Effect of different fatty acids on the proliferation and cytokine production of dairy cow peripheral blood mononuclear cells

Noémie Vanacker,^{1,2} Richard Blouin,² Céline Ster,¹ and Pierre Lacasse^{1*}

¹Sherbrooke Research and Development Centre, Agriculture and Agri-Food Canada, Sherbrooke, Quebec, Canada J1M 0C8

²Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada J1K 2R1

ABSTRACT

During the transition period, dairy cows often experience negative energy balance, which induces metabolic and immunological disturbances. Our previous work has shown a relationship between the inhibition of immune functions and increased blood nonesterified fatty acid (NEFA) levels. In this study, we evaluated the effect of 11 fatty acids (palmitoleic, myristic, palmitic, stearic, oleic, linoleic, docosahexaenoic, conjugated linoleic, lauric, eicosapentaenoic, and linolenic acids) as well as a mix that represented the NEFA profile observed during the transition period at different concentrations (0, 50, 100, and 250 μ M) on proliferation and cytokines secretion of lymphocytes. To assess lymphoproliferation, peripheral blood mononuclear cell (PBMC) from 5 healthy cows (166–189 d in milk) were isolated, stimulated with the mitogenic lectin concanavalin A (ConA), incubated for 72 h with or without fatty acids, and subjected to flow cytometry analysis. Our results showed that all fatty acids, except lauric acid, significantly reduced proliferation of PBMC in a dose-dependent manner. The most detrimental effect was observed with conjugated linoleic and stearic acids, where proliferation of PBMC was already inhibited at the lowest dose (50 μ M). For cytokine secretion, we found that levels of IL-4 in culture supernatant of ConA-stimulated PBMC were reduced after a 24-h exposure to the lowest dose (50 μ M) of oleic and palmitoleic acids. A dose of 100 μ M of eicosapentaenoic acid, NEFA mixture, and myristic acid was necessary to observe a reduction in IL-4 levels. The PBMC also showed a decrease in the secretion of IFN- γ in response to lauric, linolenic, palmitoleic, and stearic acids at 50 μ M and myristic acid at 100 μ M. Overall, polyunsaturated fatty acids were more potent inhibitors of cytokine secretions than saturated fatty acids. In addition, we detected an inverse relationship between the melting points of fatty acids and their abil-

ity to inhibit IL-4 and IFN- γ secretions, as evidenced by greater inhibition with low-melting point fatty acids. Overall, our study confirmed that NEFA have a negative effect on some lymphocyte functions, and that their inhibitory effect on cytokine secretions increases with the degree of unsaturation.

Key words: transition period, immune function, degree of saturation, lymphocyte

INTRODUCTION

For high-producing dairy cows, the transition from gestation to peak lactation is associated with a high susceptibility to infectious diseases such as mastitis and metritis, likely caused by a state of immunodepression. Indeed, parturition in dairy cattle is associated with a decreased responsiveness of blood lymphocytes to stimulation with mitogenic agents (Carbonneau et al., 2012; Ster et al., 2012; Vanacker et al., 2017) and with decreased immunoglobulin production by B cells (Nonnecke et al., 2003; Lacetera et al., 2005). Accordingly, about 75% of diseases occur during the first month following calving, and 36% of deaths occur within the first 60 d after calving (LeBlanc et al., 2006). Another study of Hammon et al. (2006) showed that there is a relationship between uterine health disorders and the energy status in Holstein cows. Therefore, it is important to determine the causes of periparturient immunodepression to improve the health of dairy cows.

In early lactation, the nutrients provided by the diet are not sufficient to provide the energy required for milk production, resulting in a negative energy balance. Consequently, cows must mobilize fat reserves to balance the energy deficit. A cause-and-effect relationship between energy deficit and the impairment of immune functions was evidenced by Nonnecke et al. (2003). Ster et al. (2012) showed that lymphocyte proliferation and interferon- γ secretion were lower when the cells were incubated with sera harvested in the postpartum period and that those parameters were inversely correlated with serum nonesterified fatty acid (NEFA) concentration. In addition, a dose-effect relation-

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*Corresponding author: Pierre.Lacasse@agr.gc.ca

ship was found between NEFA concentrations and the inhibition of both lymphocyte proliferation and interferon- γ secretion (Lacetera et al., 2004; Ster et al., 2012). These results suggest that the increase in blood NEFA concentration is implicated in the peripartum immunodepression. Accordingly, the odds of developing metritis (Ospina et al., 2010) and mastitis (Moyes et al., 2009) in early lactation increase when the blood NEFA concentration is elevated.

Previous studies have shown an inhibitory effect of different fatty acids on lymphoproliferation (Calder et al., 1990; Calder et al., 1994; Ster et al., 2012). However, the mechanism by which fatty acids affect bovine immune cell functions is still unknown. Calder and Willemsen (2016) suggested that changing immune cell membrane composition can affect the physical properties, the aptitude to form signaling platforms, and the activity of membrane lipid-derived signaling molecules. Indeed, phospholipid membrane functions are strongly involved in the ability of cells to proliferate. Therefore, any change in the fatty acid composition of the membrane lipids has an effect on the proliferative functions. Another way by which NEFA could affect immune function is through the binding and activation of receptors such as the peroxisome proliferator-activated receptor (**PPAR**).

The results vary considerably among studies evaluating the effect of NEFA on immune functions. These variations can be explained by the wide range of protocols used, including the following: different fatty acids used as either free fatty acid or as an albumin complex, and different concentrations and different exposure times of the T-lymphocyte to the fatty acids. Calder et al. (1991) studied the effect of fatty acids on the proliferation of concanavalin A-stimulated rat lymph node lymphocytes. In general, the effect was dependent on both dose and time of exposure. Unsaturated fatty acids had greater inhibitory power than SFA. To our knowledge, no studies have so far addressed the effect of fatty acids on bovine lymphocyte functions. Because

there is a great variability of the responses between species (Tsang et al., 1977), it is important to assess the effect of the fatty acids found in the blood profiles of cows during the peripartum period. Therefore, the aim of this study was to determine the effect of several fatty acids on bovine lymphocyte functions.

MATERIALS AND METHODS

Reagents

All fatty acids (palmitoleic acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, docosahexaenoic acid, lauric acid, eicosapentaenoic acid, and linolenic acid; Table 1) were purchased from Sigma-Aldrich Canada Ltd., except for CLA, which was purchased from Cederlane. A NEFA mix containing 36% stearic acid, 30% palmitic acid, 25% oleic acid, 3% linoleic acid, 2.5% palmitoleic acid, 2% myristic acid, and 1.5% docosahexaenoic acid was prepared. These proportions reflected those encountered in bovine serum during the periparturient period based on different *in vivo* studies (Leroy et al., 2011; Contreras et al., 2012; Hostens et al., 2012; Watts et al., 2013). Each fatty acid was prepared in a solution of 100% ethanol to reach a concentration of 100 mM and then stored at -20°C until used. On the day of the experiment, each fatty acid was diluted in RPMI 1640 medium supplemented with 1% BSA, glutamine, antibiotics, and 1% ethanol to reach the tested concentration and placed at 37°C on agitation for 1 h before use. The final concentration of ethanol in the wells was 0.25%, and we have previously determined that this concentration of ethanol does not affect the tested parameters.

Animals and Experimental Procedures

The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care (1993) and approved by Sherbrooke Research and De-

Table 1. Fatty acids used in this study

Fatty acid	Class	Lipid number	% Mix ¹	Melting point ($^{\circ}\text{C}$)
Lauric acid	Saturated	C12:0	0	43.2
Myristic acid	Saturated	C14:0	2	54.4
Palmitic acid	Saturated	C16:0	30	62.9
Stearic acid	Saturated	C18:0	36	69.3
Palmitoleic acid	Monounsaturated	C16:1	2.5	-0.1
Oleic acid	Monounsaturated	C18:1	25	13.4
Linoleic acid	Polyunsaturated	C18:2	3	-5
Conjugated linoleic acid	Polyunsaturated	C18:2 <i>cis-9,trans-11</i>	0	42.5
Linolenic acid	Polyunsaturated	C18:3	0	-11
Eicosapentaenoic acid	Polyunsaturated	C20:5	0	-53 to -54
Docosahexaenoic acid	Polyunsaturated	C22:6	1.5	-44.5 to -44.7

¹% Mix = percentage of each fatty acid used in the mix.

velopment Centre animal care committee (#486). Five healthy, multiparous Holstein cows (166–189 DIM, with SCC <240,000/mL) housed at Agriculture and Agri-Food Canada's Sherbrooke Research and Development Centre (Sherbrooke, QC, Canada) were used as immune cell donors. Blood samples were collected from the jugular vein in blood-collection bags containing citrate-phosphate-dextrose-adenine anticoagulant (Animal Blood Resources International). The PBMC were isolated as described by Vanacker et al. (2017). The plasma NEFA concentration for each donor cow was determined by an enzymatic colorimetric method using a Randox kit (Scientifiques ESBE, Saint-Laurent) as previously described by Vanacker et al. (2020). The plasma NEFA concentration was similar in all 5 donor cows, ranging between 210 and 228 μM .

PBMC Proliferation Assay

Proliferation of PBMC was assayed as described by Ollier et al. (2016), with some modifications. Briefly, isolated PBMC were labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester using the CellTrace CFSE Cell Proliferation Kit (Thermo Fisher Scientific). To assess the effect of the fatty acid on PBMC proliferation, labeled PBMC were incubated in triplicate within 6 h after blood collection with the fatty acid of interest or the mix at different concentrations (0, 50, 100, and 250 μM) and in presence of the mitotic agent concanavalin A (**ConA**; Sigma-Aldrich) at a concentration of 1 $\mu\text{g/mL}$ in a 96-well plate (1.5×10^5 cells per well) at 38.5°C with 5% CO_2 for 72 h. In each plate, 3 wells were incubated with PBMC stimulated with ConA as positive control, and 3 wells contained PBMC without mitogen stimulation as a negative control. After a 72-h incubation, proliferation was analyzed using the FACS-Canto II flow cytometer (BD Biosciences). The number of PBMC recorded was 15,000.

Before data acquisition, multiple validation tests were done to localize the gates for the dead cells (staining with propidium iodine). These gates were used in the present experiment to evaluate cell death percentage.

PBMC Production of IFN- γ and IL-4

To assess the effect of the fatty acid on PBMC cytokine secretion, PBMC from the same donor cows as for the proliferation assay were incubated with the fatty acid of interest or the mix at different concentrations (0, 50, 100, and 250 μM) and in presence of the mitotic agent ConA (Sigma-Aldrich) at a concentration of 1 $\mu\text{g/mL}$ in a 24-well plate (1.2×10^6 cells per well) at 38.5°C with 5% CO_2 for 24 h. The supernatant was then collected by pooling the 2 wells with the same condition

and stored at -80°C until measurement. Supernatant cytokine content was determined with commercial kits for each cytokine as follows: IFN- γ (Bovine Interferon- γ Specific ELISA Assay Kit, Cedarlane Laboratories Limited) and IL-4 (Bovine IL-4 ELISA Kit, RayBiotech Life).

Statistical Analysis

Because all doses were tested within each of the donor cows, data for percent proliferation were analyzed as a complete block design in a one-way factorial with 4 levels (doses) for each fatty acid separately. Each dose was then compared with the positive control (no fatty acid supplementation and stimulated with ConA) result using a Dunnett adjustment. For the cytokine data, due to variation among cell donor cows, the effect of each dose was tested using a Student's *t*-test on the within-cow delta values calculated between the result at a specific concentration and without added NEFA ($n = 5$). The fatty acids were separated according to their saturation degree (saturated, monounsaturated, and polyunsaturated), and their average response for each concentration was analyzed with the MIXED procedure of SAS. In addition, regression analysis was performed on the melting point of the fatty acids (Table 1) and the average response using the REG procedure of SAS. Differences were considered significant when $P < 0.05$ and were considered a trend when $P < 0.10$.

RESULTS

PBMC Proliferation

Except for lauric acid ($P > 0.1$), all fatty acids inhibited proliferation within the tested range (Figure 1). The inhibitory effect of CLA and stearic acid was already significant ($P < 0.01$) at the smallest dose tested (50 μM). At 100 μM , NEFA mixture, oleic acid, and palmitic acid significantly decreased ($P \leq 0.05$) PBMC proliferation, whereas eicosapentaenoic acid tended to decrease it ($P = 0.06$). At the highest tested concentration, 250 μM , every fatty acid except for lauric acid significantly decreased lymphoproliferation ($P < 0.01$). No specific effect on the saturation degree ($P > 0.5$) or the melting point ($P \geq 0.2$) of fatty acids was observed on lymphoproliferation.

Without fatty acids added, cell death ranged between 31.6 and 35.0%. At 50 μM , none of the fatty acids increased cell death (Figure 2). At a concentration of 100 μM , cell death was increased ($P < 0.01$) by CLA and stearic acid. At the highest concentration, CLA, docosahexaenoic acid, NEFA mixture, myristic acid, linolenic acid, oleic acid, palmitic acid, and stearic acid

significantly increased cell death ($P \leq 0.05$), whereas eicosapentaenoic acid and linoleic acid tended ($P < 0.1$) to increase it. Lauric acid and palmitoleic acid did not increase ($P > 0.4$) cell death at any of the doses tested.

Cytokine Secretion

Concentrations of IFN- γ in the supernatant of PBMC incubated without ConA were very low and below the detection limit of the test (not shown). Without NEFA added to the medium, stimulation of PBMC with ConA induced a secretion of IFN- γ (value $\pm$ SE; $3,220 \pm 641$ pg/mL). At a concentration of $50 \mu\text{M}$, lauric acid, linolenic acid, palmitoleic acid, myristic acid, and stearic acid significantly decreased IFN- γ secretion ($P \leq 0.05$) when compared with that observed in cells incubated without fatty acids (Table 2). At this same concentration, CLA, eicosapentaenoic acid, and linoleic acid tended to decrease the levels of IFN- γ in the supernatant ($P < 0.1$). Furthermore, at a concentration of $100 \mu\text{M}$, the NEFA mixture, lauric acid, linolenic

acid, and myristic acid significantly decreased IFN- γ secretion ($P \leq 0.05$), whereas all the other fatty acids except for stearic acid and oleic acid tended to decrease it ($P < 0.1$). On the contrary, palmitic acid tended to increase IFN- γ secretion ($P < 0.1$). Oleic acid as well as stearic acid had no effect on IFN- γ secretion. At the highest concentration, no significant changes were observed; however, eicosapentaenoic acid, lauric acid, myristic acid, linolenic acid, and palmitoleic acid tended to decrease IFN- γ secretion ($P < 0.1$). We observed that PUFA were more potent inhibitors of IFN- γ secretion than SFA ($P \leq 0.05$) at all concentrations (Figure 3). Similarly, the inhibitory effect of fatty acid on IFN- γ production increased when the melting point of the fatty acids decreased ($P \leq 0.05$). Polyunsaturated fatty acids were more inhibitory than SFA at $50 \mu\text{M}$ ($P = 0.02$), $100 \mu\text{M}$ ($P = 0.05$), and $250 \mu\text{M}$ ($P = 0.03$). Monounsaturated fatty acids tended to be more inhibitory than SFA at $50 \mu\text{M}$ ($P = 0.07$).

Concentration of IL-4 in the supernatant of PBMC incubated without ConA were very low and below the detection limit of the test (not shown). Without NEFA

Figure 1. Percentage of lymphoproliferation in the presence of fatty acids at different concentrations (blue = $50 \mu\text{M}$; orange = $100 \mu\text{M}$, and gray = $250 \mu\text{M}$) compared with untreated control (horizontal line; ** $P < 0.01$; * $P < 0.05$; † $P < 0.1$); SEM ≤ 10.5 . MIX = mix of nonesterified fatty acids; LAU = lauric acid; MYR = myristic acid; PAL = palmitic acid; STE = stearic acid; PTL = palmitoleic acid; OLE = oleic acid; LIN = linoleic acid; NIC = linolenic acid; EIC = eicosapentaenoic acid, DHA = docosahexaenoic acid.

Figure 2. Percentage of cell death during the lymphoproliferation in either absence (yellow = 0 μ M) or presence of fatty acids at different concentrations (blue = 50 μ M; orange = 100 μ M, and gray = 250 μ M; *** P < 0.001; ** P < 0.01; * P < 0.05; † P < 0.1); SEM $\leq$ 5.6. DHA = docosahexaenoic acid; EIC = eicosapentaenoic acid; LAU = lauric acid; LIN = linoleic acid; MIX = mix of nonesterified fatty acids; MYR = myristic acid; NIC = linolenic acid; OLE = oleic acid; PAL = palmitic acid; PTL = palmitoleic acid; STE = stearic acid.

added to the medium, stimulation of PBMC with ConA induced a secretion of IL-4 ($2,207 \pm 366$ pg/mL). At a concentration of 50 μ M, oleic acid, myristic acid, and palmitoleic acid significantly decreased IL-4 secretion ($P \leq 0.05$) when compared with the control (Table 3). At 100 μ M, NEFA mixture, myristic acid, palmitoleic acid, oleic acid, linolenic acid, and eicosapentaenoic acid significantly decreased IL-4 secretion ($P \leq 0.05$), whereas lauric and linoleic acids tended to decrease it ($P < 0.1$). At the highest concentration, NEFA mixture, myristic acid, palmitoleic acid, and oleic acid significantly decreased IL-4 secretion ($P \leq 0.05$), whereas all other fatty acids except for palmitic acid and stearic acid tended to decrease it ($P < 0.1$). Stearic acid was the only fatty acid showing no effect on IL-4 secretion at all doses tested. At a concentration of 250 μ M, we observed that PUFA tended to be inhibitors of IL-4 secretion ($P = 0.09$; Figure 4). At the concentrations of 50 and 100 μ M, the inhibitory effect of fatty acids on IL-4 production tended to increase when the melting point of the fatty acid decreased ($P < 0.1$). At a concentration of 250 μ M, that effect became significant ($P < 0.01$).

DISCUSSION

The mix of NEFA had an effect on lymphocyte functions at a concentration of 100 μ M, which is much lower than the concentration observed during the periparturient period, when blood concentrations can reach up to 700 μ M (Adewuyi et al., 2005; McCarthy et al., 2015). In vivo, most NEFA are complexed to BSA. Although BSA was added to the cell medium, its concentration in the well (0.25%) was low in comparison to its concentration in the bloodstream (3%; Labouche, 1964). Therefore, for the same concentration, the amount of free fatty acid available is likely to be higher in vitro compared with in vivo, which could explain the biological effects observed in vitro with lower doses. The negative effect of the NEFA mix on lymphoproliferation showed that the model chosen for this assay was adequate. The results supported the concept that NEFA have a role in the impairment of the immune system during the periparturient period.

In the present study, most fatty acids inhibited IL-4 and IFN- γ secretion. Although they reported an inhibitory effect of fatty acids on bovine PBMC proliferation,

Table 2. Interferon- γ concentration (pg/mL) in the culture medium of peripheral blood mononuclear cells exposed to different concentrations of fatty acids compared with untreated control; values presented in the table represent the mean obtained by all 5 cow donors

Fatty acid	50 μ M			100 μ M			250 μ M		
	Mean ¹	SEM	P-value	Mean ¹	SEM	P-value	Mean ¹	SEM	P-value
Mix of nonesterified fatty acids	-997.2	509.12	0.12	-1,731.6*	738.95	0.08	-2,459.2	1,305.93	0.13
Lauric acid (C12:0)	-678.8*	219.71	0.04	-1,340*	412.25	0.03	-2,256.8	948.39	0.08
Myristic acid (C14:0)	-1,032.2*	363.34	0.05	-952.8*	270.14	0.02	-1,825.2	811.94	0.09
Palmitic acid (C16:0)	743.6	385.28	0.13	2,365.6	979.81	0.07	303.2	1,672.83	0.87
Stearic acid (C18:0)	-612.4*	193.24	0.03	-441.6	427.38	0.36	-1,224	1,264.12	0.39
Palmitoleic acid (C16:1)	-1,088*	356.16	0.04	-1,764.8	739.34	0.08	-2,421.6	1,081.31	0.09
Oleic acid (C18:1)	-2,183.2	1,025.73	0.10	-2,586.4	1,290.61	0.12	-2,763.2	1,453.36	0.13
Linoleic acid (C18:2)	-1,598.4	703.88	0.09	-2,326.4	1,065.10	0.09	-2,713.2	1,312.81	0.10
Conjugated linoleic acid (C18:2)	-1,707.6	731.82	0.08	-2,172.4	979.28	0.09	-2,713.2	1,281.49	0.10
Linolenic acid (C18:3)	-1,692.8*	564.82	0.04	-2,176.8*	795.70	0.05	-2,585.2	1,086.56	0.08
Eicosapentaenoic acid (C20:5)	-1,840	775.70	0.08	-2,288.4	892.83	0.06	-2,640.8	1,152.59	0.08
Docosahexaenoic acid (C22:6)	-1,747.6	1,266.13	0.24	-1,728.8	656.06	0.06	-2,844.4	1,420.57	0.12

¹Calculated as the difference between the concentration of IFN- γ at a given dose and its concentration at 0 mM.* $P \leq 0.05$.**Table 3.** Interleukin-4 concentration (pg/mL) in the culture medium of peripheral blood mononuclear cells exposed to different concentrations of fatty acids compared with untreated control; values presented in the table represent the mean obtained by all 5 cow donors

Fatty acid	50 μ M			100 μ M			250 μ M		
	Mean	SEM	P-value	Mean	SEM	P-value	Mean	SEM	P-value
Mix of nonesterified fatty acids	-358.34	167.83	0.10	-771.37*	248.83	0.04	-1,388.56*	506.89	0.05
Lauric acid (C12:0)	-553.29	290.46	0.13	-1,080.1	433.43	0.07	-1,636.11	714.03	0.08
Myristic acid (C14:0)	-438.45*	159.57	0.05	-510.88*	120.20	0.01	-745.61*	272.99	0.05
Palmitic acid (C16:0)	174.56	207.44	0.45	231.91	212.91	0.34	-216.89	515.60	0.70
Stearic acid (C18:0)	-79.59	201.55	0.71	265.28	257.98	0.36	-80.73	417.38	0.86
Palmitoleic acid (C16:1)	-318.89*	86.82	0.02	-826.1*	245.67	0.03	-1,213.43*	367.39	0.03
Oleic acid (C18:1)	-930.23*	260.22	0.02	-1,152.27*	304.05	0.02	-1,524.21*	491.51	0.04
Linoleic acid (C18:2)	-203.41	179.20	0.32	-653.87	273.65	0.08	-1,148.6	454.75	0.07
Conjugated linoleic acid (C18:2)	-133.22	169.56	0.48	-408	243.42	0.17	-1,102.33	493.46	0.09
Linolenic acid (C18:3)	-966.5	377.29	0.06	-1,220.44*	445.07	0.05	-1,758.28	736.02	0.08
Eicosapentaenoic acid (C20:5)	-745.19	356.00	0.10	-1,017.29*	336.40	0.04	-1,855.27	820.63	0.09
Docosahexaenoic acid (C22:6)	-348.87	399.32	0.43	-372.49	238.55	0.19	-1,582.91	697.16	0.09

¹Calculated as the difference between the concentration of IL-4 at a given dose and its concentration without fatty acids.* $P \leq 0.05$.

Renner et al. (2013) did not report an effect of fatty acids on IL-4 and IFN- γ . The difference observed between their study and ours is likely related to the methodology used. Indeed, we measured cytokine secretion in the culture supernatant using ELISA tests, whereas Renner et al. (2013) measured cytokine gene expression in the PBMC after 72 h of incubation by real time reverse transcription-PCR. The effect that could have been observed in cytokine secretion might have been mitigated by the long incubation. In addition, the ELISA method is a direct measurement of the cytokine concentration present in the supernatant, whereas the amount of RNA doesn't equal the amount of protein that is actually translated (Alberts et al., 2002; Al-Ali and González-Sarmiento, 2016).

Inhibition of cytokine secretion was greater for PUFA than for SFA. Similarly, studies have reported that PUFA were more effective inhibitors of rat lymphocyte functions than SFA (Calder et al., 1990). Because phospholipids are the main component of the cell membrane (Nicolson, 2014), a change in the fatty acid composition alters its biophysical properties. Calder et al. (1994) showed that by adding fatty acids to the medium, the fatty acid composition and the membrane fluidity were changed. They found that long-chain SFA decreased

membrane fluidity, whereas UFA increased it. Calder and Willemsen (2016) have proposed that those changes in membrane fluidity could be responsible for the inhibition of lymphocyte functions. In support of this hypothesis, the regression analysis of our results showed an inverse relationship between the melting point and the ability to inhibit IL-4 and IFN- γ secretions. Nevertheless, the inhibitory activity of CLA, a PUFA with a relatively high melting point, was not clearly lower than that of linoleic acid, which has the same chemical composition but a much lower melting point. Fan et al. (2003) showed that PUFA changed lipid raft composition, soluble membrane phospholipids, and fatty acyl composition, which could lead to a modulation of T-cell activation. Lipid rafts are specialized plasma-membrane microdomains containing high concentrations of cholesterol and sphingolipid, and their polar lipids are predominantly formed of saturated fatty acyl residues (Martins de Lima et al., 2007). Lipid rafts are important in signal transduction pathways such as the T-cell receptor pathway, which is indirectly involved in the regulation of T-cell functions such as proliferation, differentiation, death, and cytokine release. Therefore, a change in their composition could explain the negative effect of fatty acids observed on IL-4 and IFN- γ

Figure 3. Mean value $\pm$ SE of IFN- γ production according to the class of nonesterified fatty acids (green = mix of nonesterified fatty acids, yellow = saturated, gray = monounsaturated, blue = polyunsaturated), calculated as the difference between the concentration of IFN- γ at a given dose and its concentration without fatty acids. Polyunsaturated fatty acids were more inhibitory than saturated fatty acids at 50 μ M ($P = 0.02$), 100 μ M ($P = 0.05$), and 250 μ M ($P = 0.03$). Monounsaturated fatty acids tended to be more inhibitory than saturated fatty acids at 50 μ M ($P = 0.07$). * $P < 0.05$; † $P < 0.1$.

Figure 4. Mean value $\pm$ SE of IL-4 production according to the class of nonesterified fatty acids (Green = mix of nonesterified fatty acids, yellow = saturated, gray = monounsaturated, blue = polyunsaturated), calculated as the difference between the concentration of IL-4 at a given dose and its concentration without fatty acids. Polyunsaturated fatty acids tend to be more inhibitory than saturated fatty acids at 250 μ M ($P = 0.09$). † $P < 0.1$.

production. We also found that stearic and palmitic acids were potent inhibitors of proliferation but had less effects on the inhibition of cytokine secretions, indicating that although membrane fluidity may affect lymphocyte functions, other mechanisms are also probably involved.

We found a negative dose-dependent effect of all fatty acids, except lauric acid, on lymphoproliferation. Accordingly, Ster et al. (2012) showed that NEFA had a negative effect on lymphoproliferation, oxidative burst, and IFN- γ production. Additionally, Calder et al. (1990) studied the effect of different fatty acids on the proliferation of ConA-stimulated rat lymph node lymphocytes. They concluded that all tested fatty acids inhibited mitogen response, and the extent of the inhibition was dependent on the fatty acid concentration. However, unlike Calder et al. (1990), we did not observe a link between the chain length or the saturation degree and the inhibitory activity of fatty acids on lymphoproliferation. Indeed, lymphoproliferation is inhibited by docosahexaenoic acid, a long PUFA, and by myristic acid, a short-chain SFA. Tsang et al. (1977) showed that the effect of fatty acids on lymphoproliferation differs between species. For example, in humans, there is no inhibition when cells are exposed to a mixture contain-

ing SFA and UFA. In ewes, oleic, stearic and palmitic acids inhibit proliferation of PBMC, but linoleic and palmitoleic acids did not (Lacetera et al., 2002). Studies have shown that fatty acids can even stimulate the proliferation of phytohemagglutinin-stimulated lymphocytes when using a combination of palmitate and oleate in rats. Therefore, it was not unexpected that results obtained with bovine cells were different from those obtained previously with rodent and human cells.

Our results did not show that a specific class of fatty acids was more potent at inhibiting lymphoproliferation, suggesting that other mechanisms may be involved. One possibility is activation of PPAR, a nuclear receptor that exists in 3 different isoforms, α , β , and γ . Each isoform is differentially expressed in diverse tissues and cell types, and regulates the transcription of genes implicated in metabolism, cell proliferation, differentiation inflammation control, and immune responses (Le Menn and Neels, 2018). A large variety of ligands, including UFA, lipids, and lipid-like molecules, are capable of binding the PPAR ligand-binding domain (Grygiel-Górniak, 2014). Peroxisome proliferator-activated receptor- γ has notably been demonstrated to impair T-cell proliferation in humans and mice. Although there is no direct evidence that PPAR

are involved in NEFA-induced inhibition of bovine lymphocyte functions, we found that docosahexaenoic acid and eicosapentaenoic acid, 2 known natural ligands of PPAR- γ , reduced lymphoproliferation in bovine cells.

The mechanisms causing immunodepression in cows during the periparturient period are diverse and complex. A better understanding of these mechanisms requires studies examining different parameters, such as immune function, fatty acid concentrations, and time of exposure, all of which can affect the results. Indeed, we cannot clearly identify a class of fatty acid for its negative effect on every immune function tested. However, the polyunsaturated class appears to be more detrimental, and the melting point seems to play a role. It is possible that changes in the physiological composition of the NEFA blood profile cause an imbalance, which then leads to immune function deficiencies. Overall, our study confirmed that NEFA individually have a negative effect on the tested parameters and that their inhibitory effects on cytokine secretions increases with the degree of unsaturation.

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ORCIDS

Pierre Lacasse <https://orcid.org/0000-0002-3579-7991>