

## Lactose-rich milk replacer modifies the proteome of blood plasma in 2-week-old calves

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**Abstract:** The aim of the study was to create a specific pattern of protein expression changes in the plasma of 2-week-old calves treated with excessive lactose doses. Identification of proteins, the expression of which changes during the short-term administration of lactose, may help to exclude or confirm the causes of diarrhea observed in newborn calves. The present study utilized 2-D electrophoresis combined with MALDI-TOF mass spectrometry and identified 6 blood plasma proteins, the expression of which changed under the influence of lactose added to the milk replacer. The pattern of expression of blood plasma proteins, including decreased expression of fibrinogen and apolipoprotein A-IV and increased expression of alpha-1B-glycoprotein, alpha-1-antiproteinase, and apolipoprotein E in 2-week-old calves fed with lactose-rich milk replacer, can be used to determine the causes of diarrhea in calves.

**Key words:** Excess of lactose, blood plasma, proteome, calves

### 1. Introduction

Significant mortality is observed in calves during the first 3 weeks of their life, and the predominant cause of death is diarrhea. Among the causes listed as responsible for the occurrence of diarrhea are noninfectious factors (e.g., oversupply of milk or milk replacer) and infectious factors (e.g., rotavirus, coronavirus, enterotoxigenic *E. coli*). The occurrence of diarrhea in calves is triggered by the accumulation of undigested lactose in the gastrointestinal tract due to its oversupply with milk replacer (1).

Biochemical analysis of plasma and urine is commonly used during veterinary diagnostics. The overall analysis of a particular body fluid proteome enables a deeper understanding of metabolic processes and the mechanisms regulating the expression of proteins in health and disease. Determination of qualitative and quantitative changes in the peptide-protein composition of body fluids, which is constantly changing in response to systemic or environmental factors (including experimental variables), allows for the determination of marker proteins, characteristic for a specific physiological or pathological condition (2).

The aim of the study was to create a specific pattern of protein expression changes in the plasma of 2-week-old calves treated with excessive lactose doses. Identification

of proteins, the expression of which changes during the short-term administration of lactose, may help to exclude or confirm the causes of diarrhea observed in newborn calves.

### 2. Materials and methods

The experiment was carried out on 6 Polish Holstein-Friesian var. Black-and-White male calves from the 13th to the 16th day of life. The use and handling of animals for this experiment was approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals (No. 3/2010 of 14.01.2010). During the first 3 days of life, calves were fed colostrum, and then from the 4th day milk replacer in an amount of 10% of body weight per day. Animals were fed milk replacer (Mlekovit Imupro, Polmass) containing 23% crude protein, 16% crude fat, 0.1% crude fiber, 45% lactose, 7.5% crude ash, 1.7% lysine, 0.42% methionine, 0.9% calcium, and 0.7% phosphorus. Calves were fed twice daily. On the 13th day (during the evening feeding) and on the 14th day (during the morning feeding) monohydrate lactose (Pharma Cosmetic) in the amount of 1 g/kg of body weight was added to the milk replacer.

Blood samples were collected once, always before the evening feeding. Blood was drawn from the jugular vein

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into K<sub>2</sub>EDTA precoated tubes. The blood samples were centrifuged (15 min, 4 °C, 3000 × g) and the harvested plasma was stored at –80 °C until further processing.

### 2.1. Two-dimensional electrophoresis (2-DE)

Before 2-DE analysis, plasma samples were processed by protein equalizer technology with a ProteoMiner Large-Capacity Protein Enrichment Kit (Bio-Rad).

Processed plasma samples were dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, 0.2% w/v 3-10 ampholytes, and 2 mM TBP. Plasma protein samples (650 µg) were applied to 4–7 NL (nonlinear) ReadyStrip IPG Strips (24 cm, Bio-Rad). The isoelectrofocusing (IEF) was run at a total of 90,000 Vh using a Protean IEF Cell (Bio-Rad). After IEF, the IPG strips were reduced with DTT in equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% w/v glycerol, and 1% w/v DTT) for 15 min and then alkylated with iodoacetamide (2.5% w/v) for 20 min at ambient temperature. The second dimension was performed on 12% SDS polyacrylamide gels (20 × 25 cm) at 40 V for 2.5 h and then at 100 V for 17 h at 10 °C using a Protean Plus Dodeca Cell electrophoretic chamber (Bio-Rad). After 2-DE separation, the gels were stained with CBB G-250 according to the modified Kang method (3).

### 2.2. Image analysis

The gels were scanned using a GS-800 Calibrated Densitometer (Bio-Rad). Analysis of 2-D images was performed using PDQuest Analysis software version 8.0.1 Advanced (Bio-Rad). To measure the variability before and after administration of lactose, the coefficient of variation was calculated. Qualitative and quantitative comparisons before and after administration of lactose were performed to show the differences in the pattern of protein spots and to examine changes in the protein expression level. Experiment normalization was performed using a local regression model (LOESS). Molecular mass (kDa) was computed for each identified protein spot based on the molecular weight standard.

### 2.3. Mass spectrometry and functional protein clustering

The protein spots showing statistically significant differences were manually excised from the gels and decolorized by washing in buffer containing 25 mM NH<sub>4</sub>HCO<sub>3</sub> in 5% v/v acetonitrile (ACN), followed by two washes with a solution of 25 mM NH<sub>4</sub>HCO<sub>3</sub> in 50% v/v ACN. The gel pieces were dehydrated (100% ACN), vacuum dried, and incubated overnight with trypsin (20 µL/spot of 12.5 µg trypsin/mL in 25 mM NH<sub>4</sub>HCO<sub>3</sub>; Promega) at 37 °C. The resulting peptides were extracted with 100% ACN, combined with an equal volume of matrix solution (5 mg/mL CHCA, 0.1% v/v TFA, 50% v/v ACN), and loaded onto a MALDI-MSP AnchorChip 600/96 plate (Bruker Daltonics). Peptide Mass Standard II was used (mass

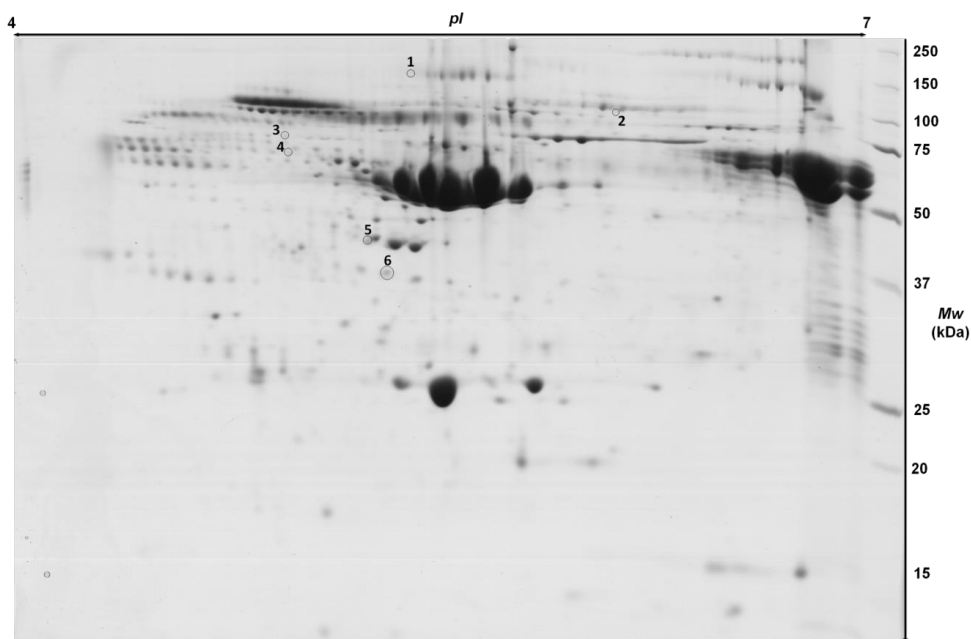
range 700–3200 Da, Bruker Daltonics) for calibration of the mass scale. Mass spectra were acquired in positive-ion reflector mode using a Microflex MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) mass spectrometer (Bruker Daltonics). Peptide mass fingerprinting (PMF) data were compared to mammalian databases (SWISS-PROT, <http://us.expasy.org/uniprot/> and NCBI, <http://www.ncbi.nlm.nih.gov/>) with the aid of the MASCOT search engine (<http://www.matrixscience.com/>). Search criteria included trypsin as an enzyme, carbamidomethylation of cysteine as a fixed modification, methionine oxidation as a variable modification, mass tolerance to 150 ppm, and a maximum of one missed cleavage site. The results were further validated by the MASCOT score (only statistically significant hits were applied) and sequence coverage.

### 3. Results

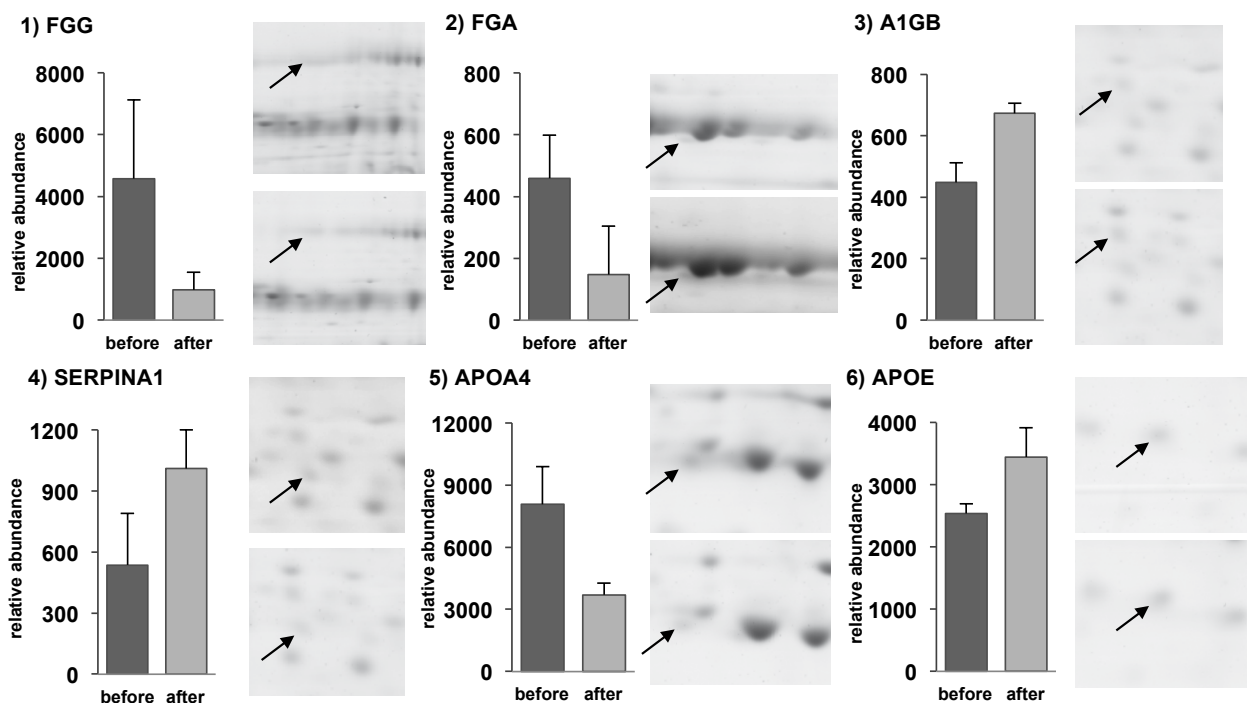
The experiments involved electrophoretic separation of blood plasma proteins of calves (Figure 1). Twelve 2-D gels were obtained, reflecting the protein profiles of the blood plasma of calves prior to the first dose of lactose and 12 h after the second dose of lactose was added to the milk replacer formulation.

Archived gels were analyzed using bioinformatics software PDQuest 8.0 Advanced (Bio-Rad). In total, 430 spots were analyzed qualitatively and quantitatively, of which 136 were present in all analyzed gels, showing the protein profiles of the blood plasma of calves both prior to administration of the milk replacer containing lactose and after the supply of the formulation. Of these, 6 showed statistically significant differences in expression. Three spots showed increased expression, while the expression of the remaining 3 was decreased. The spots, which showed differences in the expression, represented 6 proteins (Figure 2).

An example of a gel, which demonstrates the blood plasma protein spots differing in expression, is presented in Figure 1. The Table describes in detail the identified protein spots. Figure 1 depicts the pI range (corresponding to the pH of the 4–7 IPG strips) and molecular weight in the range of 20 to 250 kDa. The Table presents detailed information on protein spots differing in relative expression after analysis using PDQuest software; characteristics of blood plasma proteins are also given (name, accession number, estimated molecular weight, isoelectric point – pI, molecular weight – MW), based on the SWISS-PROT and NCBI databases. The results also include a point value, reflecting the reliability of identification (so-called SCORE), the percentage of coverage of identified amino acid sequence fragments for the analyzed proteins, and the number of fragments matched with the amino acid sequence of the protein.



**Figure 1.** The 2-DE map of differentially expressed calves plasma proteins. Presented 2-D gel was stained with Coomassie brilliant blue G-250; 650 µg of protein was applied to the IPG strip (24 cm, pH 4–7) for the first dimension and the second dimension was performed on 12% SDS-PAGE gels. Spot numbers correspond to those in the Table and Figure 2.



**Figure 2.** Differentially expressed blood plasma proteins. The short names of the identified proteins, spot numbers, graph of differences in abundance, and changes in spot intensities are presented in the expanded view of the gel. Spot numbers of the identified proteins correspond to those presented on the 2-DE map of calves' plasma proteins (Figure 1) and described in the Table.

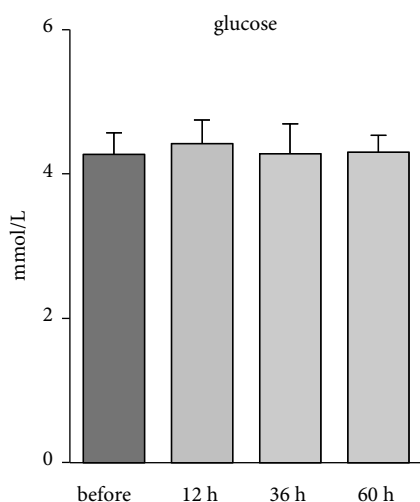
The concentration of glucose in the blood plasma of 2-week-old calves before lactose administration was 4.27 mmol L<sup>-1</sup> and its level increased to 4.42 mmol L<sup>-1</sup> 12 h after the second dose of lactose (Figure 3).

Soft stool with a delicate sour odor and small green mucus streaks was observed in all calves after administration of milk replacer with additional quantity of lactose. This may indicate a change in the rate of passage of gastric contents.

**Table.** The list of the differentially expressed calves blood plasma proteins and characterization of the parameter identification using MALDI-TOF mass spectrometer.

Spot no.	Protein name	Accession no.	Calculated Mr [kDa]	Theoretical pI/Mr [kDa]	Mean relative abundance before lactose addition	SD	Mean relative abundance after lactose addition	SD	Fold change	Significance of differences	MALDI-TOF MS		
											Mascot score	Sequence coverage [%]	Mass values matched
1.	Fibrinogen gamma-B chain isoform XI	XP_005217490	174.2	5.56/49.76	4572.4	2557.90	979.8	562.45	-4.67	P ≤ 0.05	198	54	19
2.	Fibrinogen alpha chain isoform XI	XP_005217494	6802.0	5.69/95.42	458.8	139.96	147.7	157.44	-3.11	P ≤ 0.05	102	22	14
3.	Alpha-1B-glycoprotein precursor	NP_001039708	82.8	5.29/54.09	448.5	64.53	673.5	31.90	1.50	P ≤ 0.01	113	31	13
4.	Alpha-1-antitrypsinase	P34955	74.5	6.05/46.42	535.2	255.23	1010.1	189.86	1.89	P ≤ 0.05	73	25	14
5.	Apolipoprotein A-IV	ELR56104	43.6	5.31/43.01	8075.5	1810.27	3695.1	578.09	-2.18	P ≤ 0.05	183	52	21
6.	Apolipoprotein E	CAA43479	38.2	5.44/35.96	2537.8	159.71	3444.6	476.10	1.36	P ≤ 0.05	141	46	17

Spot numbers of the identified proteins correspond to those presented on the 2-DE map of calves' plasma proteins (Figure 1) and on the graph of differences in abundance and changes in spot intensities (Figure 2).



**Figure 3.** Mean blood plasma glucose concentration in calves before and after supply of lactose.

#### 4. Discussion

The present study utilized 2-D electrophoresis combined with MALDI-TOF mass spectrometry and identified 6 blood plasma proteins, the expression of which changed under the influence of lactose added to the diet.

We have shown a decrease in the expression of plasma fibrinogen gamma-B chain isoform X1 and fibrinogen alpha chain isoform X1 after administration of lactose with milk replacer. Fibrinogen (Fb) is a glycoprotein produced by the parenchymal hepatic cells and released into the bloodstream as a dimer. Each fibrin monomer is composed of three polypeptide chains,  $\alpha$ ,  $\beta$ , and  $\gamma$ , connected by disulfide bonds (4,5).

Fibrinogen, in addition to the hemostatic function, acts as one of the acute phase proteins. Hepatic synthesis of Fb and then release into the bloodstream increases significantly under the influence of proinflammatory agents (6).

Studies of Ge et al. (7) and Guo et al. (8) indicated that fibrinogen and especially its D domain increased vascular endothelial permeability. Guo et al. (8) suggested that the C-terminus of fibrinogen  $\gamma$  was responsible for an increase in microvascular endothelial permeability. In turn, Ge et al. (7) showed that the D fragment increased endothelial permeability of bovine pulmonary artery independently of the carboxy-terminal sequence of the gamma-chain. According to Guo et al. (8), fibrinogen-induced permeability might explain macromolecule transport across the microvascular endothelium during inflammation.

The concentration of fibrinogen in the blood plasma of calves is decreasing from 13 to 20 days of age (9). In the available literature, there are no data related to the effect

of lactose supplementation to the diet on blood fibrinogen concentration. Gökçe et al. (10) and Sobiech et al. (11) observed a higher concentration of this protein in calves with diarrhea in comparison to calves of the control group. In spite of slight diarrhea, as a result of lactose supply, a decrease was observed in the expression of Fb in the blood plasma of the subject calves. Fibrinogen can be considered as an indicator protein in determining the cause of diarrhea in calves. Decreased blood Fb concentration in calves with diarrhea may indicate that it was caused by a noninfectious agent (in this case, oversupply of lactose).

Alpha-1B-glycoprotein (A1BG) is a plasma protein, the expression of which in blood plasma increased in response to the addition of lactose to milk replacer. The function of this protein is still unknown. It belongs to the immunoglobulin family and is a protein released into the blood plasma. The increase in the expression of A1BG in serum was observed in cattle and sheep during respiratory tract inflammation (12,13). It seems that diarrhea observed in calves in response to the oversupply of lactose may result in increased concentrations of A1BG in their blood plasma.

Alpha-1-antitrypsin is a glycoprotein synthesized primarily in the liver and secreted into the bloodstream. To a lesser extent, it is synthesized by macrophages, pulmonary alveolar cells, and intestinal and corneal epithelium. Alpha-1-antitrypsin belongs to the acute-phase proteins. It is released during inflammatory response and inactivates many proteolytic enzymes such as neutrophil elastases (14,15).

The concentration of alpha-1-antitrypsin in the blood plasma increases rapidly in response to inflammation or infection (14,15). It can be assumed that the increase in expression of this protein in the blood plasma of calves was caused by the lactose maldigestion because of lactose supply exceeding the enzymes' capacity to break down lactose to glucose and galactose. Undigested lactose attracts water and electrolytes to the digestive tract, resulting in loose stool.

Further proteins, the expressions of which in blood plasma of calves changed after the addition of lactose to milk replacer, were involved in the metabolism of fat: apolipoprotein A-IV and apolipoprotein E.

A study by Minehira et al. (16) showed that carbohydrate overfeeding markedly increased net de novo lipogenesis at the expense of glycogen synthesis and increased mRNA levels for the key lipogenic enzymes. Lepczyński et al. (17) observed lower blood plasma concentrations of total cholesterol, HDL-cholesterol, and LDL-cholesterol and downregulation of apo A-IV in preruminant calves fed milk replacer in comparison to calves fed whole milk. Moreover, the authors showed that the milk replacer had a much higher carbohydrate content than the milk (17).

Apolipoprotein A-IV is a glycoprotein synthesized primarily in the enterocytes of the small intestine during fat absorption. In the blood, apo A-IV occurs primarily in the free form; the remaining amount is present in chylomicrons and high-density lipoproteins. This protein is involved in absorption, transport, and metabolism of lipids, and it may also act as a postprandial satiety signal and an antioxidant (18,19). In piglets with induced experimental inflammation, decreased hepatic apo A-IV mRNA expression was observed (20). In addition, a decrease of apo-IV free form (lipoprotein-free apolipoprotein) was also demonstrated as well as a form associated with high-density lipoproteins in blood plasma.

Sato et al. (21) observed a decrease in the concentration of apo-AIV mRNA in the intestine of fasted rat pups that in the 15th hour of fasting reached a value equal to 20% of the prefasting level. Refeeding of lactose or milk replacer increased the concentration of apo A-IV in the intestine, but it had no effect on the level of apo A-IV in the serum. Only the administration of Intralipid alone to fasted pups contributed to increased levels of apo A-IV in the serum 3 h after refeeding. Satoh et al. (22) also observed an increase in the concentration of apo A-IV in serum in 14-day-old suckling rat pups fasted overnight after administration of Intralipid along with lactose, glucose, fructose, and sucrose.

Apolipoprotein E (apo E) is a class of apolipoproteins found in the chylomicron and intermediate-density lipoprotein. It binds to a specific liver and peripheral cell receptor and is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. Apo E present in the blood plasma is primarily derived from the liver. It has been shown that this protein is also synthesized in the spleen, brain, lungs, and kidneys. In addition to the regulation of lipid metabolism, apo E is involved in the modulation of the inflammatory response and oxidative stress (23).

The study of Wang et al. (24) showed in mice that exogenous apo A-IV injection decreases blood glucose levels and stimulates a transient increase in insulin secretion. Gao et al. (25) observed in mice with apo E deficiency reduced blood glucose and better insulin sensitivity. Considering the above publications, it can be assumed that the increased amount of glucose supplied with food could be the cause of the observed decreased expression of apo A-IV and increased expression of apo E in the blood plasma of tested calves.

The acute-phase response in the body occurs due to various stimuli, including metabolic disorders arising from dietary errors. Changes in the concentrations of the acute-phase proteins are dependent on the stimulus that induced them. In the present experiment, triggering the acute-phase response and proteins involved in this process (fibrinogen, alpha-1B-glycoprotein, alpha-1-antitrypsin, apo A-IV, apo E) was a reaction to the increased quantity of disaccharide-lactose administered to calves with milk replacer formulation.

The observed changes in protein expression of blood plasma in calves were caused by the direct effect of an oversupply of sugar (apo A-IV, apo E) or diarrhea (fibrinogen, alpha-1B-glycoprotein, alpha-1-antitrypsin).

The pattern of expression of blood plasma (decreased expression: fibrinogen, Apo IV; increased expression: alpha-1B-glycoprotein, alpha-1-antitrypsin, and apo E) in 2-week-old calves treated with an oversupply of lactose can be used to determine the causes of diarrhea in calves.

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