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EFFECT OF ESSENTIAL OIL COMBINATION ON PERFORMANCE, MILK COMPOSITION, BLOOD PARAMETERS AND PREGNANCY RATE IN EARLY LACTATING DAIRY COWS DURING HEAT EXPOSURE

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ABSTRACT

The objective of this study was to determine effect of an essential oil combination (EOC), which contained cinnamaldehyde and diallyl disulfide on performance, milk composition, blood parameters and pregnancy rate of early lactating dairy cows during heat exposure. Twenty five Holstein cows (days in milk= 37.4±3.09) were assigned to one of two treatment groups: a Control (n=12) and EOC fed (n=13). Cows were fed a total mixed ration comprising concentrate and silage of common vetch with triticale. The concentrate differed only in the supplementation of EOC at 25 mg/kg concentrate (as fed basis). The experiment lasted 11 weeks. Dry matter intake (DMI) and milk production were measured daily while milk samples were taken twice a week. Blood samples were collected weekly, and ultrasonography was performed at 29 d and 42 d post TAI to determine pregnancy rate. Average of ambient temperature, relative humidity and temperature-humidity index (THI) were 25.9°C, 73.4% and 76.8, respectively. The EOC supplementation had no effect (P> 0.05) on performance, milk composition and pregnancy rate. The EOC, however, increased (P< 0.01) insulin concentration, and tended to decrease (P= 0.074) serum total cholesterol concentration, and increase (P= 0.097) NEFA concentration. In conclusion, EOC supplementation in diets of early lactating dairy cows during heat exposure did not affect milk yield and composition, and pregnancy rate. The increase of insulin and reduction of total cholesterol observed in EOC group needs to be confirmed with further research.

Key words: Essential oil combination, Cinnamaldehyde, Cholesterol, Diallyl disulfide, Heat stress, Insulin.

INTRODUCTION

Heat stress is defined as any combination of environmental conditions (such as temperature, relative humidity, and solar radiation) that will cause the effective temperature of the environment to be higher than the temperature range of the animal's temperature zone / thermal neutral zone (Farooq *et al.,* 2010). Temperatures above the thermoneutral zone initiate physiological, anatomical, and behavioral responses (such as reduction of DMI intake, decline of performance, increase of respiratory rate and body temperature, increase peripheral blood flow) in the animal's body (Serbester *et al.,* 2005, Bohmanova, 2006). The purpose of these responses is to increase heat loss and reduce heat production in an attempt to maintain body temperature within the range of normality (Bernabucci *et al.,* 2010).

A number of *in vitro* experiments have shown that essential oil or active compounds have potential manipulation of ruminal fermentation (Cardoza *et al*., 2004; Kongmun *et al*., 2010). Cinnamaldehyde and diallyl disulfide are active compounds in cinnamon and garlic oils, respectively (Busquet *et al.,* 2006). Cardozo *et al.* (2005) reported that cinnamaldehyde decreased ruminal proteolysis. Reduced degradation of protein in rumen may prevent to increase body temperature under heat stress (Arieli *et al.*, 2004). Diallyl disulfide has been shown to decrease methane production approximately by 70% (Busquet *et al*., 2005a; Busquet *et al.,* 2006) and improved digestibility and energy utilization efficiency (Klevenhusen *et al.,* 2011). This action of diallyl disulfide is related to inhibitioon of 3-Hydroxymethylglutaryl Coenzyme A (HMG-CoA) reductase, one of the major enzymes involved in the cell wall synthesis of methanogens (Busquet *et al.,* 2005a,b). However, suppression of HMG-CoA reductase can decrease hepatic synthesis of cholesterol (Gebhardt and Beck, 1996). It is well known that cholesterol is a precursor of steroid hormone such as progesterone. Also, garlic and main components have vasodilatory effect which may be beneficial to dissipate body core temperature. Anim-Nyamea *et al*. (2004) reported that garlic supplementation was associated with a increase blood flow to peripheral tissues, this might be provided by the vasodilatory effect.

Combinations of phytoactive compounds of different essential oils may result in additive and/or synergetic effects (Benchaar *et al*., 2009). As a result improving of microbial fermentation and nutrient utilization in rumen may improve energy status and pregnancy rate in early lactation under heat stress condition.

The present literature has limited information about the effects of cinnamaldehyde and diallyl disulfide supplementation on serum metabolites and hormone concentrations, and pregnancy rate of early lactating dairy cows during heat stress. Therefore, the objective of this study was to determine effect of an essential oil combination, which contained cinnamaldehyde and dially disulfide on performance, milk composition, blood parameters and pregnancy rate in early lactating dairy cows during heat exposure.

MATERIALS AND METHODS

Geolocation and experimental animals: Animals were maintained under protocols approved by the University of Cukurova Institutional Animal Care and Use Committee (Adana, Turkey). The experiment was conducted at The Research and Application Dairy Unit at Cukurova University and continued from 09 May to 25 July (11 weeks). Minimum and maximum temperatures during the experimental months were 15.3°C and 33.4°C; 18.3°C and 38.2°C; 21.3°C and 35.6°C, respectively. Similarly, average relative humidities in the experimental months were 69.2%, 72.2%, and 76.7% , respectively.

Twenty five lactating Holstein dairy cows were housed in individual stalls. The mean parity number, BW, DIM, milk yield, and BCS was 2.2±0.21, 514±12.57 kg, 37.4±3.09, 29.8±1.23 kg, and 2.93±0.29, respectively (mean±SD).

Feeding and synchronization protocol: Cows were individually fed a total mixed ration comprising (on a DM basis) 40% silage of common vetch with triticale and 60% concentrate feed. The concentrate was the same for both treatment groups, and differed only in the supplementation of EOC (encapsulated combination of cinnamaldehyde and diallyl disulfide, Next Enhance® 300, Novus Int., Spain) at 25 mg/kg concentrate (as fed basis). The composition of concentrate and nutrient composition of the individual feeds are presented in Table 1. All cows were fed twice daily for *ad libitum* consumption.. The refusals were collected and weighted daily.

The experiment was started after 2 weeks of adaptation to the stalls and feeding regime. All cows were fed the control diet during the pre treatment periods. Last day of the pre treatment periods, cows were blocked according to parity, body weight, days in milk, and milk yield and were randomly assigned to one of two treatments: Control diet (n= 12) and EOC diet (n= 13).

Presynch-Ovsynch protocol (El-Zarkouny *et al.*, 2002) was conducted in the experiment. Briefly, cows in both groups were presynchronized with 2 injections of PGF2α (500 µg, im, cloprestenol sodium, Egevet, Turkey) given at -14 and 0 d at the adaptation period. Twelwe days after the last PGF2α injection, Ovsynch protocol was beginned d 12 GnRH (10 µg, im, buserelin acetate, Receptal, Intervet, Turkey); d 19 PGF2α; d 21 GnRH injection, 16 h after last GnRH cows were timed artificial insemination (TAI). Transrectal ultrasonography (Falcovet 100 with 7.5 Mhz lineer-array trans-rectal transducer, Esaote/Pie Medical Equipment Co., Maastricht, The Netherlands) was performed at 29 and 42 d post-TAI to determine pregnancy rate.

Sampling and measurements: The percentages of silage and concentrate were adjusted weekly on an as fed basis to reflect changes in DM content of the silage and concentrate. The DM content of the silage was determined at 60°C for 48 h, whereas DM content of concentrates were determined at 105°C for 24 h. Crude protein, ether extract and crude ash of the feed samples were analysed according to procedures of Association of Official Agricultural Chemists (AOAC, 2000). Neutral detergent fiber (NDF) by using heat stable α-amylase and Na-sulfite and ADF were determined with Van Soest *et al*. (1991) procedures using an Ankom apparatus (Ankom® Tech. Corp., Fairport, NY, USA) without correcting for residual ash.

Cows were milked twice daily, and milk yield was recorded at each milking for individual cows. Individual milk samples were collected twice weekly from consecutive morning and afternoon milkings and analyzed for somatic cell count (CellCount, DeLaval, Sweden), total solids, fat, protein, lactose, casein, and urea-N and citric acid (Milkoscan FT 120, FOSS Electric, Hillerd, Denmark).

Body condition score and weight were determined weekly; BCS of the cows was assessed independently by three persons on a five-point scale (1= thin and 5= fat) with interval of 0.25 (Ferguson *et al*., 1994). The average of these three assessments of BCS was used in the data analysis. Measurement of body weight was recorded on 2 consecutive days after the morning milking and before the morning feeding.

Blood samples (10 ml) were collected at approximately 4 h post morning feeding weekly from the jugular vein into two plain evacuated serum tubes (BD Vacutainer Systems, Plymouth, UK). All blood samples were transported to the lab in an ice bucket, and serum was separated by centrifugation (Universal 320R, Hettich, Germany) at 1600 x *g at* 4°C for 15 min and stored at -20°C until assays for glucose, total cholesterol, non-esterified fatty acids (NEFA), IGF-I, insulin and

progesterone. Serum was analyzed on automated clinical chemistry analyzers. Analytical specifications are presented in Table 2.

Dry bulb temperature (Tdb), dew point temperature (Tdp), and relative humudity (RH) were recorded hourly by weather stations (Vantage Pro2, Davis Instruments, USA) near the experiment unit. Temperature and RH accuracy were within ±0.5°C and 3%, respectively. Wet bulb temperature (Twb) was calculated as reported by Bohmanova (2006). The temperature-humidity index (THI) was calculated as:

THI= (0.35 x Tdb + 0.65 x Twb) x 1.8 + 32 (Bianca, 1961).

NRC (2001) equations were used to estimate energy balance and net energy for maintenance and milk energy.

Statistical analysis: Statistical analyses were done using SAS programme (SAS, 2000). All daily data were averaged to weekly means. Descriptive statistical analysis revealed that SCC, progesterone, IGF-I, and insulin concentrations were not normally distributed. Therefore, these traits were logarithmically transformed prior to analysis. The transformed data were used to calculate P values while least squares means and standard errors in table are not log-transformed.

Data (exception of pregnancy rate) were analyzed by ANOVA as repeated measures (Littell *et al*., 2000; Akbaş *et al*., 2001) using the MIXED procedure of SAS with effects of treatment, week, interaction between treatment and week. The estimation method was the REML, and the degrees of freedom method was Kenward-Rogers (Littell *et al*., 2000). The compound symmetry, unstructured, first-order autoregressive, and toeplitz variance-covariance structures were tested and best fitted the model was chosen based on the smallest Schwartz's Bayesian Criterion (Littell *et al*., 2000; Eyduran and Akbaş, 2010). Pregnacy rate were analyzed using the GENMOD procedure of SAS. Significance was declared at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$. Results were reported as least squares means.

RESULTS

Average weekly Tdb, RH and THI during the study were 25.9°C, 73.4%, and 76.8, respectively (Fig. 1). Dry matter intake averaged 21.21 kg/d and DMI were not different (Table 3) for Control and EOC cows. Milk yield, body weight, and body condition score were similar (P> 0.05) in Control and EOC cows. There was no difference in respect to body weight change and energy balance among treatments (P> 0.05).

Milk composition did not vary (P> 0.05) among treatment groups. However, an interaction (P= 0.042) between treatment and week was observed for MUN. Also, somatic cell count did not different among treatment groups.

Serum NEFA concentration was tended to increase (P= 0.097) by feeding EOC (Table 4). Concentration of serum total cholesterol tended to be lower (P= 0.074) for EOC cows compared with Control cows whereas serum glucose, IGF-I, and progesterone concentrations were unaffected (P> 0.05) by supplemental EOC. Serum insulin concentration was increased significantly (P< 0.01) by feeding EOC.

Pregnancy rates at 28 d and 42 d after TAI were not significantly (P> 0.05) affected by supplemental EOC (Table 5).

Table 1. Composition of concentrate mixed into the TMR and nutrient composition of the individual feeds

	Control	**EOC**	
Ingredients (g/kg of DM)			
Barley	120.6	120.6	
Corn	59.4	59.4	
Wheat bran	16.2	16.2	
Wheat middlings	120	120	
Corn gluten meal	60	60	
Sunflower meal	16.98	16.98	
Soybean meal	90.54	90.54	
Canola meal	60.78	60.78	
EOC	-	0.025	
Molasses	25.32	25.32	
Protected fat[a]	13.38	13.38	
Salt	3.36	3.36	
Limestone	12.78	12.78	
Vitamins/Minerals premix[b]	0.66	0.66	
Chemical composition	**Control**	**EOC**	**Silage**
Dry matter (g/kg)	891	891	475
Crude protein (g/kg of DM)	211	210	83
Neutral detergent fiber (g/kg of DM)	236	230	486
Acid detergent fiber (g/kg of DM)	100	97	328
Ether extract (g/kg of DM)	44	47	19
Crude ash (g/kg of DM)	80	76	98
NEL (Mcal/kg of DM)[c]	1.64	1.64	1.48

[a] Contained (dry matter basis): free fatty acids, 0.05%; iyot value, 20.5; palmitic acid (C16:0), 72.5%; stearic acid (C18:0), 5.4%; oleic acid, (C18:1) 16.4% and linoleic acid (C18:2), 3.4%.

[b] Contained (dry matter basis): Mn (as Mn sulphate), 50.000 mg/kg; Fe (as Fe sulphate), 50.000 mg/kg; Zn (as Zn sulphate), 50.000 mg/kg; Cu (as Cu sulphate), 10.000 mg/kg; Co, 150 mg/kg; I (as Ca Iodate), 800 mg/kg; Se (as Na selenite), 150 mg/kg; niacin, 150.000 mg/kg; vitamin A, 15.000 IU/kg; Vitamin D3, 3.000 IU/kg and vitamin E, 30.000 mg/kg.

[c] Calculated according to Cornell Net Carbohydrate and Protein Systems (V6.1.36).

Table 2. Analytical specifications for serum glucose, total cholesterol, IGF-I, progesteron, insulin, NEFA concentrations

Item	Instrument	Method	Interassay CV (%)	Intraassay CV (%)
Glucose	Cobas (Roche Diagnostics, Indianapolis, USA)	Enzymatic colorimetric	4.95	10.45
Total cholesterol	Cobas (Roche Diagnostics, Indianapolis, USA)	Enzymatic colorimetric	2.51	9.17
IGF-I	Immulite 2000 Systems Analyzer (Siemens Healthcare Diagnostics, Gwynedd, UK)	Solid phase enzyme-labeled chemiluminescent immunometric assay	5.83	12.50
Progesteron	Cobas E 170 (Cobas, Roche Diagnostics, Basel, Switzerland)	Electrochemiluminescence immunoassay	3.97	14.34
Insulin	RT-2100 (Cusabia, China)	ELISA	4.65	15.23
NEFA	Keylab (BDC+ Biosed, Italy)	Enzymatic colorimetric	4.65	8.97

Table 3. Effect of feeding essential oil combination on performance and milk composition of early lactating dairy cows during heat exposure

Item	Treatment		SED[b]	P value		
	Control	EOC[a]		T[c]	W[d]	T x W
Dry matter intake (kg/d)	20.90	21.51	0.552	0.281	<.0001	0.324
Milk yield (kg/d)	28.34	27.41	1.070	0.389	<.0001	0.641
Body weight (kg)	488.39	492.85	20.571	0.831	<.0001	0.168
Body weight change (kg/week)	-3.95	-4.92	1.123	0.392	0.004	0.306
Energy balance (Mcal/d)	4.11	5.11	0.698	0.160	<.0001	0.912
Body condition score	2.84	2.83	0.071	0.896	<.0001	0.213
Milk composition						
Total solids (%)	11.50	11.20	0.229	0.585	<.0001	0.608
Fat (%)	3.23	3.32	0.174	0.584	<.0001	0.752
Protein (%)	2.77	2.85	0.062	0.192	<.0001	0.367
Lactose (%)	4.70	4.65	0.054	0.326	<.0001	0.141
Casein (%)	2.26	2.31	0.054	0.408	<.0001	0.666
Milk urea-N (mmol/L)	7.60	7.80	0.228	0.410	<.0001	0.042
Citric acid (mmol/L)	5.52	5.73	0.338	0.538	<.0001	0.739
Somatic cell count (x 1000/mL)	203.94	182.35	71.560	0.759	0.484	0.943

[a] EOC= essential oil combination of cinnamaldehyde and dially disulfide.
[b] SED= standard error of the difference between means.
[c] T= treatment.
[d] W= week.

Table 4. Effect of feeding essential oil combination on concentrations of metabolites and hormones of early lactating dairy cows during heat exposure

Item	Treatment		SED[b]	P value		
	Control	EOC[a]		T[c]	W[d]	T x W
NEFA (µEq/L)e	876.13	904.66	21.664	0.097	<.0001	0.194
Glucose (mmol/L)	1.92	1.95	0.103	0.726	<.0001	0.913
Total cholesterol (mmol/L)	4.25	3.82	0.294	0.074	<.0001	0.867
Insulin (mIU/mL)	81.17	112.73	10.530	0.005	0.007	0.052
IGF-I (nmol/L)	9.46	9.63	1.479	0.916	<.0001	0.635
Progesterone (nmol/L)	7.25	7.20	0.090	0.646	<.0001	0.832

[a] EOC= essential oil combination of cinnamaldehyde and dially disulfide.
[b] SED= standard error of the difference between means.
[c] T= treatment.
[d] W: week.
[e] NEFA= nonesterified fatty acids.

Table 5. Effect of feeding essential oil combination on pregnancy rate of early lactating dairy cows during heat exposure

Item	Treatment			
	Control	EOC[a]	SED[b]	P value
Pregnancy rate	16.7% (2/12)	30.8% (4/13)	0.980	0.405

[a] EOC= essential oil combination of cinnamaldehyde and dially disulfide.
[b] SED= standard error of the difference between means.

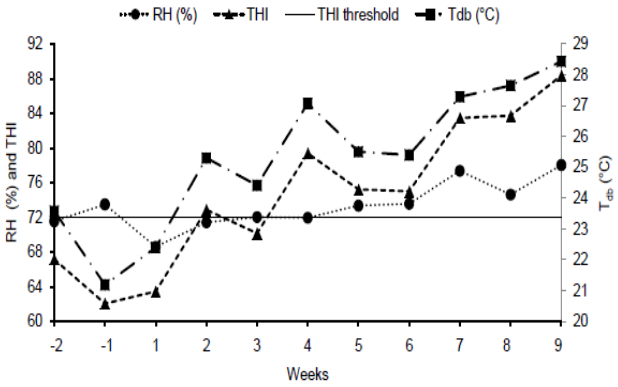

Fig 1. Average weekly dry bulb temperature (Tdb, °C), relative humidity (RH, %) and temperature-humidity index (THI)

DISCUSSION

Up to our knowledge, the present study is the first one investigating effects of cinnamaldehyde and diallyl disulfide supplementation on serum metabolites, hormone concentrations, and pregnancy rate of early lactating dairy cows under heat exposure. Average weekly THI was 76.8. It was 4.8 units above 72 that was suggested as the threshold value by Armstrong (1994). Except for pre treatment period and weeks 1 and 3 in treatment period, cows were under heat stress. This is more prominent for weeks 4, 7, 8, and 9.

In the present study, EOC did not affect feed intake, milk yield, milk composition. Few studies (Yang *et al*., 2007; Benchaar *et al*., 2008; Benchaar and Chouinard, 2009; van Zijderveld *et al*., 2011) have been investigated the effects of cinnamaldehyde and dially disulfide on feed intake, milk production and compositon of dairy cows. Similarly, they found no differences in DMI, milk yield, milk composition with the diet supplemented EOC. Yang *et al*. (2007) observed that feeding of cows with garlic (5 g/day) had no effect on milk composition. van Zijderveld *et al.* (2011) did not observe any change in milk composition when diallyl disulfide was fed at level of 56 mg/kg of DM as well. Higher dose (200 mg/kg of DM) of dially disulfide decreased only milk fat level and garlic odor was detected in milk (van Zijderveld *et al*., 2011). Higher feed intake capacity of dairy cows may mask effects of essential oil on rumen fermentation and lactational performance. The results of the *in vitro* studies using essential oil or active compounds have given promising results by improving ruminal condition and modifying ruminal fermentation (Cardoza *et al*., 2005; Busquet *et al*., 2005a,b; Busquet *et al*., 2006) but not *in vivo* studies. This inconsistency betwen *in vitro* and *in vivo* results may be attributed to dynamic rumen condition in animal experiment, dietary and environmental conditions.

The EOC cows were slightly higher MUN level than Control cows. There was an interaction between treatment and week for MUN. Relatively lower THI at 5 and 6 weeks than 4 week (Fig. 1), increased DMI 1 and 4 kg/week, respectively (data not shown). Higher MUN level could be associated with increased DMI. However, it was reported that cinnamaldehyde inhibited peptidolysis (Cardozo *et al.,* 2005). Under heat stress conditions, reduced degradation of protein can be useful nutritional modification to alleviate for body temperature increase (Arieli *et al.,* 2004). Our findings with respect to MUN did not support the results of Kamel *et al*. (2009). In the study of Kamel *et al*. (2009) feeding of EOC in early and late lactating dairy cows reduced MUN and tended to increase milk protein level. The inconsistency between our finding and Kamel *et al*. (2009) might be attributed to different feeding regime (concentrate-based diet *vs* forage-based diet).

Feeding EOC did not affect somatic cell counts in the present study. Somatic cell counts remained unchanged in other experiments. For instance, Benhaar *et al*. (2009) reported that somatic cell counts were not affected in early lactating cows fed at 1 g/d of cinnamaldehyde supplement. Nevertheless, *trans*-cinnamaldehyde reduced mastitis pathogen population (such as *Staphylococcus aureus* and *Streptococcus agalactiae*) to undetectable levels on d 12 of the *in vitro* experiment (Ananda Baskaran *et al*., 2009).

Supplementing the diet of lactating dairy cows with EOC reduced total cholesterol concentrations (EOC: 3.82 mmol/L *vs* Control: 4.25 mmol/L). Garlic oil or its orgonosulfur compounds (namely *S*-allyl cysteine, diallyl disulfide or allyl mercaptan) can interfere with cholesterol biosynthesis (Gebhardt and Beck, 1996; Miller and Wolin, 2001).). The mechanism of action garlic or its active compounds could be related to inhibition of HMG-CoA reductase (Busquet et al. 2005a; Busquet et al. 2006). Thus, HMG-CoA reductase inhibitors have potential to inhibit cholesterol biosynthesis (Kongmun *et al*., 2010).

Although concentrations of glucose, IGF-I did not significantly differ between treatments, feeding EOC increased serum insulin concentrations (EOC: 112.73 mIU/mL *vs* Control: 81.17 mIU/mL). Liu *et al*. (2005) and Xie *et al*. (2011) reported that cinnamaldehyde and

garlic oil enhanced release of insulin β cells stimulation. Also, *in vitro* incubation of pancreatic islets with cinnamaldehyde promoted insulin relaese (Anand *et al*., 2010). However, it is know that insulin has antilipolitic effect. Therefore, diets that elevate circulating insulin may have expected to inhibite lipolysis and reduce NEFA concentrations (Corl *et al*., 2006). Higher serum NEFA concentration in EOC cows did not support this expectation. Nevertheless, essential oil could reduce hepatic uptake, re-esterification, or oxidation of NEFA (Whitney and Muir, 2010). Our findings with respect to NEFA concentrations supported the results of Whitney and Muir (2010). They reported that the effect of essential oil or active compounds was primarily on hepatic functions rather than lipid catabolism.

Pregnancy rate in early dairy cows possitively related with high blood insulin and IGF-I which associated with energy balance (Santos, 2008). High plasma insulin level for EOC cows in the present study were not increased pregnancy rate. Increasing circulating insulin concentrations during the early post partum period can advance the resumption of oestrous cycles by enhancing follicular growth (Santos, 2008). However, high concentrations of insulin can be both detrimental to the developmental competence of oocytes, and also associated with the incidence and characteristics of abnormal ovarian cycles (Lucy, 2003). Futhermore, IGF-I is important factor for pregnancy rate. IGF-I may interact with insulin to influence cell proliferation and estradiol production (Garnsworthy *et al*., 2008; Garnsworthy *et al*., 2009). These hormones are related to energy balance. However energy status of EOC cows were not higher than control cows. Increase in plasma insulin level could not be, therefore, improved pregnancy rate in the present study. There is no published study about the effects of EOC supplementation to reproductive performance or pregnancy rate of dairy cows. Recently, a few studies investigated the effect of supplemental yeast, which has been used to alter rumen fermentation, on reproductive performance. Supplemental yeast did not affect resumption of ovarian activity, pregnancy rate or pregnancy losses of dairy cows under heat stress (Bruno *et al*., 2009) or thermoneutral (Kalmus *et al*., 2009; Allbrahim *et al*., 2010) conditions.

Conclusion: Feeding of EOC in early lactating dairy cows during heat exposure did not affect milk yield and composition, and pregnancy rate. However feeding of EOC increased serum insulin concentration, tended to increase serum NEFA concentration, and decrease total cholesterol concentration. The increase of insulin and reduction of total cholesterol observed in EOC group needs to be confirmed with further research.

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