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Effect of Different Fatty Acids Sources on Some Blood Factors and Interleukin Gene Expression in Finishing Lambs

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ABSTRACT

The current research was conducted to study the effects of different fatty acids sources in the diet on blood metabolites and hormones in finishing lambs experimentally infected with the virus that causes foot and mouth disease. A total of fifteen Sangsari male lambs with an average live weight of 48±2 kg and an average age of 8±1 month were randomly assigned to one of three dietary experimental treatments as follows: 1) Calcium soap of palm oil fatty acids (PO) as a source of palmitic acid (16:0); 2) Calcium soap of sunflower oil fatty acids (SO) as the source of linoleic acid (n-6 18:2); and 3) Calcium soap of linseed oil fatty acids (LO) as the source of α-linolenic acid (n-3 18:3). The lambs were housed in individual pens and offered the iso-caloric and iso-nitrogenous diets for 28 days including 21 days of adaptation period and 7 days of the sampling period. The results illustrated that the lowest and the highest expression of IL-4 mRNA were measured in LO and SO treatments, respectively. Expression of IL-8 mRNA was lower in LO and PO treatments when compared with SO. The highest level of glucose in LO treatment when compared with sunflower oil or palm oil. Lambs on the LO diet showed the highest blood concentration of insulin and the lowest blood concentration of glucagon when compared with lams on SO and PO diets. The highest blood contents of triiodothyronine and thyroxin hormones were measured in lambs on the LO diet when compared with other treatments. However, the concentration of blood glucose, insulin, glucagon, triiodothyronine, and thyroxin were the same between PO and SO groups. In conclusion, the findings of the current experiment confirmed that the inclusion of α-linolenic acid but not linoleic acid in the diet of virus-infected lambs suppressed pro-inflammation with lowering expression of IL-4 and IL-8 mRNA and increased blood glucose, insulin, T3, and T4 which may lead to higher weight gain and feed efficiency of virally infected lambs.

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Introduction

Inflammation is a vital part of the immunologic response, but the excessive expression and higher levels of pro-inflammatory cytokines (such as IL-1β, TNF-α, and IL-6) are destructive (Calder, 2003). Pro-inflammatory cytokines induce insulin resistance and adipose tissue lipolysis, increase the blood level of glucose and amplify catabolic metabolism such as muscle proteolysis (Bertoni *et al.,* 2015). Also, pro-inflammatory cytokines reduce the passage rate of digesta through the gastrointestinal tract, decrease the feed intake, cause an increase in body temperature and also decrease locomotion score (Bertoni *et al.,* 2015; Jamshidi *et al.,* 2020). At the level of the liver, pro-inflammatory cytokines enhance the synthesis of acute-phase proteins such as serum amyloid A, haptoglobin, ceruloplasmin, and C-reactive protein (Powanda, 1980) and reduce the synthesis of other proteins such as retinol-binding protein, paraoxonase, lipoproteins, and albumins (Loor *et al.,* 2013).
Common dietary sources of n-6 polyunsaturated fatty acids (PUFA) in ruminant diets are

sunflower, soybean, corn, and safflower oils. The n-6 PUFA such as linoleic acid (18:2 n-6) is the precursor of arachidonic acid (20:4 n-6), which can convert to leukotrienes, prostaglandins (such as PGE2), and their derived metabolites which have important roles in regulating immunity or inflammation occurrence (Yaqoob *et al.,* 2000; Poorghasemi *et al.,* 2017b).

The conversion of arachidonic acid to pro-inflammation mediators can be prevented by the long-chain n-3 PUFA (Calder *et al.,* 2002). The main n-3 PUFA in ruminant diets is α-linolenic acid (18:3 n-3). The studies have implied that the higher ratios of n-3 fatty acids to monounsaturated or saturated fatty acids, results in higher incorporation of n-3 fatty acids into phospholipids (Berge *et al.,* 1999; Madsen *et al.,* 1999). Feeding n-3 PUFA results in the substitution of arachidonic acid by eicosapentaenoic acid in cell membranes of monocytes, macrophages, lymphocytes, and neutrophils which are involved in inflammation. This substitution leads to decreased production of arachidonic acid-derived mediators through the lower expression of 5-lipoxygenase and cyclooxygenase-2 and competition for lipoxygenase and cyclooxygenase. Thus, n-3 PUFA feeding results in a decreased capacity of immune cells to synthesize series two prostaglandins from arachidonic acid (Yaqoob *et al.,* 2000) and induce the formation of series-3 eicosanoids, which have anti-inflammatory effects compared to series two prostaglandins (Gulliver *et al.,* 2012).

Because acute phase reaction leads to anorexia, catabolic processes of adipose and muscle tissues are one of the main resources of lipids and proteins during pro-inflammation (Ceciliani *et al.,* 2012). It was shown that a low oral dose of IFN-α in cattle decreases plasma glucose and body reserves while increases plasma β-hydroxybutyrate, non-esterified fatty acids, reactive oxygen species, ceruloplasmin, and haptoglobin (Trevisi *et al.,* 2009). High production of cytokines during severe inflammation results in lower blood T3 and T4 hormones (Huszenicza *et al.,* 2002).

One of the most devastating diseases in domesticated and wild cloven-hoofed animal species is Foot and mouth disease (FMD) (Slozhenkina *et al.,* 2020). The causative agent of this infectious disease belongs to the *Aphthoviruses* genus of the *Picornaviridae* family. Like many domesticated cloven-hoofed animals, a lamb is very susceptible to foot and mouth disease virus (Park *et al.,* 2006; Orsel *et al.,* 2007).

Since previous studies have focused on the effect of different fat sources containing omega-3 and omega-6 fatty acids on performance, nutritional and production parameters in birds, the present study on the effect of these fatty acids on safety parameters and gene expression related to immunity in livestock can be significant in terms of innovation (Halakoo *et al.,* 2020).

Also, because the results of previous reports have shown that in addition to genetic selection, non-genetic factors such as some nutrients and unsaturated fatty acids in the diet can express genes responsible for the immune response by altering immune maturity and antibody levels (Rajaei-Sharifabadi *et al.,* 2021).

Therefore, the present experiment was performed to investigate the effects of 16:0, 18:2 n-6, and 18:3 n-3 fatty acids on blood metabolites and hormones of lambs infected with foot and mouth disease agents.

Materials and Methods

Animals and management

In this study, a total of fifteen healthy Sangsari male lambs with an average body live weight of 48±2 kg and an average age of 8±1month were randomly assigned to one of three experimental treatments (5 lambs per treatment). Treatments were as follows: 1) palm oil group (PO) received calcium soap of palm oil fatty acids in the diet as the source of palmitic acid (16:0); 2) sunflower oil group (SO) received calcium soap of sunflower oil fatty acids in the diet as the source of linoleic acid (n-6 18:2), and 3) linseed oil group (LO) received calcium soap of linseed oil fatty acids in the diet as the source of α-linolenic acid (n-3 18:3).

The diets were balanced using Sheep CNCPS software. The feed ingredients and chemical composition of the experimental diets are presented in Table 1. The lambs were individually housed and offered the iso-caloric and iso-nitrogenous diet for 28 days including 21

days of adaptation period and 7 days of the sampling period. Rations were fed to the lambs three times daily. Animals have free and continuous access to fresh and clean drinking water. After the adaptation period, lambs were vaccinated against FMD.

Table 1. Feed ingredients and chemical composition of basal experimental diet.

Ingredients, g/kg of DM	
Alfalfa Hay	158
Wheat Straw	158
Barley Grain	330
Dry Corn Grain	228
Soybean Meal	82
Oils	28
Limestone	8
Di-Calcium Phosphate	2
Salt	2
Sodium Bicarbonate	4
Chemical Composition, g/kg of DM	
Metabolisable Energy (Mcal/kg)	2.85
Crude Protein	140
Neutral Detergent Fiber	257
Non-Fibrous Carbohydrate	529
Crude Fat	27
Calcium	7.9
Phosphorus	4.3

Data and sample collection

A single blood sample was collected before morning feeding on each of the 15 animals 7 days after vaccination (on day 28 of the experiment) by jugular venipuncture after proper restraint with labeled sterile serum tubes containing 1 mg ml-1 of ethylene diaminetetracetate (EDTA) for the hematological analysis. The serum was separated by centrifugation at 3000 rpm for 15 min and then were kept at −20°C for later analysis of blood metabolites and hormones.

RNA extraction and Real-time qPCR

RNeasy®Mini Kit (Qiagen, Hilden, Germany) was used to isolation of total RNA from lamb's blood sample, following the manufacturer's protocol. The ratio of 260/280 nm absorbance readings was quantified by Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) for calculation of the concentration and purity of extracted RNA. The genomic DNA was removed before the reverse transcription of RNA to cDNA. Genomic DNA quantification is performed by spectrophotometry, agarose gel electrophoresis, cleavage-by-cleavage enzymes, and polymerase chain reaction (PCR). Complementary DNA (cDNA) was synthesized with approximately 100ng/µL purified RNA using Quantitect® reverse transcription kit (Qiagen, Hilden, Germany) following the manufacturer's procedure. For the internal standard, the GADPH gene was used as a housekeeping gene to standardize the expression.

The Bio-Rad CFX96 Real-time PCR system (Bio-Rad Laboratories, CA, USA) was used to perform Real-time qPCR. Thermal cycling conditions consisted of enzyme activation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15s and annealing and extension at 60°C for 60 s (Faisal *et al.,* 2013). Primer characteristics of cytokines and reference gene are described in Table 2. At the end of the amplification cycle, the analysis of the melting curve was performed to confirm the specificity of amplification. The relative expression levels of IL-4 and IL-8 transcripts were measured by quantitative real-time PCR. The comparative ΔΔCt method was used for quantification of Real-Time PCR outputs. A 5-fold serial dilution of cDNA was used as a standard curve to determine the efficiency of amplification of housekeeping and target genes. The GenEx enterprise software was used to statistically analyze obtained fold changes.

Blood analysis

Glucose concentrations in the serum were measured by Hitachi 902 Automatic Analyser (Roche Diagnostics, Germany) using Pars Azmoon kit (Tehran, Iran). The concentrations of plasma triiodothyronine, thyroxin, insulin, and glucagon were determined using Pishtaz Teb (Arak, Iran) ELISA kits according to the manufacturer's recommendations.

Statistical analysis

A completely randomized design with 3 treatments and 5 replicates per treatment was subjected to analyze the data. All data were analyzed using SAS v9.2 (Statistical Analysis System, SAS Institute, USA). Differences between treatment means were considered significant at $P<0.05$.

Table 2. Primer characteristics of cytokines and reference gene for real-time PCR amplification.

Target gene	Primer Sequence (5′ - 3′)	Product size (bp)	NCBI accession number
IL-8	F - CGAAAAGTGGGTGCAGAAGGT	80	NM_001009401
	R - GGTTGTTTTTTCTTTTTCATGGA		
IL-4	F - CGCTCCCATGATTGTGGTAGTT	64	NM_001009313
	R - GCCCAGTGGACAGGTTTCTG		
GADPH	F - GAGAAGGCTGGGGCTCACC	129	AF030943
	R - GCTGACAATCTTGAGGGTATTGTT		

F=Forward, R=Reverse, GADPH=Glyceraldehyde3-phosphate dehydrogenase, IL-8=Interleukin-8, IL-4=Interleukin-4

Results

Results of this study revealed significant differences *(P* <0.05) in IL-4 and IL-8 mRNA levels among various treatments (Table 3). The lowest and the highest expression of IL-4 mRNA was measured in LO and SO treatments, respectively *(P* <0.05). Expression of IL-8 mRNA was lower in LO and PO treatments when compared with SO *(P* <0.01).

Table 3. The relative IL-4 and IL-8 mRNA expression (fold change) in lambs fed experimental diets.

	PO	SO	LO	SEM	P
IL-4 mRNA expression	1.76^{b}	2.73^{a}	1.01^{c}	0.270	0.0208
IL-8 mRNA expression	1.09^{b}	2.32^{a}	1.01^{b}	0.334	0.0001

PO: Palm oil treatment (palmitic acid); SO: sunflower oil treatment (n-6 PUFA, linoleic acid); LO: linseed oil treatment (n-3 PUFA, α-linolenic acid); SEM: standard error of means. Different superscript letters in a row mean statistical significance *(P* <0.05).

There were significant differences between treatments on blood glucose concentrations (Table 4). Lambs that received 18:3 n-3 fatty acids in the diet (LO group) had higher blood glucose (*P*<0.05) when compared with lambs on 16:0 (PO group) or 18:2 n-6 (SO group) diets. However, the concentration of blood glucose was similar between PO and SO groups (*P*>0.05).

In the current study, lambs on the LO diet had the highest blood concentration of insulin (*P*<0.05) and the lowest blood concentration of glucagon (*P*<0.05) when compared with lambs on SO and PO diets, perhaps due to higher blood glucose in the LO group. However, there were no significant differences in blood concentration of insulin and glucagon between PO and SO groups (*P*>0.05).

The highest concentrations of T3 and T4 hormones in blood were measured on lambs on the LO diet when compared with other treatments (*P*<0.05). However, the concentrations of blood T3 and T4 were similar between groups fed palmitic acid and linoleic acid (*P*>0.05).

Table 4. The effects of palmitic, linoleic, and α-linolenic acid diets on blood glucose and hormones level in finishing lambs.

	PO	SO	LO	SEM	P
Glucose, (mg/dL)	58.0^{b}	54.6^{b}	65.8^{a}	4.57	0.0066
Insulin, (ng/L)	134.6^{b}	133.6^{b}	184.0^{a}	9.91	0.0001
Glucago, (ng/L)	329.2^{a}	333.8^{a}	287.8^{b}	14.37	0.0005
T3, (ng/mL)	0.75^{b}	0.81^{b}	0.91^{a}	0.05	0.0008
T4, (ng/mL)	5.08^{b}	4.93^{b}	6.55^{a}	0.46	0.0002

PO: palm oil treatment (palmitic acid); SO: sunflower oil treatment (n-6 PUFA, linoleic acid); LO: linseed oil treatment (n-3 PUFA, α-linolenic acid); SEM: standard error of means. Different superscript letters in a row mean statistical significance *(P* <0.05).

Discussion

The effect of nutrition on the immune system can be specific or non-specific, some substances have an indirect effect and strengthen and stimulate the immune system including polyunsaturated fatty acids PUFA (Poorghasemi *et al.,* 2013a). Depending on the type and amount of fat, both cell-mediated and humoral immunity are affected (Poorghasemi *et al.,* 2015).

Dietary linolenic and linoleic acid and most of the omega-6 fatty acids in the feed are converted to arachidonic acid before they enter the cell membrane. Arachidonic acid is the main precursor to the production of eicosanoids (Poorghasemi *et al.,* 2013b).

Eicosanoids are hormone-like substances that play a biological role in regulating platelets, interfering with blood vessel walls, monocytes, and macrophages. Linolenic and linoleic are also seriously the metabolic origin of different types of pro-inflammatory and anti-inflammatory eicosanoids (Poorghasemi *et al.,* 2017a).

There is some evidence for different effects of α-linolenic acid on the eicosanoid (interleukin)

synthesis. Some of the most important eicosanoids include prostaglandins (PG), leukotrienes (LT), and thromboxane (TX), which are involved in many immune responses. PGE2 regulates the production of IL-2 and TNF. Leukotrienes increase T cell and B cell division and the activity of natural killer cells and the release of cytokines from T lymphocytes (Ghaderzadeh *et al.,* 2019). α-linolenic acid produces anti-inflammatory substances and anti-thrombotic components (Araújo et al., 2020).

Winnik *et al*. (2011) confirmed that T cells of animals that ate a high α-linolenic acid diet expressed less IL-4 and reduced differentiation towards Th2 cells. Mizota *et al*. (2009) and Kaveh *et al*. (2019) showed that IFNγ level and the ratio of IL-4 to IFNγ, which is an index of actual Th2/Th1 cytokine-production, was significantly lower during consumption of the n-6 versus the n-3 rich diet. In another study higher Th2 interleukins such as IL-4 and IL-5 resulted in an allergic response due to enhanced growth of mucosal-type mast cells and IgE production (Kaveh *et al.,* 2019). Zeng *et al*. (2016) found that a high ratio of α-linolenic acid to linoleic acid in the juvenile fish diet significantly suppressed pro-inflammatory cytokines (interleukin-1β, tumor necrosis factor α, interferon γ2, and interleukin-8), increased complement C3 levels, raised interleukin-10 mRNA abundance in the intestine. Darwesh *et al*. (2019) implied that the anti-inflammatory effects of n-3 PUFAs are ascribed to their ability to displace arachidonic acid in the cell membrane as an alternative substrate for PLA2, activate GPCR mediated cell signaling pathway that stimulates PPARs, inhibits NF-kB activity, and inhibit the NLRP3 inflammation cascade and so on. In agreement with our finding with interleukin-8, Hadfield (2017) showed that flaxseed supplement decreased CXCL8. Matsuyama *et al*. (2005) showed that TNFα and IL-8 levels decreased significantly in the n-3 group compared with the n-6 group.

Interleukin-8 is important as a mediator in response to the host to tissue and inflammatory damage and is also important as a neutrophil activator, neutrophil chemotactic, and basophil. This cytokine is secreted by various cells such as monocytes, T cells, neutrophils, and endothelial cells in the pathological and inflammatory stages (Poorghasemi *et al.,* 2015).

Interleukin-8 is promptly stimulated in response to pro-inflammatory cytokines, such as interferon-α and cellular pressures. Activating neutrophils by interleukin-8 produces enzymes that can cause tissue damage and ulceration. Poorghasemi *et al*. (2015) reported in their test results that α-linolenic has anti-inflammatory effects and its use by reducing the mechanism of inflammatory markers such as N-Telopeptides and a-TNF reduces inflammation and tissue damage.

Many studies have shown that a variety of specific fatty acids stimulate gluconeogenesis (Williamson *et al.,* 1966). Polyunsaturated fatty acids, especially the n-3 ones, increase the catabolism of fatty acids while decrease fat synthesis and esterification in the rodent liver (Ikeda *et al.,* 1998). Therefore, PUFA partitions fatty acids towards oxidation and prevents triglycerides and other esterified compounds production (Kumamoto and Ide, 1998). In support of this concept, lower accumulation of triglycerides was measured in rodent hepatocytes, which were exposed to PUFA (Ikeda *et al.,* 1998; Kumamoto and Ide, 1998).

Fatty acids change both neuropeptide and hormone concentrations and their receptors (Bhathena, 2006). Polyunsaturated fatty acids are substrates for thromboxanes, leukotrienes, and prostaglandins, which can act like hormones (Lakdawala and Grant-Kels, 2015). As seen in the current study, lambs on the LO diet had the highest blood concentration of insulin. Consistent with the present results, the researchers found that LO intake increased insulin levels and improved insulin resistance parameters (Cooper *et al.,* 2004). Also, they stated that LO not only releases insulin by cells but also increases insulin efficiency (Cooper *et al.,* 2004). Some studies have documented that high α-linolenic acid or a high ratio of n-3 to n-6 fatty acids stimulates insulin secretion from pancreatic β-cells (Itoh *et al.,* 2003; Wei *et al.,* 2010). Bhaswant *et al*. (2015) showed that n-3 PUFAs improves insulin sensitivity and secretion by regulating the apelin and other pathway and higher release of glucagon-like peptide 1 in the intestine. Higher glucose and insulin concentrations in virally infected animals

in the current study showed that α-linolenic acid diets suppress inflammation in finishing lambs, which consequently may lead to improved feed intake and weight gain.

Researchers reported that higher α-linolenic acid intake decreases insulin resistance (Muramatsu *et al.,* 2010). Al-Hasani and Joost (2005) showed that lowering the ratio of n-6 to n-3 fatty acids in the rodent diet can increase insulin sensitivity by increasing PPARγ activity (Al-Hasani and Joost, 2005). Bhathena (2000) showed that in contrast with PUFA, trans and saturated fatty acids decrease insulin level, which leads to insulin resistance. In a human study by Kurotani *et al.* (2012), α-linolenic acid inclusion improved glucose homeostasis and increased adiponectin level. Thus, the current study showed that when inflammation occurs in finishing lambs, n-3 fatty acid diets improve insulin sensitivity and increase blood glucose, which consequently enhances the uptake of glucose and amino acids into muscle cells which should improve daily weight gain.

In ruminants, transcription factors such as SREBP1, PPARα, and PPARγ regulate the mRNA level of the stearoyl-CoA desaturase (SCD) enzyme. Ebrahimi *et al.* (2014) showed that goats who received diets enriched with α-linolenic acid had upregulation of PPARα and PPARγ but downregulation of the SCD gene compared to goats who received a diet enriched with linoleic acid. Insulin stimulates lipogenesis and incorporation of amino acids into protein and inhibits lipolysis and proteolysis (Ladeira *et al.,* 2016; Sandri *et al.,* 2018). Also, higher plasma insulin concentrations enhance nutrient uptake by muscle and adipose tissues and partition the nutrients to the mammary gland, which is not insulin-responsive (Zhao *et al.,* 1996). Higher plasma insulin and glucose levels may result in a decrease in lipogenesis which consequently may lead to higher weight gain and lower fat to protein ratio in carcasses of virally infected finishing lambs receiving a long-term α-linolenic acid diet.

In lamb, most serum T3 and rT3 produces by mono deiodination of T4 in peripheral tissues and only a small amount of serum T3 production is done in the thyroid gland (Fisher *et al.,* 1972). It shows that a long-term high n-3 PUFAs diet intake would improve thyroid hormone action in the liver (Souza *et al.,* 2010). A high level of T3 increases feeds intake at the level of the hypothalamus (Kong *et al.,* 2004). The overall effects of T4 are to increase protein synthesis, lipid metabolism, basal metabolic rate and to provide more glucose to cells (Capen and Martin, 1989). Rozing *et al.* (2012) reported that pro-inflammatory cytokines lower peripheral thyroid hormone levels during inflammation. Some researchers have shown a negative relationship between T3 and innate interleukins (Boelen *et al.,* 1996). Thus, suppressed inflammation in finishing lambs on α-linolenic acid diet results in higher T3 and T4 hormones in lambs, which leads to improved feed intake, feed efficiency, and production performance.

Conclusion

The findings from this experiment showed that the inclusion of α-linolenic acid but not linoleic acid in diets of virus-infected lambs suppressed pro-inflammation by lowering the expression of IL-8 and IL-4 mRNA. This increased blood glucose, insulin, T3, and T4, which may lead to higher weight gain and feed efficiency of virally infected lambs. They bind to specific receptors and cause-specific biological changes in the cells and damage to them because IL-8 and IL-4 cytokines act on specific cells through a specific receptor. Therefore, the present experiment confirmed that the presence of α-linolenic in the diet of lambs by inhibiting these cytokines can affect their growth and production performance as well as their health.

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Conflict of interests

The authors declare that they have no conflicts of interest.

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