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Moretti, Débora B.; Nordi, Wiolene M.; Cruz, Thaline M.P.; Machado-Neto, Raul

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Research Article

Catalase, superoxide dismutase, glutathione peroxidase and oxygen radical absorbance capacity in the gut of juvenile pacu *Piaractus mesopotamicus* and dourado *Salminus brasiliensis* fed bovine first milk secretion

Débora B. Moretti[1], Wiolene M. Nordi[1], Thaline M.P. Cruz[1] & Raul Machado-Neto[1]
[1]Animal Science Department, Luiz de Queiroz College of Agriculture
University of São Paulo, Piracicaba, Brazil
Corresponding author: Debora B. Moretti (dmoretti@usp.br)

ABSTRACT. Besides the immunological benefits of colostrum consumption, this lacteal secretion has a great concentration of biological molecules that can affect gut physiology and fish healthy. This study evaluated the antioxidant potential in the gut of juvenile pacu, *Piaractus mesopotamicus*, and dourado, *Salminus brasiliensis*, fed twice a day with diets containing 0, 10 or 20% of lyophilized bovine colostrum (LBC). The whole medium and posterior intestine was collected after 30 or 60 experimental days and the activity of catalase, superoxide dismutase and glutathione peroxidase was determined as one international unit per milligram of protein, and the oxygen radical absorbance capacity as µM of equivalent Trolox per milligram of protein. Only the juvenile pacu were affected by the diet containing LBC ($P < 0.05$). The juvenile fed 10% LBC showed higher oxygen absorbance capacity than the juveniles fed 20% LBC ($P < 0.05$). Interaction between diet and period was observed to superoxide dismutase (SOD) activity ($P < 0.05$), juvenile pacu fed 0% LBC and 10% LBC did not change enzyme activity at 30 and 60 days, whereas juvenile fed 20% LBC showed higher value at 60 days compared to 30 days ($P < 0.05$). The present result reveal that the consumption of diet containing LBC improved SOD activity in the gut of juvenile pacu indicating a possible protective action of this lacteal secretion in an omnivorous fish.

Keywords: *Piaractus mesopotamicus*, *Salminus brasiliensis*, antioxidant, enzymes, colostrum, intestine, Neotropical fish.

INTRODUCTION

In Brazil, tilapia and carp are the major cultivated species; however, endemic and Neotropical species such as pacu and dourado have favorable attributes for industrial breeding. These species exhibit rapid growth, efficient feed conversion, wide acceptance and appreciation in the domestic market and are valuable in fishing (Urbinati & Gonçalves, 2005; Esteves & Sant´Anna, 2006). However, studies on physiology and nutritional requirements of these fish are still necessary.

The inclusion of lyophilized bovine colostrum in fish diet is an innovative idea that can possibly elicit a protective effect in the aquatic organism. In mammals, consumption of this lacteal secretion in the first hours of life plays an important role in nutritional, metabolic and endocrine status (Blum & Hammon, 2000; Blum & Baumrucker, 2002). The positive effects of colostrum consumption, such as reduction of enteric and respiratory disease, mortality and morbidity, are considered consequences of the presence of immunoglobulin in the intestinal tract acting as local protection (Nocek *et al.*, 1984; Daniele *et al.*, 1994; Baracat *et al.*, 1997). Besides the immunological benefits of colostrum consumption, this lacteal secretion has a great concentration of biological molecules that can affect gut physiology (Boudry & Thewis, 2009). According to Pandey *et al.* (2011), components of colostrum as nucleosides and nucleotides, ghrelin and lysozyme are considered health promoters. The authors report that nucleotides and nucleosides regulate bodily functions and participate in fatty acid biosynthesis. Hossain *et al.* (2016) reported that nucleotide administration influences growth, immune responses and oxidative stress resistance of juvenile red sea bream *Pagrus major*. Changes of the intestinal mucosa function results in increased uptake of luminal toxic materials such as antigens, toxins, and pro-inflammatory molecules (Söderholm & Perdue, 2001). In this regard, it is important to maintain the integrity of the intestinal

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epithelium, especially by mechanisms that fight against intestinal stress. Bovine colostrum in the lyophilized form has its original characteristics preserved, and the supply of this milk secretion to other species is a practice that may be feasible especially next to dairy production. Considering the nutraceutical properties of bovine colostrum, companies have commercialized this product.

Thus, this study investigated the effect of bovine colostrum on antioxidant potential in the gut of juvenile pacu *Piaractus mesopotamicus* and dourado *Salminus brasiliensis*.

MATERIALS AND METHODS

Experimental procedures

The first bovine milk secretion was collected from multiparous Holstein cows, pooled and frozen at 20°C. After the lyophilization process, the pool was homogenized and kept at 20°C until addition to the experimental diets. Pelleted diets, isonitrogenous and isoenergetic, were formulated to the juvenile pacu and dourado (Tables 1-2). Diets and the lyophilized bovine colostrum were submitted to chemical analysis according to AOAC methods (AOAC, 2000).

Farm-raised, feed-conditioned juvenile pacu (8.5 ± 0.7 g and 7.8 ± 0.3 cm) and dourado (13.3 ± 0.9 g and 10.8 ± 0.3 cm) were randomly distributed in 36 tanks (324 pacu in 18 tanks and 270 dourado in 18 tanks). Water quality parameters were monitored daily and the registered temperature (26.8 ± 1.5ºC), dissolved oxygen (5.8 ± 1.0 mg L^{-1}) and dissolved ammonia (<0.05 mg L^{-1}) were within acceptable range for the species.

Juvenile pacu and dourado were adapted to the experimental diets for ten and seven days, respectively, and then hand-fed the experimental diets containing 0, 10 or 20% of lyophilized bovine colostrum (three tanks per diet) to apparent satiety twice a day (08:30 and 16:30 h) for either 30 or 60 days. Fish were kept, maintained and treated according to accepted standards for the humane treatment of animals (authorized by the ESALQ/USP ethics committee).

Seven juveniles per treatment were sampled at each mentioned date for tissue sample collection. Sampling routine included 24 h of fasting and anesthesia with a benzocaine medium (0.1 g L^{-1}). After slaughter, the whole medium and posterior intestine was collected and stored at -80ºC.

The antioxidant capacity in the intestinal tissue was assessed by the determination of oxygen radical absorbance capacity (ORAC) and by the activity of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT).

The samples were diluted to 2.5 mg tissue mL^{-1} sodium phosphate buffer 75 mM pH 7.4 and analyzed for oxygen radical absorbance capacity. 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 analyzes were performed in black microplate which was added 30 µL of sample, 60 µL of 487 nM fluorescein solution and 110 µL of 76 mM AAPH (2,2'-Azobis (2-amidinopropane) dihydrochloride) solution. The analyzes 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 µM of equivalent Trolox per mg protein.

GPx reaction was performed in medium containing 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 (Wendel, 1981). The decrease in absorbance was recorded for 5 min in a spectrophotometer with a wavelength set at 340 nm. The glutathione peroxidase activity values are expressed as unit U mg^{-1} protein. One unit catalyzes the oxidation by H_2O_2 of 1 mol of reduced glutathione to oxidized glutathione per minute at 25°C, pH 7.0.

The SOD activity was measured using the Superoxide Dismutase Kit Assay Kit (Cayman Chemicals, Ann Arbor, MI). This method uses a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. The assay measures the three types of SOD (Cu/Zn, Mn and FeSOD). The SOD activity values are expressed as unit U mg^{-1} protein. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

AT activity was determined according to the methodology described by Iwase *et al.* (2013). In this analysis, 100 µL of sample 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 specificity of the reaction 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 U mg^{-1} protein units. One unit of catalase is responsible for the consumption of 1 mol of H_2O_2 per minute.

Table 1. Chemical composition of experimental pelleted diets provided to juvenile pacu, *Piaractus mesopotamicus*. [1]Guabi Nutrição Animal, Campinas, São Paulo (ingredient per kg). Vitamins: A, 2,500 UI; D3, 600.000 UI; E, 37.500 UI; K3, 3,750 mg; C, 50,000 mg; B1, 4.000 mg; B2, 4,000 mg; B6, 4,000 mg; B12, 4,000 mg; calcium pantothenate, 12,000 mg; biotin, 15 mg; acid folic, 1,250 mg; niacin, 22,500 mg. Mineral: Cu, 2,500 mg; Zn, 12,500 mg; I, 375 mg; Se, 87.5 mg; Co, 125 mg; Mn, 12,500 mg; Fe, 15,000 mg; BHT, 15,000 mg. [2]Original matter basis. Source: Moretti *et al.* (2014a).

Ingredient (g kg^{-1})	Juvenile pacu diets		
	0%	10%	20%
Bovine colostrum (679 g kg^{-1} CP)	-	100	200
Soybean meal (45 g kg^{-1} CP)	265	76.7	-
Wheat meal	238	311.8	311.8
Poultry by-product meal	200	200	131
Broken rice	188	198	200
Fish meal (55% CP)	50	50	50
Fish oil	46.2	40	45.7
DL-methionine	2.4	3.4	4.7
L-lysine HCl	-	6	11.5
BHT	2	2	2
Cellulose	-	3.4	-
Calcareous	-	-	14.7
Corn grain	-	-	9.3
Premix[1]	10	10	10.5
Chemical composition (g kg^{-1})[2]			
Dry matter	940.6	933.4	936.4
Crude protein	324.6	314.9	322
Crude fiber	30	27.7	30.7
Fat	90.5	98.2	109.3
Ash	106.4	101.1	92.8
Gross energy (MJ kg^{-1})	18	18.2	18.3

Table 2. Chemical composition of experimental pelleted diets provided to juvenile dourado, *Salminus brasiliensis*. [1]Guabi Nutrição Animal, Campinas, São Paulo (ingredient per kg). Vitamins: A, 2,500 UI; D3, 600.000 UI; E, 37.500 UI; K3, 3,750 mg; C, 50,000 mg; B1, 4.000 mg; B2, 4,000 mg; B6, 4,000 mg; B12, 4,000 mg; calcium pantothenate, 12,000 mg; biotin, 15 mg; acid folic, 1,250 mg; niacin, 22,500 mg. Mineral: Cu, 2,500 mg; Zn, 12,500 mg; I, 375 mg; Se, 87.5 mg; Co, 125 mg; Mn, 12,500 mg; Fe, 15,000 mg; BHT, 15,000 mg. [2]Original matter basis. Source: Moretti *et al.* (2014b, 2014a).

Ingredient (g kg^{-1})	Juvenile dourado diets		
	0%	10%	20%
Bovine colostrum (679 g kg^{-1} CP)	-	100	200
Soybean meal (45 g kg^{-1} CP)	230	230	230
Poultry by-product meal	204.8	119.8	24.5
Fish meal (55 g kg^{-1} CP)	320	300	300
Fish oil	95	90	85.2
Premix	10	10	10
BHT	0.2	0.2	0.2
Cellulose	20	30	30
Corn (whole grain)	120	120	120
Chemical composition (g kg^{-1})[2]			
Dry matter	924.4	936.7	325.7
Crude protein	422	425.1	444.7
Crude fiber	18.9	29.2	37.3
Fat	140.7	140.6	133.7
Ash	121.9	114.5	91.1
Gross energy (MJ kg^{-1})	20.8	21.1	20.8

Statistical analysis

Statistical analysis was performed using SAS software (SAS Institute Inc). The variables were analyzed based on a 2×3 completely randomized factorial design. The three diets (0, 10 and 20% of LBC) and feeding period (30 and 60 experimental days) were considered the main effects. After the data were confirmed for normal distribution with the Shapiro-Wilk test, analysis of variance was performed using the Proc Mixed procedure. If the F value was significant, the Tukey test was used for multiple comparisons between pairs of means at 5% of probability. Results are presented as means and standard errors.

RESULTS

In the juvenile pacu, the ORAC value was affected by diet and period ($P < 0.05$), the juveniles fed 10% LBC showed higher oxygen absorbance capacity than the juveniles fed 20% LBC and at 30 days the value was higher than at 60 days, Table 3. Interaction between diet and period was observed to SOD activity ($P < 0.05$), juveniles fed 0% LBC and 10% LBC did not change enzyme activity at 30 and 60 days, whereas juvenile fed 20% LBC showed higher enzyme activity at 60 days compared to 30 days (Fig. 1). GPx activity was not influenced by diet and period ($P > 0.05$) and CAT activity was affected only by period ($P < 0.05$), showing higher activity at 60 days compared to 30 days. In the juvenile dourado, GPx activity was affected by period ($P < 0.05$), showing higher activity at 30 days than at 60 days. SOD and CAT activity and ORAC values were not affected by diet and period ($P > 0.05$).

DISCUSSION

According to Huang *et al.* (2002), the ORAC technique can be used to evaluate the antioxidant activity in animal tissues, plant extract, natural products and foods. In this essay, the higher values of equivalent trolox indicate greater tissue ability to protect against the formation of peroxyl radicals and therefore higher antioxidant activity. In the present study, the antioxidant activity in the intestine of juvenile pacu was influenced by diet and experimental period. Regarding to the diet, juvenile fed with 10% LBC had higher ORAC value than fish fed with 20% LBC. Considering the experimental period, at 30 days the ORAC value was greater than at 60 days, showing a higher antioxidant activity against peroxyl radicals in younger fish that has faster cellular metabolism and therefore is most likely to produce reactive oxygen species. According to Martínez-Álvarez *et al.* (2005) a free-radical theory of ageing emerged in the middle of the

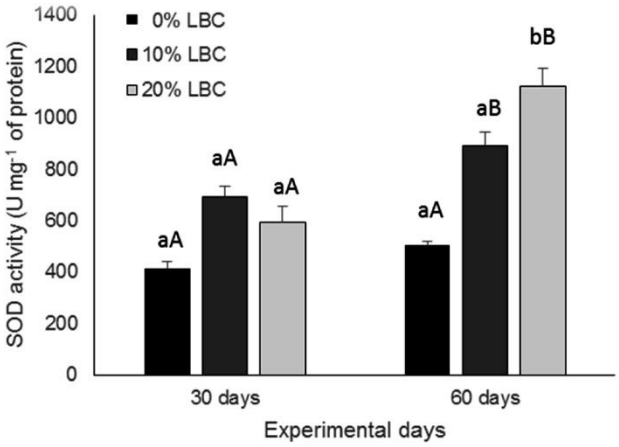

Figure 1. Superoxide dismutase activity in the intestine of juvenile pacu, *Piaractus mesopotamicus*, (mean ± SE) fed lyophilized bovine colostrum. Superoxide dismutase activity expressed as unit U mg^{-1} protein. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the radical superoxide. ^{ab}Means with different small letters in the same period are different by Tukey test ($P < 0.05$). ^{AB}Means with different capital letters in the same diet are different by Tukey test ($P < 0.05$).

20th century and postulates that while ROS generation increases with age, antioxidant defenses are impaired. The authors suggest that functional deterioration associated with ageing is derived from an accumulation of oxidative damage inflicted by non-scavenged ROS on lipids, proteins and nucleic acids.

In dourado, the higher GPx activity at 30 days than at 60 days is the probable mechanism that assist the younger juvenile in the fight against reactive oxygen species. This enzyme is responsible for the reduction of lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Vasconcelos *et al.*, 2007). Correia *et al.* (2003) observed lower antioxidant enzyme activities in whole body of adult *Gammarus locusta* compared to juveniles, suggesting that the older animals are more susceptible to lipid peroxidation and oxidative stress. The superoxide dismutase deals with superoxide radical, catalyzing the dismutation (or partitioning) of O_2^- into either oxygen (O_2) or hydrogen peroxide (H_2O_2) (Vasconcelos *et al*., 2007). In juvenile pacu, the evaluation of SOD revealed a greater ability to deal with the generation of superoxide species at 60 days, contrary to that observed for the antioxidant activity against the generation of peroxyl radicals. Influence of bovine colostrum inclusion was observed on SOD activity in pacu, juveniles fed with 0 and 10% LBC kept the enzymatic activity of SOD at 30 and 60 days, while the juveniles fed 20% LBC had higher activity of this

Table 3. Antioxidant potential in the intestine of juvenile pacu, *Piaractus mesopotamicus*, and dourado, *Salminus brasiliensis*, (mean ± SE) fed lyophilized bovine colostrum. Within rows, means followed by different letters are significantly different at $P = 0.05$. ns: not significant; $*P < 0.05$; $**P < 0.01$. [1]0% LBC: juveniles fed 0% of lyophilized bovine colostrum; LBC 10%: juveniles fed 10% of lyophilized bovine colostrum; 20% CBL - juveniles fed 20% of lyophilized bovine colostrum. [2]D: diet effect, P: period effect; DxP: interaction between diet and period. [3]SOD: superoxide dismutase (expressed as unit U mg^{-1} protein. One unit of SOD is define as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical); GPx: glutathione peroxidase (expressed as unit U mg^{-1} protein. One unit catalyzes the oxidation by H_2O_2 of 1 mol of reduced glutathione to oxidized glutathione per minute at 25°C, pH 7.0); Cat: catalase (expressed as units U mg^{-1} protein. One unit of catalase is responsible for the consumption of 1 mol of H_2O_2 per minute); ORAC: oxygen radical absorbance capacity (expressed as µM of equivalent Trolox per mg protein).

Species	Variable[3]	Diet[1]			Period (days)		Effect[2]		
		0% LBC	10% LBC	20% LBC	30	60	D	P	DxP
Piaractus mesopotamicus	SOD	458 ± 29	796 ± 56	858 ± 125	586 ± 48	884 ± 95	**	**	*
	GPx	177 ± 12	167 ± 32	175 ± 7	174 ± 18	170 ± 17	ns	ns	ns
	CAT	755 ± 238	533 ± 94	734 ± 151	437 ± 99	891 ± 86	ns	*	ns
	ORAC	446 ± 125ab	505 ± 100a	348 ± 104b	628 ± 61	234 ± 31	*	**	ns
Salminus brasiliensis	SOD	355 ± 56	314 ± 51	290 ± 45	369 ± 38	268 ± 38	ns	ns	ns
	GPx	187 ± 44	166 ± 37	125 ± 41	224 ± 30	94 ± 19	ns	*	ns
	CAT	737 ± 104	622 ± 83	759 ± 153	765 ± 102	646 ± 82	ns	ns	ns
	ORAC	227 ± 26	215 ± 30	234 ± 45	231 ± 26	220 ± 29	ns	ns	ns

enzyme at 60 days. This result indicates a positive effect of bovine colostrum intake in enteric cell protection. Kwon *et al.* (2010) also detected antioxidative effects of bovine colostrum in an intestinal ischemia/reperfusion rat model. The authors observed higher activity of SOD, GPx and CAT in rats fed bovine colostrum compared to rats fed saline. Although the intake of bovine colostrum does not determine changes in the performance of juvenile pacu and dourado (Machado-Neto *et al.*, 2016), an improvement in the physiological condition of the juveniles possibly implies a better capacity to face challenges. Tang *et al.* (2013) showed that the increase in the activity of antioxidant enzymes in the intestine of the young grass carp *Ctenopharyngodon idella* is related to decrease in lipid peroxidation and protein oxidation, resulting in enhanced intestinal defense. Similar to colostrum, amino acids also increase the intestinal activity of antioxidant enzymes (Li *et al.*, 2014; Hong *et al.*, 2015). Jiang *et al.* (2010) also observed that the inclusion of components that inhibit free radical generation (myo-inositol) in the diet of Jian carp (*Cyprinus carpio* var. Jian) improves antioxidant status, and lipid peroxidation and depress protein oxidation in muscle, intestine and hepatopancreas. For the juvenile dourado, a carnivorous species adapted to higher protein degradation in the digestive tract, the activity of SOD enzyme was not changed with the inclusion of bovine colostrum in the diet. However, this diet could be considered of great value to this specie, since Nordi *et al.* (2016) observed proper development of enteric, muscle and hepatic tissue of juvenile *S. bransiliensis* consuming diet with colostrum and Moretti *et al.* (2014a, 2014b) did not found significant implications in intestinal enzymatic activity of juvenile *P. mesopotamicus* and *S. brasiliensis* also consuming diets with the first milk secretion.

The present result reveal that the consumption of diet containing lyophilized bovine colostrum improved superoxide dismutase activity in the gut of juvenile pacu, an omnivorous fish, indicating a possible protective action of this lacteal secretion.

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