Short communication: Decreasing the dietary ratio of n-6 to n-3 fatty acids increases the n-3 concentration of peripheral blood mononuclear cells in weaned Holstein heifer calves

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ABSTRACT

Utilization of nutrients to improve overall heifer health is of interest because of the importance of replacement heifers to the dairy industry. The objective of our study was to compare the effect of supplementation of dietary n-3 and n-6 fatty acids (FA) on FA concentrations in peripheral blood mononuclear cells (PBMC) of Holstein calves. Twenty-seven Holstein heifer calves (107 ± 2.6 d of age; 142.6 ± 6.5 kg of body weight) from the university research and teaching herd were randomly assigned to a common TMR supplemented with 1 of 3 treatments: Ca salts of flaxseed FA (Virtus Nutrition, Corcoran, CA) containing 35% 18:3 n-3 (N3), Ca salts of soybean FA (Virtus Nutrition) containing 50% 18:2 n-6 (N6), or a 50:50 mix of N3 and N6. Treatments were supplemented with FA at 4% of dietary dry matter and fed for 30 d. Feed intake was recorded daily, and body weight, wither height, and body condition score were measured weekly throughout the study. On d 28 heifers were vaccinated with a *Pasteurella* vaccine and the temperature response to the vaccine was recorded. Blood was collected on d 0 and 28 for PBMC isolation. After total lipid extraction and FA methyl ester preparation, FA composition of PBMC was measured. We observed no effect of treatment on body weight gain, body condition score change, or wither height change. Heifers receiving the N3 diet had a lower temperature response to *Pasteurella* challenge compared with both the mix and N6 diets. Heifers consuming the N3 diet had a greater content of total n-3 FA, α -linolenic acid, and eicosapentaenoic acid in PBMC compared with heifers fed the N6 and mix diets. Heifers receiving the N3 diet also had a lower content of total n-6 FA, linoleic acid, and arachidonic acid in PBMC than heifers fed the N6 and mix diets. In conclusion, our study determined

that feeding weaned female Holstein heifers a diet high in n-3 FA increased concentrations of n-3 FA in PBMC. **Key words:** fatty acid, heifer, peripheral blood mononuclear cell

Short Communication

The health and survival of replacement heifers is critical to dairy operations. Dairy managers rely on replacement heifers to increase herd size, replace cows culled, increase milk production, and enhance profitability. Consequently, it is of interest to utilize nutrients to prevent or decrease incidence of disease and improve overall health of heifers.

Supplementation of α -linolenic acid (**ALA**; 18:3n-3 series) and linoleic acid (**LA**; 18:2n-6 series), both essential PUFA, can influence animal performance when included in ruminant diets. Calder (2005) reported that ALA has anti-inflammatory and proresolution properties. Feeds commonly rich in n-3 fatty acids (**FA**) fed to dairy cows include flaxseed, fish meal oil, fish oil, and fresh grass and alfalfa. Conversely, feeds higher in n-6 FA include corn grain, corn and alfalfa silage, corn distillers grain, soybean meal, and whole cottonseeds (National Research Council, 2001).

Dietary n-3 FA, such as ALA, serve as precursors to docosahexaenoic acid (**DHA**; 22:6n-3) and eicosapentaenoic acid (**EPA**; 20:5n-3). Treatment of human leukemic promonocytes with DHA and EPA decreased cell expression of several proinflammatory cytokines, including tumor necrosis factor (**TNF**)- α and IL-1 β (Chu et al., 1999). Linoleic acid has both pro- and anti-inflammatory roles (Fritsche, 2008). Arachidonic acid (**ARA**; 20:4n-6) can be synthesized from LA and can lead to the formation of prostaglandins and thromboxanes that have proinflammatory properties (Ricciotti and FitzGerald, 2011). Alternatively, production of prostaglandin E2, a derivative of ARA, inhibits production of the proinflammatory cytokines TNF- α and IL-1 (Calder, 2006).

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Calder and Yaqoob (2007) reported that FA could affect immune function in numerous ways, including controlling gene expression and modifying lipid mediator production. To date, there are limited reports on the effect of dietary FA with respect to altering the immune response in dairy calves (Hill et al., 2011a; Karcher et al., 2014). Feeding preweaned calves diets with 2% flax oil or fish oil, both high in n-3 FA, tended to moderate the fever response after *Pasteurella* vaccine challenge and reduced blood cell expression of key proinflammatory cytokines (Karcher et al., 2014).

Phospholipids are a primary component of cellular membranes, and the FA composition of the diet can influence the FA profile of the phospholipids in membranes. For example, intake of a diet high in n-6 FA can change the FA composition of the phospholipids in cell membranes such that more LA and ARA are present. This can alter gene expression toward a proinflammatory state (Calder, 2012). Conversely, consuming a diet high in n-3 FA leads to higher levels of ALA or other n-3 FA in membrane phospholipids (Calder, 2012). In this case, gene expression would favor an anti-inflammatory state (Calder, 2012).

Therefore, the objective of this study was to determine the effects of supplemental dietary n-3 and n-6 FA on the FA concentration of peripheral blood mononuclear cells (PBMC) of weaned Holstein heifer calves. We hypothesized that supplementing varying levels of n-3 and n-6 FA to heifers fed a TMR would alter the FA concentration of PBMC.

The Michigan State University Institutional Animal Care and Use Committee approved all animal procedures before the start of the experiment. Twenty-seven Holstein heifer calves (107 ± 2.6 d of age; 142.6 ± 6.5 kg of BW) were selected from the Michigan State University Dairy Teaching and Research Center and were housed in individual stalls (1.94 m deep, 1.13 m wide). Heifers were randomly assigned to a common TMR, fed once daily in the morning, and supplemented with 1 of 3 treatments (9 heifers/diet): Ca salts of flaxseed FA (Virtus Nutrition, Corcoran, CA) containing 35% 18:3n-3 (**N3**), Ca salts of soybean FA (Virtus Nutrition) containing 50% 18:2n-6 (**N6**), and a 50:50 mix of N3 and N6. Heifers were fed the common TMR for a 14-d adaptation period before the start of supplemental dietary fat inclusion and collection of data. The FA treatment was supplemented at 4% of dietary DM for 30 d and topdressed each morning at the same time the TMR was fed. Water was provided ad libitum, and calves were fed to achieve an ADG of 0.9 kg/d. The trial consisted of 3 blocks, each with a 14-d adaptation period and 30-d treatment period, with 9 heifers/block (3 heifers/treatment per block). The study was conducted from January 2012 to May 2012.

The ingredient and nutrient composition of the diets fed as a TMR and fat supplement analysis are described in Table 1. The DM concentration was determined weekly for forages, and diet was adjusted when necessary. Diet ingredients were sampled from each block and dried at 55°C in a forced-air oven for 72 h for DM determination. Dried samples were ground with a Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA) and analyzed as reported by Boerman et al. (2017). The FA concentration of the fat supplements was determined as described by Lock et al. (2013).

Intake of feed from each animal was measured daily (Table 2). Feed provided was adjusted weekly to target a gain of 0.9 kg/d. Heifers were weighed at the beginning of the treatment period and weekly at d 7, 14, 21, and 28. Withers height and BCS were also measured weekly throughout the experiment. The 1-to-5 system was used for BCS (0.25-unit increments; Wildman et al., 1982), with 1 being emaciated and 5 being obese. Scores were based on changes around the vertical and transverse processes of the spine as palpated by 2 trained technicians. On d 28, heifers were vaccinated with a *Pasteurella* vaccine (OneShot; Zoetis, Madison, NJ). Rectal temperature was measured at 0, 3, 6, 9, 12, 24, 36, and 48 h relative to vaccine administration.

Blood was collected from each heifer via jugular venipuncture on d 0 and d 28 before vaccination. At each sampling, 200 mL of blood was collected in blood collection tubes containing EDTA as the anticoagulant. Samples were then placed on ice and processed within 2 h of collection.

After PBMC isolation and purification (Contreras et al., 2010), total lipids were extracted using chloroform: methanol:water (2:2:1, by vol) according to Bligh and Dyer (1959). Fatty acid methyl esters were prepared using a combination of base-catalyzed transesterification (0.5 N KOH in methanol) followed by an acid-catalyzed methylation (BF_3 in methanol, 10% wt/wt) and dissolved in n-hexanes (Christie, 1993). Fatty acid methyl esters were further purified by solid phase extraction silica cartridges (Supelco, Bellefonte, PA) using n-hexanes:chloroform (1:1). The FA composition analysis and GC conditions were as described by Lock et al. (2013).

Growth data were analyzed using the MIXED procedure in SAS (version 8; SAS Institute Inc., Cary, NC) as a completely randomized design. The statistical model used was $Y_{ijk} = \mu + T_i + w_j + Tw_{ij} + C_k(T_i) + \varepsilon_{ijk}$, where Y_{ijk} is the observed measurement, μ represents the overall population mean, T_i is the fixed effect of treatment i , w_j is the random effect of week j , Tw_{ij} is the interaction of treatment i and week j , $C_k(T_i)$ is the random effect of calf k nested within treatment i that was used to test the effect of treatment, and ε_{ijk} is

Table 1. Diet ingredients and nutrient composition of the common TMR diet and fatty acid (FA) supplements¹

Item	N3	Mix	N6
Diet ingredient (% of DM)			
Alfalfa haylage	54.0	54.0	54.0
Ampli-Calf ²	42.0	42.0	42.0
N3 FA-enriched supplement	4.0	2.0	0.0
N6 FA-enriched supplement	0.0	2.0	4.0
Nutrient composition of common TMR (% of DM)			
DM	46.51		
NDF	34.8		
CP	19.78		
Starch	9.77		
FA	4.65		
FA profile of supplements (g/100 g of FA)			
C16:0	17.30		13.11
C18:0	4.53		3.02
C18:2	13.12		48.86
C18:3	35.12		0.73
C22:0	0.20		0.33
C20:2	0.04		0.02
C20:3	0.02		0.01
C20:4	0.09		0.04
C20:5	0.76		0.4
C22:4	0.09		0.12
C22:5	0.14		0.07
C22:6	0.49		0.21
ΣSFA	23.4		17.22
ΣMUFA	25.32		31.77
ΣPUFA	49.87		50.47

¹Diets were a TMR supplemented with Ca salts of flaxseed FA containing 35% 18:3n-3 (N3; Virtus Nutrition, Corcoran, CA), Ca salts of soybean FA containing 50% 18:2n-6 (N6; Virtus Nutrition), or a 50:50 mix of N3 and N6.

²Future Calf TCR 20 Ampli-Calf R40 (pelleted feed; Land O'Lakes Purina Feed LLC, Shoreview, MN). Proximate analysis: 40 g/ton of monensin, 20% CP (minimum), 20% fat (minimum), 13% fiber (maximum), 1% calcium (minimum), 0.55% phosphorus (minimum), and 0.30 mg/kg of selenium (minimum).

the residual assumed to be normally distributed with mean zero. Block was included but was not significant in the model and was removed. Week was modeled as a repeated measurement using an autoregressive type 1 covariance structure. First-order autoregressive was the covariate structure used for analysis because it resulted

in the lowest Bayesian information criterion for most of the variables measured. Significance was declared at $P \leq 0.05$.

To the best of our knowledge, this is the first study to report the effect of dietary supplementation of n-3 FA on n-3 concentrations of PBMC in weaned dairy

Table 2. Fatty acid (FA) composition of peripheral blood mononuclear cells from calves (n = 27) fed 1 of 3 diets for 30 d¹

Fatty acid (%)	N3	Mix	N6	SEM	P-value
C18:2 <i>cis</i> -9, <i>cis</i> -12	8.7 ^b	10.1 ^a	10.8 ^a	0.48	0.004
C20:2 <i>cis</i> -11, <i>cis</i> -14	0.53 ^c	0.68 ^b	0.91 ^a	0.08	0.001
C20:3 <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	1.42 ^c	1.66 ^b	1.84 ^a	0.07	0.003
C20:4 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	12.2 ^c	13.7 ^b	14.7 ^a	0.41	0.003
Σ n-6	24.0 ^c	27.6 ^b	30.6 ^a	0.79	0.001
C18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	1.14 ^a	0.79 ^b	0.61 ^c	0.06	0.001
C20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	1.25 ^a	0.81 ^b	0.41 ^c	0.06	0.001
C22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	1.09	1.11	0.98	0.07	0.14
Σ n-3	8.2 ^a	7.2 ^b	5.8 ^c	0.28	0.001
ΣSFA	43.4	41.6	41.2	1.24	0.44
ΣMUFA	14.2 ^a	13.6 ^{ab}	12.6 ^b	0.67	0.05
ΣPUFA	32.2 ^b	34.8 ^{ab}	36.4 ^a	0.97	0.03

^{a-c}Means within a row with different superscripts differ.

¹Diets were a TMR supplemented with Ca salts of flaxseed FA containing 35% 18:3n-3 (N3; Virtus Nutrition, Corcoran, CA), Ca salts of soybean FA containing 50% 18:2n-6 (N6; Virtus Nutrition), or a 50:50 mix of N3 and N6.

heifers. Table 2 shows the FA composition of PBMC. We observed that heifers receiving the N3 diet had greater concentration of n-3 FA in PBMC ($8.2 \pm 0.3\%$) compared with heifers receiving the N6 diet ($5.8 \pm 0.3\%$) and the mix diet ($7.2 \pm 0.3\%$). Heifers consuming the N3 diet had higher levels of ALA ($1.1 \pm 0.1\%$) in PBMC compared with heifers consuming the N6 and mix diets (0.6 and $0.8 \pm 0.1\%$, respectively). We also observed a greater concentration of EPA in PBMC of heifers fed the N3 diet ($1.3 \pm 0.1\%$) compared with heifers consuming the N6 and mix diets (0.4 vs. $0.8 \pm 0.1\%$, respectively).

Very few studies in dairy cattle have focused on the effect of dietary PUFA supplementation on phospholipid concentrations in PBMC. Moallem and Zachut (2012) fed transition dairy cows a diet supplemented with flaxseed oil (56.1 g/d of ALA) for 22 d before parturition. They reported a 2.6-fold increase in the proportion of ALA in blood plasma compared with the control cows. Additional work in other species has examined the role of dietary supplementation in phospholipid concentrations. Supplementation of fish oil to postweaned male pigs for 28 d increased EPA in PBMC compared with control pigs (Merle et al., 2011). Similarly, feeding fish oil (6 g of total n-3/100 kg of BW per day) to yearling horses for 35 and 70 d increased proportions of EPA, DHA, and sum of n-3 in plasma and red blood cells compared with the control (Vineyard et al., 2010).

A link exists between disease and the incorporation of dietary n-3 and n-6 FA into membrane phospholipids (Harris et al., 2007). Based on the implications of PUFA on human health and cardiovascular disease, a large body of literature is focused on the effect of dietary PUFA supplementation on immune response. Yaqoob et al. (2000) fed healthy humans a dietary supplement containing 2.1 g of EPA and 1.1 g of DHA/d for 12 wk. Participants receiving the n-3 supplement had greater proportions of EPA and DHA in PBMC lipids and a reduction in the total n-6 to total n-3 PUFA compared with participants receiving the control supplement. Similar results were seen when human subjects received supplementation of either fish oil (1,296 mg of EPA and 864 mg of DHA/d) or flaxseed oil (3,510 mg of ALA and 900 mg of LA/d) for 8 wk. Erythrocyte membrane EPA and DHA increased 300 and 42%, respectively, with the supplementation of fish oil (Cao et al., 2006). These results, including our current study, support the hypothesis that dietary supplementation of n-3 FA affects the concentration of n-3 in PBMC and other cellular membranes.

Fever is the result of an inflammatory response. Heifers in our study were challenged with a *Pasteurella* vaccine to determine diet influence on moderating the fever response. Overall, heifers receiving the N3 diet

had a lower rectal temperature response to *Pasteurella* vaccine challenge ($39.5 \pm 0.08^\circ\text{C}$) compared with heifers receiving the N6 diet ($39.8 \pm 0.08^\circ\text{C}$; Figure 1; $P < 0.05$). There was an effect of time on temperature response, with rectal temperatures increasing to 24 h and declining by 48 h. At 3, 6, and 9 h postvaccination, the temperatures of heifers receiving the N3 diet were lower compared with the temperatures of heifers fed the N6 diet. We did not observe an effect of vaccination on feed intake across the 3 diets during the 48-h postvaccination challenge (data not shown). Karcher et al. (2014) reported a tendency for a decreased temperature response over a 48-h period post-*Pasteurella* vaccine in preweaned bull calves fed a milk replacer (MR) supplemented with 2% flax oil for the first 28 d of life.

Cytokines regulate the development of acute and chronic inflammation, and proinflammatory cytokines include TNF- α , IL-1 β , osteopontin, and IL-8. Long-chain FA, such as n-3, are capable of influencing the inflammatory response (Calder, 2010). This influence is mediated by the altered activation of the transcription factor nuclear factor κB (Novak et al., 2003). Diets rich in n-3 reduced expression of TNF- α (Simopoulos, 2002; Gaines et al., 2003). These diets reduce the capacity of PBMC to produce proinflammatory cytokines TNF- α and IL-1 by suppressing mRNA transcription (Simopoulos, 2002; Gaines et al., 2003). Because TNF- α and IL-1 induce fever and activate the acute phase response, the inhibitory effect of n-3 FA on PBMC cytokine synthesis causes anti-inflammatory effects (Endres et al., 1989).

Additionally, Hill et al. (2011b) reported that preweaned bull calves fed MR supplemented with a commercially available blend of butyric acid, coconut oil, and flax oil had reduced expression of whole-blood TNF- α mRNA after a *Pasteurella* vaccine challenge on d 36 compared with calves fed the control MR. Calves on the treatment diet also had less of an increase in rectal temperature response to the vaccine compared with control calves. Compared with control calves, TNF- α expression was reduced in LPS-stimulated whole blood collected from preweaned bull calves fed an MR supplemented with 2% fish oil (Karcher et al., 2014).

The primary objective of our study was focused on the effect of supplemental dietary n-3 and n-6 FA on the FA concentration of PBMC in the blood of weaned Holstein heifer calves. However, the effect of diets on performance parameters is important to note. To date, limited studies have focused on the effect of feeding dietary FA on the production performance of weaned heifers. The current study focused on limit feeding weaned Holstein heifers to target a gain of 0.9 kg/d. As expected, we did not detect differences in ADG between heifers fed N3, N6, or a mix (Table 3). Additionally, no differences were detected for BCS change and wither

Table 3. Performance of calves (n = 27) fed 1 of 3 diets for 30 d^{1,2}

Item	N3	Mix	N6	SEM	P-value
Calves (no.)	9	9	9		
DMI (kg/d)	3.65	3.80	3.83	0.15	0.33
Initial BW (kg)	139.6	146.4	142.8	6.23	0.65
Final BW (kg)	162.2	167.9	166.3	6.05	0.55
ADG (kg/d)	0.810	0.767	0.839	0.057	0.91
Initial BCS ³	2.47	2.36	2.39	0.05	0.66
BCS change	0.04	0.25	0.25	0.13	0.55
Initial wither height (cm)	100.1	99.2	100.8	1.27	0.67
Wither height change (cm)	4.22	6.27	4.52	0.79	0.99

¹Diets were a TMR supplemented with Ca salts of flaxseed fatty acids containing 35% 18:3n-3 (N3; Virtus Nutrition, Corcoran, CA), Ca salts of soybean fatty acids containing 50% 18:2n-6 (N6; Virtus Nutrition), or a 50:50 mix of N3 and N6.

²On d 28, calves received a *Pasteurella* vaccine at 0 h, and rectal temperature was measured immediately before vaccination and at 3, 6, 9, 12, 24, 36, and 48 h postvaccination.

³1-to-5 system based on Wildman et al. (1982).

height among the 3 diets. The authors acknowledge that there is high variability in some of the data related to the production variables and that there may not be enough power to statistically gauge differences between some of these variables.

Hill et al. (2011a) reported the results of a study that fed weaned Holstein bull calves (60 d of age) a textured grower containing 0.25% Ca salts of flaxseed FA (Virtus Nutrition, Corcoran, CA) and blended with 5% chopped hay or a control. Calves were fed the treatment diets free choice for 56 d. There were no differences in ADG, DMI, or feed efficiency between the 2 diets. However, the addition of the Ca salts of flaxseed FA in the diet resulted in a greater BCS change compared with the control (Hill et al., 2011a). The age at which n-3 is supplemented into the diet of young calves may affect the growth response of the animal. Hill et al. (2009)

began feeding Holstein bull calves at 2 to 3 d of age MR supplemented with a control starter, a starter containing 0.125% Ca salt of flax oil, or a starter containing 0.250% Ca salts of flax oil. The calves were weaned at 28 d and continued to receive the starter ad libitum for 84 d. Chopped hay was added to the starter at 5% from d 56 to 84. Calf BCS did not differ across diets, but ADG increased linearly as flax oil increased in the starter from 28 to 56 d and 56 to 84 d. The feeding period of Ca salts of flax oil in our study was 30 d compared with 84 d in the Hill et al. (2009) study, and the FA were supplemented to weaned heifers beginning at approximately 107 d of age.

Several studies indicate that feeding starter ad libitum to preweaned dairy calves does not affect BW gain or BCS. Karcher et al. (2014) reported no increase in ADG when Holstein bull calves were fed MR with 2%

Figure 1. Rectal temperature (means $\pm$ SE) of calves (n = 27) receiving a *Pasteurella* vaccine on d 28 (0 h) and temperature response at 3, 6, 9, 12, 24, 36, and 48 h postvaccination. Diets were a TMR supplemented with Ca salts of flaxseed fatty acids containing 35% 18:3n-3 (N3; Virtus Nutrition, Corcoran, CA; solid line), Ca salts of soybean fatty acids containing 50% 18:2n-6 (N6; Virtus Nutrition; short-dash line), or a 50:50 mix of N3 and N6 (long-dash line). Different letters (a,b) indicate significant differences between diets within time ($P < 0.05$).

flax or fish oil from 0 to 28 d compared with calves fed the control diet. However, an increase in ADG and feed efficiency was observed for the 2% flax oil diet compared with the 2% fish oil diet. Calves in this study were offered a textured calf starter ad libitum. Similarly, Ballou and Depeters (2008) reported limited change in growth rate when Jersey bull calves consumed MR containing 2% fish oil. It is important to recognize that these studies fed preweaned calves MR supplemented with ad libitum starter. The weaned heifers in our study were limit fed a common TMR, which contained 54% alfalfa silage, 42% pelleted starter (Future Cow TCR 20 Ampli-Calf R40; Land O'Lakes Purina Feed LLC, Shoreview, MN), and 4% added fat (DM basis).

Manipulation of dietary factors may influence the modulation of the immune system and response to disease. The type of FA within the phospholipid membranes of white blood cells affects immune cell function (Raphael and Sordillo, 2013). This study showed that feeding weaned Holstein heifers a diet high in n-3 FA increased concentrations of n-3 FA in PBMC. Incorporation of higher levels of n-3 FA in the cell membrane may have contributed to the lower temperature response when calves were challenged with a *Pasteurella* vaccine. Future studies are needed in Holstein heifers of similar age. These studies should focus on the mechanisms by which n-3 modulates health, including ideal doses and the extent of FA protection past the rumen.

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