Serum haptoglobin and protein electrophoretic fraction modifications in buffaloes (*Bubalus bubalis)* around calving and during early lactation

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SUPPLEMENTARY FILE

Material and Methods

Farm conditions and animals

A total of 30 multiparous Italian Mediterranean Buffaloes (*Bubalus bubalis*) of mean age 114 ± 8 months and 540 ± 55kg average body weight were randomly selected from a farm of 850 animals located in Southern Italy (Corigliano Calabro, 39°36' N 16°31' E, 0 m.a.s.l, Cosenza). The average milk production in 270 DIM of the buffaloes found in this farm was 2827.5±654.6 kg per year with an average of 8.47% of milk-fat and 4.74% of milk protein. All buffaloes had a dry period of 120 days. Details of the total mixed ration (TMR) and the chemical composition of diets used during dry period and subsequent early lactation period are reported in online Supplementary Table S1. Water was available *ad libitum.* All animals enrolled in the study were clinically healthy. Their health status was evaluated on the basis of rectal temperature, heart rate, respiratory profile, appetite and fecal consistency. Animals were kept under their natural photoperiod and an average environmental temperature of 14±6 °C. Body condition score (BCS) was evaluated, by a single operator, on a scale from 0 to 5 at the same time points as blood sampling according to the method of Edmonson *et al.* (1989). The protocol of this study was reviewed and approved in accordance with the standards recommended by the *Guide for the Care and Use of Laboratory Animals* and Directive 2010/63/EU.

Blood sampling and chemistry analysis

Blood sampling was performed at 4 different time points; 7±5 days before expected calving and 7±5, 30±5 and 50±5 days after calving. Blood sampling was performed early in the morning, before daily delivery of ration, by jugular venipuncture into10 mL vacuum tubes without anticoagulant agent (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK). Serum separation was carried out immediately. The blood samples were allowed to clot for 30 min after blood sampling, then the tubes were centrifuged at 1780g for 10 minutes.The obtained serum was transported to the laboratory at 4°C and then stored at -18°C until analysis. On serum samples, the concentration of total proteins, haptoglobin, albumin, α1-globulins, α2-globulins, β1-globulins, β2-globulins and γ-globulins was evaluated.

The serum total protein concentration was measured by means of automated analyzer BT 1500 (Biotecnica Instruments S.p.a., Rome, Italy) using the Biuret method with commercially available kit (GesanS.r.l, Campobello di Mazara, Italy). Electrophoresis for protein fractions assessment was performed using an automated system (Selvet 24, Seleo Engineering, Naples, Italy) according to the procedures described by the manufacturer. For each sample, 25 µL of serum was applied to numbered sample wells on cellulose acetate films. Each holder accommodated up to 24 samples. Films were electrophoresed for 28 min. at 180 V. After electrophoresis, films were simultaneously fixed using an automated system, stained in red stain acid solution for 10 minutes and then dried at 37°C. After destaining in acetic acid and drying

completely for 15 minutes, films were scanned on a densitometer and electrophoretic curves plus related quantitative specific protein concentrations for each sample were displayed, using computer software (SelVet 24). All samples were analyzed by the same operator, who determined the lines separating fractions in the densimeter tracing. The major protein fractions were divided according to the recommendation by the manufacturer from cathode to anode into albumin, α1-, α2-, β1-, β2- and γ-globulins, respectively.

Serum haptoglobin concentration was determined by means of automated analyzer BT 1500 (Biotecnica Instruments S.p.a., Rome, Italy) using the turbidimetric method with commercially available kit (GesanS.r.l, Campobello di Mazara, Italy).

Milk sampling and analysis

Daily milk production (DMP) was measured by means of a commercial milk meter Afimilk MCP (Afifarm, Kibbutz Afikim, Israel). For each animal two milk samples were collected, one for the determination of somatic cell count (SCC) and the other one for milk composition, at the 3 *post partum* sampling points. Collection was performed aseptically according to National Mastitis Council guidelines (Hogan *et al.* 1999). The teat ends were cleaned externally first with commercial pre-milking disinfectant solutions then dried with individual towel and cleaned again with alcohol. The first few streams of foremilk were rejected and a pool of 25 mL of milk from the quarters was collected in sterile containers and preserved with sodium azide for assessing SCC with a Fossomatic cell counter (Foss Electric, Hillerod, Denmark). Milk samples were stored at 2-6 °C and cultured until 24 hours.The SCC obtained values were converted to SCS (Somatic Cell Score) by:

$$SCS = \log2 (SCC/100) + 3$$

where SCC was in units of cells per microliter (Wiggans and Shook 1987).

For the determination of milk composition, milk samples were analyzed using a lacto-scan (Milkotronic Analyzer, MCC, Nova Zagora, Bulgaria). The Net Energy of Lactation (NE_L) represents the energy contained in the milk produced. The NE_L concentration in milk is equivalent to the sum of heats of combustion of individual milk component (fat, protein and lactose). Reported heats of combustion of milk fat, protein and lactose are 9.29, 5.71 and 3.95 Mcal/kg, respectively. NE_L concentration in milk was calculated by the equation proposed by Tyrrell and Reid (1965):

$$NE_L \text{ (Mcal/Kg)} = 0.0929 \times \text{Fat\%} + 0.0547 \times \text{Crude protein \%} + 0.0395 \times \text{Lactose \%}$$

Statistical analysis

Data, expressed as mean values ± standard deviation (±SD), were tested for normality using the Shapiro-Wilk normality test. All data were normally distributed ($P > 0.05$) and the statistical analysis was performed. One-way analysis of variance (ANOVA) was used to determine a statistically significant effect of peripartum period on serum total proteins, haptoglobin, albumin and globulin fractions, and in order to verify the effect of time after calving on productive parameters and milk constituents. Bonferroni's multiple comparison test was applied for post-hoc comparison. The Person test was performed in order to assess significant correlations between haematochemical parameters and productive parameters and/or milk constituents. P value <0.05 was considered statistically significant.Statistical analysis was performed using the STATISTICA 7 software package (Stat Software Inc., Tulsa, Oklahoma, USA).

Supplementary Table S1. Feed chemical composition of total mixed ratio (TMR) used for all animals during *pre-partum* and *post-partum* period

	Dry Period	**Lactation Period**
Feed basis (kg)	19.20	23.20
Dry Matter (DM) (kg)	9.64	17.66
Dry Matter Intake (DMI) (kg per animal)	9.04	17.40
Chemical Composition (% of DM)		
Energy (UFL)	0.63	0.94
CP	9.12	15.40
PD	/	9.40
PDIN	6.08	1.55
PDIE	6.81	9.76
PDIA	2.51	4.83
UIP%DM	25.74	22.21
NDF	61.51	34.83
ADF	38.35	21.60
ADL	9.20	3.85
EE	2.61	5.44
ASH	8.52	7.22
ST	10.60	22.16
NSC	16.91	34.88
Ca	0.49	0.80
P	0.58	0.40

UFL: Unitè Fouragère Lait; CP: Crude protein; PD: protein digestible; PDIN: protein digested in the small intestine when rumen-fermentable nitrogen is limiting; PDIE: protein digested in the small intestine when rumen-fermentable energy is limiting; PDIA: dietary protein undegraded in the rumen but truly digestible in the small intestine; UIP%DM: percentage of undegradable intake protein on dry matter; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; EE: ether extract; ASH: Ashes; ST: Starch; NSC: non-structural carbohydrates; Ca: calcium; P: phosphorus