Colostrum from primiparous Holstein cows shows higher antioxidant activity than colostrum of multiparous ones

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SUPPLEMENTARY FILE

Supplementary materials and methods

The project was approved by the Ethics Committee on Animal Use ESALQ/USP, No. 2015-16, and conducted in accordance with the rules of Waste Management of ESALQ/USP. Colostrum was collected from eight primiparous and eight multiparous (second and third lactations) health black and white Holstein cows from Fazenda Santo Antonio, Piracicaba, São Paulo, Brazil. Primiparous and multiparous cows received the same diet during pregnancy, 3 kg of concentrate per cow day^{-1} divided into two meals daily and composed of corn meal (91.5 kg per cow day^{-1}), soybean meal (1.2 kg per cow day^{-1}), hill (colipearl, 0.021 kg per cow day^{-1}), adsorvent (Taxfin™, 0.009 kg per cow day^{-1}), Bovigold beta pré-parto (0.27 kg per cow day^{-1}). Furthermore, the animals were supplied 30 kg of roughage per cow day^{-1}, in both groups, composed of sorghum or maize silage (19 kg per cow day^{-1}), hay (1 kg per cow day^{-1}) and humid barley (10 kg per cow day^{-1}). The cows sampled in this study were vaccinated duing pregnancy in order to improve colostrum quality. The vaccines used were: Rotavec, J-VAC and Fortress, which act mainly to prevent the occurrence of diarrhea in newborns. The Rotavec vaccine is applied for the active immunization of pregnant females, increasing the level of antibodies against rotavirus, coronavirus and Eschericia coli (MSD Saude Animal, 2015). The Fortress vaccine is used against diseases caused by Clostridium species, responsible for the occurrence of symptomatic carbuncle, gas gangrene and enterotoxaemia (Rural Centro). The J-Vac vaccine in addition to containing antiobodies against the E. Coli

bacterium also stimulates the animal's immune response and protects the ehrd against environmental mastitis (Portal do Agronegocio, 2013).

Shortly after birth, calves were separated and 300 mL of first milking colostrum was collected. These samples were stored in labeled vials under -80 ºC for subsequent laboratory tests.

Quantification of IgG in colostrum was performed by radial immunodiffusion (Mancini et al., 1965). IgG concentrations were expressed in mg mL^{-1}. Colostrum whey was obtained after precipitation using renin and total protein concentration was determined according to the biuret method (Reinhold, 1953). The SDS-PAGE was performed according to the methodology of Laemni (1970). Then, colostrum whey diluted to 7.5 mg of protein mL^{-1} was mixed with Tris-HCl buffer (pH 6.8), glycerol, SDS, DTT and bromophenol (1:2). These prepared samples were left in a water bath at 99 °C for 10 min for protein denaturation. Electrophoresis was carried out in a separation polyacrylamide gel (12%) and a stacking polyacrylamide gel (4%). For the run, 40 mA were applied to the stacking gel and at 50 mA to the separating gel. Protein fractions were identified using a standard molecular weight marker (Bio-Rad, Hercules, CA, USA). After the run, the gels were pre-fixed, stained with Coomassie R dye. The gels were scanned and analyzed in the GelAnalyser software, and peaks from the identified bands were obtained. To quantify protein fractions, bands of each sample were compared with a standard running on the gel. The results were expressed as mg mL^{-1} of serum.

The ceruloplasmin activity was determined in colostrum whey according to Rice et al. (1963). Samples were incubated for 30 min at 37 °C with 0.15% p-phenylenediamine and, after the reaction, 0.015% azide was added to stop the reaction. In control samples, azide added before the reaction. The absorbance was registered using 540 nm wavelength. The ceruloplasmin activity was calculated in IU L^{-1}, following the formula: IU L^{-1} = (sample absorbance - control absorbance) x 137. IU (international unit) refers to the amount of enzyme degrading 1 µmol of substrate per minute at 37 °C. The

lactoperoxidase activity in colostrum whey was performed according to the method described by Mansson-Rahemtulla et al. (1986). Then, a standard curve was prepared with stock solution containing DTNB, mercaptoethanol and potassium phosphate buffer (pH 5.66). The DTNB in the presence of mercaptoethanol is reduced to NBS. Samples of colostrum whey were homogenized with NBS solution, and stock hydrogen peroxide was added. Absorbance was registered at 0 and 20 min, using 412 nm wavelength. In this analysis, lactoperoxidase in colostrum whey catalyzes the oxidation reaction of NBS with hydrogen peroxide, changing the substrate color into a colorless product. Next, the oxidant hydrogen peroxide was transformed into water, and the bromine in the NBS was oxidized. Lactoperoxidase in colostrum whey were expressed in IU mL^{-1}. The lysozyme activity was performed in colostrum whey by turbidimetric assay (Parry et al., 1965), using 0.75 mg mL^{-1} of lyophilized cells of *Micrococcus lysodeikticus* Gram-positive bacteria as substrate, diluted in 0.1 M citrate, phosphate buffer pH adjusted to 5.8. After placing 175 µL of bacteria solution into micro plate wells and 25 µL of each serum sample, the absorbance reading at 450 nm was registered after 0 and 20 min of incubation. The results are presented as U mL^{-1}, where 1.0 U of lysozyme corresponds to the number of enzymes that reduces the optical density by 0.0001 per minute. Colostrum whey samples were diluted to contain 30 mg of protein mL^{-1} and analyzed for oxygen radical absorbance capacity (ORAC), according to Melo et al. (2015). All reagents, samples and dilutions of the calibration curve were prepared with sodium phosphate buffer, 75 mM, pH 7.4. A trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) calibration curve was used. The analyses were performed in a black microplate to which a 30 µL of samples, 60 µL of 406 nM fluorescein solution and 110 µL of 76 mM AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) solution was added. The analyses were performed in triplicate with a kinetic methodology using emission absorbance of 528 nm and excitation absorbance of 485 nm for 2 h at 37 °C. The values are expressed as µmol of equivalent Trolox mL^{-1}. Twenty microliters of colostrum whey were incubated with

300 µL of a solution composed of 48 mM buffer phosphate, pH 7.7, 0.38 mM EDTA, 0.95 mM azide (to inhibit catalase), 1 mM glutathione, 0.12 mM nicotinamide adenine dinucleotide phosphate (NADPH), 3.2 U of glutathione reductase, 0.02 mM DL-dithiothreitol and 0.0007% hydrogen peroxide to determine the glutathione peroxidase activity (GPx) (Wendel, 1981). The decrease in absorbance was recorded for five min in a spectrophotometer with a wavelength set at 340 nm. The values of glutathione peroxidase activity are expressed as unit U mL^{-1}. One unit catalyzes the oxidation by H_2O_2 of one mol of reduced glutathione to oxidized glutathione per minute at 25 °C, pH 7.0. The catalase (CAT) activity was determined according to the methodology described by Iwase et al. (2013). In this analysis, 100 µL of colostrum whey or standard were incubated with 100 µL of 1% Triton-X and 100 µL of 30% of hydrogen peroxide. Within 15 min, the O_2 foam height formed was measured with a digital caliper. The reaction specificity was tested using samples containing 10 mM of sodium azide. A calibration curve was drawn with defined units of catalase activity. The values of catalase activity are expressed as units of U mL^{-1}. One unit of catalases is responsible for the consumption of 1 mol of H_2O_2 per minute. The variables iron (µg dL^{-1}), latent iron binding capacity (LIBC, µg dL^{-1}), total iron binding capacity (TIBC, µg dL^{-1}), transferrin (mg mL^{-1}) and transferrin saturation index (TSI%) were determined by commercial kits (Labtest Diagnóstica SA, Lagoa Santa, MG, Brazil).

The statistical analyses were performed using PROC MIXED procedure of SAS statistical program. The experimental design was completely randomized and after confirming the normal data distribution with the Shapiro-Wilk test, the analysis of variance and Pearson correlation analysis were performed. The probability of 5% ($P<0.05$) was considered, except where specified.

Table S1. Correlation coefficient between the analyzed variables in colostrum from primiparous and multiparous (second and third lactations) Holstein cows

	Total protein	Lactoperoxidase	ORAC	Lactoferrin	Albumin	Ig heavy chain	Ig light chain	IgG	Lysozyme	Ceruloplasmin	GPx	Catalase	Iron	LIBC	TIBC	TSI
Lactoperoxidase	-	-														
ORAC	-	-	-													
Lactoferrin	0.63	-	-	-												
Albumin	-	-	-	-	-											
Ig heavy	0.98	-	-	0.54	-	-										
Ig light	0.99	-	-	0.64	-	0.96	-									
IgG	0.83	-	-	-	-	0.83	0.81	-								
Lysozyme	-	0.53	-	0.57	-	-	0.50	-	-							
Ceruloplasmin	-	-	-	-	-	-	-	-	-	-						
GPx	-	0.58	-	-	-	-	-	-	-	-	-					
Catalse	-	-	-	-	-	-	-	-	-	-	-	-				
Iron	-	-	-	-	-	-	-	-	-	-	-	-	-			
LIBC	-0.65	-	-0.51	-	-	-0.72	-	-0.56	-	-	-	-	-	-		
TIBC	-0.77	-	-0.59	-	-	-0.86	-0.75	-0.71	-	-	-	-	-	0.99	-	
TSI	0.81	-	-	0.80	-	0.79	0.81	-	-	-	-	-	-	-0.68	-0.61	-
Transferrin	-0.77	-	-0.59	-	-	-0.86	-0.75	-0.71	-	-	-	-	-	0.99	1	-0.61

ORAC – oxygen radical absorbance capacity organic matter; GPx – glutathione peroxidase; LIBC – latent iron biding capacity; TIBC – total iron binding capacity; TSI – transferrin saturation index. Pearson correlation analysis were performed and only coefficients with $P<0.05$ are shown