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In vitro effects of conjugated linoleic acid (CLA) on bovine monocytes' inflammatory functions. By Ávila et al., 2020. A demonstration that CLA may have important roles in modulating some in vitro monocyte immune functions was provided. Only CLA mixture (50:50) exerts an anti-apoptotic activity and can increase ROS production in an inflammatory in vitro model, suggesting that the effects of each CLA isomer are different and in combination synergic effects are induced.

Running head

Immunomodulatory effect of CLA on monocytes

In vitro effects of conjugated linoleic acid (CLA) on bovine monocytes' inflammatory functions

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HIGHLIGHTS

- CLA exerts immunomodulatory effects in some in vitro functions of bovine monocytes
- CLA mixture reduces bovine monocytes' apoptosis
- CLA mixture also increases ROS production under pro-inflammatory conditions
- The mixture of the two CLA isomers is more effective than the individual isomers

ABSTRACT

The conjugated linoleic acid (CLA) isomers, a group of naturally occurring isomers of the essential fatty acid (FA) linoleic acid, have received special attention in animal and human nutrition. Although they have long been used as dietary integrators in dairy cows, the effects of CLA isomers on bovine immune cells remain yet mostly undisclosed. The present study aimed to cover this gap and investigate the in vitro effects of CLA on inflammatory functions, including chemotaxis, phagocytosis, killing capability and extracellular respiratory burst of purified bovine monocytes ($CD14^+$). The apoptosis rate of monocytes was addressed as well. Once assessed the effects of different concentrations (10, 50, 100 and 500 μM) of the two main CLA isomers, namely the *cis-9,trans-11* (c9,t11) and the *trans-10,cis-12* (t10,c12), the experiments were carried out using a concentration of 50 μM of the CLA isomers, both individually and in a mixture (50:50). The immunomodulatory activity of linoleic acid, an essential FA, and stearic acid, a saturated FA, was also investigated. Only the 50:50 CLA mixture was able to reduce monocytes apoptosis and to increase the extracellular respiratory burst during experimental pro-inflammatory conditions, as assessed by measuring reactive oxygen species (ROS) production. CLA and linoleic acid had no effects on chemotaxis, phagocytosis and killing. Remarkably, treatment of monocytes with stearic acid sensibly

reduced their chemotactic capability. In conclusion, the present results demonstrated that CLA isomers do have immunomodulatory effects on some bovine monocytes' functions, confirming that the mixture of the two CLA isomers is more effective than the CLA isomers individually.

Keywords: conjugated linoleic acid, monocyte, innate immunity, dairy cow

INTRODUCTION

Conjugated linoleic acid (**CLA**) is a group of naturally occurring positional and geometrical isomers of the essential omega-6 fatty acid (**FA)** linoleic acid, featuring conjugated double bonds in either cis or trans configuration (Bhattacharya et al., 2006). CLA is synthesized as an intermediate product during the biohydrogenation of linoleic acid by *Butyrivibrio fibrosolvens* rumen bacteria (Churruca et al., 2009) or through the endogenous conversion of trans-vaccenic acid by desaturase-9 in the mammary gland, as previously demonstrated (Griinari et al., 2000).

Among the 28 CLA isomers reported so far, the *cis-9,trans-11* (**c9,t11***)* and the *trans-10,cis-12* (**t10,c12**) are the most abundant and the ones with relevant biological activities (Pariza et al., 2001; Viladomiu et al., 2016). CLA is formed in ruminants, in particular the c9,t11 isomer that is the most predominant isomer in milk fat (80-90%). The concentration of CLA in blood has been recently measured as 0.54 mg/l, primarily c9,t11-18:2 (Lahlou et al., 2014) The c9,t11 isomer concentration in multiparous late-lactating Holstein-Friesian cows' milk fat is 0.71 g/100g of total FA, and 0.02g/100 g of blood fat, and it sensibly increases (up to 2.5 folds) after feeding with fresh pasture (Kay et al., 2005). In other studies, the mean concentration of c9,t11 isomer in bovine milk varied between 5.04 to 11.28 mg/g of fat in animals fed with different diets, showing that CLA milk content is markedly influenced by the composition of the animal's diet (Bauman et al., 1999; Fritsche et al., 1999). The two isomers differ in their biological effects, and further different activities were demonstrated when combining them in equal amounts (Pariza et al., 2001). Dietary supplementation of dairy cows with CLA is a frequent and relevant nutrition strategy, as it has been observed in in vivo studies a reduction in milk fat and glucose production, allowing a more efficient whole-body

energy utilization, and enhanced milk yield (Selberg et al., 2004; Dänicke et al., 2012; Galamb et al., 2017). Beside the effects on metabolism, supplementing CLA as well as other Polyunsaturated Fatty Acids (**PUFA**) in dairy ruminants has been identified as a potential strategy to mitigate the effects of the pro-inflammatory status associated to the oxidative stress related to metabolic and endocrine changes around calving in dairy cows (Sordillo, 2016). Although CLA isomers have been extensively used for improving milk quality and alleviating the magnitude of negative energy balance, the information about their impact on cow's immunity is limited. In vitro studies carried out on a model of mammary gland epithelial cells (**BME-UV1**) demonstrated that CLA can modulate inflammation and respiratory burst (Basiricò et al., 2015, 2017; Dipasquale et al., 2018). The effects of CLA on the activity of immune cells have been investigated on bovine peripheral blood mononuclear cells (**PBMC**) and resulted in contradictory results. Supplementing dams with a commercial CLA preparation (Lutrell Pure™, BASF-SE) in preceding lactation period exerted effects on the ex vivo stimulation ability of bovine PBMC (Dänicke et al., 2012). In an in vitro study assessing the effect of c9,t11 and t10,c12-CLA isomers, the inhibition of bovine isolated PBMC mitogen-activated proliferation was detected together with a marginal effect of c9,t11 isomer on cells' cytokine expression pattern (Renner et al., 2013). The apparent inconsistency of these results may be related to the fact that most of the studies were not carried out with comparable experimental designs and on an isolated population, but on PBMC, which included both lymphocytes and monocytes. Monocytes are myeloid cells derived from bone marrow. They play a pivotal role in immune defence against infections and injuries and are involved in almost all the phases of the immune reactions (Hussen and Schuberth, 2017). Given their pivotal role, the present study aimed to investigate the in vitro effect of c9,t11 and t10,c12-

CLA isomers, both individually and using a mixture (50:50) of the two isomers, on bovine sorted monocyte population. To examine if and how these CLA isomers could play a role in modulating monocytes' inflammatory activities, their impact on chemotaxis, respiratory burst, phagocytosis, killing capability, and apoptosis were evaluated. The effects of other unsaturated, such as linoleic acid, and saturated FA, such as stearic acid, were assessed as well.

MATERIALS AND METHODS

Materials

Ficoll-Paque PLUS (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden), EDTA 2mM, red blood cell lysis buffer, sterile-filtered Dulbecco's Phosphate Buffered Saline without calcium and magnesium (**DPBS**) (Sigma, St. Louis, USA) were used for bovine PBMC isolation. For monocytes purification, CD14 MicroBeads, LS Columns and Pre-Separation filters 30mm (Miltenyi-Biotech, Bergisch Gladbach, Germany) and Bovine Serum Albumin (**BSA**; Sigma, St.Louis, USA) were used. After isolation, cells were resuspended in complete medium, comprising RPMI 1640 Medium with 25 Mm Hepes and L-Glutamine supplemented with 1% of Non-essential Amino Acid Solution 100X and 1% Penicillin Streptomycin Solution 100X (Euroclone, Milano, Italy), and 10% Fetal Bovine Serum (**FBS**; Sigma, St. Louis, USA). Sterile 96 wells plate MICROTEST (Becton Dickinson and Company, Franklin Lake, USA), 384 well black plates, 24-well Transwell migration plates (Costar, Corning, USA) and cryogenic vials (Sigma, St. Louis, USA) were routinely used for cell culture. The FA 9(E),11(Z)-Octadecadienoic acid and 10(E),12(Z)-Octadecadienoic acid (c9,t11 and t10,c12-

CLA, respectively) (Matreya LLC, State College, USA); linoleic and stearic acid from (Sigma, St. Louis, USA) were used for cells' treatment.

Zymosan A from *Saccharomyces cerevisiae,* and Cytochrome C from equine heart, phorbol myristate acetate (**PMA**) (Sigma, St. Louis, USA), fluorescein-labelled *Escherichia coli* bioparticles K-12 strain (Invitrogen, Oregon, USA) and *Escherichia coli* American Type Culture Collection (ATCC) 25922 (strain Seattle 1946; LCG Standards) were used for chemotaxis, reactive oxygen species (**ROS**) production, phagocytosis and killing capability assays, respectively.

Purification of monocytes from blood

Peripheral blood from 33 pluriparous late lactating healthy Holstein-Friesian cows was collected during routine slaughtering procedures at a local slaughterhouse in sterile flasks containing 1.8mg K_2EDTA as anticoagulant per ml of blood. Monocytes ($CD14^+$ cells) were isolated through Ficoll 1.077 g/ml density gradient centrifugation, as previously described (Dilda et al., 2012), with few modifications. Briefly, blood was first centrifuged at 1260g, for 30 min at 4°C and the buffy coat (PBMC ring) was collected. PBMC were then diluted 1:5 in sterile cold PBS without Ca^{2+} and Mg^{2+}, containing 2mM EDTA, layered on Ficoll and centrifuged without breaks at 1700g for 30 min at 4°C. PBMC were recovered at the interface, washed with PBS without Ca^{2+} and Mg^{2+} and treated with Red Blood Cell Lysis Buffer for red blood cells elimination. Two subsequent centrifugations (500g for 7 min with cold PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA) were carried out to remove platelets. The $CD14^+$ monocytes purification was carried out using magnetic-activated cell sorting technique (**MACS**). Isolated PBMC were incubated with anti-human CD14 microbeads for 15 min at

144 4°C and $CD14^+$ cells were isolated from an MD column (LS) according to the manufacturer's
145 instructions. The homogeneity of the sorted cells (> 98%) was determined using an automatic
146 cell counter (Sysmex). The working concentration of monocytes was then adjusted with
147 complete medium.
148
149 ***Unsaturated and Saturated FA preparation***
150 Stock solutions of unsaturated and saturated FA were prepared. The two CLA isomers
151 (c9,t11and t10,c12-CLA), linoleic acid and stearic acid were reconstituted in ethanol at a
152 concentration of 357 mM for CLA isomers and linoleic acid and of 70.3 mM for stearic acid.
153 Stock solutions were stored at -20 °C and fresh dilutions with the complete medium were
154 prepared when needed. The amounts of CLA isomers used for preliminary studies ranged from
155 0 to 500 μM. Additionally, the mixture of both isomers in a 50:50 proportion, linoleic and
156 stearic acid at 50 μM were also prepared. During the first part of the study, the working
157 concentration of CLA isomers was determined by testing the different concentration of
158 individual c9,t11 and t10,c12-CLA isomers on monocytes' apoptosis. Once determined the
159 CLA working solution, this was tested on monocyte immune-related functions, including
160 chemotaxis, phagocytosis, respiratory burst, killing and apoptosis.
161
162 ***Apoptosis assay***
163 Apoptosis assay was performed in triplicate on 50×10^3 sorted monocytes seeded in 384 well
164 black plates. The experiment was carried out on cells purified from 8 animals. Firstly, to
165 determine CLA working concentration the cells were incubated overnight at 39 °C in the
166 humified atmosphere 5% CO_2 with increasing concentrations of c9,t11 and t10,c12-CLA

isomers (10, 50, 100 and 500 μM) or with 0.1% ethanol as control (vehicle), being the concentration of ethanol found in 500 μM of CLA isomers. At this concentration of ethanol, no effects on viability on bovine PBMC were observed, as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetra- zolium bromide (**MTT**)-based assay (data not shown). Once determined the CLA working concentration (50 μM), the effects of other unsaturated and saturated FA on monocytes' apoptosis were measured by incubating the cells with 50 μM of each CLA isomer, the 50:50 mixture of the two isomers, linoleic acid, stearic acid and ethanol (vehicle) as the control, with the same concentration of ethanol found in the 50 μM FA solutions (0.014%). The apoptosis rate was measured after overnight incubation by using the Apo-ONE® Homogeneous Caspase-3/7 kit (Promega, Madison, WI, USA). The caspase-3/7 reagent was added to each well and the fluorescence intensity was measured using a fluorescence plate reader Fluoroscan Ascent at 485/538 nm (absorbance/emission), every 30 minutes up to 4 hours, as previously described in bovine monocytes (Ceciliani et al., 2007).

Chemotaxis assay

Monocytes chemotaxis towards zymosan activated serum (**ZAS**) was measured as previously reported (Lecchi et al., 2008; McClelland et al., 2010) with some minor modifications. The experiment was carried out on cells purified from 8 animals. Monocytes were first pretreated overnight, in absence of chemoattractant, with 50 μM of each CLA isomer, the 50:50 mixture of the two isomers, linoleic acid and stearic acid or 0.014% ethanol as the control in 24-well Transwell migration plates, equipped with a 5 μm pore size membrane. A total of $1x10^5$ monocytes (100 μl final volume) were added in triplicates in the upper chamber, while FA and migration medium (RMPI-1640 with 1% of FBS) were added in both chambers at a final

volume of 750µl. Cells were incubated overnight at 39°C in humified atmosphere 5% CO_2. After pretreating the cells, the chemotaxis was measured by adding 3mg/ml of the chemoattractant ZAS to the lower chamber, in the presence of newly added FA (50 µM) or ethanol (vehicle) as control, and again incubated for 2 h at 39°C in humified atmosphere 5% CO_2. For the negative control, cells were incubated with the vehicle without ZAS. Finally, upper chambers were removed, non-migrated cells on the upper part of the membrane were gently eliminated using a swab moistured with PBS, and migrated cells stained with Diff-Quick (Sigma, St Louis, USA) and counted in ten different fields, using light microscopy.

Determination of respiratory burst by measuring ROS production under normal and pro-inflammatory conditions

The production of extracellular superoxide anion (O_2^-) was determined by the cytochrome C reduction method as previously described (Lecchi et al., 2016). The experiment was carried out on cells purified from 8 animals. A total of $1x10^5$ monocytes (50µl) were seeded in complete RPMI-1640 without phenol red in duplicates in 96-well sterile plates. Cells were then incubated overnight at 39°C in a humidified atmosphere of 5% CO_2, with 50 µM (50 µl) of each CLA isomers individually, the 50:50 mixture, linoleic acid and stearic acid or 0.014% ethanol (vehicle) as control at a final volume of 100 µl. At the end of the incubation time, 10 µl of cytochrome C and 90 µl of HBSS or only 100 µl of HBSS as negative control were added to each well to make up a final volume of 200 µl. Finally, to mimic possible pro-inflammatory conditions the second set of experiments was performed by adding PMA (2.5 µg/ml final concentration). The absorbance was measured every 30 min for 4 h at 550 nm with LabSystems Multiskan plate reader Spectrophotometer.

213

214 ***Phagocytosis assay***

215 Monocytes' phagocytic activity was determined as previously described (Lecchi et al., 2011).
216 The experiment was carried out on cells purified from 9 animals. First, $6x10^8$ fluorescein-
217 labelled *Escherichia coli* bioparticles (K-12 strain) were opsonized, by incubating them with
218 20% of autologous serum for 30 min at 37°C. The suspension was then centrifuged at 800 g
219 for 15 min and suspended in HBSS. Opsonized bacteria were stored at -20°C upon use. A 100
220 µl suspension containing $3x10^5$ monocytes was seeded in duplicates in 96-well sterile plates
221 with 50 µM (50 µl) of each CLA isomers individually, the 50:50 mixture, linoleic acid and
222 stearic acid or 0.014% ethanol (vehicle) as the control. The medium was then added to reach
223 a final volume of 200 µl. Afterwards, the cells were incubated overnight at 39°C in a
224 humidified atmosphere of 5% CO_2. Cells were then washed with sterile HBSS and 45
225 opsonized fluorescein-labelled *E. coli* bioparticles (100 µl) per cell were added. Monocytes
226 were then incubated again for 2 h at 39°C in a humidified atmosphere of 5% CO_2. Cells were
227 washed twice with HBSS and 0.4% of trypan blue was added to quench the fluorescence from
228 non-internalized bacteria. Finally, cells were again washed with HBSS and the fluorescence
229 intensity of fluorescein-labelled *E. coli* bioparticles was measured using a Fluoroscan Ascent
230 FL (Thermo Scientific) at 485/538 nm (absorbance/emission).

231

232 ***Killing Capability assay***

233 The intracellular bacteria-killing capability of monocytes was evaluated according to (Lecchi
234 et al., 2013). The experiment was carried out on cells purified from 7 animals. Briefly, *E. coli*
235 American Type Culture Collection (ATCC) 25922 (LCG Standards) was opsonized with 20%

bovine serum at 37 °C for 30 min. The bacteria were washed twice by centrifuging at 1500 g for 10 min and suspended in sterile HBSS. A total of $3x10^5$ bovine monocytes (100 µl) were suspended in complete medium-containing cryogenic vials (300 µl final volume) (Sigma, St. Louis, USA) and treated overnight with 50 µM (50 µl) of each CLA isomers individually, the 50:50 mixture of them, linoleic acid and stearic acid or 0.014% ethanol as vehicle at 39°C in a humidified atmosphere of 5% CO2. Cells were then washed and incubated with $1x10^7$ of opsonized live *E. coli* and incubated for 1 h at 39°C in a humidified atmosphere of 5% CO_2. Monocytes were centrifuged at 110g for 5 min to remove unbound bacteria and treated further for 1 h with 100 µg/ml of Gentamicin to kill any remaining extracellular bacteria. Gentamicin was eliminated by washing with HBSS and centrifuging the cells at 110g for 5 min. Finally, cells were lysed using 0.5% Triton X-100 (Sigma, St. Louis, USA) for 10 min and the surviving *E. coli* were counted on MacConkey agar plates. Results are expressed in colony-forming units (**CFU**).

Statistical analysis

Statistical analyses were performed in GraphPad Prism 8.0.2, San Diego, California USA. For the data normality assessment, the Shapiro Wilk test was applied. Repeated measures one-way ANOVA and Tukey's multiple comparison test were used to evaluate ROS production, under normal and pro-inflammatory conditions, in normally distributed data. Repeated measures Friedman test and Dunn's multiple comparison test were used for apoptosis, chemotaxis, phagocytosis and killing capability, in not normal distributed samples. Statistical differences were accepted at $P \leq 0.05$.

RESULTS

Effect of c9,t11and t10,c12-CLA isomers on bovine monocyte apoptosis

To determine c9,t11and t10,c12-CLA isomers optimal concentration, a preliminary study on monocytes was carried out by incubating overnight the cells with increasing concentrations of CLA isomers (10, 50, 100 and 500 μM) or with the vehicle (ethanol), and their apoptosis rate was assessed (Supplemental Figure S1; https://doi.org/10.3168/jds.20XX-XXXXX). No effects were observed with any of the CLA isomers at increasing concentrations on the sorted population. The following experiments were then carried out using a concentration of 50 μM. In the second part of the study, the differential effects of additional unsaturated (linoleic acid and the mixture 50:50 of both CLA isomers) and saturated FA (stearic acid) on monocytes apoptosis were compared. After exposing the cells with the FA overnight, apoptosis (Figure 1) was found to be reduced (P = 0.013) only by the 50:50 mixture of CLA isomers when compared with the vehicle (ethanol).

Effect of unsaturated and saturated FA on bovine monocyte chemotaxis

In this part of the study, the capability of the unsaturated and saturated FA of modulating monocyte chemotactic activity was measured by using transwell migration plates. The chemotactic activity of monocytes was activated using ZAS, after exposing purified cells to 50 μM of the FA or the vehicle (ethanol) overnight. The results are presented in Figure 2. Co-incubation with CLA and LA did not modulate monocyte chemotaxis. On the contrary, stearic acid induced an evident decrease in the chemotactic ability of monocytes in a statistically

significant way ($P = 0.032$) when compared with the control (vehicle), the 50:50 mixture of CLA ($P = 0.049$) and the t10,c12-CLA ($P = 0.013$); and ($P < 0.001$) with c9,t1-CLA.

Effect of unsaturated and saturated FA on bovine monocyte ROS production

Monocytes' production of extracellular superoxide anion at both normal and under inflammatory conditions was evaluated through the Cytochrome C reduction method. Cells were treated overnight with the FA and then the ROS production was measured every 30 min for 4 h (Supplemental Figure S2; https://doi.org/10.3168/jds.20XX-XXXXX.). As the highest ROS levels were observed in both experimental conditions at 60 min after the addition of Cytochrome C, the effects of the different FA on the cells' superoxide anion production were only further evaluated and presented at this time point (Figure 3). Cells under normal conditions did not show any difference in ROS production (Figure 3A) as compared to control. On the contrary, an increase with the 50:50 mixture of CLA isomers was detected ($P = 0.002$), after inducing a pro-inflammatory challenge with PMA (Figure 3B), when compared to the control and ($P = 0.003$) with c9,t11-CLA and linoleic acid.

Effect of unsaturated and saturated FA on bovine monocyte phagocytosis and killing capability of E. coli

The last set of experiments was aimed to study whether the co-incubation of isolated bovine monocytes with unsaturated and saturated FA affects their phagocytic and killing capability, as determined by fluorescein-labelled *E. coli* bioparticles internalization assay and intracellular *E.coli* killing assay, respectively. The capacity of monocytes to phagocyte

303 (Figure 4A) and kill live *E. coli* (Figure 4B) when treated overnight with saturated and
304 unsaturated FA was not affected when compared to the vehicle.
305
306 **DISCUSSION**
307
308 In this study, we reported the effects of c9,t11 and t10,c12-CLA isomers, separately and as
309 50:50 mixture, as well of linoleic acid, an essential FA, and stearic acid, a saturated FA, on
310 several immunoregulatory functions of bovine monocyte ($CD14^+$), including apoptosis,
311 chemotaxis, phagocytosis, respiratory burst and killing capability. Our main finding was that
312 the 50:50 mixture of the c9,t11 and t10,c12-CLA isomers, when used at a concentration of 50
313 μM, reduced the apoptosis rate of monocytes. Co-incubating cells with the 50:50 mixture of
314 the two isomers also increased the respiratory burst, as determined by an increase of the
315 production of ROS, but only in an experimental pro-inflammatory environment. On the
316 contrary, CLA does not affect any of the other monocyte immunoregulatory functions herein
317 assessed. Remarkably, we found that stearic acid was capable of a statistically significant
318 reduction of chemotaxis.
319 Conjugated linoleic acid isomers have been routinely used as a feed supplement for dairy cows
320 due to their beneficial in vivo effects. A decrease in milk fat synthesis and its consequent
321 improvement in energy balance, increase in milk production, improved reproductive
322 performance and reduction of metabolic-related diseases were reported (Perfield et al., 2007;
323 de Veth et al., 2009; Basiricò et al., 2017; Csillik et al., 2017). However, their effects on bovine
324 immunity have been scarcely addressed.

Monocytes provide a suitable in vitro model to assess the impact CLA exerts on bovine immunity. Monocytes are circulating blood leucocytes playing a major role in the host immune defence against invading pathogens (Chávez-galán et al., 2015). They fulfil their defensive roles by migrating into inflamed tissues, producing pro-inflammatory cytokines and ROS, and eventually phagocytosing and killing engulfed pathogens.The adequate display of their functions is critical for an effective immune response.

The rate of apoptosis is regarded as a way to control the activity of blood monocytes, by either increasing or reducing their presence and activity in the inflammatory environment. Therefore, apoptosis is regarded as an integral feature of the immune system (Feig and Peter, 2007). Under this premise, this study tested whether CLA isomers affect apoptosis in sorted monocytes, demonstrating that 50:50 mixture of the two CLA isomers can reduce apoptosis. The impact of CLA on apoptosis has been widely studied on cancer cell models in humans, and the results converge toward a pro-apoptotic and antiproliferative effect (Ochoa et al., 2004; Wang et al., 2008). Studies carried out on bovine cellular models resulted in apparently contradictory results. In bovine mammary cells (MAC-T cell line), co-incubating with 35μM concentrations of both CLA isomers promoted an increase of apoptosis rate (Keating et al., 2008), while in another study the c9,t11-CLA isomer (60 μM) was able to reduce the caspase-3 activity, thus decreasing apoptosis rate, in bovine aortic endothelial cells (Lai et al., 2005), suggesting the hypothesis that the effects of CLA are cell-specific.

For all the following experiments, the working concentration of CLA was set at 50 μ*M*. This concentration of CLA was selected following preliminary studies, that were carried out by co-incubating the monocytes with different concentrations of CLA isomers, demonstrating that there were no differences in modulating apoptosis rate using concentrations ranging from 10

μM to 500 μM. The use of a concentration of 50 μM was set to compare the present results with others from previous studies, that used the same CLA concentration (Basiricò et al., 2015, 2017; Dipasquale et al., 2018). Indeed, the concentration of 50 μM is also close to physiological levels found in human sera (10-70 μM) (Basiricò et al., 2015). Moreover, positive effects when using (50 μM) of CLA such as an improved redox status of bovine mammary cells (Basiricò et al., 2015) or atheroprotective properties in human monocytes have been reported (McClelland et al., 2010).

To further investigate that the effects reported were specific for CLA isomers and not related to an unspecific effect in response to treatment with FA, we compared the differential effects of linoleic acid as omega-6 PUFA control and stearic acid as saturated acid, which is also the main FA found in some commercially available CLA supplements, on bovine monocytes' apoptosis. We also incorporated a 50:50 mixture of both CLA isomers, as most of the animal studies reporting CLA benefits and commercially available CLA supplements use a mixture of these two isomers in roughly equal amounts (Song et al., 2005; Renner et al., 2012). The CLA mixture was the only treatment that caused a significant reduction in monocytes apoptosis when compared with the vehicle (ethanol), an effect not observed with the individual CLA isomers.

In the following set of experiments, the capability of CLA isomers to modulate chemotaxis was studied. We did not observe any difference in monocytes' migration toward ZAS when treated with CLA isomers and its mixture. These results differ from those previously reported in human monocytes, that demonstrated that CLA could modulate monocytes/macrophages' chemotaxis by PPAR*y* activation and COX-2 inhibition, suggesting atheroprotective properties (McClelland et al., 2010). Intriguingly, a statistically significant reduction of

chemotaxis was found when monocytes were co-incubated with stearic acid. These results are in contrast with what previously reported in human monocytes, where stearic acid was found to have a pro-inflammatory activity (Anderson et al., 2012).

In the final set of experiments, further defensive performance of monocytes in the inflammatory focus such as phagocytosis, killing and respiratory burst were measured. Treating isolated monocytes with CLA has no impact on the phagocytic and killing capability of bovine monocytes. These findings are different as compared to the reported effects of CLA on other species, including dogs and pigs, and other cellular targets like polymorphonuclear cells (**PMN**), where the t10,c12-CLA isomer increased the phagocytosis process, either indirectly (Kang et al., 2007), or directly (Kang et al., 2009). No differences in the production of extracellular superoxide anion under normal conditions were also found. On the contrary, treating the cells with PMA, to mimic inflammatory conditions, upregulated ROS production in monocytes treated with the CLA mixture. These results are in agreement with those reported in human macrophages, where both c9,t11 and t10,c12-CLA isomers upregulated ROS synthesis, through a PPARγ dependent mechanism (Stachowska et al., 2008). Remarkably, a similar effect of increasing ROS production was recently demonstrated in BME-UV1 cells (Dipasquale et al., 2018). Moreover, t10,c12-CLA isomer has already shown to increase oxidative stress in human in vivo studies (Risérus et al., 2002) and canine PMN (Kang and Yang, 2008; Kang et al., 2009).

CONCLUSIONS

CLA has shown to present a wide range of beneficial properties for cows' health, production and welfare. However, its effects on immune cells' responses and functionality have been

scarcely addressed so far. This study demonstrates that CLA exerts an anti-apoptotic activity, and can increase ROS production in an inflammatory in vitro model, suggesting that CLA may have relevant roles in modulating some in vitro monocyte immune functions. The present study provides the evidence that the effects of each CLA isomer are different and a combination of the c9,t11with t10,c12-CLA isomers induce synergic effects on at least two important monocyte immune functions, namely apoptosis and inflammatory induced respiratory burst. Besides its isomer-dependent activity, CLA effects are also strictly related to their cellular targets, as the effects observed on mammary gland and endothelial cell lines are different compared to those on immune-related cells such as monocytes. Several aspects of the potential immunomodulatory effects of omega-6 FA are still elusive, particularly the molecular basis of the different mechanisms of action of CLA. Elucidation of these mechanisms would improve our understanding of the actions of CLA in experimental in vivo systems and determine its practical biological significance, supporting a more targeted utilisation of CLA in dairy animal nutrition. Given the wide use of stearic acid in dairy animal nutrition, its immunomodulatory effect on reducing chemotaxis deserves to be further explored.

CONFLICT OF INTEREST STATEMENT

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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REFERENCES

Anderson, E.K., A.A. Hill, and A.H. Hasty. 2012. Stearic acid accumulation in macrophages induces toll-like receptor 4/2-independent inflammation leading to endoplasmic reticulum stress-mediated apoptosis.. Arteriosclerosis, thrombosis, and vascular biology 32:1687–95. doi:10.1161/ATVBAHA.112.250142.

Basiricò, L., P. Morera, D. Dipasquale, A. Tröscher, and U. Bernabucci. 2017. Comparison between conjugated linoleic acid and essential fatty acids in preventing oxidative stress in bovine mammary epithelial cells. Journal of Dairy Science 100:2299–2309. doi:10.3168/jds.2016-11729.

Basiricò, L., P. Morera, D. Dipasquale, A. Tröscher, A. Serra, M. Mele, and U. Bernabucci. 2015. Conjugated linoleic acid isomers strongly improve the redox status of bovine

mammary epithelial cells (BME-UV1). Journal of dairy science 98:7071–7082.

Bauman, D.E., L.H. Baumgard, B.A. Corl, and J.M. Griinari. 1999. Biosynthesis of conjugated linoleic acid in ruminants. Pages 1–14 in Proc. Am. Soc. Anim. Sci.

Bhattacharya, A., J. Banu, M. Rahman, J. Causey, and G. Fernandes. 2006. Biological effects of conjugated linoleic acids in health and disease. Journal of Nutritional Biochemistry 17:789–810. doi:10.1016/j.jnutbio.2006.02.009.

Ceciliani, F., V. Pocacqua, A. Miranda-Ribera, V. Bronzo, C. Lecchi, and P. Sartorelli. 2007. alpha(1)-Acid glycoprotein modulates apoptosis in bovine monocytes.. Veterinary Immunology and Immunopathology 116:145–152. doi:10.1016/j.vetimm.2007.01.006.

Chávez-galán, L., M.L. Olleros, D. Vesin, I. Garcia, and R.A. Harris. 2015. Much more than M1 and M2 macrophages , there are also CD169 + and TCR + macrophages 6:1–15. doi:10.3389/fimmu.2015.00263.

Churruca, I., A. Fernández-Quintela, and M.P. Portillo. 2009. Conjugated linoleic acid isomers: Differences in metabolism and biological effects. BioFactors 35:105–111. doi:10.1002/biof.13.

Csillik, Z., V. Faigl, M. Keresztes, E. Galamb, H.M. Hammon, A. Tröscher, H. Fébel, M. Kulcsár, F. Husvéth, G. Huszenicza, and W.R. Butler. 2017. Effect of pre- and postpartum supplementation with lipid-encapsulated conjugated linoleic acid on reproductive performance and the growth hormone–insulin-like growth factor-I axis in multiparous high-producing dairy cows. Journal of Dairy Science 100:5888–5898. doi:https://doi.org/10.3168/jds.2016-12124.

Dänicke, S., J. Kowalczyk, L. Renner, J. Pappritz, U. Meyer, R. Kramer, E.M. Weber, S. Döll, J. Rehage, and G. Jahreis. 2012. Effects of conjugated linoleic acids fed to dairy cows during

early gestation on hematological, immunological, and metabolic characteristics of cows and their calves. Journal of Dairy Science 95:3938–3953. doi:10.3168/jds.2011-4879.

Dilda, F., G. Gioia, L. Pisani, L. Restelli, C. Lecchi, F. Albonico, V. Bronzo, M. Mortarino, and F. Ceciliani. 2012. Escherichia coli lipopolysaccharides and Staphylococcus aureus enterotoxin B differentially modulate inflammatory microRNAs in bovine monocytes.. Veterinary journal (London, England : 1997) 192:514–6. doi:10.1016/j.tvjl.2011.08.018.

Dipasquale, D., L. Basiricò, P. Morera, R. Primi, A. Tröscher, and U. Bernabucci. 2018. Anti-inflammatory effects of conjugated linoleic acid isomers and essential fatty acids in bovine mammary epithelial cells.. Animal : an international journal of animal bioscience 12:2108–2114. doi:10.1017/S1751731117003676.

Feig, C., and M.E. Peter. 2007. How apoptosis got the immune system in shape.. European journal of immunology 37 Suppl 1:S61-70. doi:10.1002/eji.200737462.

Fritsche, J., R. Rickert, H. Steinhart, M.P. Yurawecz, M.M. Mossoba, N. Sehat, J.A.G. Roach, J.K.G. Kramer, and Y. Ku. 1999. Conjugated linoleic acid (CLA) isomers: formation, analysis, amounts in foods, and dietary intake. Lipid / Fett 101:272–276. doi:10.1002/(SICI)1521-4133(199908)101:8<272::AID-LIPI272>3.0.CO;2-W.

Galamb, E., V. Faigl, M. Keresztes, Z. Csillik, A. Tröscher, P. Elek, M. Kulcsár, G. Huszenicza, H. Fébel, and F. Husvéth. 2017. Effect of pre- and post-partum supplementation with lipid-encapsulated conjugated linoleic acid on milk yield and metabolic status in multiparous high-producing dairy cows. Journal of Animal Physiology and Animal Nutrition 101:1026–1035. doi:10.1111/jpn.12544.

Hussen, J., and H.-J. Schuberth. 2017. Heterogeneity of Bovine Peripheral Blood Monocytes.. Frontiers in immunology 8:1875. doi:10.3389/fimmu.2017.01875.

485 Kang, J.-H., and M.-P. Yang. 2008. In vitro evaluation of the effect of trans-10, cis-12
486 conjugated linoleic acid on phagocytosis by canine peripheral blood polymorphonuclear
487 neutrophilic leukocytes exposed to methylprednisolone sodium succinate.. American
488 journal of veterinary research 69:494–500. doi:10.2460/ajvr.69.4.494.
489 Kang, J.H., G.S. Lee, E.B. Jeung, and M.P. Yang. 2007. Trans-10, cis-12-conjugated linoleic
490 acid increases phagocytosis of porcine peripheral blood polymorphonuclear cells in vitro.
491 British Journal of Nutrition 97:117–125. doi:10.1017/S0007114507280584.
492 Kang, J.H., G.S. Lee, E.B. Jeung, and M.P. Yang. 2009. Trans-10, cis-12 conjugated linoleic
493 acid modulates phagocytic responses of canine peripheral blood polymorphonuclear
494 neutrophilic leukocytes exposed to Clostridium difficile toxin B. Veterinary Immunology
495 and Immunopathology 130:178–186. doi:10.1016/j.vetimm.2009.02.005.
496 Kay, J.K., J.R. Roche, E.S. Kolver, N.A. Thomson, and L.H. Baumgard. 2005. A comparison
497 between feeding systems (pasture and TMR) and the effect of vitamin E supplementation on
498 plasma and milk fatty acid profiles in dairy cows.. The Journal of dairy research 72:322–32.
499 doi:10.1017/S0022029905000944.
500 Keating, A.F., F.Q. Zhao, K.A. Finucane, D.R. Glimm, and J.J. Kennelly. 2008. Effect of
501 conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A
502 desaturase gene expression. Domestic Animal Endocrinology 34:284–292.
503 doi:10.1016/j.domaniend.2007.08.005.
504 Lahlou, M.N., R. Kanneganti, L.J. Massingill, G.A. Broderick, Y. Park, M.W. Pariza, J.D.
505 Ferguson, and Z. Wu. 2014. Grazing increases the concentration of CLA in dairy cow milk
506 a. Animal 8:1191–1200. doi:10.1017/S1751731114000998.
507 Lai, K.L., A.P. Torres-Duarte, and J.Y. Vanderhoek. 2005. 9-trans, 11-trans-CLA:

508 Antiproliferative and proapoptotic effects on bovine endothelial cells. Lipids 40:1107–1116.
509 doi:10.1007/s11745-005-1474-1.
510 Lecchi, C., F. Ceciliani, S. Bernasconi, F. Franciosi, V. Bronzo, and P. Sartorelli. 2008. Bovine
511 alpha-1 acid glycoprotein can reduce the chemotaxis of bovine monocytes and modulate
512 CD18 expression. Veterinary Research 39:50. doi:10.1051/vetres:2008027.
513 Lecchi, C., G. Invernizzi, A. Agazzi, M. Ferroni, L.F. Pisani, G. Savoini, and F. Ceciliani. 2011.
514 In vitro modulation of caprine monocyte immune functions by ω-3 polyunsaturated fatty
515 acids.. Veterinary journal (London, England : 1997) 189:353–5.
516 doi:10.1016/j.tvjl.2010.09.001.
517 Lecchi, C., N. Rota, A. Vitali, F. Ceciliani, and N. Lacetera. 2016. In vitro assessment of the
518 effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in
519 bovine polymorphonuclear cells. Veterinary Immunology and Immunopathology 182:89–
520 94. doi:10.1016/j.vetimm.2016.10.007.
521 Lecchi, C., A. Scarafoni, V. Bronzo, P.A. Martino, A. Cavallini, P. Sartorelli, and F. Ceciliani.
522 2013. Alpha1-Acid glycoprotein modulates phagocytosis and killing of Escherichia coli by
523 bovine polymorphonuclear leucocytes and monocytes. Veterinary Journal 196:47–51.
524 doi:10.1016/j.tvjl.2012.07.022.
525 McClelland, S., C. Cox, R. O'Connor, M. de Gaetano, C. McCarthy, L. Cryan, D. Fitzgerald, and
526 O. Belton. 2010. Conjugated linoleic acid suppresses the migratory and inflammatory
527 phenotype of the monocyte/macrophage cell. Atherosclerosis 211:96–102.
528 doi:10.1016/j.atherosclerosis.2010.02.003.
529 Ochoa, J.J., A.J. Farquharson, I. Grant, L.E. Moffat, S.D. Heys, and K.W.J. Wahle. 2004.
530 Conjugated linoleic acids (CLAs) decrease prostate cancer cell proliferation: Different

molecular mechanisms for cis-9, trans-11, and trans-10 cis-12 isomers. Carcinogenesis 25:1185–1191. doi:10.1093/carcin/bgh116.

Pariza, M.W., Y. Park, and M.E. Cook. 2001. The biologically active isomers of conjugated linoleic acid. Progress in Lipid Research 40:283–298. doi:10.1016/S0163-7827(01)00008-X.

Perfield, J.W., A.L. Lock, J.M. Griinari, A. Sæbø, P. Delmonte, D.A. Dwyer, and D.E. Bauman. 2007. Trans-9, cis-11 conjugated linoleic acid reduces milk fat synthesis in lactating dairy cows. Journal of Dairy Science 90:2211–2218. doi:10.3168/jds.2006-745.

Renner, L., S. Kersten, A. Duevel, H.J. Schuberth, and S. Dänicke. 2013. Effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid, linoleic acid, phytanic acid and the combination of various fatty acids on proliferation and cytokine expression of bovine peripheral blood mononuclear cells. Nutrients 5:2667–2683. doi:10.3390/nu5072667.

Renner, L., J. Pappritz, R. Kramer, S. Kersten, G. Jahreis, and S. Dänicke. 2012. Fatty acid profile and proliferation of bovine blood mononuclear cells after conjugated linoleic acid supplementation. Lipids in Health and Disease 11:1–7. doi:10.1186/1476-511X-11-63.

Risérus, U., S. Basu, S. Jovinge, G.N. Fredrikson, J. Ärnlöv, and B. Vessby. 2002. Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: A potential link to fatty acid-induced insulin resistance. Circulation 106:1925–1929. doi:10.1161/01.CIR.0000033589.15413.48.

Selberg, K.T., A.C. Lowe, C.R. Staples, N.D. Luchini, and L. Badinga. 2004. Production and metabolic responses of periparturient Holstein cows to dietary conjugated linoleic acid and trans-octadecenoic acids.. Journal of dairy science 87:158–68. doi:10.3168/jds.S0022-0302(04)73153-7.

554 Song, H.-J., I. Grant, D. Rotondo, I. Mohede, N. Sattar, S.D. Heys, and K.W.J. Wahle. 2005.
555 Effect of CLA supplementation on immune function in young healthy volunteers. European
556 Journal of Clinical Nutrition 59:508–517. doi:10.1038/sj.ejcn.1602102.
557 Sordillo, L.M. 2016. Nutritional strategies to optimize dairy cattle immunity.. Journal of dairy
558 science 99:4967–4982. doi:10.3168/jds.2015-10354.
559 Stachowska, E., M. Baśkiewicz-Masiuk, V. Dziedziejko, I. Gutowska, I. Baranowska-Bosiacka,
560 M. Marchlewicz, B. Dołęgowska, B. Wiszniewska, B. Machaliński, and D. Chlubek. 2008.
561 Conjugated linoleic acid increases intracellular ROS synthesis and oxygenation of
562 arachidonic acid in macrophages. Nutrition 24:187–199. doi:10.1016/j.nut.2007.10.018.
563 de Veth, M.J., D.E. Bauman, W. Koch, G.E. Mann, A.M. Pfeiffer, and W.R. Butler. 2009.
564 Efficacy of conjugated linoleic acid for improving reproduction: A multi-study analysis in
565 early-lactation dairy cows. Journal of Dairy Science 92:2662–2669. doi:10.3168/jds.2008-
566 1845.
567 Viladomiu, M., R. Hontecillas, and J. Bassaganya-Riera. 2016. Modulation of inflammation and
568 immunity by dietary conjugated linoleic acid. European Journal of Pharmacology 785:87–
569 95. doi:10.1016/j.ejphar.2015.03.095.
570 Wang, L.S., Y.W. Huang, S. Liu, P. Yan, and Y.C. Lin. 2008. Conjugated linoleic acid induces
571 apoptosis through estrogen receptor alpha in human breast tissue. BMC Cancer 8:1–14.
572 doi:10.1186/1471-2407-8-208.
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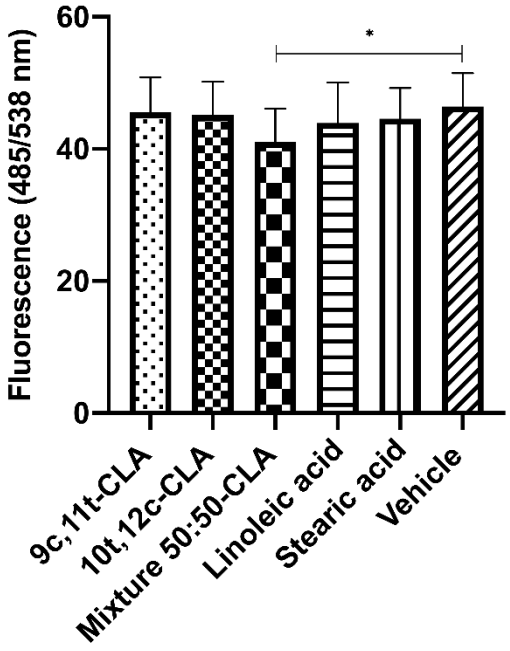
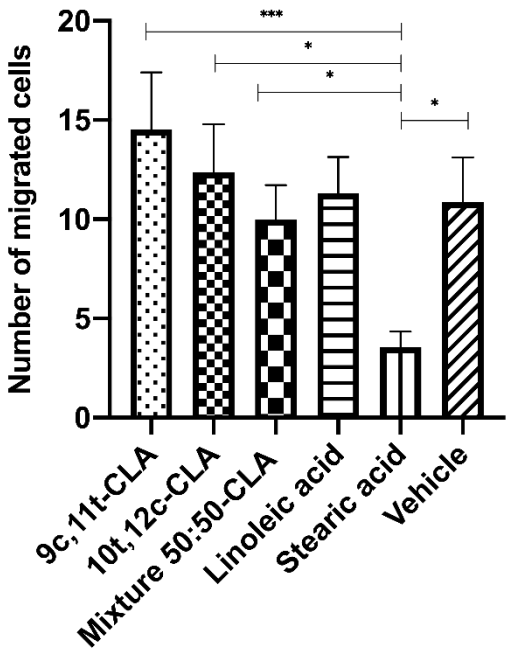
FIGURES



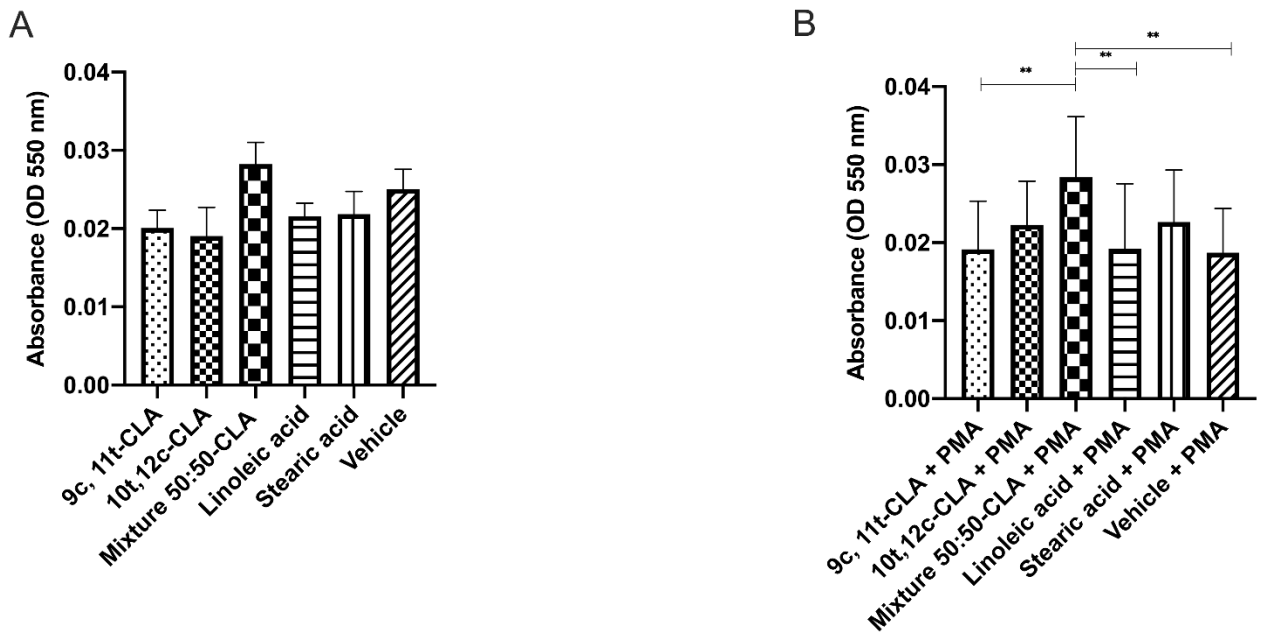
Ávila_Figure 1.

Figure 1. In vitro effect of saturated and unsaturated FA on bovine monocytes apoptosis. Caspase-3/7 enzymatic activity of bovine monocytes after overnight incubation with FA or vehicle (0.014% ethanol). Data are means ± SEM of eight independent experiments. Significance was declared for $P < 0.05$ (*).



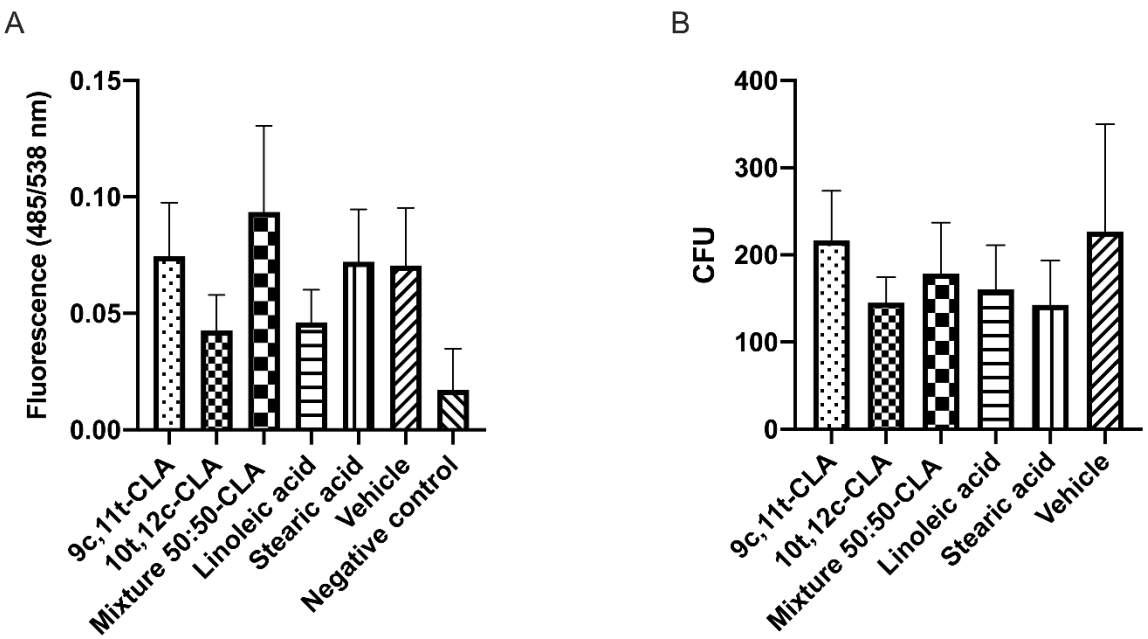
Ávila_Figure 2.

Figure 2. Differential effects of saturated and unsaturated FA on bovine monocytes chemotaxis. Cells were treated with 0.014% of ethanol as vehicle control. Data are means ± SEM of eight independent experiments. Significance was declared for $P < 0.05$ (*) and $P < 0.001$ (***).



Ávila_Figure 3.

Figure 3. In vitro effect of saturated and unsaturated FA on bovine monocytes extracellular superoxide anion generation, at 60 min after the addition of Cytochrome C, under (A) normal conditions or (B) pro-inflammatory conditions (phorbol myristate acetate (PMA) stimulation). Cells treated with 0.014% of ethanol (vehicle) were considered as control. Data are means ± SEM of eight independent experiments. Significance was declared for $P < 0.01$ (**).



Ávila_Figure 4.

Figure 4. (A) Phagocytosis of fluorescein-labelled *Escherichia coli* bioparticles and (B) killing capability of live *Escherichia coli* by bovine monocytes after overnight exposure with FA. The results are expressed as fluorescence intensity (OD 485-538 nm) and as colony forming units (CFU), respectively. Cells treated with 0.014% of ethanol (vehicle) were considered as control. Data are means ± SEM of nine and seven independent experiments, respectively.