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Oleic acid as a biomarker for early diagnosis of elevated blood levels of non-esterified fatty acids and β-hydroxybutyric acid in the early stages of lactation in high-yielding Polish Holstein cows

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Inappropriate doses and quality of dietary nutrients cause problems in providing the protein and energy balance in a feed ration. Especially, energy value of the feed ration poses many problems to dairy cattle breeders and particularly in the perinatal period, which results in increased incidence of metabolic disorders. These disorders are today one of the most frequent causes of culling of dairy cows, as they underlie most of the disease entities. The aim of this experiment was, therefore, to verify the hypothesis that oleic acid (OA) can be used as a biomarker for early diagnosis of elevated blood levels of non-esterified fatty acids (NEFAs) and β-hydroxybutyric acid (BHBA) in the early stages of lactation in high-yielding Polish Holstein (PHF) cows. The highest blood levels of NEFAs and BHBA of 1.573 and 1.116 mmolL^{-1}, respectively, was associated with the highest content of OA in milk fat. High concentrations of both NEFAs and BHBA, indicating explicitly the occurrence of the metabolic disease in cows, occur when the content of OA in milk exceeds 24g 100g^{-1} of fat. Oleic acid may be used as a biomarker for the early diagnosis of elevated blood levels of NEFAs and BHBA in the early stages of lactation in high-yielding PHF cows.

KEY WORDS: non-esterified fatty acids/β-hydroxybutyric acid/oleic acid/metabolic profiles

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Dairy cattle are one of the main species of the global animal production. High productivity of dairy cows enforces increased demand for nutrients and, thereby, for well-balanced feedstuffs adjusted for both living and production needs. Inappropriate doses and quality of dietary nutrients cause problems in providing the protein and energy balance in the feed ration. Especially, energy value of the feed ration poses many problems to dairy cattle breeders, particularly in the perinatal period, which results in an increased incidence of metabolic disorders that are today one of the most frequent causes of dairy cows' culling (18%), as they underlie most of the disease entities [Lach 2008]. They exert a negative effect upon both reproductive outcome and the production itself, and in critical cases may be the cause of death [Kowalski 2007, Jóźwik *et al.* 2012a]. Today, the main metabolic diseases include: ketosis, acidosis and alkalosis, displaced abomasums, hypocalcemia, retained placenta, and *metritis* [Nowak *et al.* 2011, Puppel and Kuczyńska 2016]. The presence of non-esterified fatty acids (NEFAs) in blood is indicative of the consumption of lipid reserves by a cow in order to balance the disproportion between the energy provided with a feed ration and the energy indispensable for milk production, whereas its high level is associated with more frequent incidence of metabolic diseases in the perinatal period [Bobe *et al.* 2004, Jóźwik *et al.* 2012b]. The concentration of NEFAs exceeding 0.6 meq L^{-1} in cows in the perinatal period is implicated in the 4-5-fold increased risk of the development of metabolic diseases [LeBlanc *et al.* 2005]. The NEFAs appear in blood considerably more rapidly than BHBA, once the organism homeostasis is disrupted. The lower blood level of BHBA in cows after calving is associated with a reduced energy value of the feed ration in the dry period [Dann *et al.* 2006]. The BHBA concentrations of <2.6 and >1.4 mmol L^{-1} in the first week postpartum are indicative of subclinical ketosis [Geishauser *et al.* 2001, Walsh *et al.* 2007]. Ospina *et al.* [2010] reported that the BHBA concentrations of ≥1.0 mmol L^{-1}. occurring between the 3rd and 14th day postpartum, were associated with an increased risk of the occurrence of clinical forms of ketosis and metritis. According to Jorjong *et al.* [2017] the increased concentration of C18:1 *cis-9* (in milk fat) in the second week of lactation may be a symptom for an early diagnosis of cows being at risk of the occurrence of elevated blood levels of NEFAs.

This study was aimed at verifying the hypothesis that oleic acid may be applied as a biomarker for the early diagnosis of elevated blood levels of NEFAs and BHBA in the early stages of lactation in high-yielding PHF cows.

Material and methods

All cows were handled in accordance with the regulations of the Polish Council on Animal Care. The experiment and all procedures carried out in the study were reviewed and approved by the Warsaw University of Life Sciences Care Committee.

The experiment was carried out at the experimental dairy farm of the Warsaw University of Life Sciences (WULS). The cows were kept in a free-stall dairy shed

and fed a total mixed ration (TMR, kg d^{-1} of an ingredient): maize silage – 24.0; alfalfa silage – 10.30; corn silage – 5.0; soybean meal – 1.80; pasture ground chalk – 0.10; vitamin mix – 0.16; rapeseed meal – 2.50. Representative TMR samples were pooled at the beginning of the experiment and stored at -20°C until analyzed for dry matter, crude protein, ash, ether extract, acid detergent fiber and neutral detergent fiber [AOAC 1990]. The chemical composition of the TMR was calculated from the chemical composition of the individual dietary constituents. The chemical composition (g kg^{-1} DM) was: Ash – 63; Crude protein – 95; Acid detergent fibre (ADF) – 230; Neutral detergent fibre (NDF) – 360.

Samples of colostrum/milk and blood were collected from 120 multiparous cows for laboratory analyses in weekly intervals (7 samplings): sampling 1 – between day 4 and 7 of lactation; sampling 2 – between day 8 and 14; sampling 3 – between day 15 and 21; sampling 4 – between day 22 and 28; sampling 5 – between day 29 and 35; sampling 6 – between day 36 and 42; and sampling 7 – between day 43 and 49.

The cows were milked daily at 05:30 and 17:30 and milk yield was recorded at each milking. The milk was placed in sterile bottles, preserved with Mlekostat CC and immediately transported to the Cattle Breeding Division (Milk Testing Laboratory of WULS) for composition analysis. Blood samples (10 mL) were taken from each cow by jugular venipuncture (by a veterinarian) into a heparinized tube, separated by centrifugation at room temperature (1,800×g, 15 min) and immediately transported to the Veterinary Centre of WULS for the analysis of blood biochemical parameters (NEFAs, BHBA).

Chemical analyses

The contents of basic milk constituents, i.e. fat, protein, casein and lactose, were determined by an automated infrared analysis with a Milkoscan FT-120 analyzer (Foss Electric, Hillerřd, Denmark).

Fatty acid methylation was performed according to the *trans* esterification method EN ISO 5509 [EN ISO 5509 2000]. Individual fatty acids were identified in crude fat using an Agilent 7890A GC (Agilent, Waldbronn, Germany) according to Puppel *et al.* [2016].

Statistical analysis

The data obtained were analyzed statistically by two –way ANOVA. Only the interactions between factors whose influence was statistically significant ($p \leq 0.05$) were considered.

The statistical model:

$$y_{ijk} = \mu + A_i + B_j + (A_i \times B_j) + e_{ijk}$$

where:

y_{ijk} – a dependent variable;

μ – the overall mean;

A_i – the week of lactation effect (i=1, 8);

B_j – the effect of oleic acid content (j= 1: 16-21 g 100g of fat^{-1}; 2: 21.5-23.5 g 100g of fat^{-1}; 3: >24 g 100g of fat^{-1}),

$A_i \times B_j$ – the interaction between weeks of lactation and concentration of oleic acid;

e_{ijk} – the residual.

Results and discussion

Table 1 presents results concerning changes in milk gross composition within the first 7 weeks of lactation. They enable confirming the thesis reported by other authors that, in this period, milk fat content is a highly variable component [Barłowska *et al*. 2006, Heins *et al.* 2008, Strzałkowska *et al.* 2010, Kuczyńska *et al.* 2011]. In our experiment, the highest content of milk fat (significant at $p \leq 0.01$) was recorded in the first week of lactation. It is due to several factors, with the key one being the production of colostrum at the beginning of lactation, which differs in its composition from the gross composition of milk. As noticed by Kuczyńska *et al*. [2011], the high fat content in milk may also indicate a high content of dietary fiber in the ration feed during the dry period [Strzałkowska *et al.* 2009]. In the successive weeks of lactation, the content of milk fat is lower than in the first week, which may be also caused by the administered feed ration and increasing production of milk as well as by physiological changes that undergo in a cow's body.

Table 1. Changes in milk gross composition within the first 7 weeks of lactation

Component		Weeks of lactation						
		1	2	3	4	5	6	7
Fat (%)	LSM	5.60^{ABCDEF}	4.60^{AGHI}	3.99^{BGJK}	4.34^{CJLL}	4.51^{DKMN}	3.60^{EHLM}	3.64^{FILN}
	SE	1.55	1.09	0.86	0.81	0.86	0.96	1.11
Protein (%)	LSM	3.79^{aBCDEF}	3.41^{aGHIJ}	3.21^{BKI}	3.03	2.90	3.02	3.09
	SE	0.45	0.28	0.20	0.29	0.17	0.21	0.35
Casein (%)	LSM	3.07^{aBCDEF}	2.80	2.69	2.58	2.47	2.55	2.60
	SE	0.302	0.23	0.18	0.22	0.18	0.18	0.24

$^{aA...}$Means in the same rows marked with the same letters differ significantly at: small letters – $P \leq 0.05$; capitals – $P \leq 0.01$.
LSM – Least-squares mean; SE – Standard error of LSM.

The content of protein differed between lactation stages similarly to fat content. It was highest in the first week of lactation, which was mainly due to the production of colostrum rich in immunoglobulins that are constituents of the milk protein. In the subsequent stages, protein content was decreasing as a possible result of: growing milk production and the ill –balanced feed ration that failed to meet cow demand for

energy; both factors could be causative of ketosis. The percentage content of protein in milk fat was decreasing till the 5th week of lactation, and afterwards it increased somewhat, again. Similar observations were reported by Ikoen *et al.* [2004], Miciński and Klupczyński [2006], and by Pecka *et al.* [2012].

Table 2 summarizes results concerning changes in the levels of biochemical blood markers of cows in the early lactation stage. Concentrations of NEFAs and BHBA are the basic elements of the metabolic profile, which are used in the diagnostics of metabolic diseases. More than 50% of herds had more than 25% of cows with elevated BHBA during the postpartum period [Ospina *et al.* 2010]. Three intervals of NEFAs and BHBA blood levels, indicating the health status of cows can be distinguished. In the case of NEFAs, these include: <0.24 mmolL^{-1} denoting an under-optimal level, 0.25-0.6 mmolL^{-1} denoting the optimal level, and ≥0.6 mmolL^{-1} denoting ketosis [LeBlanc *et al.* 2005, Ospina *et al.* 2010, **Puppel and** Kuczyńska 2016]. In the case of BHBA, the intervals are as follows: <0.5 mmolL^{-1} denoting an under-optimal level, 0.51-1.2 mmolL^{-1} denoting the optimal level, and ≥1.2 mmolL^{-1} denoting ketosis [Duffield 2000, Geishauser *et al.* 2001, Dann *et al.* 2006; Walsh *et al.* 2007; Ospina *et al.* 2010]. Summarizing, peripheral NEFA levels reflect the breakdown of body fat reserves, while elevated ketone concentrations 'visualize' the incapacity of the liver to handle the overwhelming flux of NEFA [Opsomer 2015].

Table 2. Changes in levels of blood NEFA and BHBA within the first 7 weeks of lactation

Component		Weeks of lactation						
		1	2	3	4	5	6	7
NEFA (mmol L^{-1})	LSM	0.98^{ABCDE}	0.70^{AF}	0.66^{BghI}	0.75^{BgJ}	0.73^{ChK}	0.68^{DL}	0.49^{EFIJKL}
	SE	0.12	0.11	0.10	0.15	0.12	0.18	0.15
BHBA (mmol L^{-1})	LSM	0.90^{AbcD}	0.89^{EFG}	0.60^{AEH}	0.78^{bJK}	1.16^{cFHJLL}	0.97^{IKLm}	0.75^{DGLm}
	SE	0.16	0.15	0.14	0.20	0.17	0.16	0.21

$^{aA...}$Means in the same rows marked with the same letters differ significantly at: small letters – P≤0.05 ; capitals – P≤0.01 .
LSM – Least-squares mean; SE – Standard error of LSM.

The present results show explicitly that throughout the experiment most of the cows suffered from some metabolic disorders owing to a high level of NEFAs (above 0.6 mmol L^{-1} on average). The highest concentration of NEFAs in blood occurred in the first week of lactation – 0.98 mmolL^{-1} (p≤0.01). Afterwards, it decreased quite rapidly and then stabilized in the 7th week of lactation, when it reached the lowest level of 0.49 mmolL^{-1}. A similar correlation was also reported by Jorjong *et al.* [2014]. Additionally, Ospina *et al.* [2010] indicated that increased concentrations of serum NEFA and BHBA had a detrimental effect on reproductive performance and milk production. On the other hand, chronically elevated concentrations of NEFA and BHBA have been demonstrated to affect multiple organ systems and to be in contrast to absolute milk yield [Opsomer 2015].

A similar descending tendency was observed for BHBA, the blood level of which decreased from 0.9 to 0.6 mmolL^{-1} till the 3rd week of lactation, then increased in the 4th week, and reached the highest value of 1.16 mmolL^{-1} in the 5th week. However, its mean concentration did not indicate any metabolic disorders in the cows. Duffield *et al.* [2009] concluded that health risk and reduced milk production appear to start between the threshold of 1200 to 1400 μM of serum BHBA in the first week postpartum.

Table 3 presents results of analyses of blood NEFA and BHBA metabolic profiles as affected by oleic acid content. These results confirm correlations between OA content and NEFA and BHBA concentrations (Tab. 4). As shown by the current study results, the high concentrations of both NEFAs and BHBA, clearly indicating the incidence of the metabolic disease in cows, occur when OA content in milk exceeds 24g 100 g fat^{-1}. It confirms earlier findings reported by Jorjong *et al.* [2014], who demonstrated, that NEFAs transferred to the milk are rich in long-chain FA, such as C18:1 *cis*-9, and concentrations in milk fat of these FA might be linked to severity of negative energy balance (NEB). In turn, Melendez *et al.* [2016] reported, that the early postpartum cows with plasma BHBA >0.7 mmolL^{-1} tended to have lower proportion of CLA than early postpartum cows of BHBA ≤0.7 mmolL^{-1}.

Table 3. NEFA and BHBA blood metabolic profiles as affected by oleic acid content

Component	C18:1 *cis9* (g 100g of fat^{-1})	LSM	SE
NEFA (mmol L^{-1})	16–21	0.305^{A}	0.082
	21.5–23.5	0.383^{B}	0.090
	>24	1.357^{AB}	0.079
BHBA (mmol L^{-1})	16 21	0.701^{A}	0.112
	21.2–23.5	0.753^{B}	0.122
	>24	1.103^{AB}	0.170

$^{aA,...}$Means in the same rows marked with the same letters differ significantly at: small letters – $P \leq 0.05$; capitals – $P \leq 0.01$.
LSM – Least-squares mean; SE – Standard error of LSM.

Results concerning the BHBA level demonstrate its high values as affected by oleic acid content, however in the interval including cows with high OA content, the concentration of BHBA was also high, which confirms explicitly that these cows should be diagnosed again, owing to the likely changes proceeding in their organisms after calving.

Table 4 summarizes blood levels of NEFAs and BHBA as affected by OA content in the first 7 weeks of lactation. In the first week of lactation, none of the cows had a too low blood level of both NEFAs and BHBA, which indicates that from the very beginning the whole herd was at risk of the development of ketosis. This should elicit a rapid response of the breeder aimed at improving the feed ration for cows by its enrichment with an additional source of energy and at constant monitoring of

Table 4. Blood levels of NEFA and BHBA as affected by C18:1 *cis9* content in the first 7 weeks of lactation

Component	C18:1 *cis9* (g 100g of fat^{-1})		Weeks of lactation						
			1	2	3	4	5	6	7
NEFA (mmol L^{-1})	16–21	LSM	-	0.36^{ABCDE}	0.20^{AFGHI}	0.47^{BF}	0.55^{CGJK}	0.12^{DHJK}	0.13^{EI}
		SE	-	0.22	0.20	0.22	0.22	0.22	0.12
	21.5–23.5	LSM	0.39^{abc}	0.38^{def}	0.36^{ghi}	0.46^{adg}	0.35	0.45^{beh}	0.30^{cfi}
		SE	0.20	0.22	0.18	0.28	0.24	0.14	0.34
	>24	LSM	1.57^{ABCDef}	1.37^{AgHJ}	1.43^{BgKLL}	1.31^{CKMN}	1.29^{DHLMOU}	1.47^{etOP}	1.05^{FJLNUP}
		SE	0.13	0.11	0.15	0.28	0.18	0.24	0.28
BHBA (mmol L^{-1})	16–21	LSM	-	0.68^{aBC}	0.51^{aDEFG}	0.74^{BDhI}	0.86^{CEhJk}	0.69^{FU}	0.72^{Ck}
		SE	-	0.30	0.27	0.30	0.30	0.30	0.17
	21.5–23.5	LSM	0.69^{ABCDe}	0.89^{AFGHI}	0.53^{BFJKL}	0.55^{BGLMN}	1.01^{CHJLOP}	0.83^{DKMO}	0.77^{eILNP}
		SE	0.27	0.30	0.25	0.38	0.33	0.19	0.47
	>24	LSM	1.12^{ABeDEF}	1.10^{AGHIJ}	0.76^{BGKLLM}	1.00^{eK}	1.60^{DHLOP}	1.37^{FILOR}	0.77^{FJMPR}
		SE	0.18	0.16	0.21	0.38	0.25	0.33	0.38

$^{aA,...}$Means in the same rows marked with the same letters differ significantly at: small letters – $P \leq 0.05$; capitals – $P \leq 0.01$.
LSM – Least-squares mean; SE – Standard error of LSM.

the health status of cows [Puppel and Kuczyńska 2016]. In the successive weeks of lactation, the thesis about the necessity of ceaseless monitoring of the health status of cows was confirmed based on maintaining the number of ketotic cows and on the recorded cases of cows with too low concentrations of both BHBA and NEFAs, which may be indicative of acidosis. Similar findings were reported by Ospina *et al.* [2010]. According to LeBlanc *et al.* [2005], Chapinal *et al.* [2011] and Seifi *et al.* [2011], high concentration of NEFAs in the first two months may lead to death of animals, hence constant monitoring of their health status is of the outmost significance.

It can generally be concluded that the oleic acid may be used as a biomarker for early diagnosis of elevated blood levels of NEFAs and BHBA in the early stages of lactation in high –yielding PHF cows.

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