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Original article

Relationship between pH of ruminal fluid during subacute ruminal acidosis and physiological response of the Polish Holstein-Friesian dairy cows

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Abstract

The aim of the study was to investigate the effect of ruminal fluid pH depression on biochemical indices of blood, urine, feces, and milk, and to determine which of them may be helpful as a marker for the diagnosis of subacute ruminal acidosis (SARA).

Ruminal fluid samples were obtained by *rumenocentesis* from 305 cows representing 13 commercial dairy herds. The herds were selected based on percentages of cows with an assigned value of ruminal fluid pH segregated into three groups as: SARA-positive herd, if at least 25% of the ruminal fluid samples indicated a pH < 5.6; SARA-risk herd, if less than 25% of ruminal fluid samples indicated a pH < 5.6, but at least 33% showed a pH ≤ 5.8; and SARA-negative herd, if less than 25% of the ruminal fluid samples indicated a pH < 5.6, but less than 33% exhibited a pH = 5.8. Moreover, the dairy cows were divided according to the ruminal fluid pH into three groups as follows: healthy cows (HC, pH>5.80, n = 196), risk cows (RC, pH 5.8 – 5.6, n = 51), and acidotic cows (AC, pH < 5.6, n = 58).

Almost 19% (58/305) of the cows were classified as acidotic (pH < 5.6) and 46.2% of the herds as SARA-positive. In the AC group, higher concentrations of insulin-like growth factor-I (IGF-I), non-esterified fatty acids (NEFA), rectal temperature and lower blood pH, compared with those of the HC group, were recorded. Moreover, in the SARA-positive herds, higher concentrations of IGF-I and the lowest blood pH, compared with SARA-negative herds, were observed.

The lowering of ruminal fluid pH increased the blood IGF-I and NEFA concentrations and the rectal temperature and decreased the blood pH. These measures are indicators of the physiological changes that occur as part of the pathogenesis of the condition and may be helpful for the diagnosis of the SARA syndrome when serial measurements are conducted.

Key words: SARA, diagnosis, biomarker, dairy cows, *rumenocentesis*

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Introduction

Subacute ruminal acidosis (SARA) is the most important nutritional disease in the dairy industry (Eckel et al. 2016). SARA is not easily diagnosed, as it is frequently a symptomless disease and the effects not being detectable. Difficulties with the diagnosis of SARA are caused by a lack of specific pathognomonic signs, diurnal fluctuations in the rumen fermentation and problems in obtaining representative rumen fluid samples for rumen pH measurement (Jorgensen et al. 1993). Calsamiglia et al. (2012) concluded that the diagnosis of SARA based only on ruminal pH measurements may not be accurate. The incidence of SARA and a lack of a reliable method to diagnose it generate considerable indirect and direct economic losses incurred by dairy cattle breeders and milk producers. SARA reduces milk yield by 2.7 kg milk/day and causes deterioration in its chemical composition, lowering fat content by 0.3% and protein content by 0.1%, at the same time generating indirect costs amounting to 1.12 USD per day/cow (Zebeli et al. 2012). Plaizier et al. (2009) estimated the annual cost of SARA to the U.S.A. dairy industry as $ 500 million to $ 1 billion and the occurrence of SARA affecting more than 20% of dairy cows. Moreover, indirect losses are connected, for example, with the incidence and therapy of the accompanying health disorders, that is lameness and infertility which deteriorate fertility indices and increase the culling rate to 15% (Gumez et al. 2014).

In practice, the major tools for early diagnosis of the occurrence of this metabolic disease on the farm are monitoring rumination, dry matter intake and feces appearance. However, none of these analyzes is completely satisfactory and none of them may be the basis for regular monitoring of herds, which could be useful in SARA prevention. Also, no tests have been developed based on the analyzes of blood, ruminal fluid, urine, feces, or milk, which could facilitate a rapid and precise screening of dairy cattle in the suspected herds.

In recent years, many potential biomarkers for early occurrence of SARA were studied. Biochemical and microbiological indicators, which may be useful in early, precise diagnosis of the occurrence of metabolic diseases such as concentrations of volatile fatty acids (VFA), ruminal and blood lipopolysaccharides (LPS), the ruminal bacterial community composition and blood acute phase proteins (SAA, Hp, LBP), the chemical composition of milk and milk fatty acids profile were studied, but no clear answer has been provided (Calsamiglia et al. 2012, Li et al. 2012, Plaizier et al. 2012). The results are still conflicting and do not allow for the development of an accessible and inexpensive diagnostic biomarker. Moreover, most studies have been conducted on a small numer of animals, which reduces the sensitivity of the statistical analysis or on animals in which SARA was induced experimentally, the results of which may not be generalizable and also some of these results may be inconsistent.

The most promising tool that could be helpful for SARA prevention seems to be the physically effective NDF ($peNDF_{>1.18mm}$, Mertens et al. 1997) or the relation of the physical form of TMR to readily degradable nonstructural carbohydrates, which is expressed in terms of the ratio of $peNDF_{>1.18mm}$ to starch, with the maximum recommended value of 1.45 (Zebeli et al. 2010) or as recently suggested not less than 1.00 (Stefańska et al. 2016).

The aim of the study was to investigate the effect of ruminal fluid pH depression on biochemical indices of blood, urine, feces, and milk, and to determine which of them may be helpful as a marker for diagnosis of subacute ruminal acidosis.

Materials and Methods

All procedures were approved by the Local Ethical Committee (decision no. 32/2014).

The study was conducted in 13 commercial dairy herds of the Polish Holstein-Friesian breed cows, located in Western and South parts of Poland. Farms were selected according to the milk yield (more than 10 000 kg/ 305 d lactation), size of a farm (over 100 lactating dairy cows), housing of cows (only free-stall barns), feeding (only total mixed ration; TMR) and length of dry period (50-60 days) and milk performance evaluated by the Polish Federation of Cattle Breeders and Dairy Farmers. In all selected farms the cows were fed TMR diets based on corn silage, wilted grass or alfalfa silage, ensiled high moisture corn grain and barley, wheat, triticale grains and rapeseed and soybeans meals. The TMR samples were collected for analyses by wet chemistry for dry matter (DM, method no. 6496), crude protein (CP, method no. 976.05), neutral detergent fibre (NDF, method no. 942.05) and starch (method no. 64.785) according to AOAC (2010). The particle size of TMR samples was evaluated using The New Penn State Separator, with 3 sieves having holes with a different diameter (19, 8, 1.18 mm) and a solid pan according to the technique described by Mertens (1997). The content of each sieve and the solid pan was weighed and recorded. Moreover, the starch contained in the components used in the TMR was easily degradable in the rumen, and the share of grain was between 44% and 56% (SE ± 1.4). Moreover, in the TMR analyzed the con-

tent of NDF was between 27% and 35% (SE ± 1.4), crude protein beetwen 16.0 and 16.5% (SE ± 0.45) and peNDF beetwen 31.4 and 26.3% (SE ± 1.3).

The herds were selected according to the classification shown by Garrett et al. (1999) based on percentages of cows with an assigned value of ruminal fluid pH segregated into three groups as: SARA-positive herd, if at least 25% of the ruminal fluid samples indicated a pH < 5.6; SARA-risk herd, if less than 25% of ruminal fluid samples indicated a pH < 5.6, but at least 33% showed a pH ≤ 5.8; and SARA-negative herd, if less than 25% of the ruminal fluid samples indicated a pH < 5.6, but less than 33% exhibited a pH ≤ 5.8. Moreover, in total, 305 dairy cows were selected and divided according to the classification of Nordlund and Garrett (1994) based on ruminal fluid pH into three groups as healthy (HC, pH > 5.81, n = 196), risk (RC, pH 5.8-5.6, n = 51) and acidotic cows (AC, pH < 5.6, n = 58). All the selected animals were between 40 and 150 days in milk (DIM), in lactation (primiparous, n = 139 and multiparous, n = 166), and clinically healthy (free of clinical *mastitis, metritis* and hoof disease). The health status of the cows was estimated based on their recent medical history and through a detailed clinical examination, carried out always by the same veterinarian.

Ruminal fluid samples were collected from the ventral sack of the rumen in dairy cows by *rumenocentesis* using non-pyrogenic needles (2.0 × 120 mm) and 30 mL syringes (Duffield et al. 2004). Currently, *rumenocentesis* is an accepted technique, providing the most accurate results (Duffield et al. 2004). Ruminal fluid samples (30 mL) were collected 3 – 6 hours after the morning feeding according to the methodology presented by Krause and Oetzel (2006). Ruminal fluid pH was measured using CP-104 pH-meter.

Blood samples were taken from the tail vein as described by Gozho et al. (2005). Blood samples were collected for each cow 3 – 6 h after the morning feed into 10 mL vacutainers for serum harvesting. The serum vacutainers were transported to the laboratory in a refrigerated container. The whole blood samples in the serum vacutainers were centrifuged at 3000 × G for 15 min and next the serum was separated and stored at -20°C until analyzes. The concentration of β-hydroxybutyrate (BHBA catalog numbers: H7587-58) was analyzed colorimetrically, using a Marcel Media spectrophotometer with a Pointe Scientific reagent kit. The serum samples were diluted initially at 1:1 ratio and analyzed in duplicate and absorbance values were read at 505 nm. The concentrations of non-esterified fatty acids (NEFA) were analyzed according to the Duncomble colorimetric method using a Marcel Media spectrophotometer (Duncomble 1964). Serum insulin-like growth factor-I (IGF-I) was assayed by radioimmunoassay method (RIA). The concentrations of hormones were determined after the reaction of IGF-I with a specific antibody labeled with a specific isotope I 125 (Diagnostic Systems Laboratories Inc., USA), which undergoes gamma radiation decay, using a Automatic Gamma radiation reader, on a Gamma Counter 1470. The RIA test was validated using curves with bovine serum of various dilution rates. Whole blood samples were immediately after sampling measured for pH using CP-104 pH-meter according to the methodology described by Li et al. (2012).

Milk samples (15 mL) were collected from morning and afternoon milkings into special tubes during milk performance evaluation conducted by the Polish Federation of Cattle Breeding and Dairy Farmers. Milk pH test was conducted in the Laboratory of the Polish Federation of Cattle Breeding and Dairy Farmers using CombiFoss FT 6000 with the Fourier Transform Infrared (FT-IR) method.

Rectal temperatures of dairy cows were measured using an electronic Microlife MT16C2 thermometer. Feces samples (approximately 200 g, wet weight) were collected from the *rectum* into plastic containers and pH was measured immediately after the collection. For feces pH measurements, as described by Gressley and Aramentano (2005), approximately 50 g of fresh feces and 25 g of distilled water were placed in a specimen cup. The cup was shaken for 20 s in a vortex, liquids were squeezed through cheesecloth, and the pH of the liquids was determined with CP-104 pH-meter.

The results obtained were analyzed using the SAS statysitcal software SAS 9.4 (2014). Means were analyzed using Duncan or Student t-test and the PROC GLM procedure. The PROC MEANS and PROC UNIVARIATE procedures were also used. Pearson phenotype correlation coefficients were calculated using the PROC CORR procedure. Statistical significance was declared at $p \leq 0.05$ and trends were considered when $0.05 < p \leq 0.1$. The standard error of the mean (SEM) was adopted as a measure of error.

Results

In the present study, 19% (58/305) of the cows were classified as acidotic (pH < 5.6), 46.2 % of the herds as SARA-positive, and 15.4% as SARA-risk herds (Table 1).

In the SARA-positive herds, higher concentrations of IGF-I ($p \leq 0.01$) and the lowest blood pH ($p \leq 0.01$), compared with SARA-negative herds, was noted (Table 2). No siginficant correlation between

Table 1. SARA herd-status and mean, minimum-maximum values of the ruminal fluid pH in healthy, risk and SARA cows.

Herd (n=13)	Total (n=305)	Groups[1]									Herd SARA-status[2]
		HC			RC			AC			
		No.	$\bar{x}$	Min.-Max.	No.	$\bar{x}$	Min.-Max.	No.	$\bar{x}$	Min.-Max.	
1	21	13	6.18	5.81-6.81	2	5.66	5.63-5.68	6	5.4	5.18-5.56	SARA – positive
2	20	9	6.16	5.83-6.50	5	5.71	5.61-5.80	6	5.44	5.26-5.56	SARA – positive
3	24	6	6.09	5.93-6.30	6	5.67	5.61-5.78	12	5.45	5.20-5.57	SARA – positive
4	26	12	5.97	5.81-6.35	4	5.68	5.64-5.73	10	5.39	5.30-5.55	SARA – positive
5	14	7	6.31	5.89-6.56	3	5.68	5.60-5.76	4	5.40	5.33-5.47	SARA – positive
6	28	13	6.33	5.90-6.69	4	5.69	5.65-5.77	11	5.48	5.35-5.60	SARA – positive
7	24	16	6.27	5.82-6.65	6	5.71	5.64-5.76	2	5.56	5.53-5.58	SARA – risk
8	25	16	6.25	5.86-6.88	7	5.70	5.61-5.80	2	5.54	5.50-5.58	SARA – risk
9	24	21	6.28	5.81-6.76	3	5.79	5.77-5.80	0	0	0	SARA – negative
10	25	23	7.21	6.44-7.85	1	5.85	5.85	1	5.49	5.49	SARA – negative
11	24	17	6.26	5.81-7.04	6	5.73	5.66-5.80	1	5.48	5.48	SARA – negative
12	25	25	6.30	5.83-6.93	0	0	0	0	0	0	SARA – negative
13	25	18	6.22	5.92-6.73	4	5.74	5.70-5.78	3	5.44	5.24-5.59	SARA – negative

[1] Groups of cows: healthy cows (HC, pH > 5.8); risk cows (RC, pH 5.8-5.6); acidotic cows (AC, pH < 5.6).
[2]S ARA herds status: SARA-positive herd, if at least 25% of the ruminal fluid samples indicated pH<5.6, SARA-risk herd, if less than 25% of cows has a pH of ruminal fluid <5.6, but at least 33% has a pH ≤5.8 and SARA-negative herd, less than 25% of cows in the herd has a pH of ruminal fluid <5.6 and less than 33% has a pH ≤ 5.8.

Table 2. The values of selected indices in herds with different SARA status.

Item	SARA herd status[1]					
	SARA-negative		SARA-risk		SARA-positive	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
IGF-I (ng/ml)	80.13^{B}	0.21	81.2^{B}	0.02	86.9^{A}	0.24
NEFA (mmol/L)	0.82	0.02	0.83	0.01	0.80	0.02
Blood BHBA (mmol/L)	0.28	0.01	0.31	0.01	0.29	0.01
Blood pH	7.65^{A}	0.03	7.35^{B}	0.09	7.27^{B}	0.01
Feces pH	6.72	0.03	6.59	0.05	6.53	0.02
Rectal temperature	38.4	0.04	38.5	0.04	38.9	0.051

SARA herds status: SARA-positive herd, if at least 25% of the ruminal fluid samples indicated pH<5.6, SARA-risk herd, if less than 25% of cows has a pH of ruminal fluid <5.6, but at least 33% has a pH ≤5.8 and SARA-negative herd, less than 25% of cows in the herd has a pH of ruminal fluid <5.6 and less than 33% has a pH ≤5.8. A, B, C – means with different letters in the row differ significantly, p≤0.01

SARA-herds status and NEFA and BHBA concentrations, rectal tempaerature and feces pH value were noted (p>0.05).

In the AC group, higher concentrations of IGF-I (p≤0.05), NEFA (p≤0.01), rectal temperature (p≤0.05) and lower blood pH, compared with the HC group, were recorded (Table 3). No significant correlation between different ruminal fluid pH and BHBA concentration and value of feces pH were found (p>0.05). There were significant negative correlations between the pH values of ruminal fluid, concentration of IGF-I and rectal temperatures (p≤0.01, Table 4). Also, the pH of the ruminal fluid was positively correlated with concentrations of NEFA and values of blood pH (p≤0.01).

Discussion

In the present study, almost 19% (58/305) of the cows were classified as acidotic (pH < 5.6), 46.2% of the herds as SARA-positive, and 15.4% as

Table 3. The values of selected indices in cows with different ruminal fluid.

Item	Groups[1]					
	HC		RC		AC	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
IGF-I (ng/ml)	81.57^{b}	1.19	79.97^{b}	2.22	85.64^{a}	2.39
NEFA (mmol/L)	0.29^{C}	0.01	0.26^{B}	0.01	0.27^{A}	0.01
Blood BHBA (mmol/L)	0.81	0.02	0.82	0.03	0.79	0.03
Blood pH	7.45^{A}	0.02	7.38^{AB}	0.05	7.26^{B}	0.06
Feces pH	6.76	0.02	6.74	0.04	6.73	0.07
Rectal temperature	38.35^{b}	0.03	38.49^{ab}	0.08	38.57^{a}	0.09

[1] Groups of cows: healthy cows (HC, pH > 5.81); risk cows (RC, pH 5.8-5.6); acidotic cows (AC, pH < 5.6)
a, b – means with different letters different significantly, p≤0.05
A, B, C – means with different letters different significantly, p≤0.01

Table 4. Correlation coefficient (r) between ruminal fluid pH and selected indices.

Indices	Ruminal fluid pH	
	r	p[1]
IGF-I	-0.16	**
NEFA	0.37	**
Blood BHBA		
Blood pH	0.48	**
Faeces pH		
Rectal temperature	-0.28	**

[1] p – probability
** correlation statistically significant p≤0.01

SARA-risk. The prevalence rate of SARA in the Polish dairy herds in the region examined was found to be similar at herd level as compared to that in other countries. SARA-positive cows were found at the rates of 20% (63 of 315) and 27.6% (24 of 87) in Germany and in Albania, respectively (Kleen et al. 2013, Shabani and Ceroni 2013). Moreover, in Ireland 25% (3/12), in Greece 33% (4/12) and in Germany 42% (11/26) of herds were found to be SARA-positive (Morgante et al. 2007, Kitkas et al. 2013, Kleen et al. 2013).

In our study, statistically significant higher concentrations of IGF-I at both levels – cows and herds was noted. Moreover, lower values of ruminal fluid pH were negatively correlated with increased levels of blood IGF-I. These results agreed with those of other studies (Khafipour et al. 2009a, 2009b). Some authors suggested that SARA could result in a metabolic endotoxemia that triggers a low-grade inflammation compared with acute disorders, such as septicemia (Allen 2000). In addition, metabolic endotoxemia can result in insulin resistance in the liver, hyperinsulinemia, higher blood glucose and modifications of the energy metabolism (Cani et al. 2007). These changes can alter the direction of propionate metabolism in hepatocytes from gluconeogenesis to oxidation as acetyl CoA, which causes positive energy balance (Allen 2000). This finding could similarly explain the gain in body condition of dairy cows experiencing SARA (Kleen et al. 2003). Moreover, in the current study, the highest rectal temperature compared with other groups was noted in the acidotic group of cows. Moreover, decrease in the ruminal fluid pH was negatively correlated with these parameters. In the SARA positive herds higher rectal temperature compared to other groups was noted, hovever there was no significant association. Plaizier et al. (2012) suggest that disorders associated with SARA could cause local inflammation of the rumen mucosa (*rumenitis)*, which also increases the rectal temperature. Antanaitis et al. (2016) showed that decrease of ruminal fluid pH during SARA increase the reticulorumen temperature which is correlated with the rectal temparature. Therefore, regular measurement of body temperature could be part of a routine systemic monitoring in cattle farms (Drillich et al. 2001). However, in our

observation rectal temperature was found to be consistent with the reference value, which led to the conclusion that signs associated with SARA are subclinical or often non-existent.

In the current study, in both classifications – cows and SARA herds status, decrease of ruminal fluid pH was associated with lowering concentrations of NEFA. There was a positive correlation between the ruminal fluid pH and NEFA. Also, there were no correlations between the decrease in ruminal fluid pH and the concentration of BHBA. Zebeli et al. (2012) showed that circulating NEFA and BHBA decreased as the amount of concentrate increased in the diet. The release of NEFA in the plasma of dairy cows is related predominantly to their mobilization from adipose tissue triacylglycerol stores through the process of lipolysis, whereas plasma BHBA derives mainly either from oxidation of NEFA in the hepatocytes or from the metabolism of butyrate in the rumen epithelium (Drackley 1999). Increasing the amount of concentrate in the diet improved the energy balance of the cows and led to greater concentrations of rumen propionate and plasma glucose in this study (Ametaj et al. 2010). Availability of propionate and glucose might have contributed to the lowered concentration of NEFA in the plasma due to their inhibitory effect on NEFA release by the adipose tissue (van Knegsel et al. 2007). Moreover, Guo et al. (2013) showed that the abundant availability of ruminal propionate and a higher plasma insulin concentration might have contributed to the reduced concentration of plasma NEFA due to a propionate and insulin inhibitory effect on the NEFA release by the adipose tissue. These results are consistent with our conception mode of insulin resistance (relation IGF-I and BCS, unpublished data) during the course of SARA. Moreover, a lower concentration of plasma BHBA is probably due to positive energy status of cows and reduced ketogenesis and source of origin from the digestive tract rather than the oxidation of triglycerides.

In the current study, a high corrlelation between the ruminal pH and blood pH was noted. In the acidotic cows and SARA-positive herds blood pH was measured below the reference value. Blood pH depends on the relative concentrations of bases, acids, and buffers in the solution. Bicarbonate is a primary blood buffer (Counotte et al. 1979). Absorption of ruminal VFA occurs by removing unionized acid and by the exchange of ionized VFA for bicarbonate during the absorption process, maintaining pH near neutrality. From all VFA, only acetate normally reaches the peripheral blood; much of the butyrate acids is converted into β-hydroxybutyrate during absorption through the rumen wall and all the propionate is converted into glucose by the liver. Presumably, VFA should not accumulate into blood plasma at sufficient concentrations to depress blood pH, but exactly how blood VFA concentrations change under acidotic conditions has not yet been determined. However, metabolism of the ruminal wall and the liver may be compromised during acidosis (Gianesella et al. 2010). Consequently, a reduced rate of VFA absorption causes ruminal pH to drop for two reasons: ruminal VFA accumulate and bicarbonate input from the blood stream and decreases pH (Li et al. 2012, Danscher et al. 20015).

Results of this study support those of the previous study by Gianesella et al. (2010), indicating that lowering of ruminal fluid pH affects depression in fecal pH, but in current study this was not statistically confirmed. Faecal pH is lower than normal, usually slightly acid (Morgante et al. 2009); this change is evident from alteration in stool colour, which appears brighter and yellowish. This change may be due to post-ruminal fermentation in the intestines because high level of grain feeding may results in carbohydrates including starch bypassing the rumen and reaching the intestines, with subsequent increased fermentation and VFA production in the hindgut causing decreased fecal pH (Danscher et al. 2015). However, many impaired ruminal functions could lead to the alteration of faecal aspects.

Conclusions

Lowering of ruminal fluid pH resulted in increased blood IGF-I, NEFA and rectal temperature and decreased blood pH. These measures are indicators of the physiological changes that occur as part of the pathogenesis of the condition and may be helpful for the diagnosis of the SARA syndrome when serial measurements are conducted. However, careful use of the results is warranted, as the parameters do not seem to change consistently and changes were small. None of these parameters seem to be able to stand alone as indicators of these metabolic disease syndrome. However, measures could be used with other indicator to formulate a combined risk index for compromised gut health. The biological significance of these results needs to be investigated further in larger field trials.

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