	4.1	4.1	-	-
Saturated fat source	1.3	-	2.45	2
Calcium soap of fish oil	-	1.3	-	0.45
Sodium bicarbonate	1.1	1.1	1.2	1.2
Calcium carbonate	0.7	0.7	0.9	0.9
Dicalcium phosphate	0.35	0.35	0.25	0.25
Magnesium oxide	0.2	0.2	0.2	0.2
Sodium chloride	0.35	0.35	0.35	0.35
Vitamin premix[2]	0.3	0.3	0.15	0.15
Mineral premix[3]	0.3	0.3	0.15	0.15

[1]SFA: saturated fat source (80% palmitic acid); CSFO: calcium soap of fish oil (16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)). [2]Contained: 1200 KIU/kg vitamin A, 360 KIU/kg vitamin D3, 12000 IU/kg vitamin E. [3]Contained: 2000 mg/kg Fe, 8000 mg/kg Mn, 12000 mg/kg Zn, 120 mg/kg Co, 3000 mg/kg Cu, 250 mg/kg I, 80 mg/kg Se.

high concentrate forage ratio, in order to compensate for the low quality of the available forages, similar to those used in dairy cows' diets in Iran.

Milk production was recorded for all cows in biweekly intervals and milk composition was determined monthly. The observed dry matter (DM) intake of each group of cows was recorded daily by weighing the offered total mixed ration (TMR) and orts, and averaged by week for the entire experimental period. The DM of corn silage was determined weekly for the adjustment of silage allotted. A representative TMR sample was taken biweekly for chemical analysis. On d 90 of the experiment, 10 cows from each group were randomly sampled in three consecutive milkings for milk composition; samples were composited by proportional weight and stored at -20 °C. Samples of blood were taken from the same cows on the same day and were frozen after the separation of sera by centrifuging for 15 minutes at 3000 rpm. The milk and sera samples were then used for FA determination.

Reproductive performance variables, including days to first service, days open, conception rate and number of cows pregnant until 120 and 150 DIM were recorded from the individual cow data.

Chemical analyses, and fatty acid profiles of the diet, milk and serum

Samples of TMR were mixed, subsampled and oven-dried at 65 ºC for 72 h. Dried samples were ground through a 1 mm screen and analyzed for crude protein, ether extract, ash, acid detergent fiber (ADF) and neutral detergent fiber (NDF) (Alamouti *et al.*, 2009). Ether extract was then used for FA profiling. For the analysis of milk and serum FA, samples were thawed at room temperature and 3 mL aliquots were collected. The FA in milk were methylated with 1 mL of 2 N methanolic NaOH at room temperature for 20 min, followed by 1 mL of 14% boromtrifluoride in methanol at room temperature for 20 min. The FA methyl esters were recovered in 1 mL of hexane (Loor *et al.*, 2005). The FA concentration was determined by gas chromatography (Younglin 6100, Anyang, Korea) with a 120 m (0.22 mm id) silica-fused column (BPX-70, Trajan Scientific LTD, Australia). Nitrogen was the carrier gas, and initial and final temperatures were set at 170 and 230 ºC, respectively, with the detector and injector temperatures set at 300 and 260 ºC. Sigma-Aldrich FAME mixture (EC Number 200-838-9, Sigma-Aldrich, Sigma-Aldrich Chemie, GmbH) was used as FA standard.

Statistical analyses

Treatment means for milk yield and composition and FA profile of milk and blood were compared using a model that included the fixed effect of treatment and the random effect of cow. For the repeated measurement the fixed effects of time and time× treatment interaction were also included. For this set of analysis, PROC MIXED, and for the analysis of reproductive parameters PROC GENMOD were applied (SAS, 2004). Treatment means were expressed as least square means. Statistical significance was declared when $p \leq 0.05$ and trends were noted when $0.05 < p \leq 0.10$.

Results

Production performance and FA profile of the milk and blood

By design of the trial, feeding 240 g and 120 g/head per d of the CSFO delivered to the cows 38 to 48 and 19 to 24 g/head per d of EPA and DHA during days 1 to 21 and 22 to150 of lactation, respectively. Chemical composition of the experimental diets showed that their NDF, ADF and non-fiber carbohydrates concentrations were within the values recommended by the NRC (2001) for high-producing cows (Table 2).

The experimental diets showed differences in the FA profile that were in accordance to the design of the trial. In diets supplemented with CSFO, the concentrations of oleic acid, linolenic acid, EPA, DHA, and total *n-3* FA increased, while that of palmitic acid decreased. As a result, the ratio of *n-6*/*n-3* FA decreased below 5 (Table 2).

The average of group DM intake was lower in cows on CSFO compared to cows on SFA (26.3 *vs* 27.2 kg/d). Milk production and composition did not differ between treatments, but a treatment × time interaction was noted for somatic cell count (SCC), where cows on CSFO diet showed lower SCC compared to SFA cows in the first milk sampling after calving. Also, milk protein concentration was lower in the CSFO-fed cows in the 2nd milk sampling (2.69 *vs* 2.95%) compared to the SFA-fed cows ($p<0.05$; Table 3). Noteworthy, taking into account the relative milk fat and protein concentrations, both treatments appeared to show a certain degree of milk fat depression, but without differences between treatments (Table 3).

Supplementation of the CSFO did not affect the concentrations of major short- and medium-chain FA in milk ($p>0.05$; Table 4). Palmitic acid was the most abundant FA in milk, followed by oleic acid (cis C18:1) that was significantly increased by feeding the CSFO. Feeding CSFO did not affect the concentration of total conjugated linoleic acid (CLA) isomers and trans C18:1; however, it significantly increased the EPA + DHA concentration. Concentrations of arachidonic acid, Σ*n-6*, *n-6*/*n-3* ratio, Σ<16C, Σ16C and Σ18C was unaffected by dietary treatments ($p>0.05$).

Table 2. Chemical composition and fatty acid (FA) profile of the experimental diets fed from parturition until 150 days in milk (DIM)

Item, % DM or stated otherwise	1-21 DIM		22-150 DIM	
	SFA[1]	CSFO[1]	SFA	CSFO
OM	92.6	92.8	92.8	92.7
CP	16.1	15.9	16.7	16.6
NDF	33.9	33.5	30.2	29.8
ADF	21.6	22.0	19.5	19.4
NFC	38.1	38.7	41.0	41.3
Ether extract	4.5	4.7	4.9	4.9
FA profile, g/100 g of total FA				
Σ<16 C	1.26	1.66	1.73	1.08
C16:0	35.4	21.2	49.1	44.9
C18:0	2.48	5.36	2.3	3.2
C18:1	15.4	19.8	12.5	13.9
C18:2, n-6	37.8	40.5	27.4	28.2
C18:3, n-3	4.77	5.43	3.45	3.63
Σ16 C	35.9	21.7	49.8	45.6
Σ18 C	60.7	71.4	45.9	49.0
C20:5 (EPA)	0.140	2.29	0.291	1.158
C22:6 (DHA)	0.109	1.40	0.233	0.832
Σn-3	5.06	9.03	3.95	6.82
Σn-6	38.4	40.6	27.5	28.4
n-6/n-3	7.58	4.50	6.97	5.0

[1]SFA: saturated fat source (80% palmitic acid); CSFO: calcium soap of fish oil (16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)).

In cows receiving the CSFO, serum FA profile showed greater concentrations of C12:0, C15:0 (p<0.10), C16:0, EPA + DHA and total *n-3* FA (p<0.05), which resulted in a significant decrease in *n-6*/*n-3* ratio compared to cows receiving SFA (Table 5).

Reproductive performance

All services conception rate was higher (p=0.04) and service per conception was lower (p=0.03) in the CSFO-fed cows as compared to the SFA-fed cows. In turn, days open were shorter in cows receiving CSFO (p<0.001; Table 6). However, days to first service, first service conception rate, and the proportion of cows that were diagnosed pregnant by 120 and 150 DIM did not differ between the experimental groups (p>0.05).

Discussion

Diets in the present study were representative of those fed to the high producing dairy cows in early lactation and contained high concentrations of non-fiber carbohydrates and crude fat (Table 2). Also, differences of the FA profile of the diets represented the projected design of the trial aiming at supplementation of the diets with *n-3* FA sources with an emphasis on maintaining the ratio of *n-6*/*n-3* FA below five. In fact, Greco *et al.* (2015) observed a linear increase in milk production of early lactation cows as the ratio of *n-6*/*n-3* FA decreased. However, Greco *et al.* (2015) started the feeding experiment from 15 DIM and did not address the immediate postpartum period. In contrast, another report suggested that feeding a greater amount of *n-6* FA in pre-insemination period followed by a greater amount of *n-3* FA afterwards results in an earlier cyclicity, a higher conception rate and lower early embryonic losses (Silvestre *et al.*, 2011). In the current study, we supplemented the *n-3* and *n-6* FA immediately after calving while maintained the dietary *n-6*/*n-3* ratio < 5. As shown in the results, this delivered high amounts of both *n-6* and *n-3* FA to the cows in the CSFO group until 21 DIM.

Except for weeks 12 (27.1 *vs* 26.4 kg/d), 15 (27.2 *vs* 26.2 kg/d) and 21 (27.8 *vs* 25.4 kg/d), the group DM intake of cows fed with the CSFO was almost consistently lower than those fed with the SFA throughout the

Table 3. Production performance of cows fed the experimental diets

Item	Treatments[1]		SEM	*p*-value		
	SFA	CSFO		Treatment	Time	Treatment × Time
Milk yield, kg/d	46.8	48.2	0.95	0.28	<0.01	0.70
3.5% fat corrected milk	42.4	43.2	0.83	0.51	<0.01	0.29
Fat yield, kg/d	1.34	1.35	0.03	0.71	<0.01	0.39
Protein yield, kg/d	1.42	1.44	0.02	0.68	<0.01	0.33
Milk fat (%)	2.88	2.83	0.05	0.52	<0.01	0.9
1st milk sampling[2]	3.34	3.41				
2nd milk sampling	3.03	2.99				
3rd milk sampling	2.69	2.64				
4th milk sampling	2.53	2.46				
5th milk sampling	2.82	2.68				
Milk protein (%)	3.01	2.98	0.02	0.37	<0.01	<0.01
1st milk sampling	3.14	3.23				
2nd milk sampling	2.95a	2.69b				
3rd milk sampling	2.99	2.98				
4th milk sampling	3.04	3.09				
5th milk sampling	2.92	2.93				
Somatic cell count (SCC)[3]	222	141	58	0.69	<0.01	0.04
1st milk sampling	315a	175b				
2nd milk sampling	266	140				
3rd milk sampling	143	136				
4th milk sampling	253	119				
5th milk sampling	138	134				

[1]SFA: saturated fat source (80% palmitic acid); CSFO: calcium soap of fish oil (16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)). [2]First milk sampling was done when the experimental cows were at 15 ± 8 DIM with the subsequent recordings taken place at monthly intervals. [3] Number of cells × 10^3. [a,b]Values differing in superscript letters in the same row are statistically different (p<0.05)

experimental period (on average 26.3 *vs* 27.2 kg/d). Linear decrease in DM intake was reported in a recent study as the ratio of *n-6*/*n-3* was lowered in the diet (Greco *et al.*, 2018). Similarly, it was shown that fish oil supplementation, but not extruded soybean, decreases the DM intake of lactating cows (Whitlock *et al.*, 2002). The typical decrease in DM intake of cows fed with fish oil has been attributed to the increased intake of long chain PUFA (Alizadeh *et al.*, 2012), which, in turn, promotes hormonal modulations that slow gut motility and increase transit time (Allen, 2000). Additionally, an improvement in total tract digestibility of nutrients has been observed as a consequence of feeding cows with PUFA (Boerman *et al.*, 2015). Nevertheless, there are studies that did not observe changes in DM intake in cows fed with fish oil (Elis *et al.*, 2016).

Similar milk production between treatments in this study was consistent with previous studies (Bharathan *et al.*, 2008; Dirandeh *et al.*, 2013) comparing the effects of PUFA *vs* SFA in dairy cows. Although a minimum of 30% NDF was supplied in both diets to meet the fiber requirements, cows in both treatments showed milk fat depression; values that dropped to as low as 2.5% in the fourth milk sampling time (*i.e.* around 100 DIM). Therefore, it was likely that cows suffered subclinical ruminal acidosis. Nevertheless, of particular interest was the effect of treatments on the SCC of milk that is generally regarded as a marker of udder health. Although the average SCC was only numerically lower for the CSFO diet, cows on this diet showed a significantly lower SCC in the first milk recording after parturition. The *n-3* FA have been shown to attenuate inflammation in the endothelial cells of various tissues, including mammary gland, through down regulation of protein expression of cyclooxygenase 2 (COX2) and 15 lipoxygenase (LOX). These enzymes are involved in eicosanoid production, which in turn, is responsible for the induction of a diverse range of inflammatory reactions (Contreras & Sordillo, 2011). In dairy cows, the transition period is associated with an increased activity of COX2 and 15 LOX, which can be attenuated or exacerbated

Table 4. Fatty acid (FA) profile of milk fat on day 90 of the experimental period (10 cows per treatment)

FA (% total FA)	Diets[1]		SEM	*p*-value[2]
	SFA	CSFO		
C4:0	1	0.95	0.10	0.70
C6:0	0.99	0.99	0.12	0.98
C8:0	0.87	0.73	0.06	0.13
C10:0	2.30	1.90	0.16	0.19
C11:0	**0.14**	**0.08**	**0.01**	**0.04**
C12:0	3.04	2.62	0.16	0.09
C14:0	10.80	10.25	0.30	0.22
C14:1 cis9	1.65	1.81	0.18	0.54
C15:0	1.36	1.35	0.07	0.88
C16:0	34.16	34.25	0.83	0.94
C16:1 cis9	3.30	3.70	0.21	0.19
C17:0	0.75	0.76	0.03	0.84
C18:0	7.66	7.47	0.31	0.68
C18:1 trans9	4.05	3.71	0.83	0.78
C18:1 cis9	**19.26**	**21.0**	**0.52**	**0.04**
C18:2 cis9, cis12	3.74	3.51	0.17	0.36
C18:2 cis9, trans11	1.14	1.25	0.10	0.48
C18:3 cis9, cis12, cis 15	0.54	0.57	0.08	0.79
C20:0	0.15	0.20	0.03	0.37
C22:0	0.14	0.09	0.02	0.14
C22:5 *n-3*	0.07	0.08	0.01	0.50
EPA + DHA[1]	**0.11**	**0.17**	**0.01**	**0.03**
Others	2.14	2.56	0.21	0.68
Σ<16 C	22.72	21.31	0.73	0.19
16 C	37.45	37.96	0.84	0.67
Σ18 C	36.41	37.60	0.10	0.46
SFA[1]	63.55	62.38	1.44	0.58
MUFA[3]	29.97	31.48	1.20	0.39
PUFA[3]	6.47	6.13	0.45	0.60
Σn-3	0.67	0.82	0.08	0.20
Σn-6	3.93	3.64	0.20	0.33
n-6/*n-3*	6.33	4.43	1.96	0.88

[1]SFA: saturated fat source (80% palmitic acid); CSFO: calcium soap of fish oil (16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)). [2] Statistically significant treatment means are shown in bold letters. [3]MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

depending on the availability of long chain PUFA (Contreras *et al.*, 2012). For example, when *n-3* FA replaces *n-6* FA (Aitken *et al.*, 2009) or palmitic acid (Contreras & Sordillo, 2011), the expression of COX2 is suppressed. Likewise, the inclusion of fish oil, as compared to soybean oil, reduces the concentration of inflammatory cytokines in the mammary tissues of rats (Lin *et al.*, 2013). In the current study, the lower SCC in the early postpartum period (*i.e.* the first milk sampling) was likely related to an optimized ratio of *n-6*/*n-3* FA that might have also been assisted by a decrease in palmitic acid content in the CSFO diet (Table 2). This finding supported our hypothesis that immediate supplementation of the *n-3* FA after calving attenuates inflammation, thereby enhancing the animal health.

Table 5. Serum fatty acid (FA) profile of cows on day 90 of the experimental period (10 cows per treatment)

FA (% of serum total FA)	Treatment[1]		SEM	*p*-value[2]
	SFA	CSFO		
C12:0	0.08	0.14	0.02	0.08
C14:0	0.26	0.31	0.03	0.21
C14:1 cis9	0.14	0.14	0.01	0.87
C15:0	0.42	0.51	0.03	0.06
C16:0	**16.22**	**17.95**	**0.40**	**0.01**
C16:1 cis9	1.51	1.55	0.12	0.82
C17:0	2.19	2.11	0.17	0.74
C18:0	21.26	20.38	1.17	0.60
C18:1 cis9	10.10	9.60	0.62	0.59
C18:2 cis9, cis12	35.24	34.81	1.47	0.84
C18:2 cis9, trans11	0.61	0.86	0.12	0.19
C18:3 cis9, cis12, cis 15	1.76	1.74	0.09	0.92
C20:0	0.16	0.22	0.04	0.26
C20:4 n-6	0.28	0.07	0.10	0.19
C22:0	0.20	0.17	0.01	0.21
C22:5	0.13	0.11	0.02	0.53
EPA + DHA, all cis	**1.83**	**2.84**	**0.21**	**0.005**
Others	6.43	5.45	0.73	0.85
Σ<16 C	0.82	0.96	0.15	0.52
16 C	18.14	19.50	0.52	0.08
Σ18 C	**69.28**	**67.47**	**0.58**	**0.04**
SFA	41.21	41.66	1.52	0.84
MUFA3	12.61	11.99	0.80	0.60
PUFA3	46.17	47.46	1.80	0.62
Σn-3	**3.59**	**4.59**	**0.25**	**0.01**
Σn-6	35.49	34.87	1.46	0.77
n-6/n-3	**10.19**	**7.67**	**0.62**	**0.01**

[1]SFA: saturated fat source (80% palmitic acid); CSFO: calcium soap of fish oil (16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)). [2] Statistically significant treatment means are shown in bold letters. [3]MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

As the number of cows sampled for the analysis of milk and serum FA was limited in the present study, it might have been difficult to observe a significant difference in FA profile between CSFO-treated cows as compared to SFA-treated cows. Nevertheless, unaltered profile of most FA in milk of cows fed with the CSFO was similar to previous studies (AbuGhazaleh *et al.*, 2002; Qiu *et al.*, 2004; Alizadeh *et al.*, 2012). However, concentrations of some FA, notably C11, decreased by feeding the CSFO in our study. Although a comprehensive study has previously detected C11:0 in milk samples collected from France, Germany, and the USA (Jensen, 2002), no study has so far reported the effect of such nutritional interventions as in our study on the concentration of C11:0 in milk.

Elevated concentrations of cis C18:1 in milk of cows fed the CSFO was partly the result of a greater cis C18:1 content in the respective diet. Changes in the dietary content of cis C18:1 has been associated with a corresponding change in milk fat in some studies (Loor *et al.*, 2005; Invernizzi *et al.*, 2010). Likewise, similar concentrations in milk of trans C18:1 and CLA may explain unchanged milk fat concentration between treatments. The lack of an effect of PUFA from fish oil alone or in combination with soybean oil on the concentration of CLA has been noted in another research (Invernizzi *et al.*, 2010). In our study,

Table 6. Reproductive parameters in cows fed with the experimental diets

Item	Treatment[1]		SEM	*p*-value[2]
	SFA	CSFO		
Days open	119	100	4.3	<0.001
Days to first service	62.6	64.4	1.90	0.29
Proportion of cows pregnant by 120 DIM	0.33	0.41		0.32
Proportion of cows pregnant by 150 DIM	0.48	0.53		0.53
Services per conception	2.76	2.06	0.098	0.03
First service conception rate	0.21	0.33		0.13
All services conception rate	0.20	0.31		0.04

[1]SFA: saturated fat source (80% palmitic acid); CSFO: calcium soap of fish oil (16% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)).

the reason might have been resulted from a minor impact of unsaturated FA on ruminal fermentation processes likely due to the adequate saponification of FA by calcium in the CSFO (Theurer *et al.*, 2009; Yang *et al.*, 2009). Moreover, high DM intake of cows in this study might have increased the passage rate of PUFA out of the rumen, thereby limiting the ruminal availability of unsaturated FA. Conversely, Moussavi *et al.* (2007) reported an increase in CLA content in reproductive tissues of cows fed the CSFO and fish meal (Moussavi *et al.*, 2007).

The significant increase in the concentration of C16:0 in serum of cows fed the CSFO was questionable considering that the intake of C16:0 was higher in the SFA-fed group. At least part of this result could be justified by the FA mean melting point theory (Gama *et al.*, 2008). Based on this theory, cows fed the CSFO diet increased their serum concentration of C16:0 to counterbalance the melting point that was reduced as a result of the elevated concentration of *n-3* FA. Such rise in the concentration of saturated FA has previously been reported in placental caruncles of cows fed with fish oil (Mattos *et al.*, 2004). The observed increase in serum concentrations of *n-3* FA resulted in a respective increase in milk FA of the CSFO-fed cows. This paralleled with a considerable decrease in the serum concentration of arachidonic acid in the CSFO-fed cows (0.07 *vs* 0.28% in SFA cows). It is reasonable to expect that the observed changes in the serum FA profile of the CSFO-fed cows would have reflected similar changes in other tissues such as reproductive tract. Therefore, the improved reproductive performance that was observed in the CSFO-fed cows likely represented a relatively higher abundance of the *n-3* FA compared to the *n-6* FA in their reproductive tissues (Moussavi *et al.*, 2007; Greco *et al.*, 2018).

The present study revealed that feeding the CSFO enhanced the reproductive performance of dairy cows through improving the overall conception rate, reduction of services per conception and advancement of conception following parturition in cows. These findings were in accordance with the results of the study of Dirandeh *et al.* (2013) in which the dietary enrichment of protected *n-3* FA results in higher pregnancy rates and lesser services per conception in dairy cows. Likewise, feeding fish oil to the dairy cows from 30 to 160 d postpartum improved the second service conception rate and lessened the pregnancy losses (Silvestre *et al.*, 2011). Positive effects of *n-3* PUFA on bovine reproductive processes could be attributed to the stimulated proliferation and steroidogenesis in the granulosa cells of the ovarian follicles (Maillard *et al.*, 2018), a greater oocyte developmental competence (Oseikria *et al.*, 2016), and a reduction in PGF2α synthesis by the uterine tissue (Santos *et al.*, 2008).

The results of this study suggest that a biphasic supplementation of *n-3* FA beginning immediately postpartum until 150 DIM improves reproductive variables and udder health of dairy cows. This feeding strategy recommends maintaining an *n-6*/*n-3* FA ratio below 5, the supplementation of 40 g/d per head of EPA + DHA in the first 21 DIM and the removal of *n-6* FA sources from the diet thereafter.

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