



Research article

Effect of nanocurcumin and fish oil as natural anti-inflammatory compounds vs. glucocorticoids in a lipopolysaccharide inflammation model on Holstein calves' health status

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ABSTRACT

Curcumin (CUR) and fish oil (FO) are among the most well-known types of natural anti-inflammatory compounds. The aim of this study was to assess the effect of nanocurcumin and fish oil vs. glucocorticoids on Holstein calves' health status. A lipopolysaccharide (LPS) challenge ($0.5 \mu\text{g kg}^{-1}$ BW) was used to induce an acute phase response. A total of 42 male Holstein calves were randomized into 7 groups: negative control (CON), positive control (LPS, injected once), 250 mg/kg BW per day fish oil + LPS (FO250), 350 mg/kg BW per day fish oil + LPS (FO350), 2 mg/kg BW per day nanocurcumin + LPS (NCUR2), 4 mg/kg BW per day nanocurcumin + LPS (NCUR4), and 0.3 mg/kg BW dexamethasone (injected once) + LPS (DEX). The duration of this experiment was 11 days, with application of the LPS challenge on day 8. Calves were weighed on days 0, 7, 9, 10, and 11 to record the average daily weight gain; diets offered and refused were recorded daily throughout the experiment. Blood collection and clinical scoring were conducted at successive time points until 72 h post LPS challenge. The data obtained also comprised rectal temperature (RT), respiratory rate (RR), heart rate (HR), concentrations of tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6), serum amyloid A (SAA), and haptoglobin (Hp). This experiment could not uncover significant effects of LPS, FO, NCUR, and DEX on the area under the curve (AUC) of the RT, HR, and RR; in addition, there was no difference between FO and NCUR vs. LPS in sickness behavior, however, DEX group significantly recovered faster than others ($P < 0.01$). There was no significant difference between groups in dry matter intake (DMI) and average daily gain (ADG) during three days post LPS challenge. The concentrations of TNF- α , IL-6, and SAA were lower in the DEX group ($P < 0.05$). Finally, no effects of FO and NCUR on cytokines and acute phase proteins (APPs) could be observed in this study. In conclusion, supplementation of FO and NCUR was not able to impact the acute phase response (APR) in calves, as levels of inflammatory cytokines and APPs as well as sickness behavior remained unchanged. It seems that the anti-inflammatory effects of FO and CUR on APR, as has been observed for other animal species, do not manifest that clearly in calves.

1. Introduction

The use of complementary and alternative medicines (CAM) as a substitute or supplement to conventional medicines has grown considerably over the past decades in human society (Koning et al., 2013). Popular CAM therapies comprise herbs (including Chinese medicines, curcumin, and cannabis), probiotics, vitamins, and fish oil (Cheifetz et al., 2017). Additionally, CAM has long been employed in traditional animal medicine and as a feed supplement to livestock diets (Oh et al.,

2013). Antimicrobial activities, immune enhancement, and stress reduction are the most useful properties attributed to different CAM compounds in animals (Oh et al., 2013). Among CAM, curcumin (CUR) and fish oil (FO) are two well-known anti-inflammatory compounds whose mechanisms of anti-inflammatory action are remarkably comparable to that of glucocorticoids (Rhen and Cidlowski, 2005; Calder, 2013; Prasad et al., 2014).

Curcumin is the primary active component of turmeric, a yellow compound originally isolated from the plant *Curcuma longa*, having

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strong antioxidant and anti-inflammatory activities (Menon and Sudheer, 2007). This component has been widely investigated as an anti-inflammatory compound in rodents and pigs. Studies evidenced that CUR improved the anti-inflammatory response to lipopolysaccharide (LPS) challenge by a significant decrease in pro-inflammatory cytokines such as TNF- α (Yun et al., 2010; Liu, 2012; Bible, 2013). Nevertheless, the poor bioavailability of CUR is one of its major drawbacks (Prasad et al., 2014). Most studies revealed that formulated CUR (such as nanoparticles) had better bioavailability and biological activities than its unformulated counterpart (Sun et al., 2010; Ahmadi et al., 2018; Hatamipour et al., 2019). Surprisingly, there is still a lack of knowledge on the effect of CUR and especially nanocurcumin (NCUR) as anti-inflammatory CAM compound in ruminants.

Fish oil has been extensively documented in both human and animal research, confirming its beneficial anti-inflammatory effects as a marine n-3 polyunsaturated fatty acids (PUFA) source (Ballou et al., 2008; Calder, 2013). Gandra et al. (2016) demonstrated that dairy cows fed an n-3 FA source had higher expression of T helper cells (CD4⁺) and potentially greater pro-inflammatory actions than cows fed an n-6 FA source. Ballou et al. (2008) and Greco et al. (2015) reported that n-3 PUFA could modulate the inflammatory response following LPS challenge in ruminants. Feeding diets containing n-3 FA may be beneficial for calf health, as it alters the cytokine response and regulation of the inflammatory response, hence affecting the ability of the animal to respond to disease. However, there is a lack of information regarding the effect of n-3 PUFA vs. other CAM or anti-inflammatory drugs on calves.

Although corticosteroids have an immunosuppressive effect, they have a substantial short-term effect on the acute phase response (APR) in ruminants through inhibiting the release of pro-inflammatory cytokines, supporting their popularity as a drug to treat acute inflammatory processes in cattle (Lekeux and VandeWeerd, 1997). Among corticosteroids, dexamethasone (DEX) has an approved inhibitory effect on LPS-induced TNF- α serum levels in calves. In the study of Ohtsuka et al. (1997) and Plessers et al. (2015a), pretreatment of DEX (0.3 mg/kg BW) significantly decreased the TNF- α concentration and endotoxin shock in LPS challenged calves. Therefore, using DEX as a positive control immunomodulatory drug in LPS challenged calves, allows to evaluate the effects of other anti-inflammatory compounds. Besides, in the current study, an LPS model introduced by Plessers et al. (2015b) was applied to induce APR in calves.

This research aimed at investigating the effect of nanocurcumin and fish oil as natural anti-inflammatory compounds vs. DEX in response to an LPS challenge in calves. Not only is this experiment the first to evaluate the effect of NCUR in response to an LPS challenge in calves, but it is also the first report which compared FO and NCUR vs. DEX under LPS challenge in ruminants. This research contained two hypotheses: (1) FO and NCUR could significantly inhibit the increase of inflammatory cytokines and APPs through APR in calves. (2) FO and NCUR as an adequate alternative to glucocorticoids could decrease the high demand for drugs to treat calves' diseases.

2. Materials and methods

The experiment was conducted at the dairy farm facilities of AQR Animal Husbandry and Agriculture Co. (Mashhad, Iran) in February 2019. The ethics committee of the Ferdowsi University of Mashhad, Iran approved all experimental procedures and animal manipulations (IACUC #A47228) based on the Iranian Council of Animal Care.

2.1. Calves, treatments, and study protocol

A total of 42 male Holstein calves, with a mean age of 34.9 (\pm 3.6) days and average body weight 55.3 (\pm 4.2) kg, were randomly housed outdoors in 1 \times 2 m individual pens at ambient temperature (approximately 11 °C). The interior of each pen was bedded with wheat straw, which was renewed every 24 h. The sample size and practical setup

mimicked that as proposed by Plessers et al. (2015a and 2015b). The summary of the experimental process is illustrated in Figure 1. The duration of the experiment was 11 days (Figure 1, days 1–11) with an acclimatization period of 7 days (Figure 1, days -7 to 0). One day before the start of the experiment (Figure 1, day 0), calves were weighed, and randomly divided into seven groups with six replicates per group and treated with 5 mg kg⁻¹ BW enrofloxacin (RazakPharma Co., Tehran, Iran), to avoid unexpected respiratory infections. The following seven experimental groups were included: 1. negative control (CON, the group which was subjected neither to LPS nor to other treatments), 2. positive control (LPS, injected once), 3. 250 mg/kg BW per day fish oil + LPS (FO250), 4. 350 mg/kg BW per day fish oil + LPS (FO350), 5. 2 mg/kg BW per day nanocurcumin + LPS (NCUR2), 6. 4 mg/kg BW per day nanocurcumin + LPS (NCUR4), and 7. 0.3 mg/kg BW Dexamethasone + LPS (DEX, injected once). Regarding LPS, DEX, FO and CUR, the doses as reported by Plessers et al. (2015b), Ohtsuka et al. (1997), Litherland et al. (2010), and Bible (2013), were administered, respectively. All calves received a total of 5 L of whole milk twice a day. In order to ensure that FO and NCUR passed the rumen, the compounds were mixed with the whole milk that was offered. Twenty-five mg of vitamin E.kg⁻¹ PUFA was added to the milk to avoid the incidence of muscular dystrophy. All groups received the same diet, based on NRC (2001) recommendations, which contained a mixture of starter and chopped alfalfa hay (Table 1). Animals had free access to freshwater. According to the groups, FO (Kilka oil, Parskilka Sea Products Co., Mazandaran, Iran) and NCUR were used from day 1 until the end of the experiment (Figure 1). To increase the oral bioavailability of CUR, NCUR oral capsules (SinaCurcumin, Exir Nano, Tehran, Iran) were used, which are a registered product from curcuminoids (IRC: 1228225765). Its bioavailability and stability have been confirmed in previous experiments (Ahmadi et al., 2018; Hatamipour et al., 2019). From the acclimatization period until the LPS challenge (Figure 1, days -7 to 8), the animals' clinical status was assessed once a day, including measurement of the rectal body temperature (RT), respiratory rate (RR), heart rate (HR), and visual inspection of feces. There was no record of diarrhea or any specific illness during the experiment. One day before the LPS challenge (Figure 1, day 7), calves were weighed again, following a 16 G indwelling catheter located aseptically in the right jugular vein. One hour before the LPS challenge (Figure 1, day 8, time -1), the clinical status was assessed by determination of the RT and visual investigation of the feces. An RT \geq 39.5 °C was chosen as an elimination criterion for the experiment at this time.

Except for the CON group, the other experimental groups were intravenously challenged once with 0.5 μ g kg⁻¹ BW ultrapure LPS from *E. coli* serotype O111:B4 (Sigma-Aldrich®; product NO. L2630). The CON group received a similar volume of saline solution. One hour before the LPS challenge, the calves in group DEX were treated once with 0.3 mg kg⁻¹ BW DEX (Aburairhan Pharmaceutical Co., Tehran, Iran) intramuscularly in the neck region (cervical ventral serratus muscle) to obtain the maximum influence of the drug on the innate immune response. In order to investigate the reference level of cytokines and APPs, venous blood samples were collected from the catheter one hour before the LPS challenges (Figure 1, day 8, time -1, before DEX treatment), and transferred into EDTA-containing tubes. To evaluate cytokines and APPs in inflammatory condition, blood samples were collected at 1, 2, 3, 4, 6, 12, 24, 48, 72 h, post LPS challenge (p.c.). RT, RR, and HR were recorded at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 18, 24 h p.c (Figure 1). First, RR was measured; subsequently, we entered the pens to determine RT by using a digital thermometer and HR as well.

Once the LPS challenge was performed, animals were clinically scored by two qualified veterinarians during 18 h. During this time, dyspnoea, coughing, breathing sounds, mental state, position, and appetite were evaluated. According to these parameters, the appearance of behavioral phases (respiratory, depression, and recovery phase) was evaluated (Figure 3) with the model detailed in Plessers et al. (2015b). Whenever calves exposed systemic shock symptoms, they would be humanely slaughtered. The retrieved blood samples were centrifuged at

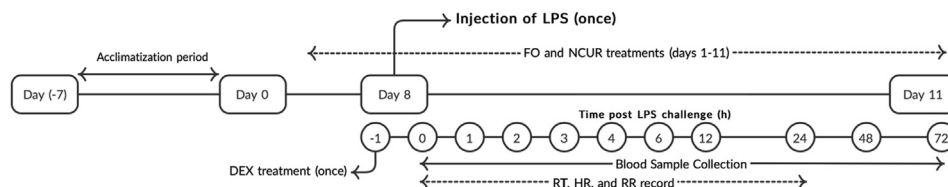


Figure 1. Schematic diagram of the experimental procedure.

1000xg for 15 min at 4 °C to obtain plasma. Then, samples were stored in aliquots at ≤ -80 °C until analysis. In order to assess the effect of treatments on average daily gain (ADG) and dry matter intake (DMI), calves were weighed on days 0, 7, 9, 10, 11, and the amount of starter diets offered and refused was recorded daily throughout the experiment (Figure 5).

2.2. Sample analyses for cytokines and acute phase proteins

In order to identify and quantify cytokines and APPs, plasma samples were analyzed in duplicate, using commercially available ELISA kits (Bioassay Technology Laboratory ELISA kits, Shanghai, China; Cat No. E0019BO, No. E0001BO, No. E0022BO, and No. E0023BO for TNF- α , IL-6, Hp, and SAA, respectively). Plasma samples were prediluted 10-fold for TNF- α , 10-fold for IL-6, and 20-fold for SAA measurements. The cytokines and APPs assays were performed according to the manufacturer's protocol, resulting in intra-assay CVs of TNF- α , IL-6, Hp, and SAA of 9.66, 9.32, 8.70, and 9.15%, respectively. The analytical sensitivities of these tests in plasma have been determined by the manufacturer as 5.85 pg ml $^{-1}$, 10.50 pg ml $^{-1}$, 0.054 μ g ml $^{-1}$, and 1.36 μ g ml $^{-1}$, for TNF α , IL-6, SAA, and Hp, respectively.

2.3. Statistical analysis

Data were checked for normal distribution, and non-normal data were analyzed using a Kruskal-Wallis test. To evaluate the effect of LPS within the different groups, the repeated-measures ANOVA was performed. Data were analyzed completely randomized with the fixed effect of treatment, time and interaction between treatment and time, as well as the random effect of calf nested within treatment by using JMP (13.2) software. The area under the curve (AUC) for RR (0–6), RT (0–24), HR (0–24), TNF- α (0–4), IL-6 (0–6), SAA (0–72), and Hp (0–24) indicated the diagnostic accuracy of the examined parameter (Gardner and Greiner, 2006). Lastly, the treatment's significance was determined by the LSMEANS Tukey HSD test. All values were expressed as mean and standard deviation (\pm SD), and $P < 0.05$ was considered as statistically significant. To investigate the factors that might influence AUC (RT, HR, RR, cytokines, and acute phase proteins), DMI, and ADG responses, a full factorial linear regression analysis was performed by using JMP (13.2) software. The individual factors (BW, DMI, age, and groups) were subjected to the backward stepwise selection process, and a significance level of $P < 0.05$ was used as a selection criterion. The DMI and BW factors were excluded from DMI and ADG models, respectively.

3. Results

3.1. Clinical signs, DMI, and ADG

The final number of calves that could be used for analysis was the following: $n = 6$ CON, $n = 6$ LPS, $n = 5$ FO250, $n = 6$ FO350, $n = 5$ NCUR2, $n = 5$ NCUR4, and $n = 6$ DEX. Because of the occurrence of severe shock symptoms, one calf was humanely slaughtered (130 min p.c. in NCUR4 groups) whereas two other calves (from the FO250 and NCUR2 groups) were excluded from the experiment and treated to avoid the appearance of severe shock symptoms. It is worth to mention that no negative effects of FO and CUR treatments on calves have been reported

before, for which the appearance of any shock symptoms is likely to relate to individual sensitivity of calves to LPS. One hour before the LPS challenge (Figure 1, day 8, time -1), the average values for RR, RT, and HR among the different groups were 34.9 ± 5 bpm, 38.6 ± 0.2 °C, 75.6 ± 8 bpm, respectively. Except for the CON group, repeated measures ANOVA revealed a significant effect of time for all parameters within each group following the LPS challenge ($P < 0.05$). After 24 h p.c., RT, HR, and RR returned to the basal level ($P > 0.05$). Figure 2 presents the effect of the groups in response to the LPS challenge on clinical parameters. In the CON group, there was no substantial difference in RT, HR, and RR for the entire duration of the experiment. A significant disparity in RT and HR between CON and other groups was present at 2–18 h p.c. ($P < 0.05$), while it was manifested in RR at 0.5–2 h p.c. ($P < 0.05$). There was no significant difference in RT between LPS, FO350, NCUR4, and DEX during the 5–24 h p.c. time interval (Figure 2A), with the exception of time 3 and 4 h p.c., at which RT in FO350 was significantly lower than DEX ($P < 0.05$). No significant difference was observed in HR between LPS, FO350, NCUR4, and DEX during 4–24 h p.c. (Figure 2B). The RR was not significantly different between LPS challenged groups, and clearly increased in all groups after LPS administration (peak at 1 h p.c.), which was followed by a swift decline in the subsequent hour (Figure 2C). In this experiment, there was no significant difference between LPS challenged groups in the AUC of the RT, HR, and RR (Table 2). Also, no interaction effects were found between BW, DMI, age, and groups.

Table 1. Ingredients and nutrient composition of the diet.

Item	Value
Ingredients %	
Alfalfa hay, medium chopped	10
Corn	45
Barley	9
Soybean meal	24
Corn gluten meal	1.8
Wheat bran	6.5
Vitamins and minerals premix ¹	0.9
Sodium bicarbonate	0.9
Calcium carbonate	0.9
Bentonite	0.5
Salt	0.5
Nutrient composition	
DM, % as fed	90
CP, % DM	19.5
NDF, % DM	17
ADF, % DM	9.1
Ether extract, % DM	3
Ca, % DM	0.7
P, % DM	0.5
Calculated ME, ² Mcal/kg of DM	3.15

¹ Contained per kilogram of supplement: 250,000 IU of vitamin A, 50,000 IU of vitamin D, 1,500 IU of vitamin E, 120 g of Ca, 20 g of P, 20.5 g of Mg, 186 g of Na, 7.7 g of Zn, 2.25 g of Mn, 1.25 g of Fe, 3 g of S, 14 mg of Co, 1.25 g of Cu, 56 mg of I, and 10 mg of Se.

² Metabolizable energy using NRC (2001) equations.

With the exception of DEX, all groups demonstrated a rather similar progression of the three consecutive behavioral phases (Figure 3). None of the calves in LPS, FO, and NCUR exhibited any deviation in diet or water before the end of the recovery phase. As expected, DEX did not present a depression phase and had a faster recovery from the challenge. The incidence of coughing and open-mouth breathing through the respiratory phase did not vary between groups. In this study, most calves were positioned sternally, while lateral decubitus during the respiratory and depression phases was expressed by 2, 1, 1, 2, and 1 calves in the LPS, FO250, FO350, NCUR2, and NCUR4 groups, respectively. The calves in the DEX group recovered faster than the other groups ($P < 0.01$). Based on the model of Plessers et al. (2015b), full recovery was recognized in the LPS, FO250, FO350, NCUR2, NCUR4, and DEX group at a mean time of 7.2, 7.5, 6.6, 7.5, 6.1 and 3.3 h p.c., respectively. At 12 h p.c., all calves easily consumed the supplied 2.5 L of milk. In addition, there was no significant difference in DMI and ADG among all groups during three days p.c. (Figure 5). No interaction effects between independent factors were observed.

3.2. Inflammatory mediators

The groups' responses to the LPS challenge on cytokines and APPs are presented in Figure 4. The highest concentrations of TNF- α , IL-6, SAA, and Hp were observed at 1, 3, 24, and 24 h p.c., respectively, in all LPS challenged groups (Figure 4). Compared to the other groups, the DEX group displayed a significant effect on TNF- α at 1 and 3 h p.c. ($P < 0.05$). At 3 h p.c., no difference was observed between FO250, FO350, NCUR4, and DEX in TNF- α . However, the level of IL-6 was significantly lower in DEX than in other groups ($P < 0.01$). By contrast, the DEX group showed the highest level of Hp at 72 h p.c. ($P < 0.01$). Also, a significant difference between LPS and DEX in SAA was seen at 24 h p.c. ($P < 0.05$). The AUC for cytokines was significantly lower in the DEX compared to other groups ($P < 0.01$), although no significant difference in APPs was observed among all groups (Table 2). Besides, there were no interaction effects between independent factors in the AUC of the cytokines and APPs.

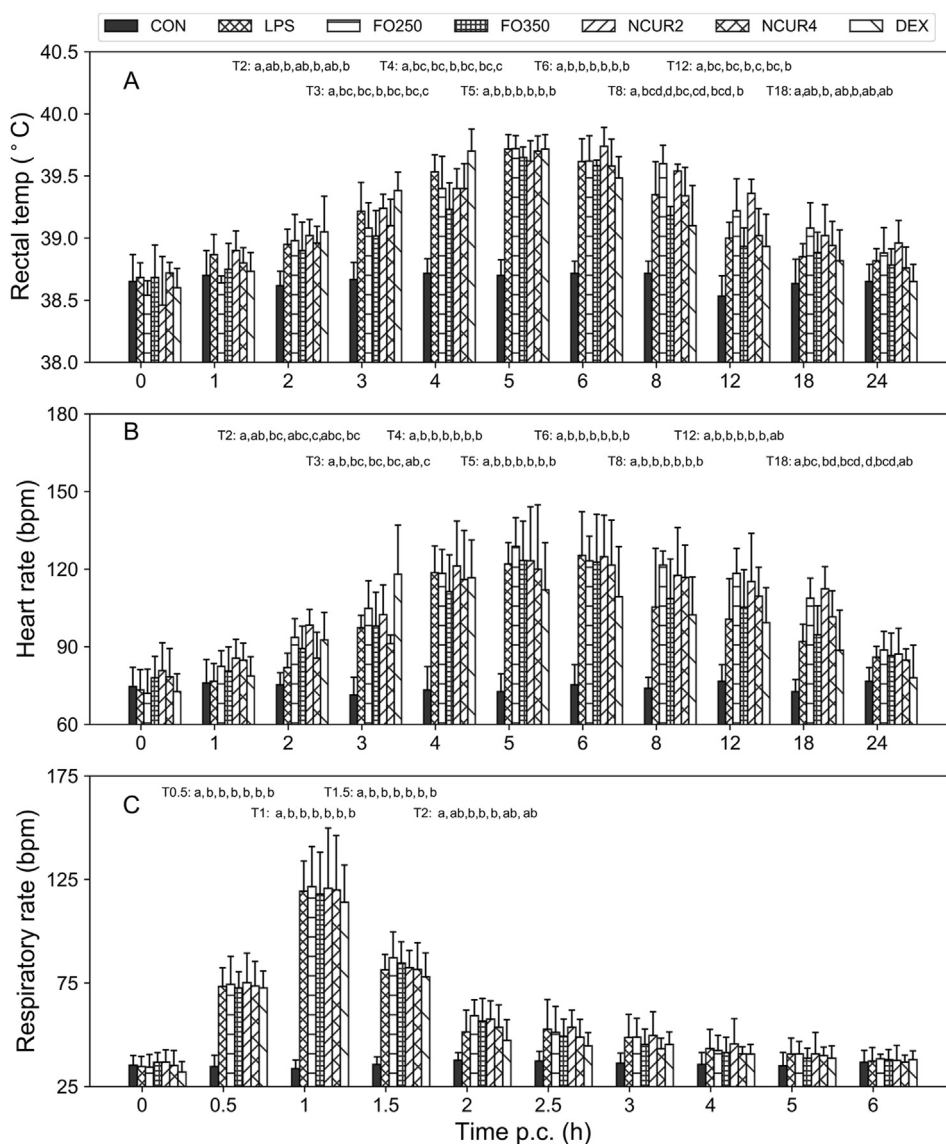


Figure 2. Effects of fish oil and nanocurcumin vs. dexamethasone on rectal temperature (A), heart rate (B), and respiratory rate (C) in Holstein calves after LPS challenge. Data is shown as mean + SD. The different letters within each time (T) indicate a significant difference between groups ($P < 0.05$).

Table 2. Effect of fish oil and nanocurcumin vs. dexamethasone in response to an LPS challenge on the area under the curve (AUC).

Item	Groups ¹						SEM	P-Value
	LPS	FO250	FO350	NCUR2	NCUR4	DEX		
RT ²	937.4	940.5	936.1	941.4	937.7	935.3	1.33	0.06
HR ³	2364	2636	2424	2635	2493	2300	150.8	0.13
RR ⁴	336.8	344.4	332.8	346.4	329.2	318.6	31.2	0.94
TNF- α ⁵	23.9 ^a	23.7 ^a	21.6 ^a	23.4 ^a	20.8 ^a	9.2 ^b	3.70	<0.01
IL-6 ⁶	57.7 ^a	59.6 ^a	54.3 ^a	59.0 ^a	52.8 ^a	16.9 ^b	8.04	<0.01
Hp ⁷	11.4	12.7	11.6	12.0	11.4	15.1	2.86	0.72
SAA ⁸	19.3	18.3	16.8	17.5	17.2	15.2	3.44	0.72

^{a,b} Means within a row with different superscripts differ ($P < 0.01$).

¹ Groups: LPS = positive control; FO250 = 250 mg/kg BW per day fish oil + LPS; FO350 = 350 mg/kg BW per day fish oil + LPS; NCUR2 = 2 mg/kg BW per day nanocurcumin + LPS; NCUR4 = 4 mg/kg BW per day nanocurcumin + LPS; DEX = 0.3 mg/kg BW dexamethasone + LPS.

² RT = rectal temperature, AUC_{0-24h} ($^{\circ}\text{C} \times \text{h}$).

³ HR = heart rate, AUC_{0-24h} (bpm \times h).

⁴ RR = respiratory rate, AUC_{0-6h} (bpm \times h).

⁵ TNF- α = TNF- α , AUC_{0-4h} (ng/mL \times h).

⁶ IL-6 = interleukin-6, AUC_{0-6h} (ng/mL \times h).

⁷ Hp = haptoglobin, AUC_{0-72h} (mg/mL \times h).

⁸ SAA = serum amyloid A, AUC_{0-72h} (mg/mL \times h).

4. Discussion

In this study, as a first research hypothesis, it was assessed whether FO and NCUR treatment could significantly inhibit the increase of inflammatory cytokines and APPs upon APR in calves. Indeed, both FO and NCUR have been acknowledged as anti-inflammatory compounds (Jacob et al., 2007; Calder, 2013), exerting protective effects against inflammatory diseases by amongst other the inhibition of the NF- κ B protein complex that is involved in the synthesis of IL-6 and TNF- α (Zhao et al., 2005). However, in the current study the results indicated no effects for both treatments on the cytokines and APPs concentration levels, following LPS challenge.

Although there is limited knowledge about the effect of CUR on the health status of ruminants, the current findings regarding CUR are in line with those of Oh et al. (2013), who reported that inflammatory cytokines (TNF- α , IL-6, and IFN- γ) were not influenced by curcumin (2 g/cow per day) in an *in vitro* LPS challenge experiment in dairy cows. The present study proves that even with a better bioavailability of CUR (using NCUR), there is still no effect of CUR on cytokines concentrations in calves. The

current research was contradictory to the findings of Xun et al. (2015), who uncovered that 300 or 400 mg/kg DM CUR supplementation in piglets, challenged by *Escherichia coli*, improved intestinal mucosal barrier integrity by decreasing the inflammatory cytokines. In addition, Bible (2013) showed that an 80 mg/kg diet of CUR significantly decreased the serum concentrations of TNF- α and APPs under LPS challenge in nursery pigs, which contrasted the present data. It seems that, in a context of APR, the effect of CUR on cytokines and APPs concentrations in ruminants is not similar to other animal species.

Also, regarding FO, the present findings are consistent with those studies that have investigated this supplement in ruminants. Indeed, in the study of McDonnell et al. (2019), it was evidenced that 40 g FO per day did not affect the calves' immune function. They did not report any effect of FO on Hp and IFN- γ and other hematology variables. However, Karcher et al. (2014) reported that using 2% FO in the milk replacer (MR) of calves tended to reduce the expression of TNF- α and IL-8 ($P < 0.1$), in an *in vitro* LPS challenge experiment (2 $\mu\text{g}/\text{ml}$ LPS), which is contradicted by the present results. Ballou et al. (2008) supplemented 2% FO in MR of Jersey calves and found that the concentration of glucose, insulin, BUN,

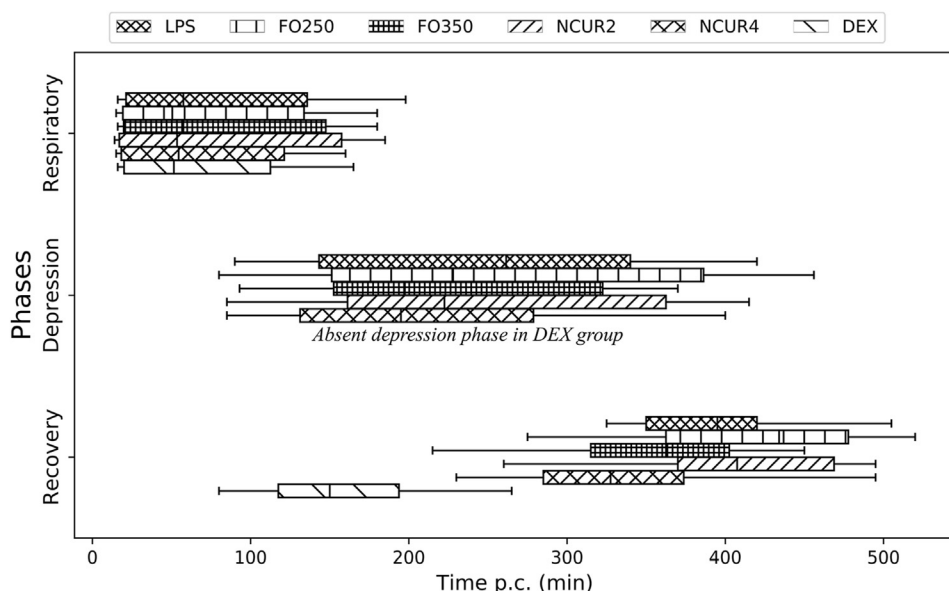


Figure 3. Effects of fish oil and nanocurcumin vs. dexamethasone on the three behavior phases in Holstein calves after LPS challenge based on the model of Plessers et al. (2015b). The boxes illustrate the mean period of the different phases, while the vertical lines indicate the minimum and maximum points in time when the respective phase starts and ends, regarding a certain animal. The beginning and the end of the respiratory phase is determined by the onset of dyspnoea and recovery from respiratory distress, respectively. Recovery from respiratory distress also indicates the start of the depression phase. This phase ends when alertness is regained, which confirms initiation of the recovery phase. When the animal stands up the end of recovery phase is assumed, indicating full recovery of the calf.

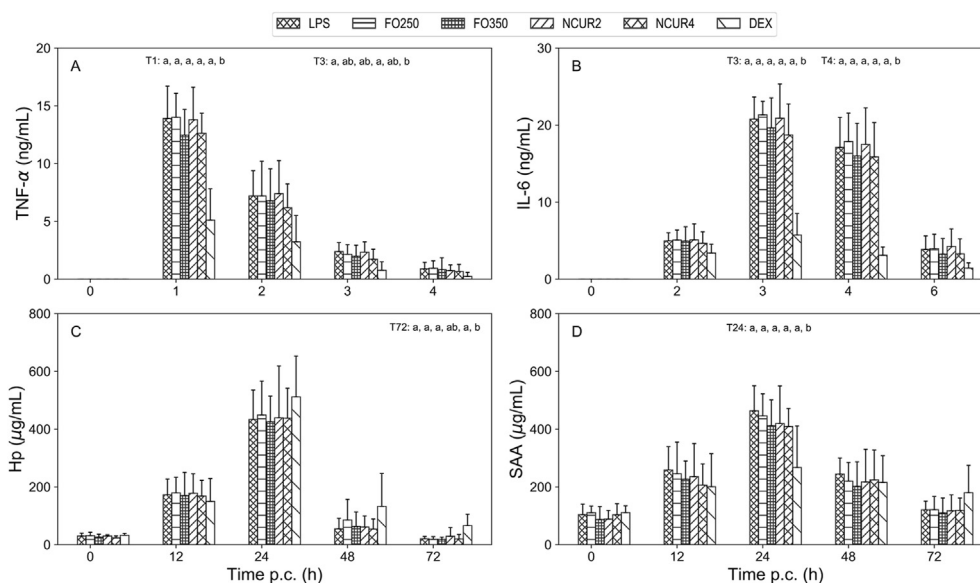


Figure 4. Effects of fish oil and nanocurcumin vs. dexamethasone on TNF- α (A), IL-6 (B), Hp (C), and SAA (D) in Holstein calves after LPS challenge. Data is shown as mean + SD. The different letters within each time (T) indicate a significant difference between groups ($P < 0.05$).

NEFA, and triacylglycerol significantly decreased following 4 $\mu\text{g}/\text{kg}$ BW of *Salmonella Typhimurium* LPS challenge. Although, in their experiment the inflammatory cytokines and APPs were not evaluated, they concluded that FO attenuates biochemical aspects of the acute phase response in Jersey calves. The insignificant effects of FO and NCUR on inflammatory cytokines and APPs in the current study, compared to the literature, could be attributed to the differences in experimental design, types of LPS challenge model, types of animal, and calf age.

Another important factor to consider is sickness behavior and its most prominent drivers. The observed fever that follows LPS challenge is considered to be mediated by the action of prostaglandin E_2 , TNF- α , IL-1 β , and IL-6 (Roth and Blatteis, 2011). Moreover, it has been explained that TNF- α is the primary mediator of acute inflammation in response to Gram-negative bacteria, which enhances the production of other pro-inflammatory cytokines such as IL-6 (Conti, 2004). In addition, sickness behavior is also associated with cytokine activation (Brymer et al., 2019). Studies have confirmed that IL-6 is positively associated with the incidence and severity of depression (Howren et al., 2009). In this research, the levels of TNF- α and IL-6 significantly increased after LPS administration, which is in line with previous work (Carroll et al., 2009; Plessers et al., 2015a, 2015b; Fernandes et al., 2019). In the present study, DEX did not exert any effect on respiratory distress and fever development but, as expected, had a definite positive influence on depression and recovery phases (Figure 3). The absence of a depression phase in DEX agrees with Plessers et al. (2015a), supporting that inhibiting inflammatory cytokines could positively affect sickness behavior in calves.

One of the most remarkable results of this study was that natural anti-inflammatory compounds did not have any significant effect on sickness behavior in LPS challenged calves (Figure 3). The insignificant effects of FO and NCUR groups vs. LPS group on sickness behavior might be related to the concentration of TNF- α and IL-6, for which no differences were observed between these groups (Figure 4). An alleviated sickness behavior has been reported in calves fed FO (Ballou et al., 2008) and in pigs fed CUR (Bible, 2013). However, in Ballou et al. (2008), FO had no significant effect on appetite which is in line with the present study. Beside cytokines, sickness behavior can also be regulated by other factors such as eicosanoids, peripheral signals, and neurotransmitters. However, the effect of FO and CUR on these factors following the LPS challenge in calves has not been assessed.

In this experiment, it was expected that groups with lower cytokines concentrations would exhibit higher ADG. However, the results showed

that DEX vs. LPS could not significantly increase ADG during three days p.c. (Figure 5). In normal physiological states, the immune system is only active to a limited extent and needs little energy and nutrients. Through infection, the immune system is strongly activated, which causes changes in nutrient partitioning and growth (Kinsbergen et al., 1994). The APR causes anorexia, which limits nutrient availability and reduces growth. Moreover, in the APR, the concentrations of cytokines increase significantly, and because the inflammatory cytokines act locally and systemically to regulate energy metabolism, this could affect body weight loss (Wang and Ye, 2015). Cytokines act in the brain, fat, and muscle tissues in a systemic process to regulate energy metabolism. In the brain, they inhibit energy input through suppression of appetite, which means inhibition of the hypothalamus. Consequently, the host has to use the energy stores in adipose tissue and skeletal muscle to satisfy the energy demands from the immune systems (Wang and Ye, 2015). In this regard, studies reported that TNF and IL-6 play a vital role in mobilizing body stores of energy which are necessary to fight infection (Molloy et al., 1993; Wang and Ye, 2015). This could explain why there were no significant differences in ADG between DEX and LPS during three days p.c., as there might be a higher energy demand to produce cytokines even in low amount in APR in DEX group. In the current study, there was no effect of natural anti-inflammatory compounds on ADG during three days p.c. compared to the LPS group, which might be related to the insignificant difference in TNF- α and IL-6 levels. This result rebuts Bible (2013), who reported that CUR was able to limit body weight loss in LPS challenged pigs. On the other hand, this research is in line with Karcher et al. (2014), who stated that FO did not have a significant effect on ADG. However, the lack of any significant effects on ADG between groups might be related to the sample size (6 replicates per group). Future research needs to be undertaken to explain the effect of FO and CUR on body weight recovery in LPS challenged calves, more than three days p.c. and with a higher sample size.

Regarding the second hypothesis, it was investigated that whether FO and NCUR could serve as an alternative to glucocorticoids in order to decrease the high usage of synthetic drugs to treat calves' diseases. Although previous studies in non ruminants reported similar anti-inflammatory mechanisms for FO, CUR, and DEX through the reduction of NF- κB transcription (Rhen and Cidlowski, 2005; Calder, 2013; Prasad et al., 2014), the present data were not able to reveal similar effects from FO and NCUR on APR in calves compared to glucocorticoids. Therefore, the data with regard to the cytokines, APPs, and sickness behavior were rejective towards the hypothesis. It has been evidenced

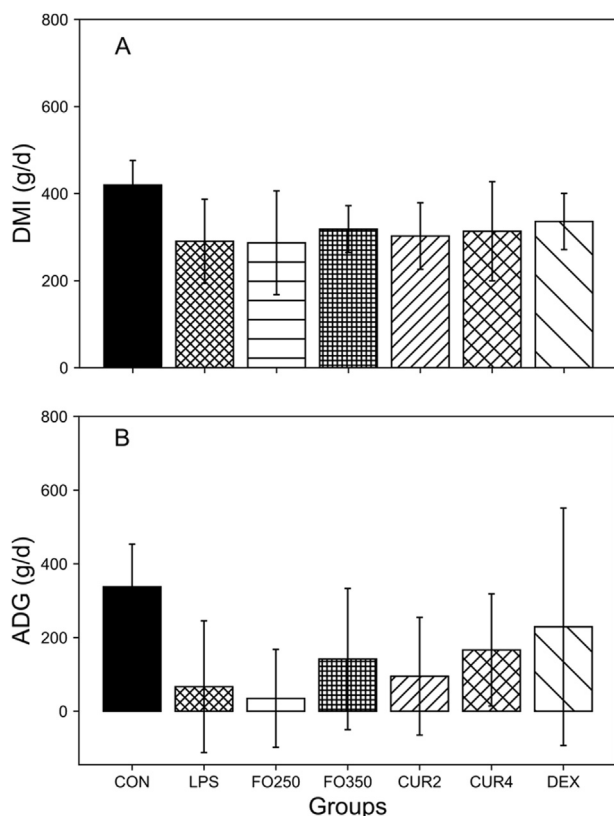


Figure 5. Effects of fish oil and nanocurcumin vs. dexamethasone on DMI and ADG in Holstein calves following three days post LPS challenge. Data is shown as mean \pm SD.

that glucocorticoids can prevent inflammation by three independent mechanisms: (1) the induction and activation of annexin I, (2) the induction of MAPK phosphatase 1, and (3) the repression of transcription of NF- κ B (Rhen and Cidlowski, 2005). With this rationale, we propose two reasons why protective effects from FO and CUR were not observed with respect to the deleterious effects of APR. First, the significant effects of DEX on cytokines could be related to other anti-inflammatory mechanisms compared with those described for FO and CUR. Secondly, although this study did not evaluate the anti-inflammatory mechanism(s) in detail, it seems that the effect of FO and CUR on the transcription of NF- κ B might not be as severe in ruminants as in other animals such as pigs. Future studies, which evaluate the effect of FO and CUR on the transcription of NF- κ B in APR in ruminants, are required.

This paper was the first study to evaluate the effect of NCUR in response to an LPS challenge in calves, also, it was the first report which compared FO and NCUR vs. DEX under LPS challenge in ruminants. We believe that the response of calves to CUR is not completely clear yet, for which more research is designated with respect to both calves' health status and performance.

An important limitation within the current study was the sample size, which appeared to be rather low to accurately assess the immune responses that were found highly variable across the animals. Although the number of animals per treatment group was based on the similar work of Charan and Kantharia (2013) and Ohtsuka et al. (1997), 6 or 5 replicates turned out to be insufficient to generalize the effects of the CAM treatments on APR in calves. Repeated sampling and data recording at specific times and intensive care of challenged calves, were some of the current study's practical difficulties. Also, the experimental costs and a large number of treatments were assigned as another limitation, which led to a decrease in the number of repeats per group. It is recommended to increase the power by decreasing the number of experimental groups in future studies. Moreover, future studies on the current topic are therefore

recommended: to identify the appropriate doses of FO and CUR as anti-inflammatory compound in APR in calves; to evaluate the effects of FO and CUR (separately or as a mixture of them) on the NF- κ B transcription in APR in calves; and subsequently, to evaluate the effect of CAM in combination with different doses of anti-inflammatory drugs on APR in calves.

5. Conclusions

The results from this study suggested that FO and NCUR could not protect calves from the deleterious effects of excessive APR. Indeed, usage of these natural anti-inflammatory compounds could not prevent the increase of inflammatory cytokines and APPs levels, as being provoked by endotoxin challenge in calves. Related to this, no beneficial effects on sickness behavior, DMI and ADG in LPS challenged calves were observed. In general, these findings may suggest that the effect of FO and CUR on NF- κ B transcription in ruminants is different compared to other animals, especially pigs. The present study confirms that dexamethasone treatment improves recovery and anti-inflammatory response in calves. It is also relevant to note that the present experiment was limited by small sample size ($n = 6$ per treatment). Therefore, a larger number of treatment replicates is strongly suggested to further assess the underlying mechanisms of natural anti-inflammatory compounds (e.g. their impact on NF- κ B transcription) and evaluate their potential in combination with low doses of drugs.

Declarations

Author contribution statement

S. Kamel Oroumieh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

L. Vanhaecke, L. Van Meulebroek: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

R. Valizadeh: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

A.A. Naserian: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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