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NMR Based Metabolomics Evaluation in Neonatal Calves with Acute Diarrhea and Suspected Sepsis: A New Approach for Biomarker/S

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Abstract

Metabolic consequences of diarrhea-induced sepsis revealed by plasma 1H-Nuclear Magnetic Resonance (NMR) quantitative metabolomics. Diarrhea and sepsis is generally the most common cause of morbidity and mortality in pre-weaned calves. Traditional biomarkers for sepsis are mainly derived from host immune/inflammatory response. Various high-throughput omics technologies facilitate comprehensive screening of sepsis-specific biomarkers in human medicine. The aim of this first study was to reveal the new potential biomarkers in diarrhea-induced septic neonatal calves evaluating by 1H-NMR based quantitative metabolomics. Clinical and laboratory data revealed all the ill calves had leukocytosis, metabolic acidosis and failure of colostral passive transfer. This study produced quantitative data sets that presented differences between patients with diarrhea–induced sepsis and healthy subjects in the level of the water and lipid soluble metabolites. All the lipid soluble metabolites (e.g., sphingomyeline, various fatty acids, etc.) were significantly decreased in diseased calves. Changes in water soluble metabolites (increases in niacinamide, choline + phosphocholine, 2-methylglutarate and isopropanol, and decreases in formate, lysine-arginine, acetate, creatine) were meaningful for pathogenic mechanisms of sepsis. This pilot study showed the implementation of plasma 1H-NMR quantitative metabolomics because it produced a physiologically relevant metabolite data set that distinguished diarrhea-induced sepsis from healthy ones. The metabolites identified and quantified in the study may be new potential biomarkers for systemic inflammatory response syndrome in calf sepsis.

Keywords: Metabolomics; NMR; Calf diarrhea and sepsis

Introduction

Calf diseases have major effects on the economic profits of cattle industry [1]. High calf mortality rates have been observed in the neonatal period covering the first months of life all over the world. Although failure of colostral transfer is important in the preventing newborn calf mortality, various available stressor factors increase calf mortality due to infectious diseases (e.g., acute diarrhea). Diarrhea is generally the most common cause of morbidity-mortality in pre-weaned calves [2-4]. Neonatal sepsis is defined as a combination of an infection (or suspected infection) and a systemic inflammatory response. The early diagnosis of sepsis remains a challenge in veterinary medicine and in human perinatology and a great challenge for clinicians, and delayed diagnosis frequently undermines treatment efforts, thereby contributing to high mortality [5,6]. The diagnosis of sepsis using currently available tests is neither early nor specific, and is most often indirect and presumptive, and is based on physical and laboratory findings consistent with this disease. New biomarkers are needed to allow early diagnosis and treatment of sepsis. Metabolomics a diagnostic tool based on the determination of metabolites contained in biofluids may open new horizons in managing critically ill newborns. It can also be considered that certain metabolomic patterns can be related with specific outcomes, thus helping in decision-making, resource allocation, and prognosis [7]. The application of new technologies and the combination of multiple omics approaches are necessary to develop tools for the effective diagnosis of sepsis and to improve a personalized approach and the prognosis for this condition [8-11]. This is the first study aiming at identification and quantification of metabolomic biomarkers in neonatal calves with diarrhea and presumed sepsis in water and lipid soluble extracts of plasma samples.

Material and Methods

Animals

The experimental design was approved by the Committee on Use of Animals in Research of the Selcuk University, Faculty of Veterinary Medicine. Forty four clinically ill calves <30 days old, presented for treatment to the teaching hospital, were subjects of study. Calves were considered suitable for inclusion in the study if they had sufficient clinical and laboratory data at admission to suggest diarrhea and septicemia. Laboratory and clinical models described by Fecteau et al. [5] were used to predict septicemia in ill calves. Eleven neonatal calves<30 days served as controls were allowed to suckle the dam for two days, and the healthy status of them were monitored daily in the first month of age.

Laboratory analyses

Laboratory workup included: Hematological analyses: complete blood count (blood cells, mean corpuscular volume, hematocrit,

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hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration) (Hemocell Counter MS4e, Melet Schloesing Laboratories, France) and blood gas analysis (pH, pO_2, pCO_2, HCO_3, base excess, O_2 saturation) (Gem Premier 3000, Instrumentation Laboratory, USA); biochemical analyses: enzymes (AST, GGT, CPK) activities, and total protein, fibrinogen, immunoglobulin G, lactate, creatinine and minerals (sodium, potassium and ionized calcium) concentrations (BT 3000 Spectrometer, Italy). Immunoglobulin G and fibrinogen concentrations were determined by use of a commercial ELISA test kits (EAST BIOPHARM).

Data collection

Physical findings, laboratory data at admission, and outcome were tabulated for all calves. The clinical abstract was constructed to include variables that reflected severity of sepsis (e.g., high plasma fibrinogen concentration and enzymes activities, failure of passive transfer, metabolic acidosis, recumbent posture, and a poor suckle reflex).

NMR based metabolomic evaluation

NMR measurements were performed at Bio-NMR Centre and Department of Chemistry, University of Florence in Florence/Italy.

Sample preparation for ^{1}H-NMR spectroscopy: Plasma samples were thawed on ice and extracted using a dual methanol-chloroform extraction (for protein precipitation and separation of hydrophilic and lipophilic fractions) as previously described [12]. This eliminates macromolecules (e.g., proteins) and establishes a fused metabolic profile for water-soluble and lipid metabolites. Briefly, 0.5 ml of ice-cold plasma was mixed with 1 ml of chloroform:methanol (1:1 vol/vol) and centrifuged. The supernatant (organic phase) was collected, and the pellet was resuspended with 0.5 ml of chloroform/methanol and centrifuged. The supernatants were combined, and 0.5 ml of ice-cold water was added to 'wash out' remaining water-soluble metabolites from the organic phase. After 15 min at -20°C, the upper (aqueous) phase was removed and added to the remaining pellet (to wash out remaining water-soluble metabolites from the pellet), 1 ml of water was added, and the sample was centrifuged and freeze-dried overnight. The water-soluble extracts were then dissolved in 0.5 ml of D_2O, centrifuged, and transferred into 5 mm NMR tubes. The lipid rich methanol-chloroform fraction (bottom phase after low temperature exposure) was evaporated using a high-speed vacuum centrifuge; dried lipid extracts were dissolved in 0.6 ml of deuterated methanol:chloroform (1:2 vol/vol) and centrifuged and transferred into 5 mm NMR tubes.

Quantitative ^{1}H-NMR spectroscopy on plasma extracts: All 1 and 2D NMR spectra were obtained on a Bruker 600 MHz spectrometer operating at 600.13 MH proton Larmor frequency and equipped a 5 mm CPTCI ^{1}H-^{13}C/^{31}P-^{2}H cryo-probe including a z-axis gradient coil, an Automatic Running-Matching (ATM) and automatic sample changer. Before measurement, samples were kept for 3-5 min inside the NMR progehead at 310 K, for temperature equilibration. For each plasma water-soluble and lipid extract, a one-dimensional (1D) NMR spectrum was acquired with water peak suppression using a standard pulse sequence (NOESY resat; Bruker), 5 dB power level, 64 scans, 64 k data points, and are laxation delay of 4stoachievefully relaxed ^{1}H-NMR spectra resulting in a total acquisition time of 7.5 min. A two-dimensional (2D)-H- C-HSQC (heteronuclear single quantum correlation) NMR technique was also acquired. The experiments were performed with 128 increments and 8 scans per increment, using 90 degree pulse and a recovery delay of 2 s. The spectral width was 16 ppm in the proton dimension and 166 ppm in the carbon dimension. The metabolites identification was performed by using 1D NOESY spectra with assistances of 2D HSQC spectra. The NMR spectral data was transformed by using Fourier transform with Top Spin 2.1 (Bruker), and all spectra were phase- and baseline-corrected automatically. The chemical shift was calibrated by aligning the TMSP signal at 0 ppm. Each 1Dspectrum was segmented into 0.02 ppm chemical shift buckets in the range between 0.2 and 10 ppm, and the corresponding spectral areas were integrated by using the AMIX 3.8.4 software (Bruker). The buckets in the region between 4.75 and 4.55 ppm were removed to avoid intensity distortions due to the water signal.

Statistical analyses

All laboratory data were presented as the mean ± SE. The data were evaluated by one-way ANOVA-Duncan using the SPSS 13 program, and the differences between the means assessed using Duncan's multiple-range test. Statistical significance was considered at $p<0.05$.

Multivariate statistical technique, orthogonal projections to latent structures-discriminant analysis OPLS-DA, was applied on the obtained spectral buckets data for dimension reduction. Statistical models were built with five-fold cross validation. Data analysis was conducted by assigning the classifier on OPLS scores. Accuracy, sensitivity, and specificity were calculated using standard definitions. All calculations were performed with R scripts developed in-house [13].

Results

Clinical and laboratory data

All ill calves had leukocytosis, metabolic acidosis and failure of passive colostral transfer. Clinical findings such as capillary refill time, scleral injection and dehydration, inability of stand and lack of suckling reflex supported their septic states. There were significant increases in enzymes levels in ill calves. The clinical and laboratory characteristics are shown in Table 1.

NMR-based metabolites

Over 20 metabolites were identified and quantified by ^{1}H-NMR from each data set (lipophilic and hydrophilic fractions) of each plasma sample (Table 2). ^{1}H-NMR spectroscopy identified biologically relevant metabolites from ill calves (Figure 1 and 2). The resulting quantitative metabolomic data showed differences in the levels of all the lipid soluble metabolites which were significantly decreased in diseased group where there were significant changes in water soluble metabolites (e.g., increases in choline, niacinamide, creatine, choline, isopropanol, 3-hyroxyubutyrate, 2-methylglutarate, lactose, and decreases in acetate, lysine-arginine) (Figure 3). Collectively, these alterations reflect the complex pathology and provide evidence for the involvement of a number of processes including metabolic shift from homolactic (lactate production) to mixed-acid fermentation, with emerging of yields other than lactate (e.g., ethanol, acetate, and formate) in some situations (e.g., aerobiosis, sugar limitation), or the availability of sugars less chosen than glucose (formate); inhibition of the activation of coagulation related to endotoxemia (niacinamide); oxidative (creatine, 2-methylglutarate); inhibition on the synthesis and release of TNF-α and high morbility (choline); anti-inflammation or immunosuppression (acetate, ω-3 PUFA, isopropanol); metabolic, immune and reparative response to trauma and sepsis (arginine); negative energy balance (3-hyroxyubutyrate) and endothelial barrier

Parameters	Healthy group (n=11) (mean ± SE)	Diseased group (n=44) (mean ± SE)	P value
Clinical			
Age, days	12,5 ± 1,59	12,3 ± 1,89	0.9700
Temperature °C	38.5 ± 0.13	37.0 ± 0.22	0.000
Pulsation, beats/min	114 ± 2.87	97.7 ± 7.65	0.057
Respiration, breaths/min	27.7 ± 1.09	32.5 ± 2.86	0,101
Dehydration, (%)	3.64 ± 0.20	9.53 ± 0.17	0.000
Capillar refill time, sec	2.09 ± 0.09	3.71 ± 0.11	0.000
Suckling reflex	-	Weak to absent	
Posture	-	Recumbent	
Scleral injection	-	↑	
Focal infection		No	
Feces	-	Watery, mucoid, bloody	
Blood Gas Analysis and CBC			
Blood pH	7.37 ± 0.01	7.20 ± 0.05	0.003
pCO_2, mmHg	49.2 ± 1.05	47.4 ± 3.30	0.609
pO_2, mmHg	22.4 ± 1.15	21.5 ± 1.70	0.676
HCO_3, mEq/L	28.6 ± 0.78	20.9 ± 2.92	0.020
Base excess, mEqlL	3.46 ± 0.58	-5.82 ± 3.43	0.016
O_2 saturation	36.5 ± 3.15	26.5 ± 3.85	0.054
White blood cells, $\times 10^3/\mu L$	10.4 ± 0.92	20.4 ± 2.82	0.003
Red blood cells, $\times 10^6/\mu L$	7.72 ± 0.34	7.93 ± 0.45	0.716
Thrombocytes,$\times 10^5/\mu L$	454 ± 50.3	726 ± 148	0.098
Mean corpuscular volume, fL	32.9 ± 0.91	35.9 ± 1.02	0.037
Mean corpuscular Hb, pg	11.9 ± 0.63	11.6 ± 0.27	0.658
Mean cell Hb concentration, g/dL	36.7 ± 2.29	32.6 ± 0.49	0.110
Hematocrit, %	25.5 ± 1.57	28.8 ± 2.17	0.227
Hemoglobin, g/dL	9.12 ± 0.30	9.34 ± 0.63	0.753
Biochemical			
Total Protein, g/dL	5,18 ± 0.15	5.32 ± 0.23	0.632
Fibrinogen, mg/dL	837 ± 63.2	293.± 23.4	0.000
ImmunoglobulinG, mg/dL	1334 ± 4.20	343 ± 12.5	0.000
Creatinine, mg/dL	1.04 ± 0.05	2.35 ± 0.75	0.096
Glucose, mg/dL	72.4 ± 4.40	64.0 ± 7.90	0.359
Lactate, mEqlL	1.56 ± 0.25	2.40 ± 0.54	0.175
AST, (U/L)	41.0 ± 3.00	132 ± 24.2	0.001
GGT, (U/L)	47.5 ± 7.52	105.6 ± 24.2	0.032
CPK, (U/L)	73.0 ± 13.4	353 ± 64.5	0.000
Na, mEqlL	142 ± 1.24	138 ± 2.55	0.192
K, mEq/L	4.21 ± 0.10	4.79 ± 0.33	0.113
Ionized Ca, mEq/L	0.96 ± 0.04	1.01 ± 0.05	0.420

Table 1: Clinical and laboratory data of healthy and ill calves

	Healthy group (n=11)	Diseased group (n=44)	P value
Water Soluble Metabolites			
Formate	0.010 ± 0.002	0.008 ± 0.002	0.021
Niacinamide	0.0002 ± 0.0003	0.003 ± 0.002	0.0005
Lactate	1.47 ± 0.54	3.18 ± 3.3	0.08
α-Glucose	0.12 ± 0.033	0.13 ± 0.132	0.83
Mannose	0.0046 ± 0.0009	0.003 ± 0.0042	0.173
Creatine	0.057 ± 0.018	0.11 ± 0.06	0.0022
Lactose	0.01 ± 0.004	0.06 ± 0.06	5.2e-5
Choline+Phosphocholine	0.026 ± 0.004	0.041 ± 0.03	0.003
Acetate	0.062 ± 0.02	0.031 ± 0.012	0.0003
Lysine+Arginine	0.026 ± 0.01	0.008 ± 0.012	0.0035
Isopropanol	0.004 ± 0.003	0.031 ± 0.024	0.0001
3-hyroxyubutyrate	0.028 ± 0.006	0.065 ± 0.03	0.001
2-Methylglutarate	0.0083 ± 0.0027	0.016 ± 0.005	1.76e-5

Lipid Metabolites			
Sphingomyeline	0.016 ± 0.003	0.002 ± 0.0006	5.59e-6
UFA	0.594 ± 0.156	0.15 ± 0.09	4.36e-5
Glycerol-P-Lipids	0.053 ± 0.013	0.023 ± 0.013	0.0004
TAG	0.043 ± 0.014	0.023 ± 0.012	0.015
Cholesterol	0.33 ± 0.04	0.089 ± 0.044	5.59e-6
Phospholipids	2.41 ± 0.18	2.046 ± 0.21	0.0004
PUFA	0.252 ± 0.065	0.073 ± 0.056	7.4e-5
FA	2.259 ± 0.408	0.508 ± 0.364	9.98e-6
Total Lipids (CH_2)n	12.9 ± 2.29	2.87 ± 1.38	5.59e-6
Total Lipids (CH_3)n	5.49 ± 0.73	0.95 ± 0.62	5.59e-6

Table 2: The numbers are relative intensity to TMSP instead of absolute concentration [µmol/ml].

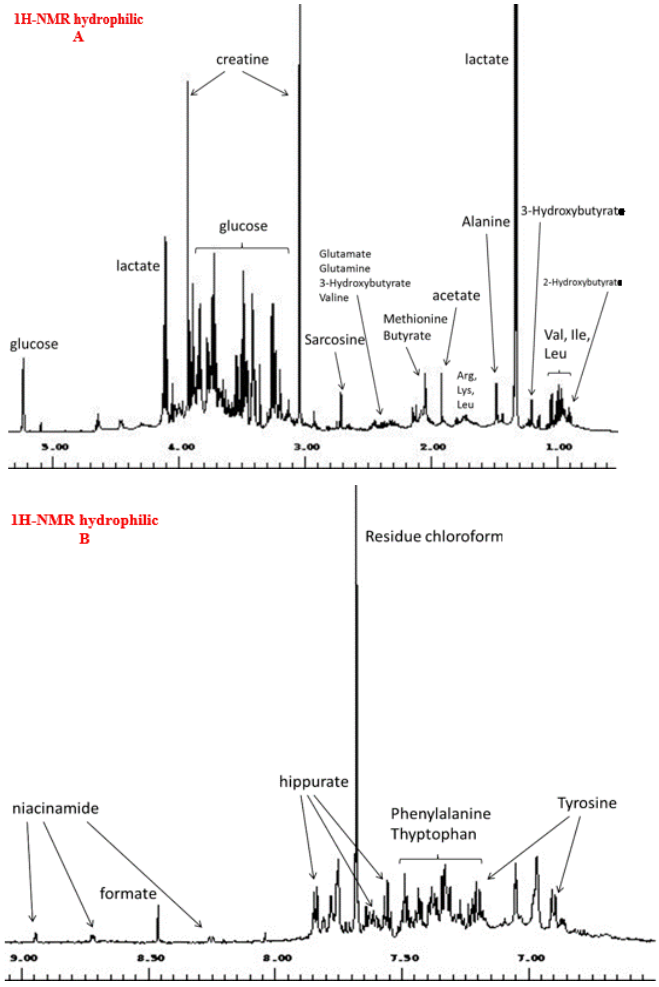

Figure 1: Representative ^{1}H-NMR spectra of plasma *water- soluble* metabolites (A and B).

function, apoptosis and organ failure (sphingomyeline) in the pathogenesis of sepsis [14-23].

Discussion

To our knowledge, this is the first study describing a NMR-spectroscopy-based metabolomic analysis in diarrheic and presumed septic calves in which obtained data support the idea that quantitative ^{1}H-NMR-spectroscopy metabolomics is a viable tool for the identification of potential novel biomarkers.

Infectious diarrhea remains one the biggest health challenges in both the beef and dairy industries [24,25]. Newborn calves are particularly at risk for developing septicemia because they are dependent on passive transfer of immunity for ultimate protection. Sepsis is verified by isolation of pathogens from blood; however, the delay in obtaining results and the high frequency of false-negative and –positive results limit the usefulness of this technique [5]. In the present study, the clinical and laboratory data from diarrheic and presumed septic calves consisted with previous studies [26-28].

Commonly used biomarkers for sepsis include C-reactive protein and procalcitonin [5,29,30]; cytokines (TNF-α, IL-1, IL-6, IL- 10, osteopontin) [27,31,32]; chemokines (macrophage migration inhibitory factor) [33,34] and soluble receptors (soluble triggering receptor expressed on myeloid cells 1; soluble urokinase-type plasminogen activator receptor) [35,36]. However, there is still no single test that satisfies the criteria as being the ideal marker for the early diagnosis of neonatal sepsis [37]. There is a need a more specific approach for sepsis care [7].

New diagnostic tools may significantly reduce morbidity and mortality in infected newborns, allowing a very early and accurate diagnosis and preventing the progression of the disease to septic shock where NMR metabolite profiling might serve as a promising approach for the diagnosis and prediction of mortality [38-40]. In experimental sepsis, potential biomarkers of sepsis from metabolomics technology are linoleic acid, oleic acid, stearic acid, docosahexaenoic acid, dopentaenoic acid and linolenic acid [41], and acetoacetate, alanine, creatine, phosphoethanolamine and formate [8]. According to Stringer et al. [42] quantitative data sets including metabolites: total glutathione, adenosine, phosphatidylserine (increased), and sphingomyelin (decreased) revealed differences between human septic patients with acute lung injury and healthy subjects. In the present study, ^{1}H NMR-based metabonomic approach was applied to differentiate the plasma metabolic profiles of neonatal calves with diarrhea-induced sepsis, in which significant decreases in all the lipid soluble metabolites (e.g., sphingomyeline, fatty acids, etc.) were observed. This finding can be explained by a heightened systemic demand for energy during sepsis, suggesting that the level of free fatty acids can serve as a useful bioindicator [8]. Moreover, a decrease in the level of polyunsaturated fatty acids was observed; this decrease can be indicative of an decreased anti-inflammatory response.

Acetoacetate enhancement may be related to increased fatty acid oxidation in septic rats [43]. The serum level of formate decreased significantly in septic rats, which might have resulted from increased consumption of formate due to increased biosynthesis of purine nucleotides in sepsis [44]. Creatine is a nitrogenous organic acid

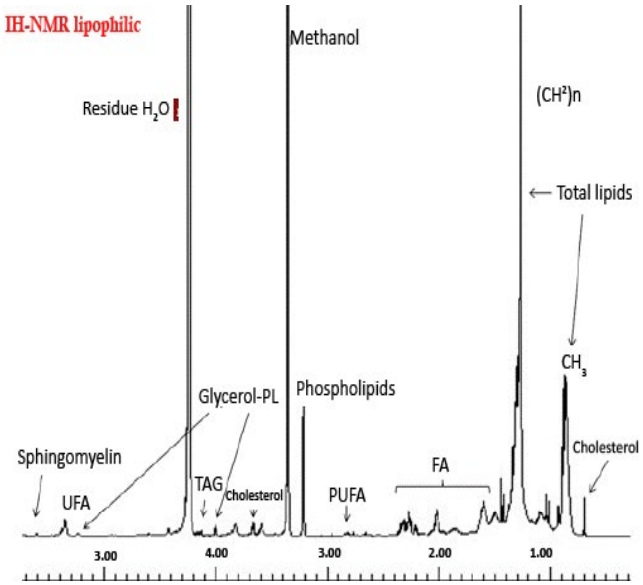

Figure 2: Representative ^{1}H-NMR spectra of plasma lipid-soluble metabolites.

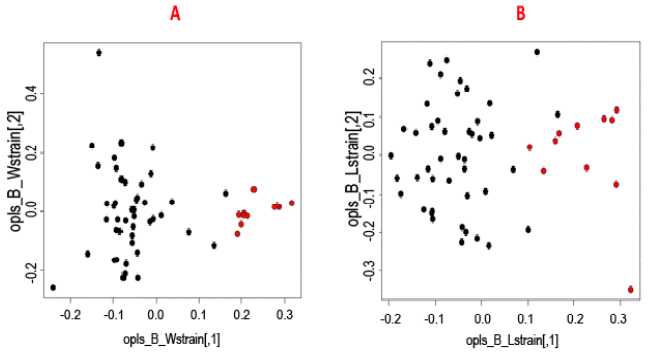

Figure 3: The OPLS-DA scoring performed on ^{1}H-NMR spectra of plasma water soluble (A) and lipid soluble (B) extracts. Samples from both groups are clearly separated. Black: diseased group; red: healthy group.

associated with muscular protein turnover and energy supply to muscles. It is enhanced in inflammatory responses including muscle damage and rhabdomyolysis [45]. Niacinamide inhibits the activation of coagulation related to endotoxemia [15]. Choline is a natural specific α-7 nicotinic receptor agonist [46]. It has therapeutic effects on inflammatory pain and experimental sepsis in animals, due to its marked inhibition on the synthesis and release of TNF-α [47, 48]. Septic patients have shown the inclination for comparably low plasma arginine and increased levels of other aminoacids, which may support the concept of a high extravascular flow and metabolic requirement of arginine in sepsis [49]. Isopropanol is a powerful negative regulator of the inflammatory cytokine TNF-α [50]. The concentrations of 3-methylglutaric acid dramatically increase during acute metabolic crises, which are clinically characterized by severe hypoketotic hypoglycaemia, acidosis, hyperammonaemia, vomiting, hypotonia, coma, and seizures [51]. Our study is accordance with previous reports on metabolic changes in natural (humans) and experimental (rats) sepsis. In the present study, characteristic metabolites changed markedly in presumed septic calves: formate, acetate, lysine arginine, sphingomyeline, ω-3 PUFA and other FAs decreased whereas creatine, 2-methylglutarate, choline, isopropanol, niacinamide and 3-hyroxyubutyrate levels increased in plasma extracts. They may indicate the processes (oxidative stress, apoptosis, organ failure, endothelial barrier function, immunosuppression, etc.) involved in a complex pathology of sepsis. These metabolites may be useful for potential markers related to a series of processes caused by a systemic inflammatory response syndrome in septic calves.

Conclusion

Routine parameters involving blood analyses and biochemical profile used in this study for sepsis provided a framework on the disease. NMR-based metabolomic evaluation is a potentially useful in the discrimination of pathogenic mechanisms in calves with suspected sepsis. Decrease in the whole lipid soluble metabolites such as sphingomyeline and fatty acids including PUFA may at least indicate a great systemic energy deficit during sepsis. Other characteristic metabolites reflecting the systemic inflammatory response syndrome a serious condition related to systemic inflammation, organ dysfunction and organ failure may be meaningful for the early diagnosis and prognosis of diarrhea induced septic calves.

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