Maize distillers dried grains with solubles alter dietary digestibility and improve intestine health of pacu, *Piaractus mesopotamicus* juveniles

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Abstract

Two trials were conducted to evaluate the effects of soyabean meal replacement by maize distillers dried grains with solubles (DDGS) in diets for pacu juveniles. Five diets were formulated with 0, 100, 200, 300 and 400 g of DDGS/kg diet replacing up to total dietary soyabean meal. In trial 1, the experimental diets were fed to five groups of fish to evaluate the apparent digestibility coefficients (ADC). In trial 2, four groups of fish were fed each experimental diet for 100 d to evaluate the effects of these diets on digestive enzyme activity, intestine oxidative stress and intestine morphology. The ADC of DM and energy was reduced with dietary DDGS inclusion, while the ADC of lipids was increased, and no differences were observed for the ADC of protein. Independent of dietary treatment, pH increased from anterior to the distal intestine with dietary DDGS inclusion. Digestive enzyme activities were higher on anterior than the distal intestine. Dietary DDGS decreased lipase, amylase, chymotrypsin and trypsin activities, while no differences were observed for total protease activity. Intestine glucose-6-phosphate dehydrogenase was reduced in fish fed the DDGS diets, while catalase activity increased. Lipid peroxidation was lower in fish fed DDGS diets than the control. Intestine histomorphology improved with dietary DDGS inclusion. Overall, the negative effects of soyabean meal could be decreased by dietary replacement with maize DDGS which may have a prebiotic effect, improving intestine health.

Key words: Antioxidant capacity: Digestibility: Digestive enzymes: Intestine health: Prebiotics

Pacu, *Piaractus mesopotamicus*, is an omnivorous characin native of rivers, floodplains, lakes and flooded forests of South America. It is considered one of the best candidates for aquaculture production in tropical areas, due to the high growth rates (it may reach more than 1 kg in 1 year), good meat quality and good consumer acceptance[1].

Pacu diets are mainly based on local ingredients, including soyabeans. However, due to soyabean costs, its replacement in diets by more economical ingredients, such as maize distillers dried grains with solubles (DDGS), may improve production profitability. DDGS is the by-product of maize grain fermentation, by the addition of enzymes and yeast, to produce ethanol. After fermentation, the remaining product goes through a set of centrifugations, separating the thin and thicker portion, that goes to drying processes. To the thicker mass, commonly known as DDG (distillers dried grains), can be included the thin portion, or soluble portion, to originate the DDGS[2]. Maize DDGS has moderate protein content (330 g/kg) and low starch content (<30 g/kg) and lacks anti-nutritional factors, except for high content of NSP[3]. Besides, part of the enzymes and yeast used during fermentation remains into the soluble portion of the final product[4].

Fish response to DDGS inclusion in the diets varies according to DDGS composition, dietary composition regarding processing (majorly extrusion), fish species and food habits[5]. Recommended dietary inclusion of DDGS ranges from 200 to 330 g/kg for carnivorous fish such as olive flounder, *Paralichthys olivaceus*[6], rainbow trout[7,8], red seabream[9] and hybrid striped bass[10]. However, DDGS is particularly attractive for omnivorous fish as it has a protein content that is closer to their dietary protein requirement, and because omnivorous fish also tolerate better the high fibre content of DDGS[11].

Thus, dietary inclusion of DDGS usually ranges from 300 to 700 g/kg for omnivorous fish like tilapia (*Oreochromis niloticus*)[12–15], channel catfish (*Ictalurus punctatus*)[16,17], blunt nose black bream (*Megalobrama amblycephala*) and gibel carp (*Carassius gibelio*)[18].

To better understand the modulatory action of DDGS on feed utilisation, growth performance and health of fish, it is necessary to study its effects on intestinal tract integrity and digestive function[19–21]. However, up to now, few studies have focused on the effects of dietary DDGS on digestive enzyme activity and intestine morphology in fish. Recently, Magalhães *et al.*[11] and Rahman *et al.*[6] evaluated DDGS inclusion in diets for carnivorous fish, and both reported no differences in digestive enzyme activities. However, studies focusing on the effects of DDGS incorporation in diets at the intestinal level for omnivorous species are still lacking. Dietary ingredient composition may also affect the oxidative status of the fish intestine[22–26]. Oxidative stress is characterised by an imbalance between reactive oxygen species (ROS) formation and removal in the organism by antioxidant defences[27], which lead to the oxidation of the main biomolecules that can affect cell function leading to tissue injuries, like damages in the intestine epithelium[28]. However, few studies focused on the oxidative stress associated with dietary DDGS inclusion. Nevertheless, the high content of PUFA[29,30] and the exposure to high temperatures during processing may increase DDGS susceptibility to lipid peroxidation (LPO)[31]. On the other hand, other components in DDGS such as its high sulphur content[32] have been highlighted to provide adequate antioxidant protection. Still, dietary inclusion of DDGS may affect the intestine oxidative status and intestine morphology.

Thus, the aim of this study was to evaluate the effects of dietary replacement of soyabean meal by maize DDGS on diet digestibility, digestive enzyme activity, oxidative status and morphology and cytoarchitecture of intestine in pacu juveniles.

Material and methods

The experimental protocol used in this study was approved by the Ethics Committee of Animal Welfare of the College of Agricultural and Veterinary Sciences of Sao Paulo State University, protocol number 1910/16.

Two trials evaluated the effects of dietary replacement of soyabean meal by maize DDGS in diets for pacu, *P. mesopotamicus*. Trial 1 evaluated the apparent nutrient digestibility of diets with increasing DDGS levels, whereas trial 2 evaluated the effects of these diets on digestive enzyme activity, intestine oxidative stress and intestine morphology.

Experimental diets

Five diets were formulated to be isoproteic (290 g/kg digestible protein) and isoenergetic (14·5 MJ/kg digestible energy) and with increasing DDGS levels (0, 100, 200, 300 and 400 g/kg) replacing soyabean meal and maize meal. The same diet formulation was used in trial 1 and trial 2; however, 5 g/kg of chromic oxide (Cr_2O_3) was added as an external marker to the diets used in trial 1. Composition and proximate analyses of the diets are shown in Table 1.

Dietary ingredients were ground (1 mm), thoroughly mixed and processed in a single screw extruder (Imbramarq, model Labor PQ-30). The extrusion temperature was kept at 124°C, screw speed at 676·5 rpm, feeding rate of about 500 g/min, and the circular matrix was set at 2 mm. Pellets were dried at 50°C for 12 h and then stocked at −20°C until used.

Fish and experimental design

Pacu (*P. mesopotamicus*) juveniles were obtained from a commercial fish farm and acclimatised for 30 d in a quarantine tank (5000 litres) upon arrival to the experimental facilities, at the Aquaculture Nutrition Laboratory of Food Engineer and Animal Science Faculty, São Paulo University, Pirassununga, SP, Brazil. During this period, fish were fed a commercial diet (Pira 32, Guabi, 320 g/kg of crude protein) twice daily until apparent satiation. Trials 1 and 2 were conducted simultaneously, within the same recirculation aquaculture system, with fish from the same livestock.

Trial 1. To set up the trial, 150 *P. mesopotamicus* juveniles (27·1 (sd 0·11) g, mean weight) were distributed to five cylindrical-conical fibreglass tanks (100 litres), at a density of thirty fish/tank. Tanks were supplied with aeration, thermoregulated to 26°C and photoperiod of 12 light–12 dark. Each tank was considered an experimental unit, and the experiment was lain out in Latin square design 5 × 5 (five treatments and five experimental periods), where each tank received each diet only for a unique time. The experimental period consisted of 11 d, the sum of the time taken for feed adaptation and faeces collection, given an entire period of 55 d.

Each period started with fish being fed for 7 d, twice daily (09.00 and 17.00 hours) with the experimental diets, until apparent satiation. Faeces were collected using a modified Guelph system as early described[33] with modifications. One hour after the last daily feeding, the groups of fish were transferred to five collecting tanks, conic fibreglass tanks (100 litres capacity) with a valve connecting the bottom of the tank to a Falcon tube kept on ice. At 10 h later, faeces sedimented into the tubes were collected, fish were relocated to the initial tanks and the tanks used for faeces collection were cleaned and water renewed. The procedure was repeated for 4 d until enough amounts of faeces were collected. Fish were fed in an independent system to avoid contamination of faeces with feed[33]. After collection, faeces were centrifuged (1800 *g*, 10 min), bulk stored for each tank at −20°C and afterwards lyophilised before chemical analysis. The apparent digestibility coefficients (ADC) of DM, protein, lipids and energy of the experimental diets were measured by the indirect method[34] as follows: ADC = $100 × (1 − (\% Cr_2O_3 \text{ diet}/\% Cr_2O_3 \text{ faeces}) × (\% \text{ nutrient or MJ/g energy faeces}/\% \text{ nutrient or MJ/g energy diet}))$.

Trial 2. Fish (21 (sd 0·13) g, mean weight) were randomly distributed to twenty fibreglass tanks of 100 litres capacity, at a density of fifteen fish per tank. Tanks were supplied with aeration, thermoregulated to 26°C and covered with nets to prevent fish to escape. A photoperiod of 12 light–12 dark was adopted. Each tank was considered an experimental unit and arranged in a

Table 1. Composition and proximate analysis of the experimental diets

Diet	Control	100 DDGS	200 DDGS	300 DDGS	400 DDGS
Ingredients (as fed basis) (g/kg)					
DDGS*	0	100	200	300	400
Soyabean meal†	231	173	115	58	0
Maize‡	254	218	181	145	108
Soyabean oil	34	28	23	17	11
Poultry meal§	66	66	66	66	66
Fishmeal‡	110	110	110	110	110
Maize gluten‡	83	83	83	83	83
Wheat meal†	130	130	130	130	130
Rice bran†	77	77	77	77	77
L-Lysine‖	5	5	5	5	5
BHT	1	1	1	1	1
Vitamin and mineral premix¶	10	10	10	10	10
Total	1000	1000	1000	1000	1000
Proximate composition (g/kg DM basis)					
DM (g/kg)	931	916	934	913	938
CP	328	321	327	321	327
Gross energy (MJ/kg)**	18·6	19·1	19·4	19·6	19·6
Lipid	32	52	58	71	76
Ash	82	78	69	69	66
NDF	249	281	328	387	442
Starch	228	196	181	157	143
Digestible protein	295	276	294	282	291
Digestible energy (MJ/kg)	14·7	14·7	14·9	14·5	14·5
DP:DE	2·0	1·9	2·0	2·0	2·0

DDGS, distillers dried grains with solubles; BHT, butyl hydroxytoluene; CP, crude protein; NDF, neutral insoluble-detergent fibre (determined using the Ankom® Filter Bag Technique and heat stable α-amylase (973·18)); DP:DE, digestible protein:digestible energy ratio.
* DDGS (CP, 336 g/kg; 20·9 MJ/kg), Libra Etanol.
† Soyabean meal (CP 448 g/kg; 17·1 MJ/kg), maize meal (CP 84 g/kg; 16·2 MJ/kg), wheat meal (CP 148 g/kg; 16·7 MJ/kg), rice bran (CP 132 g/kg; 19·4 MJ/kg), Cargill, Sao Paulo, Brazil.
‡ Maize gluten (CP 715 g/kg; 21·1 MJ/kg), fishmeal (CP 580 g/kg; 14·6 MJ/kg), InVivo, Sao Paulo, Brazil.
§ Poultry meal (CP 489 g/kg; 13·1 MJ/kg), Agromix.
‖ Lysine, Anjinomoto LTDA.
¶ Vitamin and mineral premix: vitamin A – 500 000 IU; vitamin D$_3$ – 250 000 IU; vitamin E – 5000 mg; vitamin K$_3$ – 500 mg; vitamin B$_1$ – 1500 mg; vitamin B$_2$ – 1500 mg; vitamin B$_6$ – 1500 mg; vitamin B$_{12}$ – 4000 mg; folic acid – 500 mg; calcium pantothenate – 4000 mg; vitamin C – 10 000 mg; biotin – 10 mg; inositol – 1000 mg; nicotinamide – 7000 mg; choline – 10 000 mg; Co – 10 mg; Cu – 1000 mg; Fe – 5000 mg; iodine – 200 mg; Mn – 1500 mg; Se – 30 mg; Zn – 9000 mg (Agromix LTDA).
** Values calculated according to FAO (2012) and NRC (2011).

completely randomised design with five dietary treatments and four replicates. Dissolved oxygen, total ammonia, nitrate and pH were monitored daily using a multiparameter Horiba (model U – 10). Fish were fed with the experimental diets for 100 d, twice daily (09.00 and 17.00 hours), until apparent satiation.

Sampling procedure

At the end of trial 2, fish were fasted for 24 h and then eight fish from each treatment (two fish per replicate) were randomly sampled, anaesthetised with benzocaine (50 mg/l), euthanised by spinal cord section and dissected on chilled trays. The intestines were carefully removed, freed from the adjacent adipose tissue and divided into three portions (anterior, mid and distal intestine). The distal intestine was distinguished from the medium intestine by its increased diameter, darker mucosa and annular rings. The anterior and mid potions were obtained by division of the remaining intestine in two identical portions. Each intestine portion was individually stored in Eppendorf tubes, immediately frozen in liquid N$_2$ and then stored at −80°C until enzymatic analyses.

Mid intestine samples were also collected from two other fish per tank, gently washed with saline water (0·9%) to remove biological fragments and fixed in Bouin's solution for 12 h. Bouin's solution was made following the proportions: 75 ml of picric acid (2·1 %), 25 ml of formaldehyde (40 %) and 5 ml of glacial acetic acid.

Enzyme activity

Each intestine portion was weighted, and the pH measured *in situ* using a pH metre (pH Eutech Instrument). Samples were homogenised (1:5 dilution, w/v), on ice-cold buffer (100 mM Tris–HCl, 0·1 mM EDTA and 0·1 % Triton X-100 (v/v), pH 7·8). Then, samples were centrifuged at 3300 ***g*** for 30 min at 4°C, and the supernatant was collected and stored at −80°C until analyses. All enzyme activities were measured at 37°C using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments). For oxidative status, distal intestine samples were used as it is the intestine portion where most of the oxidative damages are usually observed[35–37].

Digestive enzyme activity

Total proteases activity was measured by the casein hydrolysis method according to Hidalgo *et al.*[38], with 0·1M Tris–HCl buffer (pH 7·8). The reaction mixture containing casein (1 % w/v; 0·125 ml), buffer (0·125 ml) and homogenate supernatant was incubated for 1 h at 37°C and stopped by adding 0·6 ml trichloroacetic acid (8 %, w/v) solution. After being kept for 1 h at 2°C, samples were centrifuged at 1800 ***g*** for 10 min and the absorbance read at 280 nm against blanks. A blank for each sample was prepared by adding the homogenate supernatant after incubation. Chymotrypsin activity was determined follow-

ing Hummel[39], with Rao & Lombardi[40] modifications, using *N*-benzoyl-L-tyrosine ethyl ester as a substrate in Tris-HCl 41·4 mM and $CaCl_2$ 10·4 mM (pH 8·1) and the absorbance read at 256 nm. Trypsin activity was measured according to Bergmeyer *et al.*[41] using 10 nM *Nα-p*-toluenesulfonyl-L-arginine methyl ester as a substrate in Tris-HCl 41·4 mM and $CaCl_2$ 10·4 mM (pH 8·1) and the absorbance read at 247 nm. Amylase (*EC* 3.2.1.1) and lipase (*EC* 3.1.1.3) activities were measured with kits (Spinreact reference 41201 and reference 1001274, respectively), adapted for fish intestine samples. The substrate 2-chloro-4-nitrophenyl-*α*-D-maltotrioside was used for amylase and absorbance read at 405 nm. Lipase was determined using 1–2-O-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin)-ester as substrates and absorbance read at 580 nm.

Enzyme activity was expressed as specific activity, defined as μmol of product generated per minute. Soluble protein concentration was determined according to Bradford[42] with bovine serum albumin solution as standard.

Oxidative status

Superoxide dismutase (SOD) activity was measured at 550 nm by the ferricytochrome C method, using xanthine oxidase as a source of superoxide radical[43]. Catalase (CAT) activity was determined according to Aebi[44] by measuring the decrease in H_2O_2 concentration at 240 nm. Glutathione peroxidase activity was assayed as described by Flohé & Günzler[45]. Glutathione reductase (GR) activity was determined at 340 nm by measuring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH), as described by Morales *et al.*[46]. GSSG generated by glutathione peroxidase was reduced by GR, and the NADPH consumption rate was monitored at 340 nm. Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured by the reduction of $NADP^+$ at 340 nm as described by Morales *et al.*[46]. LPO was measured at 535 nm, as the reaction of malondialdehyde and thiobarbituric acid, as described by Buege & Aust[47].

Enzyme activity was expressed as units (SOD and CAT) or milliunits (glutathione peroxidase, GR and G6PDH) per mg of soluble protein. Except for SOD, one unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per min under the assay conditions. One unit of SOD activity was defined as the amount of enzyme necessary to produce 50 % inhibition of the ferricytochrome c reduction rate. LPO was expressed as nmol of malondialdehyde per g of intestine tissue.

Histological assays and analysis

Samples were washed and stored in alcohol at 70 % for 30 d until processing by classical histological techniques and stained with haematoxylin–eosin, briefly, dehydration with ethanol, paraffin inclusion, obtention of histological sections with microtome, deparaffinisation, hydration and staining. Histological preparations were photographed using a light microscope (Carl Zeiss) for analysis of histological modifications. The following parameters were used as criteria of intestine alterations: mucosa fold height (shortening, widening and fusion of intestinal folds), supranuclear vacuolisation in the absorptive cells (enterocytes)

of the intestine epithelium; increased cellularity of connective tissue and widening of lamina propria and submucosa; leucocytes infiltration in lamina propria and submucosa, number of goblet cells (GC) and nucleus position within the enterocytes[48]. Blinded evaluation of the histological samples was performed by disposing scores from 1 to 3, where score 1 indicates normal conditions and 3 major deformities. The overall value of enteritis degree was calculated by averaging scores of all parameters.

Statistical analyses

Data are expressed as mean values with their pooled standard errors. Normality and homogeneity of variances were tested using the Shapiro–Wilk and Levene tests, respectively. All statistical procedures were performed using the SPSS software package[49], and probability level of 0·05 was used for rejection of the null hypothesis. Statistical analysis of digestive enzymes and pH was done by two-way ANOVA, with diet and intestine portion as factors. When necessary, Tukey's test was used to detect differences in digestive enzyme activity among intestine portions within each dietary treatment. Significant differences among dietary DDGS levels within each intestine portion were determined by orthogonal polynomial contrast to identify linear and quadratic effects. Second, data from apparent digestibility, sum and ratios (A/P, L/P and A/L) of digestive enzyme activities, oxidative stress enzymes and histology were analysed by one-way ANOVA, and in case of significance, orthogonal polynomial contrast was applied to identify linear and quadratic effects of dietary DDGS inclusion. The number of fish per group was established following Thorarensen *et al.*[50] recommendations.

Results

The effect of the diets on growth performance and feed utilisation was not the object of the present study and was published elsewhere[51]. In short, no differences in growth performance were observed due to diet composition but feed conversion and protein efficiency ratios were positively affected by dietary DDGS inclusion.

Trial 1

The ADC of the experimental diets are presented in Table 2. Increasing dietary DDGS levels linearly decreased the ADC of DM from 73·9 to 67·4 % and of energy from 79 to 73 %, while the ADC of lipids linearly increased from 87·8 to 94·1 % and the ADC of protein was not affected, averaging 89 %.

Trial 2

Intestinal pH. Intestine pH values were affected both by dietary treatments and intestine portion. No differences were observed on the anterior intestine pH of fish fed diets with increasing DDGS levels. On the contrary, there was a linear increase of mid intestine pH with dietary DDGS incorporation, while a quadratic effect was noticed for the distal intestine. Independently of dietary treatments, pH values were higher in the distal intestine portion (Table 3).

Table 2. Apparent digestibility coefficients (ADC) of nutrients in experimental diets containing increasing levels of distillers dried grains with solubles (DDGS) inclusion for pacu (*Piaractus mesopotamicus*) juveniles (*n* 5)†
(Mean values and pooled standard errors)

	Diets						Polynomial contrasts	
%	Control	100 DDGS	200 DDGS	300 DDGS	400 DDGS	SEM	Linear	Quadratic
DM	73·9	71·7	71·7	62·9	67·4	1·7	**	NS
Crude protein	89·8	88·6	90·1	87·9	88·9	0·7	NS	NS
Energy	79·1	77·0	76·9	69·4	73·0	1·4	***	NS
Crude lipids	87·8	91·5	92·6	92·5	94·1	1·4	**	NS

Cr_2O_3, chromium oxide.
** $P < 0.01$, *** $P < 0.001$.
† ADC = $100 \times (1 - (\% Cr_2O_3 \text{ diet}/\% Cr_2O_3 \text{ faeces}) \times (\% \text{ nutrient or MJ/g energy faeces}/\% \text{ nutrient or MJ/g energy diet})$.

Table 3. pH values and digestive enzymes specific activity in different intestine portions of pacu (*Piaractus mesopotamicus*) fed the experimental diets (*n* 8)†
(Mean values and pooled standard errors)

	Diets						Main effects			Contrast	
Item	Control	100 DDGS	200 DDGS	300 DDGS	400 DDGS	SEM	D	IP	D × IP	Linear	Quadratic
pH											
Anterior	6·8^A	6·7^A	6·8^A	6·9^A	6·8^A	0·02	***	***	NS	NS	NS
Mid	6·8^A	6·8^A	6·8^A	7·0^A	6·9^A	0·03				**	NS
Distal	7·2^B	7·1^B	7·0^B	7·3^B	7·4^B	0·04				*	*
Average	6·9	6·9	6·9	7·1	7·0	0·03				*	NS
Lipase											
Anterior	12·3^B	10·5^C	10·5^B	9·8^B	7·0^B	0·4	***	***	***	***	NS
Mid	2·7^A	3·4^B	1·0^A	3·5^A	1·0^A	0·2				***	NS
Distal	1·2^A	0·5^A	1·1^A	0·4^A	0·6^A	0·1				**	NS
Sum	16·2	14·4	12·5	13·6	8·5	0·8				***	NS
Amylase											
Anterior	247·6^C	210·8^C	192·7^B	132·4^B	67·1^B	13·6	***	***	***	***	NS
Mid	114·1^B	110·8^B	59·5^A	76·9^A	66·7^B	5·4				***	NS
Distal	53·5^A	60·9^A	67·6^A	42·6^A	29·0^A	3·2				***	***
Sum	415·2	382·6	319·8	251·9	162·7	18·4				***	NS
Proteases											
Anterior	538·2^A	719·7	687·6	1671·8^B	650·0	124·7	**	***	**	NS	NS
Mid	460·0^A	536·8	426·8	489·3^A	553·2	23·6				NS	NS
Distal	834·0^B	577·2	675·6	743·3^{A,B}	468·9	46·0				NS	NS
Sum	1832·2	1833·7	1790·0	2904·3	1672·0	327·2				NS	NS
Chymotrypsin											
Anterior	58·2^B	35·7^C	45·8^B	29·0^C	25·7^B	2·8	***	***	***	***	NS
Mid	17·3^A	23·7^B	9·8^A	13·9^B	17·8^B	1·0				*	***
Distal	4·4^A	1·2^A	2·8^A	1·3^A	0·9^A	0·4				**	NS
Sum	79·9	60·6	58·5	44·2	44·4	4·5				***	NS
Trypsin											
Anterior	6·1^B	6·1^B	6·0^B	3·6	5·2^B	0·9	**	***	**	NS	NS
Mid	4·2^{A,B}	6·7^B	2·1^A	3·4	3·7^{A,B}	0·5				**	NS
Distal	2·0^A	1·8^A	2·0^A	1·9	1·9^A	0·2				NS	NS
Sum	12·3	14·6	10·1	8·9	10·8	1·1				*	NS
A:P	0·24	0·22	0·19	0·11	0·10	0·02				***	NS
L:P	0·009	0·008	0·007	0·006	0·006	0·0				**	NS
A:L	25·7	27·0	26·4	18·4	19·6	1·7				**	NS

D, diet; IP, intestine portion; A:P, amylase:total proteases ratio; L:P, lipase:total proteases ratio; A:L, amylase:lipase ratio; proteases, total proteases.
^{A,B,C} Values sharing a common superscript letter are not significantly different at Tukey's test within enzyme ($P > 0.05$).
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way ANOVA and contrasts).
† Values of proteases, lipase, amylase and chymotrypsin expressed as IU/mg protein, and trypsin as mU/mg protein.

Digestive enzyme activity. Digestive enzyme activities in the different intestine portions are presented in Table 3. Total protease activity was not affected by the intestine portion, except for higher values in the distal intestine of fish fed the control diet and in the anterior intestine of fish fed the 300 DDGS diet. On the other hand, lipase, amylase, chymotrypsin and trypsin activities decreased linearly from the anterior to the distal intestine portion, except for fish fed diet 200 DDGS where no differences on enzyme activities were observed for mid and distal intestine. Also, anterior and mid intestine portions had similar amylase activities in fish fed the 400 DDGS diet and trypsin activities in fish fed the 100, 300 and 400 DDGS diets.

Table 4. Specific activity of antioxidant enzymes and lipid peroxidation on the distal intestine of pacu (*Piaractus mesopotamicus*) fed the experimental diets (*n* 8)†
(Mean values and pooled standard errors)

Item	Control	100 DDGS	200 DDGS	300 DDGS	400 DDGS	SEM	Linear	Quadratic
			Diets				Polynomial contrasts	
LPO	11·4	8·2	8·4	6·7	7·1	0·5	***	NS
CAT	51·4	69·7	61·1	67·0	63·2	1·4	**	***
SOD	132·4	160·7	106·8	133·0	183·0	9·5	NS	NS
GPX	69·6	67·7	61·8	72·6	73·7	7·9	NS	NS
GR	34·8	35·3	31·9	29·8	31·8	2·0	NS	NS
G6PDH	222·0	211·3	225·2	164·4	159·2	7·1	***	NS

LPO, lipid peroxidase; CAT, catalase; SOD, superoxide dismutase; GPX, glutathione peroxidase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase.
** $P < 0.01$, *** $P < 0.001$.
† Results of G6PDH, GPX and GR expressed as mU/mg protein, SOD and CAT expressed as IU/mg protein, and LPO expressed as nmol malondialdehyde per g tissue.

Table 5. Histological evaluation of pacu (*Piaractus mesopotamicus*) mid intestine fed the experimental diets (*n* 8)†
(Mean values with pooled standard errors)

Parameter evaluated	Control	100 DDGS	200 DDGS	300 DDGS	400 DDGS	SEM	Linear	Quadratic
			Diets				Polynomial contrasts	
FH	1·8	2·0	2·1	2·0	2·1	0·07	NS	NS
LP	2·0	2·0	1·8	1·8	1·6	0·06	*	NS
SM	1·6	1·8	2·2	2·3	2·1	0·08	**	NS
LI	1·9	2·1	2·1	1·9	2·0	0·04	NS	*
GC	1·8	2·3	1·6	1·3	1·7	0·08	*	*
ENT	1·8	1·9	2·3	2·1	1·8	0·08	NS	*
SNV	2·5	2·2	1·4	1·2	1·0	0·10	***	NS
Mean score	1·9	2·1	1·9	1·8	1·7	0·03	***	NS

FH, mucosa fold height (shortening, widening and fusion of intestinal folds); LP, increased cellularity of connective tissue and widening of lamina propria; SM, increased cellularity of connective tissue and widening of submucosa; LI, leucocytes infiltration in lamina propria and submucosa; GC, number of goblet cells; ENT, nucleus position within the enterocytes; SNV, supranuclear vacuolisation in the absorptive cells (enterocytes) of the intestine epithelium.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
† Score from 1 to 3, with 3 indicating major alterations. Mean scores calculated by averaging the scores of the evaluated parameters.

With a few exceptions, there was a linear decrease of lipase, amylase and chymotrypsin activities in all intestine portions with the increase of dietary DDGS levels. However, a quadratic effect was observed for amylase on the distal intestine and chymotrypsin on the mid intestine. Trypsin activity linearly decreased in the mid intestine with dietary DDGS level.

Except for total proteases, total digestive enzyme activities, calculated as the sum activity of the three intestine portions, decreased linearly with dietary DDGS level. Amylase:total proteases (A:P), lipase:total proteases (L:P) and amylase:lipase (A:L) ratios also linearly decreased with increasing dietary DDGS level.

Oxidative status

There were no differences between dietary groups in glutathione peroxidase, GR and SOD activities (Table 4). There was a quadratic effect of dietary DDGS on CAT activity, while G6PDH activities and LPO linearly decreased with dietary DDGS level.

Histology

The scores of parameters used to assess gut morphology and the damage mean score are presented in Table 5. Dietary DDGS linearly reduced lamina propria width and cellularity, enterocytes vacuolisation and the number of GC, while it increased submucosa width and cellularity. A quadratic effect was noticed for intraepithelial leucocyte infiltration and enterocytes' nucleus position. Intestine global damage, represented by mean score, reduced linearly with DDGS inclusion in the diets (Table 5; Fig. 1).

Discussion

Diet digestibility

The evaluation of DM digestibility coefficients allows to get an estimative of the general digestibility of a diet or ingredient. In the present study, the observed decrease of DM digestibility with increasing DDGS levels may suggest an increment in the amount of non-digestible nutrients into the diets, also confirmed by the decrease of dietary energy digestibility. Similarly, Cheng & Hardy[8] in rainbow trout and Li *et al.*[52] in channel catfish observed a reduction of DM and energy digestibility with increasing dietary DDGS levels. The authors correlated the results with the high amount of fibre present in DDGS.

Among factors capable of inducing changes in dietary nutrient digestibility in DDGS-rich diets, fibre is probably the most important. Indeed, Krogdahl *et al.*[53] mentioned as an adverse feature of DDGS use in animal feeds its high amount
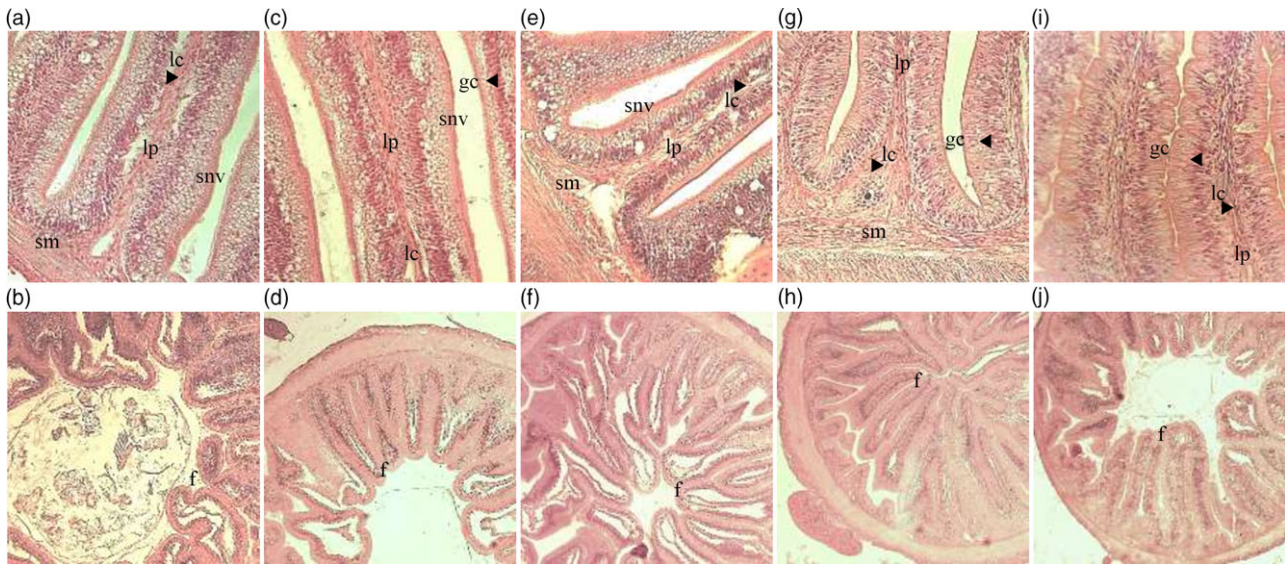
Fig. 1. Histological modifications on intestines of pacu (*Piaractus mesopotamicus*) juveniles fed diets with increasing levels of maize distillers dried grains with solubles inclusion and soyabean meal replacement. LP, increased cellularity of connective tissue and widening of lamina propria; SM, increased cellularity of connective tissue and widening of submucosa; GC, number of goblet cells; SNV, supranuclear vacuolisation in the absorptive cells (enterocytes) of the intestine epithelium.

of cellulose and hemicellulose. High fibre content may interfere with the capacity of digestive enzymes to attach to its substrates, thus reducing the ADC of dietary nutrients[54] or increasing endogenous losses[55,56]. Dietary fibre, specifically NSP, may interfere with feed utilisation by modulating digesta viscosity, gut emptying time and chime passage rate, changes in gut morphology, digestive physiology and microbiota[57].

Although high dietary fibre and nitrogen-free extract content may lead to decreased protein digestibility[58], in the present study, increased dietary DDGS levels, and a concomitant increase of dietary NSP, did not affect protein digestibility. Similarly, Magalhães *et al.*[11] also did not find differences in protein digestibility in meagre (*Argyrosomus regius*) and seabass (*Dicentrarchus labrax*) fed diets with DDGS. The ADC of lipids increased with the dietary inclusion of DDGS, though this effect should be related to the increased dietary lipid levels in the diets with DDGS and not to the dietary DDGS inclusion. Indeed, other studies reported lower ADC of lipids in rainbow trout[8], sunshine bass[59], meagre and European seabass[11] fed diets with DDGS. Increased ADC of lipids with the increase of dietary lipid content was also previously reported for rainbow trout[60], red tilapia[61], sea bass[62] and rockfish, *Sebastes schlegeli*[63].

Intestinal pH

Intestinal pH values of pacu ranged from 6·7 to 7·4, which is a smaller range than values (6·5–8·2) found in a variety of fish species[19]. Though intestinal pH is expected to increase as it distances from the stomach, in this study, intestinal pH was similar between anterior and mid intestine portions and higher only in the distal intestine. Similarly, Hlophe *et al.*[64] also did not find major differences in pH of different intestinal portions in other omnivorous species, namely *Tilapia rendalli*, *Oreochromis mossambicus* and *Clarias gariepinus*, though pH values were slightly higher in the distal part of the intestine.

Diet composition may affect intestine pH, as dietary components may have distinct buffering capacities[65,66]. In this study, increasing dietary DDGS levels led to significant increase in pH in the mid and distal intestine portions. This may be related to the increased dietary fibre as it is known that dietary fibre content and source, as well as potential interactions between fibre and minerals, may affect intestinal pH[67]. To our knowledge, this is the first study in fish evaluating the effect of DDGS in an omnivorous intestinal pH but, similar to the present study, an alkalisation on anterior and mid intestine of turbot[68] fed DDGS diets replacing fishmeal, and on caecum and apex in pigs fed diets with 300 g/kg DDGS[69], was observed. Contrarily, Youssef *et al.*[70] observed that increasing DDGS levels up to 150 g/kg in broilers' diets provoked acidification of duodenum and ileum and the same was true for swine fed a diet containing 300 g/kg of maize DDGS compared with a diet with 300 g/kg of wheat middling[71]. Differences in amino acid composition and digestibility capacity over the intestine may interfere with intestine pH[72].

Digestive enzyme activity

Overall, digestive enzyme activities decreased along the intestinal tract. This was expected, as digestive enzymes measured are produced by the exogenous pancreas and are therefore released in the first portion of the fish intestine. Thus, higher enzyme activities are expected to be found in the anterior intestine, as previously reported in different species, independent of food habits, as for the omnivorous Nile tilapia, *O. niloticus* L.,[64,73] and the carnivorous African catfish, *C. gariepinus*[64]. Similarly, in rainbow trout fed either a control diet or a high protein DDGS diet, trypsin activity was much higher in the proximal than in the distal intestine[74]. However, no difference of total protease activity was observed between intestine portions for all treatments, unless for fish fed the 300 DDGS diet that had

higher total protease activity in the anterior than the mid intestine.

The negative effect of DDGS on the activity of digestive enzymes might be related to the rich content in NSP. The increase on digesta viscosity and water content in the gut, as a result of the increase in soluble NPS intake, will reduce digestive enzyme activities by reducing their contact with the substrate and/or impairing the transport of nutrients and digestive secretions, thus reducing diet digestibility[75], as observed. The increased water content in the gut also promoted to less extent by insoluble NSP can increase chime DM and enhance the passage of the digesta along the intestine, reducing once more the efficacy of digestive enzymes[76]. The negative effect of NSP on the digestive process and the digestibility of dietary ingredients has been reported for a variety of fish species such as Atlantic salmon, *Salmo salar*[77], catfish[78], Nile tilapia[79,80] and rainbow trout, *Oncorhynchus mykiss*[81].

Unlikely amylase and lipase, total protease activity does not seem to be very dependent on fish nutritional habits[82]. Accordingly, in this study, as well as in the study of Magalhães *et al*.[11], total protease activity was not affected by dietary DDGS level. This is contrary to lipase, amylase, chymotrypsin and trypsin activities, which decreased as dietary DDGS level increased. Even though trypsin and chymotrypsin activities are accounted for the total protease activity, it also includes other alkaline proteases activities, as carboxypeptidases and di- and tri-peptidases. Moreover, some NSP components may impact negatively in the activity of some proteolytic enzymes, while other enzymes may have its activity increased and consequent effect being observed on protein digestibility[81], as it seems to have occurred in the present study. Lack of dietary DDGS effect on trypsin, amylase and lipase activities was however reported for olive flounder juveniles (*P. olivaceus*) fed rice-dried distillers grain (DDG)[6], suggesting that the effect of dried distillers grain on the activity of digestive enzymes may be dependent on ingredient source or processing technology. Accordingly, Magalhães *et al*.[11] observed both in European sea bass and meagre that, comparatively to the control fish-based diet, one of the maize DDGS products tested promoted digestive enzyme activity, while the other DDGS product did not affect digestive enzyme activity.

Due to the differences in lipid digestibility of the experimental diets, it was expected a higher lipase activity in the intestine of fish fed diets with increasing DDGS levels. However, the opposite was observed. When detailing diet lipid composition and digestibility, it is possible to observe that the increase in lipids digestibility was far below the increase in dietary lipids due to dietary DDGS inclusion. While dietary lipids increase on over 50 % from diet control to 400 DDGS, lipids digestibility increased <7 %. Thus, the increase in apparent lipid digestibility seems to reflect the increase in dietary lipid content.

Oxidative status

Up to now, studies of dietary DDGS effects on intestine oxidative status in fish were lacking. In broilers fed diets with 150 g/kg DDGS, total antioxidant activity and SOD activity in plasma and liver appeared to decrease[83]. On the contrary, Hanson

et al.[84] did not find an effect of DDGS-based diets on the oxidative status of nursery pigs.

In the present study, among the antioxidant defence, enzymes assayed only CAT activity was induced in the intestine of fish fed DDGS diets, indicating that it was the preferred enzymatic pathway for eliminating the hydrogen peroxide produced. The metabolism pathway of SOD involves the supply of substrate for CAT (H_2O_2), and usually, those enzymes have associated activity. That was not observed in the present study as by other authors[85,86] and might be related to changes on SOD isoforms. Besides the two forms present in eukaryotes organisms, Mn SOD and Cu/Zn SOD, there is evidence that isoforms can be expressed by the enzyme reaction with other components like malondialdehydes, amino acids[87], proteins and carbohydrates[86]. The patterns and specific activity might vary depending on the isoform[88,89].

Another enzyme that is related to oxidative status in animal organisms is G6PDH that converts glucose-6-phosphate into 6-phosphogluconate generating NADPH. NADPH supports the action of the enzyme GR that restores the levels of reduced glutathione in the organism[90]. However, in the present study, reduction of G6PDH does not seem to be related to antioxidant defence but with lipid content in diets. G6PDH is one of the major rate-limiting enzymes from pentose phosphate pathway and has high relation with lipogenesis, since its product, NADPH, is very important in biosynthesis of fatty acids and cholesterol. Once lipid contents were increasing and starch decreases in experimental diets as DDGS was being included, lipogenesis was reduced due to high lipids availability, which was represented by reductions on G6PDH activity.

Nonetheless, LPO in the intestine of pacu fed DDGS diets was significantly reduced, and this seems to indicate the potential of DDGS. This may be related to the NSP and the yeast cell components in DDGS, which may directly or indirectly affect oxidation by inducing changes in fish gut microbiota[80,91,92] and intestine ecosystem[57]. For instance, it was shown that low dietary inclusion of soluble NSP (guar gum) reduced gut oxidative stress in white sea bream[93]. Also, yeast is known to have distinct protective oxidative stress responses in fish species such as rainbow trout[94], gilthead sea bream[95] and European sea bass[96]. Not only whole yeast but also its cell wall components such as mannans and glucans have been shown to provide antioxidant benefits[97,98] and to modulate gut microbial communities[99,100] allowing changes in ROS production in the organism. DDGS components have been studied as prebiotic for several production animals[101,102]. Nevertheless, the reduction of soyabean meal in the diets cannot be completely discarded as having also contributed to the reduction of oxidative stress in the pacu intestine. Indeed, anti-nutritional factors present in soyabean were shown to be responsible for promoting the increase of free radicals, and an imbalance between ROS production and removal in mice fed raw soyabean-based diets by promoting inflammatory process[103]. Inflammation is always accompanied by lipid oxidation, due to excessive ROS production.

Due to the addition of sulphuric acid during ethanol production, the sulphur content in DDGS can be higher than that in common ingredients used in aquafeeds[104]. Sulphur and some sulphur-containing compounds have been reported to have

antioxidant proprieties[32,105–107]. Song et al.[32] feeding nursery pig with an oxidised DDGS diet (300 g/kg) suggested that the high sulphur content of maize DDGS, as well as the high serum sulphur-containing antioxidants observed in animal fed DDGS, may be enough to prevent metabolic oxidative stress. Thus, the sulphur content of DDGS may have also contributed to the reduction of oxidative stress in pacu intestine.

Intestine morphology

The replacement of soyabean meal by maize DDGS improved pacu intestinal morphology. It is known that soyabean may induce inflammation in the distal portion of the fish intestine, particularly in salmonids[35–37]. This soyabean-induced enteritis is associated with anti-nutritional factors present in soyabean, such as lectins and saponins, which were shown to induce histological alterations in gastrointestinal tract cells affecting mucosal cell membrane permeability[48,108]. Though salmonids are carnivorous and eventually more prone to have intestinal problems when dealing with plant feedstuff-based diets[109], enteritis was also reported in omnivorous fish, such as common carp[110] or zebrafish[111]. On the contrary, carnivorous species such as the European sea bass or gilthead seabream seem to be more tolerant of soyabean anti-nutritional factors[112,113]. Nevertheless, although no overt inflammation was noticed, when fed diets containing soyabean meal gilthead seabream and European sea bass showed some alterations in the distal intestine morphology[114,115].

Instead, the accumulation of vacuoles in the intestine of fish fed lower or none DDGS diets seems to be related to lipid accumulation and/or inhibition of re-esterification of fatty acid, common in plant ingredients[116], it was noticed an excess of lipid drops and clear irregularity on the size of the vacuole, which can modify the absorptive capacity of the enterocyte in the brush border[117]. Reduction in GC promoted by DDGS inclusion and soyabean meal replacement is also evidence of a reduction in intestine damage since GC are involved in the synthesis of mucus as lubricants against mucosa degradation[118].

Adding to that, yeast cells have been shown to improve gut morphology of some fish species and this may also contribute to explain the improved gut histology of pacu fed the DDGS diets. For instance, in common carp, Omar et al.[119] observed increased GC number in the posterior gut of fish fed increasing levels of yeast protein concentrate, and Huang et al.[120] observed increased microvillus length, which suggests higher nutrient absorption capability. In Nile tilapia, live yeast (but not inactivated yeast) also improved gut microvilli length and density[121]. The beneficial effects of yeast on gut morphology may be obtained not only with live yeast cells but also with yeast polysaccharides, which may function as prebiotics[122]. Further, the reduction of oxidative stress of pacu intestines fed DDGS diets can also contribute to explain the improvement of the intestinal histological morphology, as ROS imbalance can cause cell structures damages[123–125].

In summary, maize DDGS inclusion in diets for pacu juveniles reduced DM, energy and lipids digestibility, and the intestinal amylase, lipase trypsin and chymotrypsin activities. However, the lack of differences in protein digestibility and total proteases activity, together with the improvements of intestine morphology and oxidative status, may contribute to explain the improvements on feed conversion ratio and protein efficiency ratio reported by Oliveira et al.[51] in pacu fed the same diets. Thereafter, the negative effects induced by soyabean meal were decreased by dietary replacement with maize DDGS, which may have a prebiotic effect, contributing to the improvement of the intestine health. Further studies evaluating maize DDGS for fish should include analysis of microbiota, to clarify DDGS effects in nutrient metabolism and immunity.

Acknowledgements

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