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BLOOD HORMONES, METABOLIC PARAMETERS AND FATTY ACID PROPORTION IN DAIRY COWS FED CONDENSED TANNINS AND OILS BLEND*

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Abstract

The aim of this study was to track the changes in blood parameters of mid-lactation multiparous Polish Holstein-Friesian cows in response to a diet supplemented with a mixture of fish-soybean oils blend and tannin-containing lingonberry shrub (*Vaccinium vitis idaea*; VVI) extract. Twelve lactating cows were randomly assigned to a crossover design of two treatments (6 cows per treatment) which consisted of a control diet containing no supplement (CON) and CON supplemented with a mixture of 99 g of VVI leaves extract and 660 g of blended fish-soybean oils (MIX) daily. The obtained results showed a significant increase in plasma glucose level, as well as C18:1*t11* and *n-3* fatty acids proportion. A significant decrease was also observed in insulin concentration, triglyceride and C18:0 proportion. Generally, the saturated fatty acid proportion decreased while the unsaturated fatty acid significantly increased with the MIX diet. In conclusion, using supplements of a mixture of VVI extract and fish-soybean oils blend modulated the unsaturated fatty acid proportion in blood, without affecting the dairy cows' blood parameters which were all within the normal ranges.

Key words: ruminants, condensed tannin, oil, blood

There is a growing interest worldwide in so-called functional food, i.e. food containing components that offer beneficial effects on the human health (Lopez-Huertas,

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2010; Kiczorowska et al., 2017). Recently, research has demonstrated promising ways to increase favourable health components in animal products, commonly by the supplementation of ruminant diets with oils (Wencelova et al., 2016) or secondary plant metabolites, such as tannins (Toral et al., 2013). Marine and plant oils as dietary supplements are good sources of energy and unsaturated fatty acids (UFA) for ruminants (Cieslak et al., 2015). Furthermore they are able to alter the activity of rumen microorganisms and to modulate the composition of fatty acids (FA) in animal products (Cieslak et al., 2015). Plant secondary metabolites supplementation to ruminant diets, in turn, may influence on food intake, metabolic parameters and hormone regulation (Shingfield et al., 2013). Dietary tannins represent major plant secondary metabolites which show an ability to modulate ruminal fermentation, amino acid flow to the duodenum (Mueller-Harvey, 2006), muscle deposition and milk production (Vasta et al., 2008).

Noticeably, many studies clarified the effects of tannins or oils supplied separately into the diet on blood parameters (Steppa et al., 2014; Shakeri, 2016). To our knowledge there are no studies investigating the effects of a mixture of condensed tannins and oils blend on blood parameters, hormones and FA proportions. Based on previous *in vitro* study (Szczechowiak et al., 2016) it can be hypothesized that simultaneous application of condensed tannins and blended fish-soybean oil will also be effective in modulating the activity of rumen microorganisms and consequently FA proportions, hormones and metabolic blood parameters content *in vivo*. Thus, the objective of this study was to investigate the effect of a diet supplemented with a combination of extract from lingonberry shrub (*Vaccinium vitis idaea;* VVI), as a source of condensed tannins with a blend of fish-soybean oils, as the source of UFA, on metabolic parameters, FA proportions and concentrations of hormones regulating metabolic processes in the blood of dairy cows.

Material and methods

All experimental procedures were performed in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). The study was approved by the Local Ethical Commission (license permit no. 59/2013).

Experimental design, cows and treatments

The present study was carried out on a commercial farm in Szemborowo (Poland) using lactating dairy cows. Twelve multiparous Polish Holstein-Friesian cows (600±30 kg body weight) during their 5th–6th months of lactation were used in crossover design. Cows were randomly assigned to two dietary treatments, based on parity (2.4±0.2; mean ± SD) and milking days (155±9 d) at the start of the experiment (6 cows per treatment). The cows used in the present study were part of a larger *in vivo* experiment to investigate the effect of condensed tannins and fish-soybean oils blend mixture (MIX diet) on rumen fermentation, methane concentration, as

well as milk production and milk composition in dairy cows (Szczechowiak et al., 2016). The diets used *in vivo* were selected based on previous *in vitro* study, where condensed tannin-containing lingonberry shrub and fish-soybean oils blend were provided separately or as a mixture. Based on the obtained *in vitro* results, in the *in vivo* study only the mixture of tested supplements was evaluated because of the most promising results (elevating VFA in the fermented rumen fluid and decrease in methane concentration) obtained in *in vitro* trial. Hence, in the present experiment two diets were evaluated: control (CON) and control supplemented with mixture of condensed tannins and fish-soybean oils blend (MIX).

In the present experiment cows in CON were fed a partial mixed ration (PMR) plus 2 kg of concentrate (C). Cows in MIX were fed the same PMR plus 2 kg of concentrate but additionally supplemented with 99 g (4.83 g/kg dry matter (DM) of total diet) of extract from leaves of VVI and 660 g (32.2 g/kg DM of total diet) of blended oils (EC). The PMR consisted of maize silage (7.67 kg DM), lucerne silage (2.67 kg DM), ensiled beet pulp (2.40 kg DM), ensiled brewers grains (2.10 kg DM), extracted rapeseed meal (1.21 kg DM), grass silage (0.68 kg DM), mineral-vitamins mixture (0.20 kg DM) and a commercial concentrate (1.78 kg DM; 19 g of crude protein/100 g concentrate).

For an efficient administration, both supplements (leaves of VVI and blended oils) were mixed with concentrate. The EC was prepared at the beginning of each period and stored in a cold place. The concentrates (C and EC) were supplied using a feeder station (De Laval, type FP 204, Tumba, Sweden).

The extract from leaves of VVI, as the source of condensed tannins, was as one of the supplements. The plant material was purchased from a commercial company (Herbapol Poznań, Poland). The chemical composition of powdered VVI was as follows (in g/kg DM): organic matter (OM) (928±3.1), crude ash (72±2.3); crude protein (CP) (17±1.0); ether extract (EE) (3±1.3); no neutral detergent fibre (NDF) was detected. In order to determine the content of tannins in crude extract, a procedure described by Szczechowiak et al. (2016) was used. The extract of *Vaccinium vitis idaea* contained 37 g of tannins per kg DM. A blend (ration 1:1; v/v) of fish oil (AGRO-FISH, Kartoszyno, Poland) and soybean oil (ROLPASZ, Strzalkowo, Poland) was used as the second supplement. The PMR chemical composition, as well as the FA proportions of the PMR and concentrates (C and EC) used in the *in vivo* experiment are presented in Table 1.

Each of the two experimental cycles lasted 26 days, with 23 days of adaptation period and 3 days of sampling period. During the first 8 days of each adaptation period, the cows were fed PMR *ad libitum*. From day 9 onwards, the cows' feed was restricted to 95% of the average daily voluntary DM intake based on days 3 to 8 of the cow assigned to each treatment to avoid the confounding effects of DM intake. The daily DM intake was constant (20.0 ± 0.5 kg) during the experiment. Moreover, during the first 8 days of each adaptation period, only half amounts of the experimental factors were used. From day 9 onwards, the cows were fed PMR and 2 kg of concentrate with full amounts of respective supplement.

Table 1. Chemical composition and fatty acid proportion of the partial mixed ration (PMR), concentrate (C) and experimental concentrate (EC) (Szczechowiak et al., 2016)

Item	PMR	C	EC
Chemical composition (g/kg DM)			
organic matter	906	902	928
crude ash	93.9	97.9	72.3
crude protein	179	212	158
ether extract	28.6	30.3	260
neutral-detergent fiber	481	245	181
Fatty acid proportion (g/100 g FA)			
C14:0	0.72	0.29	2.50
C16:0	19.4	16.8	15.5
C18:0	2.65	2.24	3.59
C18:1*c9*	18.5	26.6	35.6
C18:2*c9,c12*	40.2	42.5	20.0
C18:3*c9,c12,c15*	8.35	3.27	3.77
C20:5*n-3*	nd	nd	0.08
C22:5*n-3*	nd	nd	0.38
C22:6*n-3*	nd	nd	1.97
SFA	26.0	22.0	23.6
UFA	74.0	78.0	76.4
MUFA	23.0	30.0	44.0
PUFA	51.0	48.0	32.4
n-6	42.2	44.3	24.4
n-3	8.58	3.56	7.93

SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, nd = not detected.

Sampling and chemical analysis of feed

Representative samples of PMR, C and EC were collected two times during each sample collection period and stored at −20°C until analysis. Samples of the PMR, C and EC were analyzed according to the AOAC (2007) for DM (method no. 934.01) and crude ash (method no. 942.05). The CP was determined by Kjel-Foss Automatic 16210 analyzer (method no. 976.05) and EE by Soxhlet System HT analyzer (method no. 973.18). The aNDF was estimated with amylase and sodium sulfite and expressed without residual ash, determined by the method of Van Soest et al. (1991). The OM was calculated from the difference between dry matter and ash. Fatty acid proportion in the feed was analyzed according to Szczechowiak et al. (2016).

Blood sampling and analyses

Blood samples were collected from the jugular vein into tubes containing clot accelerator and granule serum separator (serum separation, Deltalab, Barcelona,

Spain) from days 23 to 26 of the experiment, three hours after the morning feeding. Blood was left at room temperature for blood clot formation and then centrifuged at 2500 rpm for 10 min at 21°C to obtain serum. Serum was stored at −20°C until analyses for hormones, metabolites and FA. The glucose concentration was analyzed colorimetrically by an enzymatic method using peroxide-phenol-aminophenazone (Trinder, 1969). Free fatty acid (FFA) concentration in serum was determined according to Duncombe (1964), while triglyceride (TAG) concentration by a colorimetric method (Megraw et al., 1979). Total cholesterol was assayed by an enzymatic method developed by Trinder (1969) with cholesterol esterase and oxidase. Blood urea nitrogen (BUN), total-protein, β-hydroxybutyrate (BHBA), albumin as well as aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined spectrophotometrically using commercially available kits (Pointe Scientific, Warsaw, Poland). Serum insulin-like growth factor 1 (IGF-1) was measured by a competitive enzyme immunoassay, using the IGF-1 ELISA kit from DRG (DRG IGF-I 600 ELISA, USA). The kit was based on the principle of competitive binding. The specificity of the assay antibodies was 100% for IGF-1 and insignificant cross-reactivity was 1.2%. The minimum detectable concentration was 9.75 ng/mL. Concentrations of insulin, glucagon and leptin were determined radio-immunologically using RIA kits from Millipore (St. Charles, MO, USA). Triiodothyronine (T3) and tetraiodothyronine (T4) were assayed by RIA kits from Beckmann Coulter (California, USA). The FA proportion in blood samples was analyzed according to Szczechowiak et al. (2016), but initially three milliliters of 2 M NaOH was added to 1000 mg of serum.

Statistical analysis

The experiment was conducted according to assumptions of the crossover study. The influence of treatment on fatty acids proportion, hormone levels and other biochemical components in cow's serum was evaluated using a linear mixed model with the lmer procedure from lme4 library under R environment (R Development Core Team, 2008). The model included treatment, group (dietary treatment sequence), period as fixed effects and random effect of cow within a group. Normality of distribution of residuals in an ANOVA model was tested using the Shapiro-Wilk test (Shapiro test procedure under R). Results were presented as arithmetic means, which were determined for all traits. In addition, the pooled standard errors of means were calculated (standard error procedure from plotrix library, R environment). Differences were considered statistically significant at $P<0.05$.

Results

Glucose was significantly elevated when the MIX diet was fed ($P<0.001$; Table 2). Nevertheless, TAG was reduced ($P<0.001$), whereas FFA, BHBA and total cholesterol were unaltered when animals were fed MIX (Table 2). The MIX

diet resulted in a significantly decreased insulin concentration (Table 2; P<0.015), whereas the glucagon concentration remained unchanged. The IGF-1 concentration was also decreased after the MIX (P<0.038). Leptin concentration was significantly elevated (P<0.025) after the MIX diet and T3 and T4 were not influenced. Moreover, a significant decrease (P<0.048) of AST and ALT activities were observed.

Table 2. Effect of a mixture of *Vaccinium vitis idaea* and oils blend (MIX) diet on metabolic parameter concentrations in blood serum

Serum parameters	CON	MIX	SEM	P-value
Protein and energy markers				
total protein (g dl^{-1})	6.27	6.33	0.046	0.701
albumin (g dl^{-1})	3.17	3.12	0.021	0.210
blood urea nitrogen (mmol l^{-1})	9.21	9.30	0.336	0.912
glucose (mmol l^{-1})	3.31	3.50	0.035	<0.001
free fatty acids (mmol l^{-1})	0.68	0.67	0.503	0.259
β-hydroxybutyrate (mmol l^{-1})	0.87	0.89	0.021	0.402
triglyceride (mmol l^{-1})	0.25	0.19	0.007	<0.001
Hepatic and lipid markers				
aspartate aminotransferase (IU l^{-1})	40.0	37.6	1.171	0.048
alanine transaminase (IU l^{-1})	19.2	17.4	0.438	0.009
total cholesterol (mmol l^{-1})	4.65	4.69	0.028	0.628
Hormone markers				
insulin (μU ml^{-1})	20.6	17.3	0.775	0.015
glucagon (pg ml^{-1})	197	194	3.723	0.228
leptin (ng ml^{-1})	4.05	4.59	0.170	0.025
triiodothyronine (ng ml^{-1})	2.61	2.77	0.049	0.110
tetraiodothyronine (ng ml^{-1})	50.0	47.7	0.759	0.157
insulin-like growth factor 1 (ng ml^{-1})	46.4	43.2	0.896	0.038

SEM = standard error of the mean.

There was a significant decrease (P<0.001) in C18:0 proportion after the MIX diet treatment (Table 3). In contrast, C16:1*c9*, *C18:1t10*, C18:1*t11* and C18:1*c9* proportions were significantly increased (P<0.007) by 16%, 21%, 66% and 7%, respectively. Likewise, C18:2*c9,c12* proportion in blood was decreased by MIX (P<0.038), and in contrast, C18:3*c9,c12,c15*, C20:4*n-6*, C20:5*n-3*, C22:5*n-3* and C22:6*n-3* increased (P<0.001). The saturated fatty acids (SFA) after the MIX diet were decreased (P<0.001), whereas the UFA and monounsaturated fatty acids (MUFA) increased (P<0.001) (Table 3).

Table 3. Effect of a mixture of *Vaccinium vitis idaea* and oils blend (MIX) diet on the blood serum fatty acid proportion (g/100 g FA)

Item	CON	MIX	SEM	P-value
Saturated				
C8:0	0.17	0.16	0.010	0.621
C12:0	0.37	0.41	0.022	0.313
C14:0	0.77	0.84	0.031	0.260
C16:0	12.0	12.0	0.104	0.737
C18:0	18.8	17.7	0.150	<0.001
Monounsaturated				
C16:1*c9*	0.58	0.67	0.013	<0.001
C18:1*t10*	0.34	0.41	0.011	0.007
C18:1*t11*	0.84	1.39	0.038	<0.001
C18:1*c9*	8.65	9.26	0.118	<0.001
Polyunsaturated				
C18:2*c9,c12*	37.6	36.5	0.366	0.038
C18:2*c9,t11*	0.15	0.15	0.008	0.619
C18:2*t10,c12*	0.02	0.02	0.001	0.823
C18:3*c9,c12,c15*	2.96	3.19	0.034	<0.001
C20:4*n-6*	0.51	0.60	0.010	<0.001
C20:5*n-3*	0.04	0.51	0.023	<0.001
C22:5*n-3*	0.04	0.75	0.032	<0.001
C22:6*n-3*	0.04	0.22	0.010	<0.001
long chain FA	84.2	84.1	0.154	<0.001
medium chain FA	15.1	15.3	0.136	0.551
SFA	40.5	38.7	0.270	<0.001
UFA	59.5	61.3	0.270	<0.001
MUFA	14.1	15.6	0.189	<0.001
PUFA	45.6	45.7	0.358	0.407
n-6	42.1	40.9	0.359	0.043
n-3	3.42	4.96	0.080	<0.001
n-6/n-3	12.6	8.33	0.234	<0.001

SEM = standard error of the mean, SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

Discussion

The previous experiments (Szczechowiak et al., 2016) and present *in vivo* study only partially confirmed tested hypothesis. The MIX treatment selectively influ-

enced the microbial population, however, changes in tested blood parameters and hormones were observed. Trying to explain the results obtained in the present study it is necessary to take into account either changes in rumen microbial population or the possibility of the absorption of condensed tannins in the rumen, their passage from rumen fluid to the abomasum and the duodenum and their absorption in the intestine of ruminants (Perez-Maldonado and Norton, 1996).

In the current experiment significant changes in the FA proportion in blood serum were noticed without changes in the total FFA concentration in blood. As reported by Halmemies-Beauchet-Filleau et al. (2013) concentrations of some FA in arterial blood are associated with changes in the flow of FA at the omasum. The higher proportion of C18:1*t11* in MIX diet simultaneously with lower proportion of C18:2*c9,c12* indicated that biohydrogenation of FA by rumen bacteria could be the determinants of these FA in arterial blood of lactating cows. In addition, other studies (Halmemies-Beauchet-Filleau et al., 2013; Shingfield et al., 2013) reported that selective retention, elongation, desaturation, deposition, and mobilization of FA in several tissues including adipocytes and enterocytes may also influence plasma C18:0 and C18:1*c9* proportion. The higher proportion of C18:3*c9,c12,c15* indicated that MIX treatment could not influence the accessibility of these FA for absorption (Halmemies-Beauchet-Filleau et al., 2013). The C18:3*c9,c12,c15* can be converted in the liver to C20:4*n-6* by desaturases and elongases. Therefore, the noticed increase in C20:4*n-6* proportions in plasma FA, might be partly attributed to the increase in accessibility of precursors for elongated FA in the liver (Zachut et al., 2010). It is widely known that tannins bind to proteins, but what is less well known is that they also affect other feed components (i.e. lipids) to different degrees (Frutos et al., 2004). In the present study, the observed increase for C20:5*n-3,* C22:5*n-3* and C22:6*n-3* and other FA in blood, can indicate that the tannins from VVI have very little if any binding power.

Although FFA blood concentration and total cholesterol after the MIX diet were unchanged, a marked decrease in TAG concentration was observed. This effect could be the consequence of the activity of dietary ingredients since either tannins or fish-soybean oils blend have been documented to be important blood lipid decreasing factors in many species in physiological and pathophysiological conditions, reducing either TAG or cholesterol concentrations (Velayutham et al., 2012).

Blood glucose concentration was analyzed since its proper concentration in the blood of dairy cows is important for energy balance and lactose production in the mammary gland. Increased blood glucose in ruminants may be the consequence of elevated propionate production in the rumen. It is obvious that this volatile fatty acid (VFA) in ruminants is a precursor of glucose synthesis. Moreover, it stimulates glucagon secretion from the pancreas (Danfaer et al., 1995) and liver gluconeogenesis (Zhang et al., 2015; Klebaniuk et al., 2016). Therefore indirectly it is an important factor elevating the glucose concentration in blood. The elevated glucose concentration in the current experiment was independent of VFA concentrations (Szczechowiak et al., 2016), but could possibly be the consequence of changes in other FA proportions. More studies are needed to evaluate the effect of MIX in dairy cows on glucose concentration in blood.

Although insulin concentration in this study was markedly decreased, it is rather impossible that in dairy cows the elevated glucose concentration could be a consequence of this change. Generally, in most mammalian species including bovines, elevated plasma glucose stimulates elevated levels of insulin in blood. Thus it is not clear why in this experiment the cows fed the MIX diet had elevated plasma glucose concentrations, accompanied with decreased plasma insulin concentrations. Besides insulin, IGF-1 concentration was also decreased due to MIX diet administration. In the experiment of Moussavi et al. (2007) blood IGF-1 concentration was determined in dairy cows after feeding fish meal (rich in C20:5*n-3* and C22:6*n-3*) or Ca salts of fish oil. In these animals IGF-1 plasma concentration tended to be higher, as did insulin and milk yield. In turn, Fouladi-Nashta et al. (2009) found that after feeding dairy cows with full-fat toasted soybean neither blood IGF-1 nor insulin concentrations were altered. In this experiment the concentrations of both hormones were decreased. The MIX diet contained, in addition to fish and soybean oils, another important and bioactive ingredient – condensed tannins from VVI – which could be responsible for the obtained decrease in hormone concentration. However there are no data confirming the direct effect of tannins on IGF-1 concentration.

There is a lack of data concerning the effects of tannins on the leptin concentration in blood in any species. Long chain *n-3* PUFA probably manifest their activity as PPARγ activators and in this way increase the leptin concentration. In the current experiment either elevated leptin concentration or decreased TAG concentration could be a consequence of the increased *n-3* PUFA in blood serum.

Tannins may reduce protein degradation in the rumen by the formation of tannin-protein complexes and the reduction of the proteolytic bacteria population (Mueller-Harvey, 2006). Consequently, duodenal non-ammonia N increases as well as its absorption from the intestine (Barry et al., 1986). Thus, animal productivity is improved (Patra and Saxena, 2011). However, the MIX diet did not affect total protein, albumin and BUN concentrations in the blood of dairy cows, which suggests unaltered nitrogen metabolism. The explanation of this effect may be the presence of fish-soybean oils in the MIX diet, which may limit tannin action. It was found that fish oil can reduce the percentage of protein in cow milk (Petit et al., 2002), but there is no report about such an effect in blood. However, the source, the dose and the duration of treating animals with dietary tannins cannot be excluded when considering their effect on nitrogen metabolism. One of the consequences of unaltered blood protein concentration in this experiment may be unchanged thyroid hormone concentration since protein intake can modify this parameter (Gerrits et al., 1998). However, there was also one report that tannins decreased T3 concentration in sheep (Barry et al., 1986). Aminotrasferases – AST and ALT – are responsible for amino acid metabolism, moreover, their activity depends on the energy status of the organism. Higher activity of these enzymes in ruminants, especially AST activity, is manifested as a result of fatty liver syndrome (Cebra et al., 1997), frequent in dairy cows during early lactation. A positive effect of the diet used in this experiment, due to keeping the dairy cows in high lactation, is the decrease of blood AST and ALT activity after feeding MIX diet.

Another hallmark confirming the welfare of animals fed the MIX diet and lack of metabolic disorders was the unchanged BHBA concentration. The presence of fish and soybean oil in the MIX diet and to a lesser extent tannins could modulate the metabolic status of animals but no parameter reflecting lipid metabolism, which could evoke increased BHBA synthesis in liver, was increased.

Conclusions

In conclusion, despite the possible impact of tannins on lipids, it does seem that the blended oils were not completely inactivated by the tannins which resulted in elevated level of *n-3* long chain fatty acids and decreased the *n-6* PUFA proportion in blood. Hence, the condensed tannins and fish-soybean oils blend mixture may be a promising supplement directed to modulate the blood *n-3* PUFA, without noticeable negative effects on other blood parameters. As the final effect more *n-3* PUFA reach the mammary gland and may be secreted in milk.

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