Effects of feeding rumen-protected linseed fat to postpartum dairy cows on plasma n-3 polyunsaturated fatty acid concentrations and metabolic and reproductive parameters

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ABSTRACT

High-yielding dairy cows experience a negative energy balance and inflammatory status during the transition period. Fat supplementation increases diet energy density, and plasma n-3 polyunsaturated fatty acids (PUFA) have been proposed to improve immune function. This study tested the hypothesis that dietary supplementation with a rumen-protected and n-3 PUFA-enriched fat could ameliorate both the energetic deficit and immune status of postpartum high-yielding dairy cows, improving overall health and reproductive efficiency. At 11 d in milk (DIM), cows were randomly allocated to groups (1) n-3 PUFA (n = 29), supplemented with encapsulated linseed oil supplying additional up to 64 g/d (mean 25 ± 4 g/d) of α-linolenic acid (ALA), or (2) control (n = 31), supplemented with hydrogenated palm oil without ALA content. Fat supplements of the n-3 PUFA and control groups were available through an automated, off-parlor feeding system, and intake depended on the cow's feeding behavior. Plasma ALA concentrations were higher in n-3 PUFA than control cows, following a linear relation with supplement ingestion, resulting in a lower n-6/n-3 ratio in plasma. Metabolic parameters (body condition score and glucose and β-hydroxybutyric acid blood concentrations) were unaffected, but milk yield improved with increased intake of fat supplements. Plasma total adiponectin concentrations were negatively correlated with ingestion of n-3 PUFA-enriched fat supplement, following a linear relation with intake. Conception rate to first AI increased with higher intake of both fats, but a decrease of calving-to-conception interval occurred only in n-3 PUFA cows. Postpartum ovarian activity and endometrial inflammatory status at 45 DIM were unaffected. In conclusion, this study evinced a positive linear relation between rumen-protected linseed fat intake and plasma n-3 PUFA concentrations, which modulated adiponectin expression and improved reproductive parameters.

Key words: n-3 PUFA, adiponectin, linseed, dairy cow

INTRODUCTION

The high-yielding dairy cow transition period is characterized by a state of negative energy balance, which adversely affects cow health, fertility, and milk yield (Raboisson et al., 2014; Abdelli et al., 2017; Sheldon et al., 2019). Fat supplementation has become a common strategy to increase the energy density of diets and minimize the effects of negative energy balance (Palmquist and Jenkins, 2017; Bionaz et al., 2020). However, this may reduce DMI, disturb rumen function, and originate fatty acid (**FA**) isomers that depress milk fat (Chamberlain and DePeters, 2017; de Souza and Lock, 2019; Manriquez et al., 2019). Because FA, namely PUFA, have essential physiologic roles, the beneficial effects of fat supplementation may rely more on FA type than on increased energy intake (Herrera-Camacho et al., 2011). In this context, the PUFA from n-3 (α-linolenic acid, **ALA**; eicosapentaenoic acid, **EPA**; docosapentaenoic acid, **DPA**; and docosahexaenoic acid, **DHA**) and n-6 (linoleic acid, **LA**; and arachidonic acid) families receive special attention due to their roles in reproductive and immune function (Moallem, 2018; Moallem et al., 2020). Increased intake of n-6 PUFA increases the proportion of LA and arachidonic acid in cell membranes, favoring eicosanoid synthesis toward a proinflammatory state (Wolf et al., 2019). In contrast, increased intake of n-3 PUFA increases the proportion of EPA and DHA in cell membranes, which favors eicosanoid synthesis toward an anti-inflammatory state (Greco et al., 2015; Wolf et al., 2019).

Transition dairy cows are good candidates for the modulation of inflammation through nutrition, because they experience a physiologic inflammatory state dur-

ing the puerperal period (Bradford et al., 2015). A pro-inflammatory state characterizes initial uterine involution directed to elimination of endometrial pathogens by immune cells (LeBlanc, 2012). Then, it switches to a pro-resolving state, where restoration of endometrial homeostasis occurs. Failure to regulate this shift results in persistent chronic endometrial inflammation (subclinical or cytological endometritis, **CYTO**; Pascottini and LeBlanc, 2020). Recently a link was unveiled between the metabolic and immune functions at the endometrial level through adipokine signaling (adiponectin and chemerin; Pereira et al., 2020). In neonatal calves, transfer of n-3 PUFA into the plasma may also take place via placenta during late gestation or via colostrum (Uken et al., 2021a), and n-3 PUFA supplementation of colostrum increases plasma n-3 PUFA content, with beneficial effects on the inflammatory response (Opgenorth et al., 2020) and metabolic and endocrine development (Uken et al., 2021b).

This study evaluated the effects of feeding a rumen-protected linseed fat supplement to postpartum high-yielding dairy cows on metabolic and reproductive parameters. The hypothesis to be tested was that feeding a rumen-protected linseed fat rich in ALA would increase blood n-3 PUFA concentrations, resulting in enhanced endometrial homeostasis at the end of the voluntary waiting period and increased fertility.

MATERIALS AND METHODS

Ethics Statement

The project was approved by the Institutional Animal Care and Use Committee (CEIE: Comissão de Ética para a Investigação e Ensino, Lisboa, Portugal; reference no. 36/2019). All clinical procedures were conducted in compliance with the European Union legislation to use animals for experimental purposes (Directive 2010/63/UE).

Animals and Experimental Design

The field study took place in a commercial dairy herd milking 550 Holstein-Friesian cows 3 times per day, with an average milk yield of 13,800 L/cow over 305 d of lactation. Once calved, blood was sampled to assess cows' total calcium (at 0 DIM) and nonesterified fatty acids (**NEFA**) (at 0, 7, 14, and 21 DIM). Cows experiencing dystocia, retained fetal membranes, puerperal metritis or mastitis, clinical hypocalcemia, clinical ketosis, or severe lameness until 11 DIM were removed from the study. At 11 ± 0.3 DIM, cows (n = 60) were stratified by parity and randomly assigned to 1 of 2 groups, receiving different fat supplements during 9 wk: (1) the n-3 PUFA group (n = 29; 14 primiparous and 15 multiparous) received a rumen-protected n-3 fat (linseed oil encapsulated with a proprietary coating technology by SIPENA, Saint Malo, France) mixed with excipient (Eurocereal); and (2) the control group (n = 31; 16 primiparous and 15 multiparous) received an isoenergetic supplement with saturated FA (hydrogenated palm oil mixed with the same excipient). The number of enrolled cows was chosen to allow detection of a 25% increase in plasma ALA concentrations ($\alpha =$ 0.05), and operators were blinded to cow group allocation. Milk samples were collected weekly until 11 wk postpartum (**WPP**) and analyzed for milk composition. Body condition score was evaluated and blood samples collected once a week, from 11 DIM until 11 WPP (Figure 1) for analysis of FA and glucose, BHB, and progesterone (**P4**) concentrations. At 23 ± 0.5 and 44 ± 0.6 DIM, vaginal discharge scoring, ultrasound genital tract evaluation, and endometrial cytology were performed, and blood samples were addressed for total adiponectin concentrations (Figure 1).

Diet, Fat Supplements, and DMI

Cows were kept as a single group and offered a TMR diet, with an estimated DMI of 25.0 kg/cow per day. Ingredients of TMR as well as TMR nutrient concentrations were analyzed by near-infrared spectroscopy (Table 1). Individual supplementation was provided through an automated, off-parlor feeding system (DairyFeed C-8000, GEA Farm Technologies), allowing 1.25 kg/d of supplement in 4 feeding windows, and the daily intake was recorded by the cow-collar electronic system. The basal TMR diet supplied 38 g/d per cow of ALA, and n-3 PUFA cows received up to 64 g/d (mean 25 ± 4 g/d) of additional ALA, whereas control cows received no additional ALA.

Blood and Milk Sampling

Blood samples were aseptically collected by venipuncture of the coccygeal vein into 10-mL dry tubes and 10-mL tubes containing K3 EDTA (13060, Vacutest Kima). Tubes were centrifuged (2,000 g for 15 min) within 30 min of collection, and serum and plasma were aliquoted into 1.5-mL tubes and stored at −80°C until analysis. Composite milk samples were automatically collected during the afternoon milking, dispensed into a 50-mL tube with bronopol, and stored at 4°C until milk composition analysis.

Table 1. Ingredient composition and nutrient concentrations of the TMR and fat supplements delivered to cows enrolled in the supplementation trial

Item	TMR	Fat supplement Control	n-3 PUFA
Ingredient (% of DM)			
Corn silage	29.1	—	—
Ryegrass hay	12.6	—	—
Barley grain	27.8	—	—
Brewers yeast	2.1	—	—
Soybean meal	13.9	—	—
Beet molasses	3.0	—	—
Rice bran	3.5	—	—
Palm kernel meal	3.9	—	—
Palmitic acid 98%	1.1	—	—
Mineral and vitamin premix	2.9	—	—
Nutrient concentration[1] (% of DM)			
% DM	51.6	90.7	91
CP	16.9	14.4	13.5
Crude fat	4.3	15.6	14.6
Starch	26.4	18.4	20.6
NDF	32.5	21.4	20
ADF	18.9	—	—
ADL	2.9	—	—
Ash	8.6	10.2	11.4
NE_L (Mcal/kg of DM)	1.702	—	—
Fatty acids (g/100 g of total fatty acids)			
14:0	0.91	1.5	0.1
16:0	43.4	46.5	5.3
18:0	3.63	47.5	30.8
18:1 *cis*-9	17.8	4.5	11.8
18:1 *cis*-11	1.38	—	0.4
18:2 n-6	28.5	—	10.5
18:3 n-3	3.32	—	40
20:0	0.38	—	0.6
Others	0.59	—	0.5

[1]Analyzed by near-infrared spectroscopy.
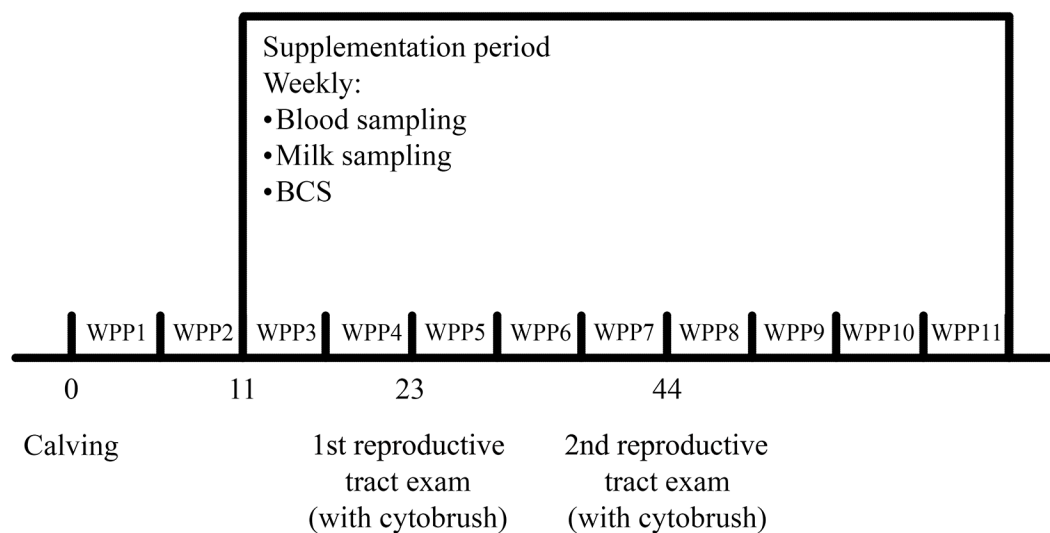
Figure 1. Experimental design. WPP = week postpartum.

Plasma FA Profile

Fatty acid methyl esters from freeze-dried plasma samples were prepared by transesterification with sodium methoxide in methanol (0.5 M, at 50°C for 30 min), followed by HCl in methanol (1.25 M, at 80°C for 20 min), with the addition of methyl nonadecanoate (1 mL of a 1 mg/mL solution) as internal standard (Alves et al., 2017). Fatty acid methyl esters were analyzed by gas chromatography with flame ionization detection using a Shimadzu GC 2010-Plus equipped with an SP-2560 capillary column (100 m × 0.25 mm, 0.20-µm film thickness, Supelco Inc.). The chromatographic conditions were as follows: injector and detector temperatures were set at 220 and 250°C, respectively; helium was used as the carrier gas at 1 mL/min constant flow; the initial oven temperature of 50°C was held for 1 min, increased at 50°C/min to 175°C and held for 35 min, increased at 2°C/min to 220°C and held for 30 min. Identification of FAME was achieved by comparison of the FAME retention times with those of commercial standards (FAME mix 37 components from Supelco Inc.) and by mass spectrometry using a GC-MS Shimadzu 2010-Plus. Integration of peaks was performed using GCsolution Version 2.41.00 (Shimadzu, 2000–2011). Theoretical relative flame ionization detection response correction factors for FAME were used to correct peak areas, according to Ackman (2002).

BCS and Energy Balance Indicators

Cows were scored for BCS using a 1-to-5 scale with 0.25 increments (Ferguson et al., 1994). Within 30 min of blood collection, glucose and BHB concentrations were assessed with a handheld device (Precision Xtra, Abbott Diabetes Care) in blood with EDTA added. Serum NEFA concentrations were determined by a colorimetric method (kit no. FA 115, Randox) using Randox RX Daytona equipment, as described by Pereira et al. (2020). The analytical sensitivity of the assay was 0.072 mmol/L, and the interassay coefficient of variation was <5%. Serum total calcium concentrations at calving were determined by a colorimetric method (kit no. CA 3871, Randox), with a 0.09-mmol/L analytical sensitivity.

Adiponectin Assay

Plasma total adiponectin concentrations were assessed as previously described (Mellouk et al., 2017; 2019; Pereira et al., 2020) with an ELISA commercial kit (E11A0125, BlueGene). The intra- and interassay coefficients of variation were 4.2% and 15%, respectively.

Milk Yield and Composition

Milk yield was retrieved from the milking parlor recording system (3 times daily). Milk composition, including fat mass percentage (% m/m) and CP mass percentage (% m/m), were analyzed by mid-infrared spectroscopy (MilkoScan 7, Foss), and SCC (cells/mL × 1,000) was analyzed by flow cytometry (Fossomatic, Foss) within 24 h of collection. The fat- (4%) and protein- (3.3%) corrected milk (**FPCM**) was calculated according to Kok et al. (2017) as follows: FPCM = milk (kg) × (0.337 + 0.116 × fat % + 0.06 × CP %).

Genital Tract Evaluation and Endometrial Cytology

Vaginal fornix discharge was collected through a Metricheck device, and the score was evaluated as described by Williams et al. (2005). Then, the genital tract was assessed through transrectal palpation and ultrasonography, and an endometrial swab was performed to evaluate endometrial cytology, as described by Pereira et al. (2020). Briefly, a cervical brush (Bastos Viegas SA) was aseptically adapted to the inner stylet of an AI gun (IMV Technologies) and gently rolled against the endometrium in the uterine body. Slides were stained with a modified Wright-Giemsa stain (Diff-Quick, MAIM SL), and the percentage of PMN was assessed from 400 total cells. The PMN cut-offs for identification of CYTO were set at ≥18% (Kasimanickam et al., 2004) and ≥5% (Gilbert et al., 2005) at 23 and 44 DIM, respectively.

Progesterone Assay

Plasma progesterone concentrations were assayed by a chemiluminescent immunoassay, using an IMMULITE 1000 analyzer (Siemens Healthcare Diagnostics) and a commercial kit (IMMULITE 1000 Progesterone Kit, Siemens Healthcare Diagnostics), as validated for bovine samples by Martin et al. (2007). The analytical sensitivity of the assay was 0.2 ng/mL, and the interassay coefficient of variation was <10%. The onset of postpartum ovarian luteal activity was considered to occur at the first P4 measurement >1 ng/mL (Colazo et al., 2008), and postpartum ovarian patterns were defined as described by Lamming and Darwash (1998).

Reproductive Management

Cows were inseminated at the first observed estrus after 45 DIM. Cows not exhibiting estrus by 75 DIM were enrolled in an OvSynch plus FTAI protocol. Pregnancy was detected by ultrasound at 39 d after AI.

Statistical Analysis

Data were compiled using Excel 2013 (Microsoft Corp.) spreadsheets before being imported into SAS (version 9.4, SAS Institute Inc.) for data analysis. The continuous response variables with repeated measures (FPCM, milk fat and protein percentages, BCS, glucose, BHB, NEFA, FA, and adiponectin) were analyzed by fitting linear mixed models with PROC MIXED of SAS. Serum calcium at calving and total fat supplement ingestion were analyzed by fitting linear models with PROC GLM. Binary response variables (CYTO at 23 and 44 DIM, pregnancy status after first AI, and postpartum P4 pattern) were analyzed by fitting generalized linear mixed models with a logit link and a binomial distribution, using PROC GLIMMIX. As recommended by Lean et al. (2016), intervals from calving to onset of postpartum ovarian luteal activity and from calving to conception (**CCI**) were analyzed by multivariable survival analysis using Cox proportional hazard regression (PROC PHREG).

The repeated-measures models had the cow specified as subject and included parity (primiparous or multiparous), fat supplement (n-3 PUFA or control), conception at first AI (yes or no), pregnancy at 150 DIM (yes or no), and CYTO at 23 and 44 DIM (yes or no) as fixed effects, and the amount of fat supplement ingested weekly (kg) and DIM as covariates. The fat supplement intake was required because animals from both treatments presented intake values that varied continuously from zero to the maximum allowed. Thus, the effect of fat supplementation was evaluated by the interaction term of supplement type × supplement ingested weekly. The model of serum calcium at calving included the fixed effects of parity, fat supplement, conception at first AI, and CYTO at 23 and 44 DIM. This model was generated to evaluate putative effects of calcemia in uterine health and fertility, as suboptimal serum calcium concentrations at calving can be detrimental to uterine health (Martinez et al., 2012) and fertility (Caixeta et al., 2017), and can alter FA metabolism (Chamberlin et al., 2013) of postpartum dairy cows. The conception at first AI and postpartum P4 pattern generalized linear mixed models, and the interval from calving to the onset of postpartum ovarian luteal activity and CCI survival analysis models, included the fixed effects of parity, fat supplement, CYTO at 23 and 44 DIM, total fat supplement ingested (kg) as a covariate, and the supplement type × total supplement ingested interaction term. For the survival analysis, cows were right-censored if not diagnosed pregnant at 240 DIM or if removed from the herd.

As suggested by Heinze and Dunkler (2017), manual backward elimination with a P-value criterion of 0.157,

without a preceding univariable prefiltering, was performed to obtain the final models. The assumption of homoscedasticity was evaluated visually using the plots of studentized residuals against predicted values, and the assumption of normality of residuals was evaluated using the Q-Q plots. To satisfy these assumptions, milk SCC, plasma adiponectin, and blood BHB concentrations were natural log-transformed. For models with repeated measures, the covariance structure that resulted in the lowest Akaike information criteria was selected. In the final models, differences were considered significant when $P \leq 0.05$, whereas a tendency was defined as $0.05 < P \leq 0.10$.

RESULTS

Fat Supplement Intake and Plasma FA Profile

Cows from the n-3 PUFA group had higher ($P = 0.004$; Table 2) total fat supplement intake than the cows of the control group. Mean total supplement intake was 33.2 ± 3.8 kg and 17.7 ± 3.7 kg for the n-3 PUFA and control groups, respectively. Plasma concentrations of total, n-3, and n-6 PUFA increased with DIM ($P \leq 0.001$). Plasma total FA concentrations were higher ($P = 0.041$) in multiparous than in primiparous cows, and were also affected by fat supplement ingestion ($P = 0.001$), increasing 1.26 ± 0.39 mg/dL per kilogram ingested. Plasma n-3 PUFA concentrations were higher in the n-3 PUFA group than in the control group, increasing ($P \leq 0.001$; Table 3) by 0.142 ± 0.026 mg/dL per kilogram of n-3 PUFA supplement ingested, whereas no increase ($P = 0.292$) was observed in the control group. Plasma n-6 PUFA concentrations were increased ($P = 0.002$) by 0.676 ± 0.212 mg/dL per kilogram of supplement ingested, irrespective of fat source. The n-6/n-3 ratio decreased with DIM ($P = 0.001$), also decreasing by 0.183 ± 0.023 units per kilogram of n-3 PUFA ingestion ($P \leq 0.001$; Figure 2). Plasma ALA concentrations increased by 0.124 ± 0.021 mg/dL with n-3 PUFA ingestion ($P \leq 0.001$; Figure 2) and DIM ($P \leq 0.001$). Increased intake of control fat produced no effect on the n-6/n-3 ratio ($P = 0.988$) or ALA ($P = 0.393$) plasma concentrations. Similarly to total n-6 PUFA, plasma LA concentrations increased with DIM ($P \leq 0.001$) and fat supplement ingestion ($P = 0.002$), with no difference between the 2 fat supplements (Figure 2). Plasma EPA and DPA concentrations also increased with DIM ($P \leq 0.001$) and supplement ingested ($P = 0.004$ and $P = 0.033$ for EPA and DPA, respectively), whereas DHA concentrations were not affected by either DIM or supplement ingested. Average concentrations of plasma FA at the end of the supplementation period (11 WPP), solved

Table 2. Final models with statistical significance of parity, DIM, treatment (group; trt), fat supplement ingestion (fat sup. intake), cytological endometritis (CYTO), and interval from conception to first AI

Dependent variable[1]	P-value[2]							
	Parity	DIM	Trt	Fat sup. intake	Trt × Fat sup. intake[1]	CYTO at 23 DIM	CYTO at 44 DIM	Conception to first AI
Total fat supplement ingested (kg)	—	—	0.004	—	—	—	—	—
FPCM yield (kg/wk)	<0.001	0.042	—	0.042	—	—	—	—
Milk fat percentage (%)	—	<0.001	—	—	—	—	—	—
Milk protein percentage (%)	—	0.003	—	—	—	—	—	—
Milk SCC (×1,000 cells/mL)	—	—	—	—	—	—	—	—
BCS	—	<0.001	—	—	—	—	—	—
Blood glucose (mmol/L)	<0.001	—	—	—	—	—	0.024	—
Blood BHB (mmol/L)	—	—	—	—	—	—	—	—
Plasma adiponectin (μg/mL)	—	0.002	—	0.072	<0.001	—	0.040	—
Serum calcium at calving (mmol/L)	<0.001	—	—	—	—	—	—	—
NEFA (mmol/L)	0.048	0.088	—	—	—	—	—	0.098
Total FA (mg/dL)	0.041	<0.001	—	0.001	—	—	—	—
Total n-3 FA (mg/dL)	0.066	<0.001	—	0.001	0.010	—	—	—
Total n-6 FA (mg/dL)	0.045	<0.001	—	0.002	—	—	—	—
n-6/n-3 FA ratio	—	0.001	—	<0.001	<0.001	—	—	—
Linoleic acid (mg/dL)	0.068	<0.001	—	0.002	—	—	—	—
α-Linolenic acid (mg/dL)	0.020	<0.001	—	<0.001	0.005	—	—	0.054
EPA (mg/dL)	—	<0.001	—	0.004	—	—	—	—
DPA (mg/dL)	—	<0.001	—	0.033	—	—	—	—
DHA (mg/dL)	—	—	—	—	—	—	—	—

[1]FPCM = fat- and protein-corrected milk; NEFA = nonesterified fatty acid; FA = fatty acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

[2]Variables not included in the final models are represented by dashes (—).

for 1 kg/d ingestion of fat supplements, are supplied in Table 4.

Milk Yield and Metabolic Variables

Fat- (4%) and protein- (3.3%) corrected milk yield increased ($P = 0.042$) with DIM, and was lower ($P \leq 0.001$) in primiparous than multiparous cows (Supplemental Figure S1, https://data.mendeley.com/

Table 3. Effects of control and n-3 PUFA fat supplement ingestion on plasma concentrations of fatty acids (FA) in postpartum dairy cows

Plasma fatty acid[1] (mg/dL)	Control	n-3 PUFA	P-value for differences
Linoleic acid	0.565*	0.640*	0.8516
α-Linolenic acid	0.024†	0.124*	0.0052
EPA	0.008†	0.011*	0.6037
DPA	0.006†	0.009†	0.7387
DHA	0.000†	0.000†	0.5866
Total n-3 FA	0.035†	0.142*	0.0110
Total n-6 FA	0.657*	0.694*	0.9339
n-6/n-3 FA ratio	0.000†	−0.183*	<0.0001
Total FA	1.090*	1.377*	0.7250

[1]Values are slopes for 1 unit change in ingestion, kg/wk. EPA = eicosapentaenoic acid. DPA = docosapentaenoic acid. DHA = docosahexaenoic acid.

*Slope differs from zero ($P < 0.10$); † slope does not differ from zero ($P \geq 0.10$).

datasets/j2bfm4tf9x/1; Pereira, 2021). Both milk fat ($P \leq 0.001$) and protein ($P = 0.003$) percentages decreased with DIM. Intake of both fat supplements (control and n-3 PUFA) increased FPCM yield by 1.95 kg per kilogram ingested ($P = 0.042$), whereas milk fat and protein percentages remained unaffected.

Serum total calcium concentrations at calving (2.21 ± 0.06 mmol/L vs. 1.96 ± 0.05 mmol/L, $P < 0.001$) and plasma glucose concentrations throughout the study were higher ($P \leq 0.001$) in primiparous than multiparous cows. Plasma NEFA concentrations were lower ($P = 0.048$) in primiparous than multiparous cows, and BCS decreased ($P < 0.001$) with DIM (Supplemental Figure S1, https://data.mendeley.com/datasets/j2bfm4tf9x/1; Pereira, 2021). Plasma total adiponectin concentrations were increased in cows with CYTO at 44 DIM ($P = 0.040$; Figure 3). Plasma total adiponectin concentrations displayed a negative linear relation with intake of n-3 PUFA supplement ($P = 0.001$), being 0.93 times lower per each kilogram of n-3 PUFA supplement ingested during the week of sampling (Figure 3).

Reproductive Variables

Final reproductive outcome models are presented in Table 5. Fat supplementation had no effect on postpartum P4 profiles, which were normal in 28 of 60 (47%)

cows, whereas the remaining 32 cows presented atypical profiles (delayed ovulation or persistent luteal phase). The interval from calving to onset of postpartum ovarian luteal activity was not affected by fat supplementation and averaged 34.5 ± 1.5 d. Diagnosis of CYTO at 23 DIM affected the interval from calving to onset of postpartum ovarian luteal activity (CYTO cows had an ovulation hazard ratio of 0.425 compared with healthy cows). The conception rate at first AI was 25% (15 of 60 cows) and increased with fat supplementation ($P = 0.015$). The odds of pregnancy at first AI increased 1.037 times for each kilogram of fat supplement ingested, irrespective of fat supplement. Regarding the CCI, primiparous cows had a higher ($P = 0.034$) hazard ratio of conception (1.832; 95% Wald confidence limits: 1.045–3.211) than multiparous cows. After adjusting

for the effect of parity, the hazard ratio of conception for cows ingesting n-3 PUFA increased ($P = 0.004$) 1.009 (95% Wald confidence limits: 0.996–1.022) times for each kilogram ingested, whereas cows ingesting increased amounts of control fat supplement tended ($P = 0.062$) to exhibit a lower hazard ratio of conception (0.977; 95% Wald confidence limits: 0.954–1.001).

DISCUSSION

This study evaluated the effects of feeding a rumen-protected n-3 PUFA-enriched fat supplement on metabolic and reproductive indicators of high-yielding dairy cows. Treatment increased n-3 PUFA plasma concentrations, modulated plasma adiponectin concentrations, and shortened the calving-to-conception interval.
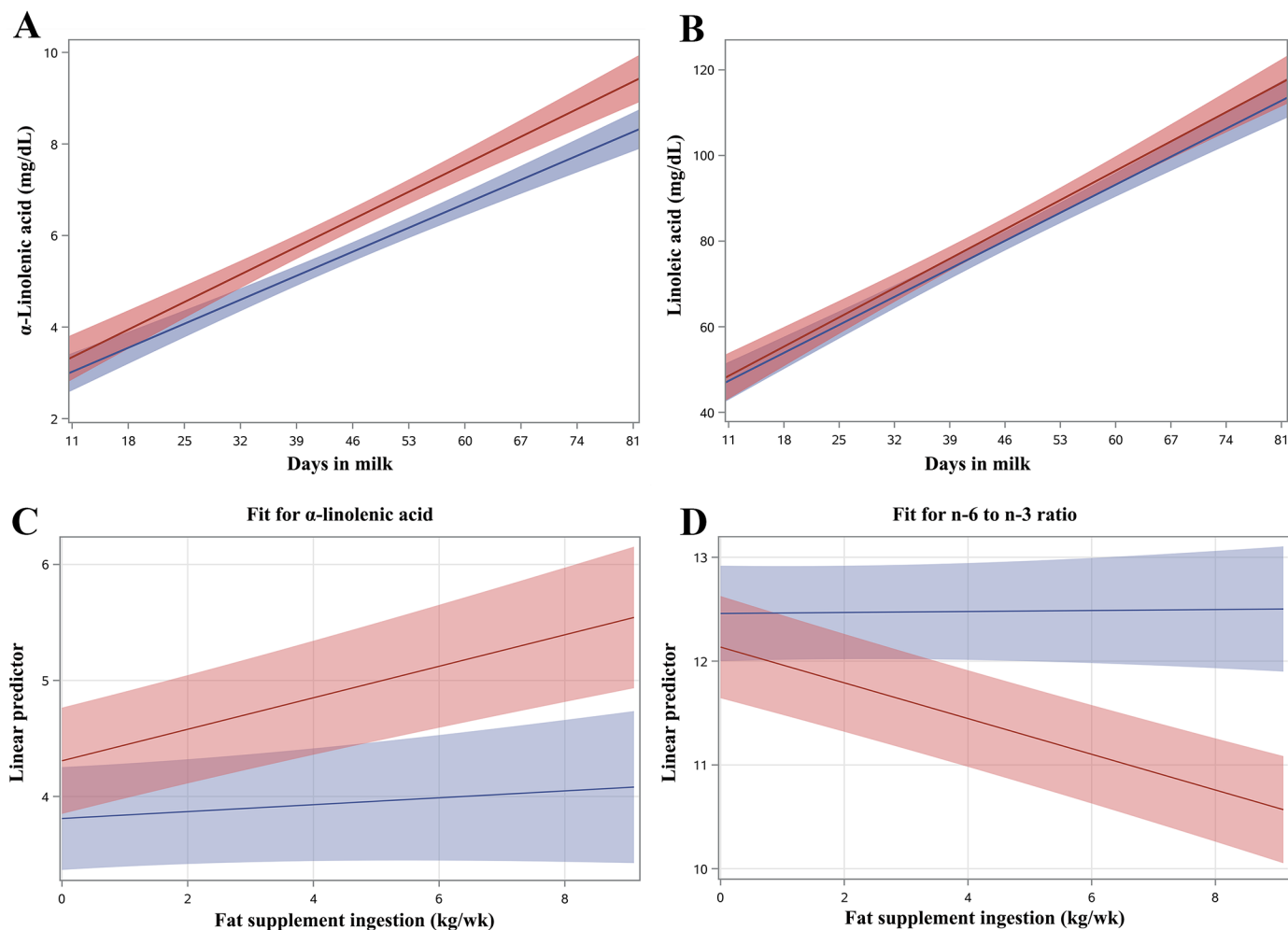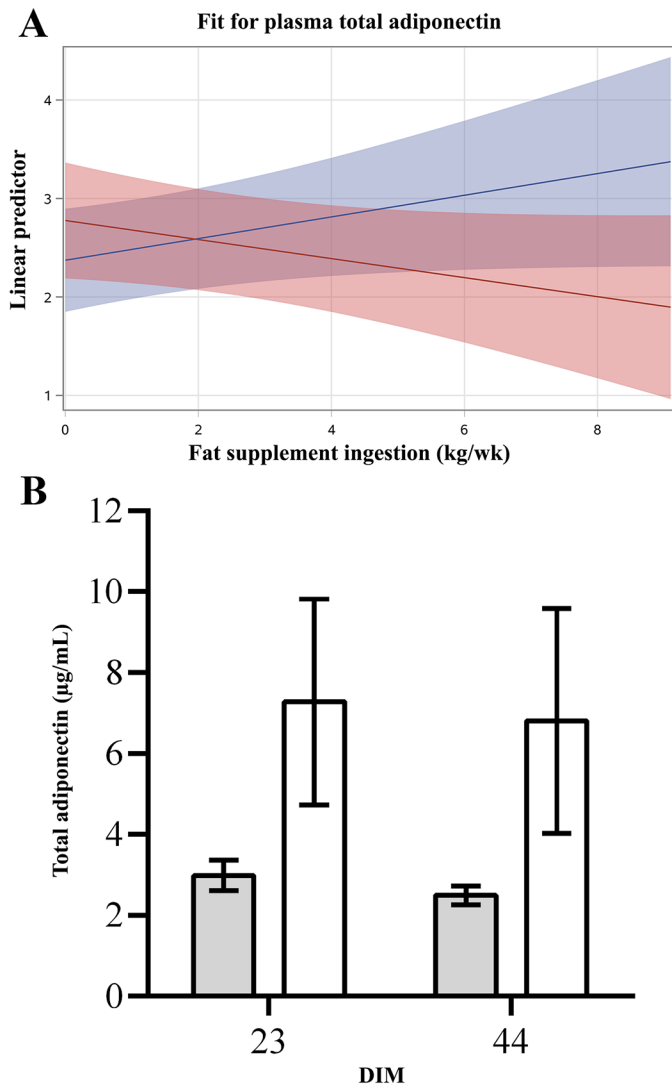

Figure 2. Plot of regression lines with respective 95% confidence limits of plasma α-linolenic acid concentrations (A), linoleic acid concentrations (B), effect of varying rates of weekly fat supplement ingestion on plasma α-linolenic acid concentrations (C), and plasma n-6 to n-3 ratio (D) in postpartum dairy cows ingesting control (blue) or n-3 PUFA (red) fat supplements.

Table 4. Least squares means ($\pm$ SE) and P-values for the differences of plasma fatty acid (FA) concentrations of dairy cows at 11 wk postpartum ingesting 1 kg/d of control or n-3 PUFA fat supplements

FA[1] (mg/dL)	Control	n-3 PUFA	P-value for differences
12:0	0.22 $\pm$ 0.02	0.26 $\pm$ 0.02	0.1532
14:0	1.69 $\pm$ 0.07	1.79 $\pm$ 0.06	0.2656
iso 15:0	0.49 $\pm$ 0.02	0.49 $\pm$ 0.02	0.9032
anteiso 15:0	1.08 $\pm$ 0.06	1.11 $\pm$ 0.05	0.6766
14:1 *cis*-9	0.65 $\pm$ 0.06	0.51 $\pm$ 0.06	0.0813
15:0	1.69 $\pm$ 0.09	1.59 $\pm$ 0.08	0.3825
16:0 DMA	0.37 $\pm$ 0.11	0.50 $\pm$ 0.11	0.3667
iso 16:0	0.32 $\pm$ 0.02	0.34 $\pm$ 0.02	0.4728
16:0	28.82 $\pm$ 1.06	28.04 $\pm$ 0.99	0.5404
16:1 *trans*	0.44 $\pm$ 0.03	0.44 $\pm$ 0.02	0.9583
iso 17:0	1.07 $\pm$ 0.04	1.09 $\pm$ 0.04	0.6929
16:1 *cis*-7	1.16 $\pm$ 0.13	1.11 $\pm$ 0.13	0.7678
16:1 *cis*-9	3.46 $\pm$ 0.20	3.51 $\pm$ 0.20	0.8021
anteiso 17:0	0.03 $\pm$ 0.01	0.02 $\pm$ 0.01	0.1643
17:0	0.99 $\pm$ 0.05	1.00 $\pm$ 0.04	0.7964
18:0 DMA	0.45 $\pm$ 0.03	0.48 $\pm$ 0.03	0.4280
iso 18:0	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.4391
17:1 *cis*-9	0.52 $\pm$ 0.04	0.58 $\pm$ 0.04	0.2208
18:0	31.00 $\pm$ 1.24	32.31 $\pm$ 1.14	0.3504
18:1 *trans*-6/*trans*-7/*trans*-8	0.20 $\pm$ 0.02	0.20 $\pm$ 0.02	0.8540
18:1 *trans*-9	0.27 $\pm$ 0.03	0.29 $\pm$ 0.02	0.6546
18:1 *trans*-10	0.55 $\pm$ 0.09	0.71 $\pm$ 0.08	0.1732
18:1 *trans*-11	0.44 $\pm$ 0.03	0.45 $\pm$ 0.03	0.7324
18:1 *trans*-12	0.33 $\pm$ 0.03	0.38 $\pm$ 0.02	0.1466
18:1 *trans*-13/*trans*-14	1.31 $\pm$ 0.06	1.41 $\pm$ 0.06	0.1577
18:1 *cis*-9	20.32 $\pm$ 1.0	19.07 $\pm$ 0.9	0.3139
18:1 *trans*-15	0.09 $\pm$ 0.01	0.08 $\pm$ 0.01	0.6228
18:1 *cis*-11	1.65 $\pm$ 0.07	1.62 $\pm$ 0.07	0.7689
18:1 *cis*-12	0.91 $\pm$ 0.06	0.98 $\pm$ 0.05	0.3604
18:1 *cis*-13	0.07 $\pm$ 0.01	0.05 $\pm$ 0.01	0.2176
18:1 *trans*-16/*cis*-14	0.19 $\pm$ 0.02	0.16 $\pm$ 0.01	0.2719
18:1 *cis*-15	0.022 $\pm$ 0.004	0.017 $\pm$ 0.003	0.2884
18:2 *trans,trans*	0.08 $\pm$ 0.01	0.08 $\pm$ 0.01	0.6738
18:2 *trans,cis*/*cis,trans*	0.49 $\pm$ 0.04	0.53 $\pm$ 0.03	0.2761
18:1 *cis*-16	0.16 $\pm$ 0.02	0.14 $\pm$ 0.02	0.5605
18:2 *trans*-9,*cis*-12	0.14 $\pm$ 0.02	0.13 $\pm$ 0.01	0.6314
18:2 *trans*-11,*cis*-15/*trans*-10,*cis*-15	0.09 $\pm$ 0.02	0.08 $\pm$ 0.02	0.6814
18:2n-6	107.46 $\pm$ 4.24	109.31 $\pm$ 4.02	0.6781
20:0	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01	0.7779
18:3n-6	2.22 $\pm$ 0.12	2.20 $\pm$ 0.11	0.8846
20:1 *cis*-11	0.027 $\pm$ 0.007	0.022 $\pm$ 0.005	0.4471
18:3n-3 (ALA)	8.06 $\pm$ 0.37	8.97 $\pm$ 0.35	0.0100
cis-9,*trans*-11 CLA	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01	0.2271
trans,trans CLA	0.24 $\pm$ 0.02	0.28 $\pm$ 0.02	0.0510
20:2 n-6	0.036 $\pm$ 0.008	0.031 $\pm$ 0.007	0.5267
18:3 *cis*-9,*trans*-11,*cis*-15/20:3n-9	0.23 $\pm$ 0.02	0.24 $\pm$ 0.02	0.5829
22:0	0.041 $\pm$ 0.009	0.034 $\pm$ 0.006	0.5313
20:3n-6	5.23 $\pm$ 0.26	5.40 $\pm$ 0.25	0.5405
20:3n-3	0.011 $\pm$ 0.006	0.021 $\pm$ 0.004	0.2004
20:4n-6	4.90 $\pm$ 0.28	4.63 $\pm$ 0.28	0.4319
20:5n-3 (EPA)	1.00 $\pm$ 0.07	1.12 $\pm$ 0.06	0.1339
22:4n-6	0.61 $\pm$ 0.05	0.64 $\pm$ 0.05	0.6137
22:5n-3 (DPA)	1.08 $\pm$ 0.07	1.16 $\pm$ 0.07	0.3288
22:6n-3 (DHA)	0.018 $\pm$ 0.008	0.012 $\pm$ 0.006	0.5109
Total n-3 FA	10.14 $\pm$ 0.46	11.08 $\pm$ 0.44	0.0316
Total n-6 FA	120.25 $\pm$ 4.69	122.06 $\pm$ 4.47	0.7145
n-6/n-3 ratio	12.19 $\pm$ 0.31	10.66 $\pm$ 0.28	0.0002
Total FA	231.82 $\pm$ 8.39	234.12 $\pm$ 7.99	0.8037

[1]DMA = dimethyl acetal; ALA = alpha-linolenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

A

B

Figure 3. (A) Plot of regression lines with respective 95% confidence limits of varying rates of weekly fat supplement ingestion effect on plasma total adiponectin concentrations in postpartum dairy cows ingesting control (blue) or n-3 PUFA (red) fat supplements. (B) Mean (± SE) plasma concentrations of total adiponectin at 23 and 44 DIM of healthy cows (gray bars) and cows with cytological endometritis (white bars).

Fat Supplement Intake

We found a large individual variation in intake of both fat supplements, but n-3 PUFA had higher total ingestion than the control fat supplement. In fat-supplemented cows, DMI is affected by forage-to-concentrate ratio, supplement inclusion rate, palatability, chain length, and saturation of FA (Bionaz et al., 2020). Because the forage-to-concentrate ratio and supplement inclusion rate were kept constant, the differences in supplement intake observed between groups were probably due to palatability or to FA chain length and saturation. The irregular intake pattern of both supplements resulted in the planned daily and total dosage not being attained uniformly. Although this jeopardized the magnitude of the metabolic and reproductive effects in several cows, it allowed evaluation of the interaction between type of supplement and the amount ingested.

Effects on Plasma FA Composition

Total plasma FA concentrations increased as lactation progressed, an expected consequence of higher DMI, resulting in a higher outflow of FA from the rumen, as observed by others (Penner and Oba, 2009; Elis et al., 2016). Corroborating this, primiparous cows with lower DMI capacity (Neave et al., 2017) also had lower plasma total FA concentrations than multiparous cows. As projected, the plasma FA profile was affected by fat supplement intake. Dietary ALA from the n-3 PUFA supplement was successfully transferred into plasma following a linear relation with intake, which agrees with studies using other n-3 PUFA sources (Gonthier et al., 2005; Zachut et al., 2010; Moallem et al., 2013). As a consequence of increased plasma ALA and total n-3FA concentrations, the n-6/n-3 ratio was lower in n-3 PUFA than in the control group. Both supplements increased plasma total n-6 and LA concentrations. This was unexpected because, unlike the n-3 PUFA supplement, the control supplement had no PUFA content. Because dairy cows do not synthesize LA, the increase in plasma LA concentrations in the control group resulted from an increased TMR intake. This contrasts with the common observation that feeding fat to dairy cows decreases DMI (Moallem, 2018). Nevertheless, palmitic acid supplements have been reported to increase DMI (Mosley et al., 2007; de Souza and Lock, 2019). In fact, palmitic acid accounted for almost half of the FA present in the control fat supplement. Thus, control cows that ingested more fat supplement probably also ingested more TMR, which was likely the cause for their elevated total n-6 FA and LA in plasma. In addition, both fat supplements equally increased EPA and DPA plasma concentrations, which was unexpected as only the n-3 PUFA fat supplement increased ALA plasma concentrations, and both EPA and DPA result from the elongation of ALA (Saini and Keum, 2018). This increase in EPA and DPA plasma concentrations in cows of the control group might also be attributed to an increased TMR intake, which contained ALA. In mammals, elongation of ALA and LA to the respective n-3 and n-6 long-chain PUFA families share the same enzymatic pathways, and many tissues convert dietary ALA to EPA and DHA by desaturation and elongation

Table 5. Reproductive outcome models with statistical significance

Outcome variable[1]	*P*-value[2]					
	Parity	Trt	Fat sup. intake	Trt × fat sup. intake	CYTO at 23 DIM	CYTO at 44 DIM
P4 pattern (normal/abnormal)	—	—	—	—	—	—
Calving to first ovulation (d)	—	—	—	—	0.004	—
Conception first AI (yes/no)	—	—	0.015	—	—	—
Calving to conception (d)	0.034	—	0.062	0.004	—	—
CYTO at 23 DIM (yes/no)	—	—	—	—	NA	NA
CYTO at 44 DIM (yes/no)	—	—	—	—	NA	NA

[1]P4 = progesterone; CYTO = cytological endometritis.

[2]Trt = treatment group (n-3 PUFA or control). Fat sup. intake = effect of intake of fat supplement; 1 unit = 1 kg. Variables not included in the final models are represented by dashes (—). NA = not applicable.

combined with peroxisomal chain shortening (Cook and McMaster, 2002). One explanation could be that, due to a limited capacity of dairy cows converting ALA to an n-3 family long PUFA, excess ALA would remain in circulation. Alternatively, because the distribution of dietary n-3 PUFA is organ-specific (Wolf et al., 2019), blood plasma might not be the best tissue to detect putative increased EPA and DHA availability. In mammals, the pathway of ALA elongation, which has DHA as the final product, is considered highly inefficient, and ALA supplementation frequently fails to affect plasma DHA concentrations (Brenna et al., 2009). Therefore, the absence of the effect of fat supplementation on DHA concentrations was somewhat expected.

Effects on Metabolic Parameters

Fat supplementation increased milk yield but did not affect BCS. Additional energy intake can be partitioned either toward body adipose depots or toward milk production. Energy channeling toward adipose tissue occurs when there is a lack of glucose or amino acids to support increased milk synthesis (Weiss and Pinos-Rodríguez, 2009) or when the metabolic limits (growth hormone and insulin) for milk production are reached (Bines and Hart, 1982). Weiss and Pinos-Rodríguez (2009) suggested that in a 40% forage diet, as in this study (42%), the supplemental fat-induced increase in energy intake was largely partitioned to milk synthesis. This is in accord with results from this study, where fat supplementation did not affect glucose and BHB blood concentrations, supporting the concept that, when energy is a limiting factor, additional energy intake from fat supplementation is directed toward additional milk synthesis (Zachut et al., 2010; Haubold et al., 2020). The saturated or unsaturated nature of FA from the supplements had no effect on BCS, blood glucose, BHB, and plasma NEFA concentrations, which

is in accordance with other studies (Petit et al., 2007; Zachut et al., 2010; Hutchinson et al., 2012; Dirandeh et al., 2013).

Total adiponectin plasma concentrations decreased in association with n-3 PUFA fat supplementation in a dose-dependent manner. Supplementing ALA and CLA to mid-lactation dairy cows also decreased adiponectin plasma concentrations (Haubold et al., 2020), and n-3 PUFA-supplemented cows displayed overexpression of the adiponectin gene in adipose tissue compared with n-6 PUFA-supplemented cows (Elis et al., 2016). Taken together, these data indicate that n-3 PUFA affects the adiponectin pathway, namely through adipose tissue, opening opportunities to modulate adipokine expression through feeding. However, because adiponectin promotes insulin sensitivity (Haubold et al., 2020), by reducing adiponectin plasma concentrations, n-3 PUFA supplementation may have the undesirable effect of extending a state of insulin resistance and lipid mobilization (Singh et al., 2014). Plasma total adiponectin concentrations were unaffected by BCS, which, together with data from other studies (Krumm et al., 2017; Mann et al., 2018), indicates that plasma adiponectin concentrations are remarkably irresponsive to BCS in postpartum dairy cows. In contrast, De Koster et al. (2017) found that adiponectin serum concentrations were negatively associated with BCS during the dry period. Altogether, this information favors the concept that, in postpartum dairy cows, the relation between body fat reserves and adiponectin might be uncoupled, maintaining insulin resistance and adipose tissue mobilization (Singh et al., 2014). The endometrial inflammatory status at 44 DIM affected plasma total adiponectin concentrations, with CYTO cows displaying increased concentrations. This is consistent with a previous study (Pereira et al., 2020), evidencing that postpartum increased adiponectin expression is a biomarker of persistent uterine inflammation.

Effects on FPCM Yield

Feeding n-3 FA gave conflicting results regarding milk yield effects, some studies reporting increased yields (Moallem, 2009; Zachut et al., 2010), whereas others found no effect (Ferlay et al., 2013; Neveu et al., 2013). Reviewing the subject, Moallem (2018) suggested that contradictory results might occur from different levels of fat intake and varying degrees of rumen protection. Also, Ariza et al. (2019) suggested that, contrary to high-fat diets, supplementing moderate quantities of PUFA (extruded linseed) might increase the diet's energy density without lowering DMI or reducing fiber digestibility. Supplementation with saturated FA from palm oil also gave contrasting results, again some authors reporting increased milk yield (Mosley et al., 2007; Piantoni et al., 2013), whereas others found no effect (de Souza et al., 2016), which may reflect the basal diet composition or fat supplement characteristics (de Souza et al., 2016). These reports highlight the dose-dependent effect of fat supplementation in dairy cows' milk yield. At a lower dose, fat supplementation increases energy density and consequently milk yield, but above a certain level of supplementation, fat depresses feed intake and milk yield (Palmquist and Jenkins, 2017). In this study, both fat supplements increased FPCM yield and had no adverse effect on milk fat. A reduction in milk fat percentage can be observed following n-3 FA supplementation if the rumen biohydrogenation pathways shift to the production of 18:1 *trans*-10, 18:2 *trans*-10,*cis*-12, and other related intermediates known to depress milk fat (Moallem, 2018).

Effects on Reproductive Parameters

Despite decreasing the n-6/n-3 ratio and increasing n-3 PUFA plasma concentrations, n-3 PUFA supplementation did not affect endometrial inflammation status at 23 and 44 DIM. Following PUFA supplementation, tissue concentrations take longer to increase than plasma concentrations (Cook and McMaster, 2002), making the time required to incorporate dietary n-3 FA into target tissues an important point when developing supplementation approaches (Moallem, 2018). Persistent CYTO can result from an unbalanced production of proinflammatory and anti-inflammatory cytokines during the first WPP (Arias et al., 2018), when reduced activation of proinflammatory cytokines impair the clearance of endometrial bacteria, which, coupled with persistent proinflammatory stimuli during later stages, maintain a hostile environment for the embryo (Wagener et al., 2017; Sheldon et al., 2019; Pascottini and LeBlanc, 2020). Because PUFA supplementation started at 11 DIM, one may hypothesize that it was not

effective in modulating cytokine production. This could potentially be achieved by beginning fat supplementation either in the prepartum or at calving, allowing for essential PUFA to accumulate in the endometrium. Additionally, a higher dietary inclusion of PUFA, namely ALA, could prove beneficial, as daily values here registered (102 g/d) are lower than those reported by others (162 g/d, as per Moallem, 2009; 402 g/d, as per Zachut et al., 2010; 131 g/d, as per Moallem et al., 2013). However, the interval from calving to first ovulation was affected by CYTO at 23 DIM. Uterine bacterial infection impairs early postpartum follicular development, as LPS and tumor necrosis factor-α suppress follicular growth and estradiol production, making affected cows less likely to ovulate the first dominant follicle (Sheldon et al., 2002; Gilbert, 2011).

Fat supplementation did not affect the onset of postpartum ovarian luteal activity, nor postpartum progesterone profiles. Dietary ratio of n-6 to n-3 PUFA can modulate prostaglandin synthesis and metabolism (Greco et al., 2018), and prostaglandins play a relevant role in uterine involution and onset of postpartum estrous cycles. Again, starting supplementation at 11 DIM might have jeopardized a beneficial effect on endometrial prostaglandin synthesis.

First-service conception rate increased in association with higher intake of both fat supplements, which agrees with data reviewed by Rodney et al. (2015). This improvement in conception rate to first AI following PUFA supplementation was related to increased LA concentrations, which would inhibit prostaglandin synthesis in the presence of trophoblast interferon, thus preventing early embryonic mortality (McNamara et al., 2003). As both n-3 PUFA and control fat supplements increased plasma LA concentrations, this could explain the observed increase in the first AI conception rate. However, cows that conceived to first AI had higher plasma concentrations of ALA, suggesting that n-3 PUFA supplementation may have exerted an additional positive effect. Interestingly, the influence on the first AI conception rate was reflected in a shorter CCI in n-3 PUFA cows but not in control-group cows. This effect in n-3 PUFA cows was proportional to ingested supplement. Although n-3 PUFA are believed to weaken the uterine inflammatory status, leading to higher chances of embryo survival (Leroy et al., 2014), the effects of n-6 and n-3 PUFA supplementation on progesterone and prostaglandin metabolism are inconsistent (Otto et al., 2014).

CONCLUSIONS

Supplement feeding with PUFA or saturated FA of postpartum high-yielding dairy cows increased FPCM

yield. Although PUFA and saturated FA supplements increased plasma LA, only the n-3 PUFA supplement increased plasma ALA concentrations and regulated adiponectin expression. Fat supplementation increased first AI conception rate, and n-3 PUFA supplement shortened the CCI in a mechanism independent of early return to postpartum ovarian activity, postpartum progesterone patterns, or persistent endometrial inflammation. These findings highlight the potential of n-3 PUFA to improve high-yielding dairy cows' fertility by favoring early conception in the postpartum period. However, to prevent postpartum persistent endometrial inflammation, alternative n-3 PUFA supplementation regimens must be developed, namely with rumen-protected EPA and DHA sources, due to the inefficient elongation of ALA. Moreover, the mechanisms behind the improvement of fertility following n-3 PUFA supplementation warrant further studies.

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