Subacute ruminal acidosis affects fermentation and endotoxin concentration in the rumen and relative expression of the *CD14*/*TLR4*/*MD2* genes involved in lipopolysaccharide systemic immune response in dairy cows

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ABSTRACT

The first objective of this study was to investigate the effects of subacute ruminal acidosis (SARA) on fermentation, ruminal free lipopolysaccharides (LPS), and expression of the cluster of differentiation 14 (*CD14*), toll-like receptor 4 (*TLR4*), and myeloid differentiation protein 2 (*MD2*) complex in white blood cells involved in the systemic immune response in dairy cows. The second objective was a study of whether increased expression of the LPS receptor complex led to increases in the concentrations of plasma high-density lipoprotein (HDL) and serum Ca. Three hundred five dairy cows located in 13 Polish high-yielding dairy commercial farms were selected according to their days in milk (40–150 d; average = 75), 305-d milk yield (10,070–12,041 kg; average = 10,940), and number of lactations (primiparous, $n = 139$ and multiparous, $n = 166$). Next, the herds were segregated into 3 groups based on the percentages of cows with an assigned value of ruminal fluid pH: SARA-positive, SARA-risk, and SARA-negative herds. Moreover, 305 selected dairy cows were divided according to the classification based on ruminal fluid pH into 3 groups as healthy (pH >5.81), risk (pH 5.8–5.6) and acidotic cows (pH <5.6). Rumen fluid samples were collected via rumenocentesis. In the AC group, we recorded higher concentrations of ruminal free LPS [4.57 Log₁₀ endotoxin units (EU)/mL; 42,206 EU/mL] compared with the healthy group (4.48 Log₁₀ EU/mL; 34,179 EU/mL). Similarly, the concentration of ruminal free LPS was higher in SARA-positive herds

(4.60 Log₁₀ EU/mL; 43,000 EU/mL) compared with SARA-negative herds (4.47 Log₁₀ EU/mL; 32,225 EU/mL). The relative mRNA abundance of genes associated with the function of LPS receptors, such as *CD14*, *TLR4*, and *MD2*, in white blood cells differed between all experimental groups on both cow and herd levels. In the acidotic group, we recorded higher concentrations of HDL (78.16 vs. 68.32 mg/dL) and serum amyloid A (10.80 vs. 9.16 µg/mL) and lower concentrations of Ca (8.26 vs. 10.16 mg/dL) and haptoglobin (470.19 vs. 516.85 ng/mL) compared with the healthy group. Similar results were obtained in the SARA herd status analysis, but the concentration of lipopolysaccharide-binding protein differed statistically. Moreover, the pH of ruminal fluid was negatively correlated with relative mRNA abundance of genes such as *CD14*, *TLR4*, *MD2*, and concentrations of serum HDL and serum amyloid A, although positively correlated with serum Ca. The results indicated that decreases in ruminal fluid pH increased the release of free LPS into the rumen and stimulated the expression of the LPS receptor complex and immune response. Moreover, an increase in the expression of the LPS receptor led to higher concentrations of plasma HDL and lower serum Ca, which may be a protective mechanism against endotoxemia. However, the biological significance of these results needs to be investigated further in larger field trials.

Key words: rumenocentesis, LPS, gene expression, inflammation

INTRODUCTION

It is common practice to feed high-yielding dairy cows high-grain diets. However, the disproportion between a high share of rapidly fermented carbohydrates and low physically effective NDF (**peNDF**) may cause impaired ruminal health through variation in the VFA

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concentrations and decreased ruminal fluid pH, which leads to SARA (Plaizier et al., 2008). According to Stefańska et al. (2017), almost 14% of the cows investigated in Poland were acidotic, which is an indication of SARA (pH <5.6; rumenocentesis). Moreover, 44% of the high-yielding commercial Polish herds were classified as SARA-positive (if at least 25% of the rumen fluid samples indicated pH <5.6). Several studies have investigated the etiology and pathophysiology of SARA (Gozho et al., 2007; Li et al., 2012; Danscher et al., 2015), but still no clear definition of SARA exists. Gozho et al. (2005) defined SARA as ruminal fluid pH depression below 5.6 that lasts for more than 180 min/d. Additionally, current recommendations for diagnosis of SARA on the dairy herd level are mainly based on ruminal pH measured in fluid sampled by rumenocentesis (Duffield et al., 2004). However, due to the constraints imposed by farm management and health problems (e.g., abscesses), these methods cannot be used as a routine monitoring tool on dairy farms. Moreover, many authors have suggested that the use of ruminal pH as the only indicator of SARA should be avoided (Li et al., 2014; Rodríguez-Lecompte et al., 2014; Danscher et al., 2015). Many cases of SARA may not be detected, as the current field diagnosis of SARA is not clearly defined and depends either on point ruminal pH measurements, which are invasive and not sufficiently accurate due to fluctuations in pH, or on continuous measurements, which require costly equipment and are primarily suited to research purposes. Therefore, Gozho et al. (2005) suggested that, to refine the definition of SARA based on rumen fluid sampling, concentrations of LPS and acute phase proteins (**APP**) should also be considered. The new refined definition, associated with the presence of LPS in the systemic circulation, could involve the LPS receptors affecting leukocyte populations and triggering the production of proinflammatory cytokines and APP (Rodríguez-Lecompte et al., 2014; Eckel and Ametaj, 2016). However, many differences have been found between concentrations of these biochemical indexes (LPS, VFA, APP) during SARA, and so this still offers limited diagnostic possibilities (Plaizier et al., 2012; Schlau et al., 2012; Guo et al., 2016). In most studies, the differences were caused by the small number of animals, which reduces the sensitivity of statistical analysis, and, in most of the presented research, results associated with SARA were induced experimentally, which may not be representative of the diagnostic occurrence of this metabolic disease on dairy farms. Moreover, over the past several years, significant progress has been made in identifying and characterizing several key molecules [LPS receptor: cluster of differentiation 14 (**CD14**), toll-like receptor 4 (**TLR4**), and myeloid differentia-

tion protein 2 (**MD2**)] and signal pathways involved in the regulation of macrophage functions by LPS and systemic immune response (Rodríguez-Lecompte et al., 2014). However, the associations between the presence of LPS in serum and plasma, innate immune response, and biochemical abnormalities in dairy cattle have not been fully explained.

We hypothesized that decreases in ruminal fluid pH associated with SARA may affect fermentation, increasing the level of ruminal free LPS and expression of the CD14/TLR4/MD2 complex in the blood, which may lead to systemic immune response in dairy cows. We also hypothesized that increases in expression of the LPS receptor complex led to higher concentrations of plasma high-density lipoprotein (**HDL**) and serum Ca. The first objective of our study was to investigate the effects of SARA on fermentation, ruminal free LPS, and expression of the CD14/TLR4/MD2 complex in white blood cells involved in the systemic immune response in dairy cows. The second objective was a study of whether increased expression of the LPS receptor complex led to increases in the concentrations of plasma HDL and serum Ca.

MATERIALS AND METHODS

All procedures were approved for the study and were performed in accordance with the “Act on the protection of animals used for scientific purpose” of the Republic of Poland, which complies with the EU directive (no. 2010/63/EU) for the protection of animals used for scientific purposes (decision no. 32/2014; European Commission, 2010).

Farms

The study was conducted on 13 commercial dairy farms of the Polish Holstein-Friesian cows located in western and southern Poland (Table 1). Farms were selected according to milk yield (more than 10,000 kg/305-d lactation), size of a farm (over 100 lactating dairy cows), housing of cows (only freestall barns), feeding (only TMR), and length of dry period (50–60 d). 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. Moreover, the starch contained in the components used in the TMR was easily degradable in the rumen.

Animals

In total, 14 to 28 Polish Holstein-Friesian breed, clinically healthy dairy cows were selected according to

Table 1. Herd general information, chemical composition, and particle size of TMR used in the observed herds¹

Item	Herd												
	1	2	3	4	5	6	7	8	9	10	11	12	13
General information													
No. of animals in herds	330	563	760	210	516	530	630	270	750	310	820	565	400
No. of tested animals	24	25	20	25	21	25	25	24	24	14	24	26	28
No. of primiparous cows/farm	18	63	75	14	66	25	54	12	35	22	65	48	34
Average lactation number/farm	3.00	2.48	2.40	3.00	2.30	3.00	2.20	3.00	3.08	2.55	2.55	2.74	2.65
Average 305-d milk production (kg)/farm	11,986	10,081	11,573	10,271	10,620	11,064	11,215	12,041	10,383	10,987	10,070	10,339	11,620
Average DIM/farm	76	76	79	73	76	75	75	74	73	74	76	79	74
Ingredient (% DM)													
Corn silage	51.6	44.7	57.3	59.1	53.9	57.3	47.8	54.4	57.2	52.6	52.7	50.1	51.3
Alfalfa silage	16.4	15.9	5.5	16.1	14.7	13.2	18.7	18.1	11.0	11.9	12.0	9.1	9.8
Wilted grass	11.7	12.4	2.7	2.7	2.4	2.2	6.2	6.0	6.6	7.2	7.2	12.5	11.0
Corn grain, ensiled	6.6	2.5	2.7	1.3	1.2	2.2	2.1	4.0	5.5	4.8	4.8	4.6	3.7
Barley grain,	4.7	9.9	10.9	4.6	4.2	3.7	5.0	3.0	3.3	3.6	4.1	3.9	4.2
Wheat grain	1.2	3.7	5.5	5.4	4.9	4.4	5.0	3.4	3.7	4.8	5.3	5.0	5.4
Triticale grain	1.2	1.2	1.4	1.9	7.3	6.6	5.6	4.0	3.5	4.8	4.8	4.6	4.9
Rapeseed meal	2.8	3.0	6.8	4.0	3.7	3.3	3.1	2.6	3.3	4.1	4.1	3.9	3.4
Soybeans meal	3.5	6.2	6.8	4.6	7.3	6.6	6.2	4.0	5.5	6.0	4.8	6.1	6.1
Mineral and vitamin mix ²	0.35	0.37	0.41	0.40	0.37	0.33	0.31	0.30	0.33	0.36	0.36	0.34	0.37
F:C ³	58:42	53:47	47:53	58:42	49:51	56:44	51:49	60:40	56:44	52:48	52:48	53:47	53:47
Nutrient composition (%)													
DM	40.8	47.2	52.4	45.2	45.4	44.6	45.5	39.6	44.7	47.6	48.5	46.6	46.3
CP	15.6	16.4	18.0	16.0	16.8	16.6	17.7	15.6	16.2	17.1	16.3	16.9	16.4
NDF	35.0	30.0	27.3	32.1	30.0	31.0	30.5	34.9	33.7	29.5	30.1	29.8	28.7
Starch	26.1	27.1	29.8	27.8	27.8	30.3	29.5	29.0	30.3	28.6	29.9	28.4	27.5
Starch to CP ratio	1.67	1.65	1.66	1.74	1.65	1.83	1.67	1.86	1.87	1.67	1.83	1.68	1.68
Particle size of diets (%)													
Sieve 19 mm	4.7	7.2	10.6	6.6	7.3	6.5	7.5	3.4	7.5	8.3	8.5	10.2	7.9
Sieve 8 mm	46.1	35.7	35.7	42.2	35.0	64.0	39.3	59.2	46.2	36.4	34.5	36.3	35.9
Sieve 1.18 mm	44.9	46.5	47.6	40.0	48.7	27.7	48.2	31.0	38.0	47.7	46.6	47.8	46.8
Pan	4.3	10.6	6.1	11.2	9.0	1.8	5.0	6.4	8.3	7.6	10.4	5.7	9.4
peNDF ⁴ >1.18 mm	33.5	26.8	25.6	28.5	27.3	30.4	29.0	32.7	30.9	27.3	27.0	28.1	26.0
peNDF >1.18 mm:starch ratio	1.28	0.99	0.86	1.03	0.98	1.00	0.98	1.13	1.02	0.95	0.90	0.99	0.95
Average pH per farm	6.30	6.05	5.80	6.24	5.91	6.06	6.05	6.25	6.23	5.85	5.81	5.92	6.06
SARA herd status ⁵	SARA-N	SARA-R	SARA-P	SARA-N	SARA-P	SARA-N	SARA-R	SARA-N	SARA-N	SARA-P	SARA-P	SARA-P	SARA-P

¹Part of results presented in Table 1 were used in Stefańska et al. (2017).²Composition: 21.5% Ca, 4.0% P, 6.5% Na, 5.5% Mg, 1,200 mg/kg of Cu, 4,000 mg/kg of Mn, 15 mg/kg of Co, 10,000 mg/kg of Zn, 60 mg/kg of Se, 1,200,000 IU/kg of vitamin A, 180,000 IU/kg of vitamin D, 6,000 IU/kg of vitamin E.³F:C = forage-to-concentrate ratio.⁴peNDF = physically effective NDF.⁵Herds were SARA-positive (SARA-P) if at least 25% of the ruminal fluid samples indicated pH <5.6; SARA-risk herds (SARA-R) had less than 25% of cows with a pH of ruminal fluid <5.6 but at least 33% with a pH ≤5.8; and SARA-negative herds (SARA-N) had less than 25% of cows in the herd with a pH of ruminal fluid <5.6 and less than 33% with a pH ≤5.8.

their DIM (40–150 d; average = 75), 305-d milk yield (10,070–12,041 kg; average = 10,940), and the number of lactations (primiparous, $n = 139$, and multiparous, $n = 166$). The health of cows was assessed according to recent medical history and through a detailed clinical examination always by the same veterinarian 1 d before starting sampling.

The herds were selected according to the classification proposed by Garrett et al. (1999) based on the percentages of cows with an assigned value of ruminal fluid pH and segregated into 3 groups. The SARA-positive herds had at least 25% of the ruminal fluid samples with pH <5.6; SARA-risk herds had less than 25% of ruminal fluid samples with pH <5.6, but at least 33% showed pH ≤ 5.8 ; and SARA-negative herds had less than 25% of the ruminal fluid samples with pH <5.6, but less than 33% exhibited pH ≤ 5.8 . Moreover, 305 selected dairy cows were divided according to the classification of Nordlund and Garrett (1994) based on ruminal fluid pH into 3 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, $n = 58$). Ruminal fluid samples were collected via rumenocentesis.

Ruminal Fluid Sampling and Analysis

Ruminal fluid samples were collected from the ventral sack of the rumen by rumenocentesis using pyrogen-free needles (2.0 $\times$ 120 mm) and 30-mL syringes (Duffield et al., 2004). The samples (30 mL) were collected 3 to 6 h after the morning feeding according to the methodology presented by Krause and Oetzel (2006). The ruminal fluid pH was measured using a CP-104 pH-meter (Elmentron, Zabrze, Poland). The calibration of the CP-104 pH meter was performed on each dairy farm before sampling relative to the reference buffer as a standard with value of pH 4, 7, and 9 (Alchem, Poznan, Poland).

Ruminal fluid samples were then divided into 3 parts. The first part was transferred into a 5-mL sterile, pyrogen-free glass bottle (Lonza Group Ltd., Basel, Switzerland) and kept on dry ice for transport to the laboratory for the initial processing before free LPS determination, as described by Gozho et al. (2007). These rumen fluid samples were centrifuged at 10,000 $\times g$ for 45 min at 4°C and the supernatant was aspirated gently to prevent its mixing with the pellet and passed through a disposable 0.22- μ m LPS-free filter (Millex; Millipore Corporation, Sigma-Aldrich, Poznan, Poland). The filtrate was collected in a sterile, pyrogen-free glass tube and heated at 100°C for 30 min. Samples were cooled at room temperature (19°C) for 10 min (Li et al., 2012). Concentrations of rumen-free LPS were

determined by chromogenic limulus amoebocyte lysate end-point assays (QCL-1000, Lonza Group Ltd.) in a 96-well microplate using an incubating microplate spectrophotometer with absorbance read at 405 nm (Synergy 2, BioTek Biokom, Warszawa, Poland). Pretreated samples were diluted until their free LPS concentration was 0.1 to 1 endotoxin units (**EU**)/mL relative to the reference endotoxin as a standard (*Escherichia coli* O111:B4). Ruminal fluid samples were diluted at 1:100,000, with the final dilution being made of 50% diluted sample and 50% β -glucan blocker (Lonza Group Ltd.).

The second part of ruminal fluid sample was transferred into a 5-mL plastic probe for VFA determination, and 0.5 mL of 85% formic acid was added to deproteinize the rumen fluid; after mixing, supernatants were centrifuged for 10 min at 10,000 $\times g$ at room temperature (Barszcz et al., 2011). Next, 500 mL of supernatants were transferred into chromatographic vials and mixed with isocaproic acid (internal standard; **IS**) at a ratio of 15 mL of IS to 100 mL of supernatant. Samples were analyzed in duplicate, using a HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with a flame-ionization detector and Supelco Nukol fused silica capillary column (30 m $\times$ 0.25 mm i.d.; 0.25 mm; Supelco, Bellefonte, PA). Helium was used as the carrier gas with a flow rate of 103 mL/min. The oven was initially kept at 100°C for 2 min, then heated at 10°C/min to 140°C and held for 20 min. The injector temperature was maintained at 220°C, whereas the detector was kept at 250°C. The total run time was approximately 27 min. Concentrations of individual VFA were estimated in relation to IS using a mixture of VFA standard solutions.

A third part of ruminal fluid sample was used for microscopic analysis of protozoa (*Entodiniomorpha* and *Holotricha*) according to the methodology described by Michalowski et al. (1986). Moreover, the total count of bacteria was determined in a Thoma chamber (Blau Brand, Wertheim, Germany) according to the method described by Ericsson et al. (2000). Identification of ciliates was performed as described by Hungate (1966).

Blood Sampling and Analyses

Blood samples were collected for each dairy cow at 3 to 6 h after the morning TMR delivery from the tail vein in a blank 10-mL Vacutainer for serum and a Vacutainer with EDTA for plasma harvesting (KABE, Poznan, Poland). The vacutainers with serum and plasma were transported to the laboratory in a refrigerated vehicle. Later, the serum and plasma vacutainers were centrifuged at 3,000 $\times g$ for 15 min at 4°C and the

serum and plasma were separated and stored at -20°C until analyzed. The serum was used for determination concentrations of lipopolysaccharide-binding protein (LBP), haptoglobin (Hp), serum amyloid A (SAA), and Ca. Similarly, the plasma was used to determine concentration of high-density lipoproteins (HDL). The concentrations of Hp, SAA, and LBP were determined using ELISA kits (Tri-Delta Diagnostic Inc., Immuniq, Zory, Poland). The serum samples were diluted initially at 1:5 for Hp, 1:500 for SAA, and 1:1000 for LBP. They were analyzed in duplicate and absorbance values were read at 630 nm for Hp and 450 nm for SAA and LBP using a microplate spectrophotometer (Synergy 2, BioTek Biokom). The minimum detection limits of these assays were 50 $\mu\text{g/mL}$, 0.133 $\mu\text{g/mL}$, and 0.216 mg/L for Hp, SAA, and LBP, respectively.

The concentrations of HDL and Ca were measured by using the colorimetric method and a microplate spectrophotometer (Synergy 2, BioTek Biokom) in reagent initially diluted at 1:1 (Pointe Scientific, Warsaw, Poland). They were analyzed in duplicate and absorbance values were read for HDL at 500 nm and for Ca at 650 nm.

RNA Isolation and Reverse Transcription

The total RNA from blood was isolated by use of Tri Reagent isolation (Molecular Research Center, Inc., Cincinnati, OH) according to manufacturer's instructions (<https://www.mrcgene.com/wp-content/uploads/2016/01/TRI-RT-3pageJan2016.pdf>). The RNA isolation method was based on the method described by Chomczynski and Sacchi (1987); RNA amount and quality were analyzed by using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific Wilmington, DE). The quality of RNA used for reverse transcription quantitative PCR reactions was between 1.9 and 2.2 (260/280 ratio). After isolation, 2 μg of total RNA was used for reverse transcription (RT) with the High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific). In this reaction, random hexaprimers were used; reaction was performed according to supplied instruction (<https://www.thermofisher.com/order/catalog/product/4368814?SID=srch-srp-4368814>). Negative control (RT-) without reverse transcriptase was included to exclude the contamination with genomic DNA. Positive control (RT+) including all reagents except RNA was also performed to check purity of reagents. Both controls were used in PCR analysis.

Real-Time PCR

Quantitative PCR (qPCR) reactions were performed for TLR4, CD14, MD2, and GAPDH. Obtained cDNA

was diluted (1:10) and used for RT-qPCR. Quantification was performed using 5 $\times$ Hot Firepol Eva Green qPCR Mix with ROX Passive Reference Dye (Solis Biodyne, Tartu, Estonia). During PCR reaction, Quant Studio 12K Flex system (ThermoFisher Scientific) was used. Program for qPCR reaction was prepared according to the instructions (https://www.sbd.ee/pics/7722_Data_Sheet_HOT_FIREPol_EvaGreen_qPCR_Mix_Plus_ROX.pdf) supplied with Eva Green MasterMix with some modifications. Namely, initial denaturation at 95°C for 15 min, then 40 cycles of denaturation at 95°C for 15 s, annealing at 61°C for 35 s, and elongation at 72°C for 15 s with fluorescence collection. To verify specificity of PCR products, melting curve analysis was also done according to protocol (95°C for 15 s; 60°C for 1 min; 95°C for 15 s), with fluorescence collection at 0.1°C intervals. Relative gene expression level was analyzed using the comparative cycle threshold ($2^{-\Delta\Delta\text{Ct}}$) method, with calculation the standard deviation of the ΔCt value, by using algorithm of Quant Studio 12K Flex Software supplied with the PCR instrument. For each sample, expression levels of the target genes were normalized to the reference gene, *GAPDH*. The reference gene was chosen according to the study by Zhao et al. (2016) and Gao and Oba (2016). Primers pairs were designed using Primers3 program (<http://bioinfo.ut.ee/primer3-0.4.0/>). Gene-specific primers sequences that span exon-exon junction (UCSC Genome Browser BLAT tool; <https://genome.ucsc.edu/cgi-bin/hgBlat>) are presented in Table 2.

Statistical Analysis

The means were subjected to ANOVA and Duncan's multiple range test using the PROC GLM procedure of SAS 9.4 (SAS Institute, 2014). The PROC MEANS and PROC UNIVARIATE procedures were also applied. The significance of the influence of the investigated experimental factors was analyzed by multivariate covariance analysis. The model included

$$y_{ijklm} = \mu + f_i + l_j + g_k + \beta_1 dl_i + \beta_2 my_m + e_{ijklm},$$

where y_{ijklm} = phenotypic value of the trait, μ = overall mean of the trait of the population, f_i = fixed effect of the farms ($i = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13$), l_j = fixed effect of the next number of lactations ($j = 1, 2, 3, 4, 5, 6, 7, 8$), g_k = fixed effect of the experimental group ($k = 1, 2, 3$), β_1 and β_2 = partial linear regression coefficients, dl_i = DIM, my_m = milk yield, and e_{ijklm} = random error. Pearson phenotype correlation coefficients were calculated using the PROC CORR procedure. Statistical significance was declared

Table 2. Gene names and primer sequences for real-time quantitative PCR analysis

Gene (accession number)	Sequence	Product size (bp)
Toll-like receptor 4 (<i>TLR4</i> ; nm_174198.6)	Forward - 5' CCTTGCGTACAGGTTGTTCC 3' Reverse - 5' GCCTAAATGTCTCAGGTAGTTAAAGC 3'	129
Cluster of differentiation 14 (<i>CD14</i> ; d_84509.1)	Forward - 5' CACCACATTGCACACCTGTT 3' Reverse - 5' CACCACATTGCACACCTGTT 3'	124
Myeloid differential protein 2 (<i>MD2</i> ; dq_319076.1)	Forward - 5' GGAGAATCGTTGGGTCTGCT 3' Reverse - 5' GCTCAGAACGTATTGAAACAGGA 3'	92
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPD</i> ; nm_001034034.2)	Forward - 5' TCATTGAAGCCTTCACTACATGGTCT 3' Reverse - 5' TGATGTTGGCAGGATCTCG 3'	147

at $P \leq 0.05$ and trends were considered when $0.05 < P \leq 0.1$. The standard error of the mean was adopted as a measure of error.

RESULTS

In our study, we noted higher total counts of bacteria, *Holotricha*, *Entodiniomorpha*, ratio of rumen acetate to propionate acid concentration ($C_2:C_3$; $P \leq 0.01$) and free ruminal LPS ($P \leq 0.05$) at both levels: in the HC versus RC and AC cow groups, and in the SARA-negative versus SARA-risk and SARA-positive herd status groups. In both classifications, in the AC group cows and SARA-positive herds, we recorded higher concentrations of acetate, propionate, n-butyrate, isobutyrate, n-valerate, isovalerate, and total VFA ($P \leq 0.01$; Tables 3 and 4). A positive correlation existed between the pH of rumen fluid and the total count of bacteria, *Holotricha*, *Entodiniomorpha*, and $C_2:C_3$ ratio

($P \leq 0.01$; Table 5). Also, the pH of ruminal fluid was negatively correlated with concentrations of acetate, propionate, n-butyrate, n-valerate, and the total VFA ($P \leq 0.01$).

We found a higher relative mRNA abundance of genes associated with the function of the LPS receptor complex, such as *CD14*, *TLR4*, and *MD2* at both levels: in the AC versus RC and HC cow groups, and in the SARA-positive versus SARA-risk and -negative herd status groups ($P \leq 0.01$; Tables 6 and 7).

In the AC group, we recorded higher concentrations of SAA ($P \leq 0.05$) and HDL ($P \leq 0.01$), as well as a tendency to a higher level of serum LBP ($P = 0.06$) and lower levels of Hp ($P \leq 0.05$) and Ca ($P \leq 0.01$) compared with the HC group (Table 6). Similar results were obtained in the SARA herd status analysis, but concentrations of LBP differed between SARA-positive and -negative herds at $P \leq 0.05$ (Table 7). Moreover, we noted a negative correlation between the pH of ru-

Table 3. Rumen microbiota composition, ruminal free LPS content, and ruminal fermentation indices in group of cows differing in ruminal average fluid pH

Indexes	Treatment ¹			SEM
	HC	RC	AC	
Ruminal fluid pH	6.35	5.71	5.56	0.09
Total bacteria count (10^9 /mL)	7.89 ^A	7.72 ^B	7.73 ^B	0.11
<i>Holotricha</i> (10^3 /mL)	4.88 ^A	4.65 ^B	4.63 ^B	0.32
<i>Entodiniomorpha</i> (10^5 /mL)	3.84 ^A	3.54 ^B	3.50 ^B	0.35
Ruminal LPS ($\log_{10}$ EU ² /mL)	4.48 ^a	4.57 ^b	4.57 ^b	0.41
Acetate (mmol/L)	33.5 ^A	39.6 ^B	44.5 ^C	3.15
Propionate (mmol/L)	15.1 ^A	20.5 ^B	25.4 ^C	0.14
N-butyrate (mmol/L)	6.7 ^A	8.6 ^B	9.9 ^C	0.01
Isobutyrate (mmol/L)	0.3 ^A	0.4 ^B	0.5 ^C	0.01
N-valerate (mmol/L)	1.1 ^A	1.5 ^B	2.0 ^C	0.01
Isovalerate (mmol/L)	0.8 ^A	1.0 ^B	1.2 ^C	0.01
Total VFA (mmol/L)	57.5 ^A	71.6 ^B	83.5 ^C	2.09
$C_2:C_3$ ratio ³	2.33 ^A	2.00 ^B	1.77 ^B	0.19

^{a,b}Means within a row with different letters differ at $P \leq 0.05$.

^{A-C}Means within a row with different letters differ at $P \leq 0.01$.

¹Treatment: HC = healthy cows, pH >5.8 ; RC = risk cows, pH 5.8–5.6; AC = acidotic cows, pH <5.6 .

²EU = endotoxin units.

³ $C_2:C_3$ = the ratio of rumen acetate to propionate acids concentrations.

ruminal fluid and CD14, TLR4, MD2, and concentrations of serum HDL, LBP, and SAA, whereas we found a positive correlation with Ca ($P \leq 0.01$; Table 8).

DISCUSSION

In the current study, we hypothesized that decreases in ruminal fluid pH associated with SARA may affect fermentation and increase the level of ruminal free LPS and expression of CD14/TLR4/MD2 complex, which may lead to systemic immune response in dairy cows. The average values of rumen fluid pH were 6.35, 5.71, and 5.56 in experimental cow groups HC, RC, and AC, respectively. The threshold value used for the definition of SARA was <5.6 , as previously defined (Nordlund and Garrett, 1994); however, the cows were fed a TMR with starch level from 26.1 to 30.3% DM, which could be the main reason for the intensity of the variation in the ruminal fermentation and might have led to the less frequent occurrence of SARA on the observed farms. For comparison, Schlau et al. (2012) used a diet consisting of 85% grain on a DM basis to induce SARA; the mean ruminal fluid pH was 6.01 for LS (low SARA risk) and 5.41 for HS (high SARA risk).

In the AC group and SARA-positive status herds (pH <5.6), the total counts of bacteria and ciliated protozoa, such as *Holotricha* and *Entodiniomorpha*, were decreased. Moreover, the total count of bacteria and both protozoa groups were positively correlated

with the pH of the rumen fluid. Bovine rumen is a classical host-microbe symbiotic system, which mainly consists of 3 groups of 10^9 bacteria, 10^6 protozoa, and 10^4 fungi per milliliter of rumen fluid (Krause et al., 2013). Ruminal fluid pH and its daily fluctuation characteristics are important factors in the regulation of the microbiome structure. In our study, we noted a reduction in the total number of bacteria from 15.1 to 8.1×10^9 /mL with a decreasing ruminal fluid pH in HC and AC, respectively. Dehority (2005) reported the death of in vitro protozoa at pH values below 5.4. Also, Owens et al. (1998) cited that, in cows on high-starch diets [150% of the NRC (2001) daily feed allowance], the prevalence of protozoa in the rumen typically declines, probably due to the lack of a floating fibrous mat in the rumen where the ciliate remain attached to multiply. According to Nagaraja and Titgemeyer (2007), a reduction in the number of the rumen ciliate population may be a good indicator of acute and subacute ruminal acidosis. Moreover, the intensive ruminal fermentation rate by ciliated protozoa was accompanied by reduced ruminal fluid pH and total count of bacteria (Owens et al., 1998). Mackie et al. (1978) observed that long-term intensive ruminal fermentation during SARA leads to an increase in concentrations of propionate, isobutyrate, and valerate, and a decrease in ruminal fluid pH and total count of ciliates and bacteria.

In the present results, we noted higher concentrations of acetate, propionate, n-butyrate, isobutyrate,

Table 4. Rumen microbiota composition, ruminal free LPS content, and ruminal fermentation indices classed by herd SARA status

Indexes	Treatment ¹			SEM
	SARA-negative	SARA-risk	SARA-positive	
Ruminal fluid pH	6.22	6.05	5.89	0.16
Total bacteria count (10^9 /mL)	7.87 ^A	7.72 ^B	7.70 ^B	0.09
<i>Holotricha</i> (10^3 /mL)	4.94 ^A	4.75 ^B	4.72 ^B	0.12
<i>Entodiniomorpha</i> (10^5 /mL)	3.89 ^A	3.65 ^B	3.60 ^B	0.16
Ruminal LPS (Log_{10} EU ² /mL)	4.47 ^a	4.49 ^b	4.60 ^b	0.07
Acetate (mmol/L)	33.5 ^A	36.3 ^B	43.4 ^C	4.10
Propionate (mmol/L)	15.1 ^A	18.7 ^B	22.5 ^C	2.17
N-butyrate (mmol/L)	6.2 ^A	7.6 ^B	8.1 ^C	0.01
Isobutyrate (mmol/L)	0.3 ^A	0.4 ^B	0.5 ^C	0.01
N-valerate (mmol/L)	1.2 ^A	1.5 ^B	1.5 ^C	0.01
Isovalerate (mmol/L)	0.8 ^A	0.9 ^B	1.0 ^C	0.01
Total VFA (mmol/L)	57.1 ^A	65.4 ^B	77.0 ^C	4.11
C ₂ :C ₃ ratio ²	2.22 ^A	1.94 ^B	1.93 ^B	0.16

^{a,b}Means within a row with different letters differ at $P \leq 0.05$.

^{A-C}Means within a row with different letters differ at $P \leq 0.01$.

¹SARA-positive herds had at least 25% of the ruminal fluid samples at pH <5.6 ; SARA-risk herds had less than 25% of cows with pH of ruminal fluid <5.6 , but at least 33% with pH ≤ 5.8 ; and SARA-negative herds had less than 25% of cows in the herd with pH of ruminal fluid <5.6 and less than 33% with pH ≤ 5.8 .

²EU = endotoxin units.

³C₂:C₃ = the ratio of rumen acetate to propionate acids concentrations.

Table 5. Correlation coefficients (r) between ruminal fluid pH and rumen microbiota, ruminal free LPS, and ruminal fermentation indexes

Item	TCB	<i>Holotricha</i>	<i>Entodiniomorpha</i>	LPS	Acetate	Propionate	N-butyrate	N-valerate	Total VFA	C ₂ :C ₃ ratio
Ruminal fluid pH	0.51**									
TCB ¹		0.39**	0.39**	-0.02	-0.75**	-0.79**	-0.70**	-0.70**	-0.84**	0.47**
<i>Holotricha</i>			0.45**	-0.45**	-0.30**	-0.39**	-0.19**	-0.36**	-0.38**	0.32**
<i>Entodiniomorpha</i>				-0.20**	-0.31**	-0.36**	-0.24**	-0.26**	-0.35**	0.20**
LPS ²				-0.24**	-0.26**	-0.33**	-0.07	-0.26**	-0.29**	0.22**
Acetate					0.01	0.35**	0.03	0.32**	0.35**	-0.21**
Propionate						0.71**	0.75**	0.58**	0.94**	-0.20*
N-butyrate							0.60**	0.81**	0.89**	-0.75**
N-valerate								0.60**	0.81**	-0.20**
Total VFA									0.76**	-0.60
C ₂ :C ₃ ratio ³										-0.42**

¹TCB = total count of bacteria.
²LPS = free ruminal LPS.
³C₂:C₃ ratio = the ratio of rumen acetate to propionate acids concentrations.
*P ≤ 0.05; **P ≤ 0.01.

n-valerate, isovalerate, and total VFA in the AC group and SARA-positive herd status group. The concentrations of total VFA were lower than in results shown by Morgante et al. (2007; 115 mmol/L in the healthy vs. 150 mmol/L in the acidotic cows), but similar to those given by Agle et al. (2010; 89.4 mmol/L and 91.4 mmol/L in the healthy and acidotic cows, respectively). The reasons for this variation are not fully understood. In our opinion, the differences in acid production may be associated with deterioration in the digestibility of OM, a faster passage from the rumen to the lower parts of the digestive tract, or an increase in the intensity of VFA metabolism during SARA occurrence. Moreover, the pH of ruminal fluid was negatively correlated with the concentrations of acetate, propionate, n-butyrate, n-valerate, and total VFA and positively correlated with C₂:C₃ ratio. Similar to our results, Golder et al. (2014) showed that diets with high NFC (50–70% of DM basic) and low NDF (16–26% of DM basic) increase the risk of ruminal acidosis, a condition associated with increased ruminal propionate, valerate, and butyrate. Bannink et al. (2008) observed, at ruminal pH lower than 6.0, an increased production of propionate and butyrate relative to acetate; moreover, Poorkasegaran and Yansari (2014) showed that a low ruminal pH (<5.6) was associated with lower acetate and C₂:C₃ ratio. In the present study, a decrease in the rumen fluid pH was associated with a decrease in the acetate-to-propionate ratio, which in AC cows and SARA-positive herds was lower than 2. Also, Krause and Oetzel (2005) reported that the C₂:C₃ ratio lower than 2 was associated directly with high-grain diets and indirectly with SARA.

Free ruminal LPS are bacterial endotoxins that are a component of the outer membrane of the most predominant group in microbial populations in the rumen, such as gram-negative bacteria. The cows in our study differed in their ruminal free LPS concentrations, with higher levels found in the AC group (4.57 Log₁₀ EU/mL; 42,206 EU/mL) and lower in the HC group (4.48 Log₁₀ EU/mL; 34,179 EU/mL). Similarly, in the SARA-positive herds we noted higher concentrations of ruminal free LPS (4.60 Log₁₀ EU/mL; 43,000 EU/mL) compared with SARA-negative herds (4.47 Log₁₀ EU/mL; 32,225 EU/mL). The differences between groups were significant, but were small in contrast to results from other studies [5.11 Log₁₀ EU/mL vs. 4.39 Log₁₀ EU/mL (Ghazo et al., 2007); 107,152 EU/mL vs. 28,184 EU/mL (Khafipour et al., 2009)]. We suggested that the differences could be associated with the content and quality of starch in the diet, and specifically with the efficiency of VFA metabolism in rumen epithelial cells as a potential mechanism for decreasing the risk of ruminal acidosis and the slow death and lysis of gram-negative bacteria and free ruminal LPS.

Table 6. Comparison of mRNA abundance of genes associated with function of LPS receptor complex and biochemical blood index concentrations in cows differing in pH of ruminal fluid

Indexes ¹	Treatment ²			SEM
	HC	RC	AC	
CD14	5.07 ^A	9.78 ^B	12.10 ^C	1.43
TLR4	1.20 ^A	1.82 ^B	2.63 ^C	0.16
MD2	1.34 ^A	2.63 ^B	9.92 ^C	0.74
LBP (mg/mL)	12.88	12.89	13.86	0.28
SAA (µg/mL)	9.16 ^a	10.70 ^b	10.80 ^b	0.23
Hp (ng/mL)	516.85 ^a	485.41 ^b	470.19 ^b	8.49
HDL (mg/dL)	68.32 ^A	68.17 ^{AB}	78.16 ^B	1.01
Ca (mg/dL)	10.16 ^A	9.86 ^{AB}	8.26 ^B	0.12

^{a,b}Means within a row with different letters differ at $P \leq 0.05$.^{A-C}Means within a row with different letters differ at $P \leq 0.01$.¹CD14 = cluster of differentiation 14; TLR4 = toll-like receptor 4; MD2 = myeloid differential protein 2; LBP = lipopolysaccharide-binding protein; SAA = serum amyloid A; Hp = haptoglobin; HDL = high-density lipoproteins.²Treatment: HC = healthy cows, pH >5.8; RC = risk cows, pH 5.8–5.6; AC = acidotic cows, pH <5.6.**Table 7.** Comparison of mRNA abundance of genes associated with function of LPS receptor complex and biochemical blood index concentrations classed by herd SARA status

Indexes ¹	Treatment ²			SEM
	SARA-negative	SARA-risk	SARA-positive	
CD14	5.49 ^A	10.67 ^B	15.83 ^C	1.43
TLR4	1.23 ^A	1.64 ^B	2.76 ^C	0.16
MD2	1.03 ^A	2.87 ^B	7.92 ^C	0.74
LBP (mg/mL)	12.03 ^a	12.87 ^{ab}	14.92 ^b	0.28
SAA (µg/mL)	8.58 ^a	10.72 ^b	11.30 ^b	0.23
Hp (ng/mL)	529.89 ^a	510.34 ^{ab}	475.94 ^b	8.49
HDL (mg/dL)	67.53 ^A	68.13 ^{AB}	77.28 ^B	1.01
Ca (mg/dL)	10.83 ^A	9.97 ^{AB}	8.68 ^B	0.12

^{a,b}Means within a row with different letters differ at $P \leq 0.05$.^{A-C}Means within a row with different letters differ at $P \leq 0.01$.¹CD14 = cluster of differentiation 14; TLR4 = toll-like receptor 4; MD2 = myeloid differential protein 2; LBP = lipopolysaccharide-binding protein; SAA = serum amyloid A; Hp = haptoglobin; HDL = high-density lipoproteins.²Treatment: HC = healthy cows, pH >5.8; RC = risk cows, pH 5.8–5.6; AC = acidotic cows, pH <5.6.**Table 8.** Correlation coefficients (r) between rumen fluid pH and comparison of mRNA abundance of genes associated with function of LPS receptor complex and biochemical blood index concentrations¹

Item	TLR4	MD2	CD14	HDL	Ca	LBP	SAA	Hp
Ruminal fluid pH	−0.20**	−0.22**	−0.29**	−0.33**	0.20**	−0.06	−0.37**	−0.16
TLR4		0.21**	0.34**	−0.20**	0.22**	0.28**	0.27**	0.09
MD2			0.25**	−0.006	0.13	0.06	0.06	0.03
CD14				−0.22*	0.17**	0.05	0.01	0.46**
HDL					−0.07	−0.10	−0.06	−0.03
Ca						−0.11	−0.02	−0.14
LBP							0.62**	0.0005
SAA								0.20**

¹TLR4 = toll-like receptor 4; MD2 = myeloid differential protein 2; CD14 = cluster of differentiation 14; HDL = high-density lipoproteins; LBP = lipopolysaccharide-binding protein; SAA = serum amyloid A; Hp = haptoglobin.* $P \leq 0.05$; ** $P \leq 0.01$.

Shen et al. (2017) reported higher concentrations of LPS in a group fed wheat rather than a corn diet (35.5 vs. 22.1×10^3 EU/mL). In the same study, animals fed a high-starch diet (39% starch, DM basis) versus a low-starch diet (24% starch, DM basis) also had a greater ($P < 0.05$) LPS concentration in ruminal fluid (40.3 vs. 17.3×10^3 EU/mL). Similarly, Guo et al. (2016) showed that feeding cows a high-starch diet (32% NDF, 27% starch, DM basis) led to increased LPS concentrations in rumen fluid, indicating a greater risk of compromised rumen health and inflammation. In the current study, we found a relationship between the quality of starch and the frequency of SARA occurrence. The SARA-positive herds were generally fed diets with a rapidly fermented starch (e.g., barley, wheat, triticale grain) than corn, and also had a greater ($P < 0.05$) LPS concentration in ruminal fluid. Moreover, the results of our study showed that the analysis of the chemical compositions of diets is important to evaluate the prepared TMR and cover the nutritional needs of high-yielding dairy cows, but their use (especially only the content of starch in the diet, as the only indicator of SARA occurrence) should be avoided. The average content of NDF was 29.4 versus 33.3% DM and starch was 28.6 versus 28.3% DM in the SARA-positive and -negative herds, respectively.

Although NRC (2001) recommends maximum NFC and minimum NDF levels (% of DM) to decrease the occurrence of SARA, and even cows are fed the same diet, there is a variation in ruminal fluid pH and degree of acidity and LPS concentrations (Chen et al., 2012; Gao and Oba, 2016; Stefańska et al., 2017). Mertens (1997) explained that feed particles longer than 1.18 mm were more effective in simulating chewing activity, and therefore in increasing the secretion of saliva and ruminal buffering capacity compared with smaller particles. On the other hand, a reduced forage particle size improved the uniformity of TMR, resulting in less sorting behavior, which might reduce the risk of ruminal disorders. Mertens (1997) also defined peNDF as the specific effectiveness of NDF for stimulating chewing activity in relation to particle size. Zebeli et al. (2012) suggested that 31.2% peNDF >1.18 mm is sufficient to prevent SARA. The concept of physically effective fiber proposed by Mertens (1997) was considered more efficient in decreasing the risk of SARA than only dietary fiber level. In our study, in SARA-positive herds we noted a lower peNDF >1.18 mm level (from 25.6 to 28.1%) than the recommended dietary level. Moreover, Zebeli et al. (2012) showed that the ratio of the dietary peNDF >1.18 mm to rapidly degradable grain is highly correlated with the ruminal pH value, and a diet with a ratio of 1.45 could prevent the occurrence of SARA.

However, this ratio may be difficult to reach when the diet is based on rapidly rumen-degradable starch such as barley or wheat. According to our results, the ratio of peNDF >1.18 mm to starch could be a better potential indicator of a well-balanced diet, useful in the prevention of SARA occurrence, and its value should be no lower than 1.00 for high-yielding dairy cows.

In the current study, in both cow and herd classifications, we found a higher expression of genes associated with the function of LPS receptors, such as *CD14*, *TLR4*, and *MD2*, together with higher serum LBP. The LBP is APP, which plays important roles in modulating the innate immune response against bacteria (Zweigner et al., 2006); LBP is a 60-kDa glycoprotein that is predominantly synthesized by hepatocytes. As an APP, its production is upregulated after infection and largely dependent on IL-1 β , IL-6, and tumor necrosis factor- α (TNF- α ; Schumann et al., 1996). By recognizing the lipid A component of LPS, LBP can be considered the first step of LPS detection and reaction by the host. The LBP binds to the outer membrane of gram-negative bacteria, where LPS resides. This binding depends on both calcium and albumin. Upon efficient binding, LBP assembles LPS to both soluble and membrane-bound CD14, activating the LPS signal. Without LBP, the sensitivity of LPS and gram-negative bacteria diminishes up to 1,000 times (Weiss, 2003). Classical function of serum LBP includes binding to the amphipathic lipid of a moiety of LPS that facilitates the process of LPS presentation to membrane-bound CD14 on monocytes (Fujihara et al., 2003) and forms a complex: LPS/LBP/CD14 (Park and Lee, 2013). Both LBP and CD14 are accessory proteins that enhance the detection of LPS by the TLR4/MD2 complex by extracting and monomerizing LPS before its presentation to TLR4/MD2. This, in turn, leads to the activation of the TLR4/MD2 complex pathway and cytokine production, thus triggering a proinflammatory response (Zweigner et al., 2006). The presentation of LPS to CD14 through LBP is believed to increase the LPS-mediated innate immunity pathways from 100 to 1,000 fold (Rahman et al., 2010). Deficiency of LBP leads to high mortality and reduced immune response, especially the recruitment of neutrophils and the production of inflammatory cytokines and chemokines, such as IL-6, macrophage inflammatory protein-2, and TNF- α . By contrast, very high concentrations of LBP inhibit the LPS signal, displaying a modulatory function (Zhou et al., 2016).

It is well documented that, during acute phase response, significant changes occur in the protein synthesis of the liver, such as the production of APP. The most reactive APP in cattle are SAA, LBP, and Hp

(Alsemgeest et al., 1994). Khafipour et al. (2009) and Zebeli and Ametaj (2009) showed that SARA could affect inflammation, which is observed in the increasing production of APP such as SAA and LBP, but not Hp. In the current study, higher concentrations of SAA were noted in the AC group and SARA-positive status herds. Gozho et al. (2005) explained that SAA is considered to be the most sensitive APP that responds faster to inflammation stimuli, because it is detected earlier in blood than Hp. No increase in Hp, despite increased SAA during SARA, might be explained by differences in the cytokines involved in initiating the synthesis of these acute phase proteins (Jacobsen et al., 2004). Either IL-6 or TNF- α is required for the synthesis of SAA, but both of these cytokines are needed for Hp synthesis (Alsemgeest et al., 1996). Moreover, the differences occur in the acute phase response, which varies between acute and chronic inflammations (Alsemgeest et al., 1994). It is widely acknowledged that repeated or chronic exposure to LPS leads to a state of responsiveness characterized by a marked reduction in the magnitude or duration of LPS responses, such as inflammation (Jacobsen et al., 2004). Chronic inflammation leads to increased levels of APP in circulation, but concentrations are not elevated to the same extent as during acute inflammatory processes (Horadagoda et al., 1999). Therefore, SAA is generally perceived as an indicator of acute inflammation in cattle, whereas haptoglobin reacts more slowly and thus reflects the presence of chronic inflammatory conditions (Plaizier et al., 2008).

In the current study, we hypothesized that an increase in the expression of the LPS receptor complex leads to higher concentrations of plasma HDL and serum Ca. Cholesterol biosynthesis is an alternative pathway of VFA metabolism in rumen epithelial cells (Steele et al., 2009). High cholesterol concentrations in the cells could be associated with an increased amount of VFA substrate in the rumen when faced with a high-grain diet, and this may increase the intracellular cholesterol concentration and cause possible abnormalities in cholesterol homeostasis and higher concentrations in the blood (Penner et al., 2011).

In our study, acidotic cows and SARA-positive herds demonstrated higher concentrations of plasma HDL compared with the healthy groups, and these levels exceeded reference values. The HDL was the most abundant of the lipoproteins in bovine plasma (>85%; Bauchart, 1993). Increasing evidence points to HDL or other lipoproteins helping to control the host response to free LPS. Numerous studies have shown that a complex of free LPS with plasma HDL and other lipoproteins has little or no stimulatory activity toward

immune cells and the response of macrophages in vitro and in vivo (Wu et al., 2004), and evidence strongly indicates that lipopoliproteins can neutralize LPS in vivo (Contreras-Duarte et al., 2014). When LPS from gram-negative bacteria are incubated with whole blood, the majority of the LPS are bound to HDL. This binding to HDL inhibits the ability of LPS to interact with toll-like receptors and activate macrophages (Khovidhunkit et al., 2004). Toll-like receptor activation of macrophages stimulates the production and secretion of cytokines and other signaling molecules, which, if produced in excess, can lead to septic shock and death (Beutler et al., 2003). In addition to binding LPS, studies have shown that HDL also facilitates the release of LPS that is already bound to macrophages, reducing macrophage activation (Kitchens et al., 1999). The phospholipid content of lipoproteins correlates with the ability of lipoproteins to neutralize LPS, whereas the content of cholesterol or triglycerides does not (Khovidhunkit et al., 2004). Additionally, phospholipids alone have been shown to protect animals from LPS-induced toxicity. Thus, both apolipoproteins and phospholipids can play important roles in the ability of HDL to neutralize LPS (Khovidhunkit et al., 2004). Read et al. (1993) showed that plasma lipopoliproteins, particularly HDL, bind LPS and preferentially shunt it to hepatocytes and away from hepatic macrophages, thereby increasing LPS excretion via bile (67%) and preventing any immune response.

On the other hand, presentation of LPS by CD14 to TLR4/MD2 could be blocked by higher concentrations of the plasma HDL. When CD14 are combined with LPS and then incubated in plasma, they release over 70% of cell-associated LPS into HDL (which prevents an immune response), whereas in serum the LPS remains tightly associated with the cells and stimulates the activity of LPS/LBP/CD14 activation of LPS/LBP/CD14 and TLR4/MD2 complexes (Kitchens et al., 2001). Other experiments have revealed that CD14 enhances LPS release from monocytes (Kitchens et al., 1999) and that LBP can facilitate the transfer of LPS from LPS-CD14 complexes to HDL (Wurfel et al., 1995). Essentially all of the LPS on the cell surface of monocytes could be released, reducing monocyte responses to LPS and attenuating the immune response of the host.

In the AC group and SARA-positive herds, we found a lower concentration of serum Ca compared with other groups and herds, below the reference value (Cozzi et al., 2011). Although the mechanistic details related to a declining response of plasma Ca to increasing concentrations of rumen endotoxin or plasma SAA are not well understood, it is speculated that withdrawal

of serum Ca might be part of the immune response to facilitate detoxification of endotoxins (Zebeli et al., 2010). In support of this, Rosen et al. (1958) indicated that adding or removing Ca from serum decreases or increases, respectively, the endotoxin-detoxifying capability of serum. Interestingly, Kula et al. (1977) also demonstrated that serum Ca, at physiological concentrations, facilitates aggregation of SAA and deposition of SAA amyloid fibrils in major organs, leading to amyloid A amyloidosis. The findings of our study and other investigations suggest that withdrawal of Ca during an acute phase response or an inflammatory condition might be part of the host strategy to maintain a stable SAA structure and help expedite the neutralization and removal of endotoxins from plasma. Waldron et al. (2003) reported that infusion of free LPS is associated with decreased concentrations of total serum Ca in a dose-dependent manner, such that greater amounts of free LPS cause more severe reduction to serum Ca. Eckel and Ametaj (2016) hypothesized that Ca inhibits binding of LPS to lipopoliproteins and suggested that hypocalcemia might be a protective response to free LPS, creating conditions for the disaggregation of free LPS and its binding to HDL and, therefore, neutralization and elimination of free LPS from circulation. We agree with their hypothesis, because we noted negative HDL and positive Ca correlations with the LPS receptor complex: CD14 and TLR4.

CONCLUSIONS

The results of the current study indicated that decreasing ruminal fluid pH associated with SARA increased the release of free LPS into the rumen and stimulated the expression of LPS/LBP/CD14 and TLR4/MD2 complexes and concentrations of SAA. In addition, plasma HDL could inhibit the ability of LPS to interact with toll-like receptors and activate macrophages, which can help to control the host immune response to free LPS. Similarly, reduction of serum Ca may be an effective protective mechanism against endotoxemia. However, the biological significance of these results needs to be investigated further in larger field trials.

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