Received: 2 October 2018 | Revised: 30 April 2019 | Accepted: 13 May 2019

DOI: 10.1111/anu.12949

ORIGINAL ARTICLE

Aquaculture Nutrition WILEY

The use of bromelain as a feed additive in fish diets: Growth performance, intestinal morphology, digestive enzyme and immune response of juvenile Sterlet (*Acipenser ruthenus*)

G. Wiszniewski[1] | Sylwia Jarmołowicz[1] | Mohamed S. Hassaan[2] | Eman Y. Mohammady[2] | Mohamed R. Soaudy[2] | Joanna Łuczyńska[3] | Elzbieta Tońska[3] | Elzbieta Terech-Majewska[4] | Teresa Ostaszewska[5] | Maciej Kamaszewski[5] | Marek Skrobisz[5] | Antoni Adamski[5] | Patrycja Schulz[6] | Edyta Kaczorek[6] | Andrzej Siwicki[6,7]

[1]Department of Ichthyology, Hydrobiology, and Aquatic Ecology, Stanislaw Sakowicz Inland Fisheries Institute, Olsztyn, Poland

[2]Aquaculture Division, Fish Nutrition Research Laboratory, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt

[3]Chair of Commodity and Food Analysis, University of Warmia and Mazury, Olsztyn, Poland

[4]Department of Epizootiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland

[5]Department of Ichthyobiology and Fisheries, Faculty of Animal Science, Warsaw University of Life Sciences, Warsaw, Poland

[6]Department of Microbiology and Clinical Immunology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland

[7]Department of Fish Pathology and Immunology, Stanislaw Sakowicz Inland Fisheries Institute, Olsztyn, Poland

Correspondence
Mohamed S. Hassaan, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt.
Email: ms.hassaan@niof.sci.eg

Abstract

Bromelain is a proteolytic enzyme extracted from *Ananas comosus* and has great potential to affect several physiological functions. The current study examined the effect of bromelain added to commercial feed at concentrations of 0 g (control), 10 g (B1) and 20 g (B2) per kg diet on growth, feed utilization, intestinal morphology, digestive enzyme and immune response of juvenile Sterlet (*Acipenser ruthenus*) for 56 days. At the end of this experiment, the highest final body weight was detected in fish fed diet of B2 compared to control. The protein content of whole fish was higher in fish fed diet of B1 and B2, but the content of calcium, iron, copper and zinc was noted lower in fillets of fish fed diet of B1 and B2. Supplementation with bromelain significantly increased the height of mucosal folds, enterocytes and the supranuclear zone of the epithelial cells compared to control diet. The enzymatic activity of lipase and pepsin was significantly ($p < 0.05$) higher in fish fed diet of B1 and B2. The highest activity of lysozyme, total protein level and total immunoglobulin and the proliferative activity of T and B cells were detected in fish fed diet of B2 compared to control, where no significant ($p > 0.05$) difference was found in ceruloplasmin, metabolic activity of spleen macrophages and potential killing activity of spleen phagocytes between different treatments.

KEYWORDS

Acipenser ruthenus, bromelain, enzymatic activity, growth, immunological response

1 | INTRODUCTION

Improving the bioeconomic efficiency of aquaculture industry is dependent on advances in biology, nutrition and environmental management of the production cycle (Hassaan et al., 2019; Hassaan Mahmoud et al., 2018; Hassaan & Soltan, 2016). Antibiotics and other veterinary drugs are administered regularly as additives, therapeutics or growth promoters and immunostimulant in fish feed (Rico et al., 2013). Recently, the use of veterinary drugs is becoming more restricted since they present several side-effects for the environment

 © 2019 John Wiley & Sons Ltd

and health safety (Hassaan, Soltan, & Ghonemy, 2014; Reverter, Bontemps, Lecchini, Banaigs, & Sasal, 2014). Additionally, drug resistance development is associated with the excessive use of antibiotics that leads to decrease in therapeutic potential over time (Anderson, 1992). Therefore, preventing disease should be based on limiting the possibility of infection and stimulating the non-specific and humoral and cell-mediated immunity of fish (Kolman, Kolman, & Siwicki, 1998). Currently, there is an increase in using bioactive materials as growth promoters in fish feed, especially after the ban of antibiotic feed additives within the European Union countries in 2006 and discussion to restrict their use outside Europe (Christaki, Bonos, Giannenas, & Florou-Paneri, 2012; Hassaan et al., 2018). The criteria for choosing a given supplement are obviously safety and high active compound content. In this regard, the medicinal properties of bromelain have been recognized since the 1970 and are worthy of consideration.

Bromelain is a raw aqueous extract of pineapple stems and fruit (*Ananas comosus*) (Smith-Marshall & Golden, 2012). This aqueous extract contains thiol endopeptidase and non-protein compounds such as carbohydrates, cellulase, glycosidase, phosphatase glycoproteins, peroxidase and protease (Arshad et al., 2014). With respect to the activity and stability of natural bromelain, Hale, Greer, Trinh, and James, (2005) reported that the proteolytic activity of concentrated bromelain solutions remains relatively stable for at least 1 week at room temperature as well as the concentrated bromelain solutions are more resistant to spontaneous inactivation of their proteolytic activity than dilute solutions in feed. Also, Rathnavelu, Alitheen, Sohila, Kanagesan, and Ramesh, (2016) show that bromelain remains biologically active with a half-life of 6–9 hr in human body. The enzymatic activity of bromelain covers a wide pH spectrum from 5.5 to 8.0 (Pavan, Jain, Shraddha, & Kumar, 2012), while the optimum range is 6.0–7.0 at a temperature range of 50–60˚C (Manzoor, Nawaz, Mukhtar, & Haq, 2016). There are many studies were carried out to investigate the separation, extraction and purification of bromelain from pineapple residues (Coêlho, Silva, Machado, Silveira, & Tambourgi, 2015; Ketnawa, Chaiwut, & Rawdkuen, 2012; Martins et al., 2014; Novaes, Ebinuma, Mazzola, & Júnior, 2013). Bromelain has a great potential to affect several physiological functions. Primarily, as an enzyme, it facilitates protein digestion by partially hydrolysing molecules into smaller peptides and increasing their availability in food (Fennema, 1996). When formulating fish feeds with high contents of plant-based ingredients, the addition of the enzyme can significantly improve fish plant protein utilization (Liebert & Portz, 2005; Singh et al., 2011). Furthermore, bromelain is considering a good alternative to microbial proteases like subtilisins from *Bacillus licheniformis* and *B. amyloliquifaceins* that are enzymes of choice for detergents (Van Beckhoven, Zenting, Maurer, Van Solingen, & Weiss, 1995). Bromelain also has immunomodulatory affects which activates the natural killer cells and modulates the immune response of T and B cells in the blood (Engwerda, Andrew, Ladhams, & Mynott, 2001). Bromelain prevents excessive platelet adhesion that reduces the risk of thrombosis (Orsini, 2006; Padma, Jayakumar, Mathai, Chintu, & Sarath, 2012). Additionally, it is anti-oedematous and anti-carcinogenic, and it has anti-inflammatory and antibiotic properties (Reddy, Grossman, & Rogers, 2013). Bromelain is also used in the food industry, for example, in meat processing and in brewing (Maurer, 2001; Soares, Vaz, Correia, Pessoa, & Carneiro-da-Cunha, 2012), and in the textile and cosmetic industries (Babu, Rastogi, & Raghavarao, 2008; Ketnawa, Sai-Ut, Theppakorn, Chaiwut, & Rawdkuen, 2009; Lima, Simões, Vieira, Silva, & Ruzene, 2018). However, the effect of bromelain particularly on the physiological status of aquatic animal has not been studied yet. Therefore, the current study was conducted to evaluate the effect of bromelain supplemented with 10 and 20 g/kg diet on fish growth rate, body chemical composition and mineral contents, liver and gastrointestinal tract histology, the activity of selected digestive enzymes, and the most important parameters of non-specific (humoral and cell-mediated) immunity on juvenile Sterlet (*Acipenser ruthenus*). Today, the sturgeon is recognized as one of the world's most precious commercial fish, mainly prized for its caviar, but increasingly also for its meat and as ornamental fish. Many sturgeon species are threatened with extinction. Aquaculture, including growing, nursing and reproduction, offers the solution for sustainable sturgeon production. Furthermore, sturgeon culture is also considered as business commodity with great economic potential.

2 | MATERIALS AND METHODS

2.1 | Fish and rearing conditions

The experiment was conducted at the Department of Ichthyology, Hydrobiology, and Aquatic Ecology, Inland Fisheries Institute (IFI) in Olsztyn. A total of 180 Sterlet with an average initial body weight of 56 ± 3.59 g were selected and acclimatized to the experimental tanks for 15 days before the start of the feeding experiment. During this period, fish were fed a commercial diet (540 g/kg crude protein, 22.6 MJ/kg gross energy). At the start of the experiment, fish were weighed, and 20 fish were randomly stocked in each of nine tanks (280 dm^{-3} each tank) connected in recirculating aquaculture system (RAS). Water quality parameters during this study were (means ± standard deviation) as follows: temperature, 20 ± 0.2°C; dissolved oxygen, 6.15 ± 0.59 mg/L; total ammonia nitrogen (TAN = NH_4^+-N + NH_3-N) and nitrate (NO_2-N), 0.163 ± 0.098 mg/L and 0.014 ± 0.004 NO_2-N mg/L, respectively; pH, 7.4–7.6. The photoperiod applied was LD 12:12. Light intensity measured at the surface of the rearing tanks was 50–60 lx. The length of the experiment was 56 days.

2.2 | Experimental design and diets

Fish were fed with three types of diets for 56 days: diet 1 (C) was a commercial diet for this species (control diet) (Nutra T-2.0, Skretting, France, 54% protein and 18% lipids), whereas diet 2 (B1) and diet 3 (B2) were supplemented with (Sigma-Aldrich; 900 U/g) 10 g and 20 g of kg^{-1} of bromelain, respectively. To prepare the experimental diets, the commercial diet was milled, and then, the bromelain levels were added. The quantity of the used enzyme was mixed with water (30 ml of distilled water; 28°C for 500 g feed)

and added to the diet and mixed until a homogeneous mass was obtained. After homogenized, the diets re-pelletized in a pellet mill using an AGA Labor vacuum device (Lublin, Poland) with a 3 mm diameter which was appropriate for the size of the fish used in the experiment. Diets were dried at room temperature, and then packed in cellophane bags and stored at 4°C until use. The proximate composition of the experimental diets was analysed according to the procedures described by AOAC methods (1995) (Table 1). Fish were fed 12 times per day using an automatic feeder at a feeding rate of 1.5% of biomass.

2.3 | Bromelain activity in the formulated feed

The activity of bromelain enzyme was estimated according to Hassaan et al. (2019). In brief, 2 g of diets (with or without bromelain supplementation) was mixed with 1 g of fish meal. Each group after mixed was incubated with buffer solution ($Na_2B_4O_7$ $(H_2O)_{10}$-H_2BO_3, pH 8.5) containing penicillin and streptomycin (200 U/ml) for 2 hr at a temperature of 35°C. Total free essential amino acid was analysed by comparing with the ammonium sulphate (the standard solution) standard curve using a spectrophotometer at OD 570 nm. The amount of free essential amino acid hydrolysed by the bromelain in the diets (with or without bromelain supplementation) and occurred naturally in fishmeal was compared. The difference in free amino acid content between diet with and without bromelain supplementation is shown in Table 2. Relative activity of bromelain % = (free amino acid in with bromelain − free of amino acid without bromelain) ×100.

2.4 | Effect of bromelain supplementation on the physical qualities of feed

The diets after re-pelleted with or without bromelain were tested for water stability according to Baeverfjord, Refstie, Krogedal, and Åsgård, (2006) (Table 3). Also, pellet durability after supplementation of bromelain was evaluated according to the procedure of Ayoola (2016) (Table 3).

TABLE 1 Proximate composition (g/kg of dry weight) of experimental diets contains 10 g (B1) and 20 g (B2) per kg of bromelain

	Dietary treatments		
	Control	B1 (9,000 U/kg)	B2 (18,000 U/kg)
Crude protein	540.0	540.0	540.0
Crude lipid	180.0	180.0	180.0
Crude fibre	10.0	10.0	10.0
Crude ash	115.0	115.0	115.0
NFE[a]	155.0	155.0	155.0
Gross energy[b] (MJ/kg)	22.6	22.6	22.6
Bromelain	0	10	20

[a]Nitrogen-free extracts = 100 − (crude protein + crude lipid + crude fibre + crude ash) (Shearer, 1994).
[b]Gross energy calculated from the chemical composition using the following energy conversion factors: 24 kJ/g proteins, 39 kJ/g lipids and 17 kJ/g NFE (Jobling, 1994).

2.5 | Growth performance and feed utilization

Initial body weight (g), initial body length (cm), initial body weight (g) and final body weight (g) of individual fish were recorded for all fish/each tank at the initiation and the termination of the experiment. Monitoring measurements of mean weight were done every seven days to determine the feed ration for each tank. At the end of the experiment, five fish from each replicate were randomly collected and the viscera and liver were weighed to determine the values of the HSI and VSI indexes. Growth performance and feed utilization parameters were calculated with the following formulas:

DGR (daily growth rate, g/day) = $(W_f - W_i) \times T^{-1}$;
SGR (specific growth rate, % per day) = $100 \times [(\ln W_f - \ln W_i) \times T^{-1}]$;
ICF (initial condition factor) = $(W_i \times 100) \times TL^{-3}$;
FCF (final condition factor) = $(W_f \times 100) \times TL^{-3}$;
FCR (feed conversion ratio) = $TFI \times (W_f - W_i)^{-1}$;
PER (protein efficiency ratio) = $(W_f - W_i) \times TFP^{-1}$.
HSI (hepatosomatic index, %) = $100 \times (LW \times W^{-1})$
VSI (viscerosomatic index, %) = $100 \times (VW \times W^{-1})$

where W_i = initial mean body weight (g), W_f = final mean body weight (g); T = rearing time (days). W = body weight (g); TL = total length (cm); FB = final stock biomass (g); IB = initial stock biomass (g); TFI = total feed intake (g); TFP = total feed protein (g); LW = liver weight (g); VW = viscera weight (g).

2.6 | Chemical composition and mineral composition in fish muscles

At termination of the trial, a random sample of five individual of whole fish and fillet was sampled from each tank, oven-dried at 105°C for 24 hr, ground and stored at −20°C for subsequent analysis. Proximate analysis was conducted on both diet and fish samples. Dry matter, total lipids, crude protein and ash contents were all determined with standard methods AOAC (1995). Dry matter was determined after drying the samples in an oven (105°C) for 24 hr. Ash was determined by incineration at 550°C for 12 hr, according to method number 942.05. Crude protein was determined with the micro-Kjeldahl method, $N \times 6.25$ (using a Kjeltech 1030 auto-analyser, Tecator, Höganäs, Sweden) according to method number 984.13, and crude fat was determined by Soxhlet extraction with diethyl ether (40–60°C), according to method number 920.39 using an E-816HE automatic extractor. Fibre content of the experimental diets was determined according to the method of Van Soest, Robertson, and Lewis (1991) using Tecator Fibertec System M 1020. Nitrogen-free extract (NFE) was computed by taking the sum of

TABLE 2 Relative bromelain activity % in the experimental diets based on free amino acid (FAA) hydrolysed mg/ml (Means ± *SD*; *n* = 4)

	Dietary treatments		
	Control	B1 (9,000 U/kg)	B2 (18,000 U/kg)
FAA without bromelain (mg/ml)	21.91	21.99	21.86
FAA With bromelain (mg/ml)	–	26.16	27.13
Difference of FAA (mg/ml)	–	4.17	5.27
Relative activity of bromelain[a] %	–	118.96	124.11

Abbreviation: FAA, free amino acid.
[a]Relative activity of bromelain % = (free amino acid in with bromelain − free of amino acid without bromelain) ×100

TABLE 3 Effect of bromelain blend as feed additive on water stability (%) and durability (%) of feed

	Dietary treatments		
	Control	B1 (9,000 U/kg)	B2 (18,000 U/kg)
Water stability (30 min)	91.05 ± 1.07[c]	93.7 ± 1.17[b]	95.95 ± 1.6[a]
Durability	86.16 ± 1.78[c]	88.98 ± 1.22[b]	90.13 ± 1.03[a]

Note: Means followed by different letters in the same row are significantly different ($p < 0.05$).

values for crude protein, crude lipid, crude fibre and ash and by subtracting this sum from 100.

The samples of fish muscles were dried to a constant weight at 105˚C and then ashed at 450°C for 12 hr. The white ash was dissolved in 1 M HNO_3 (Suprapur-Merck), and then, each sample was transferred quantitatively into 25-ml volumetric flasks with deionized water. The concentrations of six elements (Fe, Mn, Cu, Zn, Mg and Ca) were measured with flame atomic absorption spectrometry (Unicam Solar 939) and corrected with a deuterium lamp. The absorption wavelengths were as follows: 248.3 nm for iron; 213.9 nm for zinc; 324.8 nm for copper; 279.5 nm for manganese; 285.2 nm for magnesium; and 422.7 nm for calcium. A solution of lanthanum chloride was added to all samples when determining calcium to eliminate the influence of phosphorus (Whiteside & Miner, 1984). Sodium and potassium were assayed using flame photometry (Flapho 4, Carl Zeiss Jena) at 589.0 and 766.5 nm, respectively (Rutkowska, 1981). Phosphorus was determined using the colorimetric method of Mattsson and Swartling (1954). The absorbance of phosphorus was determined at 610 nm (VIS 6000 Spectrophotometer).

2.7 | Histological analysis

On the last day of the experiment, the liver and mid-section of the gastrointestinal tract were collected from five fish from each tank and were analysed histologically (Alvarez-González et al., 2008; Zawistowski, 1986). The tissues were fixed in Bouin solution, dehydrated with ethanol, cleared with xylene, embedded in paraffin blocks and then sectioned with a microtome (Leica, Bensheim, Germany) into 5-μm sections. The sections were stained in haematoxylin and eosin (H&E) and then analysed under a light microscope (Olympus Cx31, Japan). MultiScanBase (Computer Scanning System Ltd., Warsaw, Poland) was used to examine and take structural measurements on each specimen as follows: liver—hepatocyte size and that of its nucleus (based on which the nuclear/cytoplasmic index was calculated) and intestines—muscularis thickness, mucosal fold height, enterocyte height, supranuclear zone height and enterocyte nucleus size (μm). Histological measurements were taken of 50 cells and of the nuclei of the tissues analysed and collected from each specimen.

2.8 | Enzyme activity

The intestine and stomach were collected from five specimens from each experimental group, frozen in liquid nitrogen and stored at −80°C until use. The material for analysing enzyme activity and the protein content of the sample was homogenized in buffers according to the procedures described below. The samples were centrifuged at a temperature of 4°C for 15 min (15,000 *g*). All enzyme activity analyses were performed in three replicates. Measurements of absorption were performed with a Camspec M501 spectrophotometer (Camspec Ltd.). Alkaline phosphatase (ALP) activity was determined with the method by Wenger, Kaplan, Rubaltelli, and Hammerman (1984). Acid phosphatase (AcP) activity was determined with the modified Hillmann method (Abbott, 1984). Leucine aminopeptidase (LAP) activity was determined with the method by Nagel, Willig, and Schmidt (1964). Amylase activity was determined with the Foo and Bais (1998) method. Lipase activity was determined with the method by Winkler and Stuckmann (1979). Trypsin activity was determined with the method by Erlanger, Kokowsky, and Cohen (1961). Pepsin activity was determined with the Anson (1938) assay using haemoglobin as the substrate. The activity of each enzyme was analysed in five replicates (at 25°C) and calculated for 1 mg of protein of the enzymatic extract (μmol of product

per 1 min). The total protein content in the sample was determined with the method by Lowry, Rosebrough, Farr, and Randall (1951).

2.9 | Immunological assays

2.9.1 | Non-specific humoral immunity

At the end of the experiment, blood was drawn from the caudal vein of five fish from each treatment and control group. After centrifuging (5,000 *g*, 10 min), selected humoral immunity parameters of the blood plasma were determined. The total protein level was determined with the spectrophotometric method described by Anderson and Siwicki (1994), and the level of total immunoglobulin (Ig) was determined according to the method described by Siwicki and Anderson (1993). Lysozyme activity was determined with the turbidimetric method (Parry, Chandan, & Shahani, 1965) modified by Siwicki and Anderson (1993). The content of ceruloplasmin (Cp; IU) was determined spectrophotometrically (Rice, Wagman, & Takenaka, 1963).

2.9.2 | Non-specific cellular immunity

The same fish of blood sampled were dissected to collect the spleen tissue for using to estimate the immunocompetent cells. Spleen leucocytes were isolated by centrifugation (2,000 *g*, 30 min) at a temperature of 4˚C in the lymphocyte separation mediums of Gradisol G and Gradisol L (Polfa), rinsed three times in PBS and then placed again in RPMI 1640 (Sigma) medium supplemented with 10% FCS (foetal calf serum, Gibco-BRL) at a concentration of 2×10^5 cells per ml. Supravital staining with 0.1% trypan blue was used to check cell viability. Two hundred cells were counted. Samples with at least 90% of living cells were used in the analysis. Macrophage metabolic activity was determined with the spectrophotometric method after the cells had been stimulated with phorbol myristate acetate (PMA). The macrophages were isolated from the spleens by centrifuging the cells in Gradisol G (Polfa) medium. The potential killing activity (PKA) of the phagocytes was determined with the spectrophotometric method after the cells were stimulated with *Aeromonas hydrophila* according to the method described in Siwicki and Anderson (1993). The proliferative activity of lymphocytes was determined based on the proliferative response of T cells stimulated with concanavalin A (ConA, Sigma) and B cells stimulated with lipopolysaccharide (LPS) with the MTT test described by Siwicki and Anderson (1993).

2.10 | Statistical analysis

Data were analysed statistically with ANOVA using the GraphPad Prism (Soft. Inc.). The data were submitted to one-way classification variance analysis. When differences were statistically significant ($p \leq 0.05$), Tukey's post hoc test was applied.

3 | RESULTS

3.1 | Relative rate of exogenous protease activity

The relative activity of bromelain in formulated diets after supplemented with bromelain B1 and B2 was 118.96% and 124.11%, respectively (Table 2).

TABLE 4 Rearing parameters of Sterlet fed experimental diets for 56 days (mean ± *SD*)

	Dietary treatments		
	Control	B1 (9,000 U/kg)	B2 (18,000 U/kg)
Initial total length (cm/fish)	26.28 ± 0.56	26.13 ± 0.23	26.21 ± 0.78
Final total length (cm/fish)	33.05 ± 0.11^b	33.63 ± 0.06^a	33.87 ± 0.64^a
Initial body weight (g/fish)	56.04 ± 3.90	54.01 ± 3.09	56.29 ± 3.72
Final body weight (g/fish)	147.27 ± 10.5^c	156.23 ± 1.23^b	162.19 ± 5.75^a
Daily growth rate (DGR; g/day)	1.27 ± 0.18^c	1.40 ± 0.06^b	1.45 ± 0.12^a
Specific growth rate (SGR; % per day)	1.34 ± 0.16^c	1.37 ± 0.22^b	1.45 ± 0.13^a
Initial condition factor	0.31 ± 0.01	0.30 ± 0.01	0.31 ± 0.01
Final condition factor	0.41 ± 0.03	0.41 ± 0.01	0.42 ± 0.03
Feed conversion ratio (FCR)	1.12 ± 0.05^a	1.04 ± 0.06^b	1.04 ± 0.09^b
Protein efficiency ratio (PER)	1.65 ± 0.08	1.79 ± 0.11	1.79 ± 0.17
Viscerosomatic index VSI (%)	3.72 ± 0.47^b	4.60 ± 0.73^a	4.50 ± 0.57^a
Hepatosomatic index HSI (%)	1.05 ± 0.36^b	1.30 ± 0.42^a	1.40 ± 0.53^a

Note: Means followed by different letters in the same row are significantly different ($p < 0.05$).

TABLE 5 Proximate and mineral composition of Sterlet fed experimental diets (mean ± *SD*)

	Dietary treatments		
	Control	B1 (9,000 U/kg)	B2 (18,000 U/kg)
Proximate of whole fish composition (g/kg ww)			
Protein	166.1 ± 10.2[a]	146.7 ± 13.6[b]	137.8 ± 15.6[b]
Lipid	96.8 ± 8.1	94.3 ± 33.0	91.5 ± 8.8
Moisture	693.8 ± 22.4	712.4 ± 25.4	710.7 ± 16.4
Proximate of fillet composition (g/kg ww)			
Protein	194.5 ± 5.4[a]	184.1 ± 20.0[ab]	166.2 ± 16.0[b]
Lipid	32.0 ± 1.8	31.0 ± 11.6	28.6 ± 7.4
Moisture	751.3 ± 25.7[b]	762.4 ± 3.4[b]	778.3 ± 3.4[a]
Mineral of fillet composition (mg/kg ww)			
Potassium	319.33 ± 296.3	341.96 ± 29.4	343.03 ± 144.9
Calcium	314.6 ± 51.6[a]	177.6 ± 36.7[b]	173.2 ± 37.0[b]
Magnesium	206.7 ± 14.2[a]	198.7 ± 12.8[b]	177.9 ± 2.6[c]
Sodium	685.5 ± 94.8[a]	554.2 ± 51.9[b]	671.7 ± 58.2[a]
Phosphorus	193.37 ± 75.5[b]	231.00 ± 488.8[a]	223.63 ± 152.0[a]
Manganese	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
Iron	2.50 ± 0.49[a]	1.77 ± 0.50[b]	1.84 ± 0.13[b]
Copper	0.47 ± 0.03[a]	0.33 ± 0.06[b]	0.36 ± 0.05[b]
Zinc	6.18 ± 0.64[a]	4.66 ± 0.18[b]	4.70 ± 0.50[b]

Note: Means followed by different letters in the same row are significantly different ($p < 0.05$).

3.2 | Effect of bromelain supplementation on the physical qualities of feed

The data for the effect of bromelain addition on feed water stability and durability are presented in Table 3. Feed water stability and durability were significantly ($p < 0.05$) different among the treatment diets. Diet supplemented with B2 (20 g/kg bromelain) had the highest value, which were significantly different as compared to diet supplemented with B2 (10 g/kg bromelain) and control diet.

3.3 | Growth analysis and feed utilization

No fish mortalities were observed during the experiment in any of the dietary treatment groups. Growth performance and feed utilization parameters are significantly affected by supplementation of bromelain in diets. Final body weight (162.19 g) was significantly ($p < 0.05$) higher than the control group (147.27 g), and diets of B1 and B2 recorded the highest final body length (Table 4). Fish fed diet of B1 and B2 had the highest DGR and SGR ($p < 0.05$), while the FCR in groups B1 and B2 was significantly statistically lower ($p < 0.05$; Table 4). No significant ($p > 0.05$) differences were found in condition factor or PER between treatment groups (Table 2). The values of HSI and VSI were significantly ($p < 0.05$) higher in groups B1 and B2 (Table 4).

3.4 | Proximate composition

Table 5 shows the chemical composition of the whole body and fillet of fish. No significant ($p > 0.05$) differences were found in lipid content of whole fish and fillet as well as the moisture content of whole-body fish. Diet supplementing with bromelain had a significant ($p < 0.05$) impact on the protein content of whole fish and fillet. The protein content of whole fish was significantly ($p < 0.05$) lower in fish fed diet of B1 and B2 than control group. The level of protein and moisture was significantly ($p < 0.05$) lower in the fillets of starlet fed diet B2 at the end of the experiment. No significant ($p > 0.05$) differences were found in potassium and manganese content among experimental diets, whereas the content of calcium, magnesium, iron, copper and zinc was significantly ($p < 0.05$) lower in fish fed diet supplemented with 10 or 20 g/kg of bromelain. The contents of sodium and phosphorous in fish fed diet of B2 were significantly ($p < 0.05$) higher in comparison with the control diet.

3.5 | Histological analysis

No significant ($p > 0.05$) differences were found in the mean size of hepatocyte size of nuclei, hepatonuclei index and muscularis thickness of fish fed the dietary treatment (Table 6). The mucosal fold height and the supranuclear zone height of the epithelial cells were significantly ($p < 0.05$) higher in fish fed diet of B1 and B2 (Table 4), whereas the intestinal epithelial cell height was significantly ($p < 0.05$) higher in fish fed diet of B2 (44.54 μm) than the control group (35.42 μm) (Table 6). There was no significant ($p > 0.05$) difference in the size of enterocyte nuclei among treatment (Table 6). Macroscopic and microscopic analyses of the liver and mid-section of the intestines did not reveal any pathological

changes in the control group or in any of the fish fed feed with increasing doses of bromelain.

3.6 | Enzyme activity analysis

Alkaline phosphatase, leucine aminopeptidase, amylase and trypsin activities of the anterior segment of the gastrointestinal tract were significantly decreased in fish fed diet supplemented with 10 or 20 g/kg (Table 7). Only the activity of alkaline phosphatase was similar in all of the groups analysed ($p > 0.05$, Table 7), while the activity of lipase increased, and in group B1, it was significantly ($p < 0.05$) higher by 3.7 IU/g and in fish fed diet of B2 by 5.24 IU/g in comparison with the control group (Table 7). The activity of the digestive enzyme pepsin also increased with increasing doses of the feed supplement by 29.49 and 26.49 IU/g in comparison with the control group (Table 7).

3.7 | Immunological analysis

Immunological indices are presented in Table 8. Non-specific humoral and cell-mediated immunity were increased significantly ($p < 0.05$) in fish fed diet of B1 and B2, except ceruloplasmin. Lysozyme activity, total protein and immunoglobulin (Ig) were significantly higher by 3.66 mg/L, 4.46 and 2.82 g/L, respectively, in fish fed diet of B2 than the control group. The proliferative response of spleen T cells was higher in fish fed diet of B2 in comparison with group B1, and the spleen B cells were higher in comparison with the control group when stimulated with ConA and LPS, respectively. B-cell activity significantly ($p < 0.05$) increased in fish fed diet of B1 compared to the control group. Blood, ceruloplasmin, spleen macrophage metabolic activity and spleen phagocyte potential killing activity did not differ significantly ($p > 0.05$) in comparison with the control group.

4 | DISCUSSION

Bromelain, which is one of the cysteine proteases such as papain and ficain, belongs to the group of sulfhydryl protease enzymes that are used as phytochemical treatments (Leung-Toung, Li, Tam, & Kaarimian, 2002). The application of cysteine proteases in the animal feed industry can increase the digestibility, acceptable flavour and palatability ingredients (Grzonka, Kasprzykowski, & Wiczk, 2007; Hassaan et al., 2019). Bromelain is able to hydrolyse the feed proteins into smaller protein in peptides with higher digestibility (Manosroi, Chankhampan, Pattamapun, Manosroi, & Manosroi, 2014; Nilsang, Lertsiri, Suphantharika, & Assavanig, 2005; Sawant & Nagendran, 2014). To achieve retention of the physical integrity of feed, with minimal disintegration and nutrients leaching into water, is not easy (Abdollahi, Ravindran, & Svihus, 2013). The current study describes the effect of graded levels of dietary bromelain (1 or 2 g 100 g^{-1}) on the growth performance of and nutrient utilization in Sterlet (Table 4). Bromelain supplementation led to higher weight gain in Sterlet compared with the fish fed the control diet. The fish fed either 1 or 2 g 100 g^{-1} of bromelain attained significantly higher growth and had lower FCR values than did the fish fed the control diet. The best SGR and FCR were recorded in fish fed the diet supplemented with 20 g/kg of bromelain. The higher feed utilization in the present study could stem from the proteases in the bromelain enzyme derived from pineapple hydrolysing dietary proteins into smaller protein peptides with higher digestibility (Fennema, 1996; Nilsang et al., 2005). To the best of our knowledge, very few studies report on the positive effects of the exogenous dietary enzyme bromelain on growth performance and feed utilization in fish. The addition of a 1% or 2% mixture of bromelain and papain significantly increased the SGR, PER and apparent net protein utilization of grass carp and grey mullet (Choi, Lam, Mo, & Wong, 2015). In a study on shrimp, Divakaran and Velasco (1999) found that the apparent digestibility of crude protein was significantly higher (74.3%) in Pacific white shrimp, *Litopenaeus vannamei*, fed a 0.4% ENZECO® bromelain diet than those fed the control diet (65.3%). The commercial enzyme Ronozyme™ VP also improved the net protein utilization of tilapia fed a feed based on palm kernel meal (Boonyaratpalin, Promkunthong, & Hunter, 2000). To use pineapple wastes containing exogenous enzymes, Deka, Sahu, and Jain (2003) report that the SGR and PER of *Labeorohita* were significantly higher in the diet containing 25% pineapple waste than the controls with other fruit waste feeds such as orange and lime, and this application could be suitable for reducing production costs.

TABLE 6 Histological morphometrics of liver and gut samples of Sterlet fed experimental diets (mean ± *SD*)

	Dietary treatments		
Morphometric data	**Control**	**B1 (9,000 U/kg)**	**B2 (18,000 U/kg)**
Size of hepatocyte (μm)	15.43 ± 0.67	15.74 ± 0.49	15.23 ± 0.79
Size of nuclei (μm)	4.89 ± 0.16	4.92 ± 0.23	4.68 ± 0.41
Hepatonuclei index	0.31 ± 0.07	0.31 ± 0.03	0.30 ± 0.05
Muscularis thickness (μm)	165.03 ± 40.51	172.45 ± 42.34	174.63 ± 33.33
Height of mucosal fold (μm)	501.21 ± 42.56^{b}	595.82 ± 24.37^{a}	590.24 ± 48.51^{a}
Height of enterocytes (μm)	35.42 ± 5.21^{b}	37.56 ± 4.81^{b}	44.54 ± 3.41^{a}
Height of supranuclear zone (μm)	13.28 ± 1.23^{b}	14.87 ± 0.61^{a}	15.24 ± 1.16^{a}
Size of nuclei (μm)	5.01 ± 0.45	5.20 ± 0.45	4.99 ± 0.17

Note: Means followed by different letters in the same row are significantly different ($p < 0.05$).

TABLE 7 Digestive enzyme activity of Sterlet fed experimental diets (mean ± *SD*)

		Dietary treatment		
	Initial	Control	B1 (9,000 U/kg)	B2 (18,000 U/kg)
Alkaline phosphatase ALP[a] (IU/g)	52.20 ± 7.58[c]	72.72 ± 7.55[a]	63.54 ± 10.39[b]	51.42 ± 5.56[c]
Acid phosphatase AcP[a] (IU/g)	2.54 ± 0.30[b]	3.16 ± 0.64[a]	3.26 ± 0.54[a]	3.24 ± 0.69[a]
Leucine aminopeptidase LAP[a] (IU/g)	3.77 ± 0.92[a]	3.64 ± 0.40[a]	2.79 ± 0.35[b]	2.94 ± 0.39[b]
α-amylase[a] (IU/g)	44.37 ± 11.29[ab]	53.70 ± 10.81[a]	41.98 ± 4.01[ab]	34.00 ± 10.06[b]
Trypsin[a] (IU/g)	56.88 ± 15.34[b]	79.70 ± 2.72[a]	37.87 ± 7.33[c]	36.00 ± 12.58[c]
Lipase[a] (IU/g)	3.11 ± 0.53[c]	2.84 ± 0.79[c]	6.54 ± 2.11[b]	8.08 ± 2.46[a]
Pepsin[b] (IU/g)	6.80 ± 2.04[c]	35.45 ± 11.61[b]	64.94 ± 9.57[a]	61.94 ± 19.09[a]

Notes: Means followed by different letters in the same row are significantly different ($p < 0.05$).
[1]Enzyme activity in the anterior section of the gastrointestinal tract.
[2]Pepsin activity in stomach.

TABLE 8 Influence of experimental diet on the non-specific cellular and humoral defence mechanisms in Sterlet (mean ± *SD*)

	Dietary treatments		
	Control	B1 (9,000 U/kg)	B2 (18,000 U/kg)
Non-specific humoral immunity			
Lysozyme activity (mg/L)	8.78 ± 2.00[b]	8.39 ± 2.10[b]	12.44 ± 3.05[a]
Ceruloplasmin (IU)	54.51 ± 2.91	53.08 ± 2.75	57.17 ± 5.15
Total protein level (g/L)	29.21 ± 4.02[ab]	26.67 ± 2.94[b]	31.12 ± 3.54[a]
Total immunoglobulin (Ig) level (g/L)	9.91 ± 1.77[ab]	9.05 ± 1.90[b]	11.85 ± 2.47[a]
Non-specific cellular immunity			
Metabolic activity of spleen macrophages	0.93 ± 0.22	0.71 ± 0.25	0.92 ± 0.49
Potential killing activity of spleen phagocytes	0.71 ± 0.19	0.74 ± 0.17	0.97 ± 0.34
Proliferative response of lymphocytes T stimulated by mitogen concanavalin A (ConA) and stimulation index (IS)	0.12 ± 0.02[b] (IS 0.89)	0.12 ± 0.01[b] (IS 1.15)	0.14 ± 0.01[a] (IS 1.71)
Proliferative response of lymphocytes B stimulated by lipopolysaccharide (LPS) and stimulation index (IS)	0.10 ± 0.01[a] (IS 0.73)	0.08 ± 0.01[b] (IS 0.11)	0.08 ± 0.01[b] (IS 1.02)

Note: Means followed by different letters in the same row are significantly different ($p < 0.05$).

In the current study, the histological examination of hepatocytes and the sizes of their nuclei showed no significant changes in them in the fish fed diets supplemented with bromelain. This could indicate that dietary bromelain at concentrations of 10 or 20 g/kg improved fish health condition, and no hepatic tissue pathology was noted among treatments. Our histological evaluations of gut tissue also showed improvement whether the fish had received a supplement of 10 g bromelain per kg or 20 g bromelain 1 per kg (Table 6). All our results pertaining to health indicators were associated with growth performance. The improvement in intestinal morphology noted in this study could have been the result of complementary enhancement to meet the increased rate of digestion and assimilation after the intake of the diets. Intestinal enterocytes are covered with a mucus layer that is secreted by goblet cells throughout the gastrointestinal tract (Johansson et al., 2011). In the present study, the enterocyte absorptive area of Sterlet fed diets supplemented with bromelain was larger, which resulted in improved feed utilization. Nutrient digestion, absorption, intestinal barrier function and mucosal function were affected by its thickness and fluidity (Smirnov, Sklan, & Uni, 2004). In this study, no morphological changes in the muscularis thickness in Sterlet fed diets supplemented with bromelain indicated that the additive had not affected the thickness of the intestinal muscle. The function of the muscularis mucosa is to promote intestinal peristalsis; a thinner muscularis decreases the rate of peristalsis and the length of time food remains in the small intestine is increased, thus increasing the time during which nutrients are absorbed (Liu, Wu, Li, Duan, & Wen, 2018).

Digestive enzymes are the most important factor influencing nutrient utilization in the gastrointestinal tract, and they are used to evaluate digestive capacity (Ribeiro et al., 2008). In the present study, dietary bromelain did not significantly improve α-amylase or trypsin activity, but it significantly enhanced that of pepsin and lipase (Table 7). Additionally, dietary bromelain decreased the enzyme activity of ALP and LAB, but it had no significant effect on the AcP enzyme. No doubt, fish did not secrete the bromelain when feed supplemented with exogenous enzymes such as bromelain which

have the same mode of action of proteases and trypsin may decrease the secretion of endogenous enzyme especially trypsin. This reason may be explained why the endogenous trypsin was decreased in fish diet supplemented with bromelain. The activities of the intestinal enzymes ALP and LAP are used as markers of enterocyte development (He et al., 2012; Kvåle, Mangor-Jensen, Moren, Espe, & Hamre, 2007). Digestive enzymes in animals are present in the gastrointestinal lumen and are associated with the intestinal epithelial cell brush-border membrane (Bakke, Glover, & Krogdahl, 2010). While digestive and metabolic functions of fish are clearly correlated with digestive and brush-border enzyme activities (Hakim Uni, Hulata, & Harpaz, 2006), no studies to date have evaluated the effect of bromelain on the activities of the digestive organs or those of the digestive and intestinal enzymes. Liu et al. (2018) showed that midgut and hindgut protease activity in Gibel carp were not affected by dietary supplementation with pure protease. In contrast, the activity of amylase in the intestine of Nile tilapia fed a diet supplemented with a mixture of enzymes (neutral protease, ß-glucan and xylanase) increased in comparison with that in fish fed a control diet (Lin, Mai, & Tan, 2007).

No significant differences were detected in the lipid and moisture contents of whole Sterlet and fillets when the diet was supplemented with bromelain, but crude protein tended to decrease with increased bromelain supplementation. The decrease in protein content of fish, probably, the use of bromelain, affects the moisture level in fish. This body composition finding concurs with Song et al. (2017) who report that there were no differences in the dry matter, crude lipid or crude ash contents of shrimp fed diets supplemented with a protease complex; however, the crude protein content of whole shrimp was higher than that in shrimp fed diets not supplemented with the protease complex. No significant differences were noted in any of the proximate composition parameters of Nile tilapia fed diets containing pineapple (*Ananas comosus*) (Inaolaji, 2011). Furthermore, the present study showed that micro- and macroelement deposition in fillets was slightly significantly lower. To date, no reports have indicated the effect of bromelain supplementation on mineral deposition in fish.

In fish, the non-specific defence mechanism is more important than specific immune defence, because the latter requires a longer time for antibodies to build up and for activation (Anderson, 1992). Adding bromelain to the Sterlet diet in quantities of 20 g/kg enhanced all of the non-specific humoral immunity parameters estimated, except for that of ceruloplasmin (Table 8). The same bromelain dose also increased the proliferative responses of T and B cells. Bromelain modulates the function of adhesion molecules on blood and endothelial cells, macrophages and natural killer cells, and it also regulates and activates various immune cells and their cytokine production (Maurer, 2001). Bromelain bolsters the immune system by increasing cytokine production, which are hormones produced by white blood cells to improve immunity. Several studies have established the ability of bromelain to remove T-cell CD44 molecules from lymphocytes (Amid, Ismail, & Arshad, 2015). Furthermore, total serum protein in fish is responsible for the innate immune response, and higher levels of this provide stronger responses (Sahu, Das, Mishra, Pradhan, & Sarangi, 2007). In this context, Choi et al. (2015) reports that levels of plasma total protein and total immunoglobulin (IgI) in common carp and mullet fed diets supplemented with a 2% of mixture of bromelain and papain were significantly higher than that in fish fed control diets. It can be concluded from this study that the use of bromelain at level of 10 or 20 g/kg could improve the growth performance, feed utilization, non-specific humoral and cell-mediated immunity of Sterlet. However, further studies are recommended to confirm these interesting effects of the dietary bromelain.

ORCID

Mohamed S. Hassaan https://orcid.org/0000-0002-6725-1715

REFERENCES

Abbott, L. (1984). *Acid phosphatase. Kaplan A et al.* Clin Chem The CV Mosby Co., St Louis. Toronto. Princeton, 1079–1083.

Abdollahi, M., Ravindran, V., & Svihus, B. (2013). Pelleting of broiler diets: An overview with emphasis on pellet quality and nutritional value. *Animal Feed Science and Technology*, *179*, 1–23. https://doi.org/10.1016/j.anifeedsci.2012.10.011

Alvarez-González, C. A., Moyano-López, F. J., Civera-Cerecedo, R., Carrasco-Chávez, V., Ortiz-Galindo, J. L., & Dumas, S. (2008). Development of digestive enzyme activity in larvae of spotted sand bass Paralabrax maculatofasciatus. 1. Biochemical analysis. *Fish Physiology and Biochemistry*, *34*, 373–384. https://doi.org/10.1007/s10695-007-9197-7

Amid, A., Ismail, N. A., & Arshad, Z. I. (2015). *Case Study: Recombinant Bromelain Selection*. Recombinant Enzymes - from Basic Science to Commercialization, 143–157, https://doi.org/10.1007/978-3-319-12397-4_10

Anderson, D. P. (1992). Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. *Annual Review of Fish Diseases*, *2*, 281–307. https://doi.org/10.1016/0959-8030(92)90067-8

Anderson, D. P., & Siwicki, A. K. (1994). *Simplified assays for measuring non-specific defense mechanisms in fish*. In Seattle, WA: Fish Health Section/American Fisheries Society Meeting, 26–35.

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *The Journal of General Physiology*, *22*, 79. https://doi.org/10.1085/jgp.22.1.79

AOAC, Association of Official Analytical Chemists (1995). *Official methods of analysis* (p. 1117). Virginia: Arlington.

Arshad, Z. I., Amid, A., Yusof, F., Jaswir, I., Ahmad, K., & Loke, S. P. (2014). Bromelain: An overview of industrial application and purification strategies. *Applied Microbiology and Biotechnology*, *98*, 7283–7297. https://doi.org/10.1007/s00253-014-5889-y

Ayoola, M. O. (2016). *Application of dietary bentonite clay as feed addictive on feed quality, water quality and production performance of African catfish (Clarias gariepinus)*. Doctoral dissertation, Stellenbosch: Stellenbosch University.

Babu, B. R., Rastogi, N., & Raghavarao, K. (2008). Liquid–liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system. *Chemical Engineering and Processing: Process Intensification*, *47*, 83–89. https://doi.org/10.1016/j.cep.2007.08.006

Baeverfjord, G., Refstie, S., Krogedal, P., & Åsgård, T. (2006). Low feed pellet water stability and fluctuating water salinity cause separation and accumulation of dietary oil in the stomach of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *261*, 1335–1345. https://doi.org/10.1016/j.aquaculture.2006.08.033

Bakke, A. M., Glover, C., & Krogdahl, Å. (2010). Feeding, digestion and absorption of nutrients. The Multifunctional Gut of Fish,

Fish physiology, (Vol. 30, pp. 57–110). https://doi.org/10.1016/S1546-5098(10)03002-5

Boonyaratpalin, M., Promkunthong, W., & Hunter, B. (2000). *Effects of enzyme pre-treatment on in vitro glucose solubility of Asian plant by-products and growth and digestibility of oil palm expeller meal by* Oreochromis niloticus *(Nile tilapia)*. In Proceedings of the Third European Symposium on Feed Enzymes, TheNetherlands, 86–92.

Choi, W. M., Lam, C. L., Mo, W. Y., & Wong, M. H. (2015). The use of food wastes as feed ingredients for culturing grass carp (*Ctenopharyngodon idellus*) in Hong Kong. *Environmental Science and Pollution Research*, *23*, 7178–7185. https://doi.org/10.1007/s11356-015-5465-8

Christaki, E., Bonos, E., Giannenas, I., & Florou-Paneri, P. (2012). Aromatic plants as a source of bioactive compounds. *Agriculture*, *2*, 228–243. https://doi.org/10.3390/agriculture2030228

Coêlho, D. D. F., Silva, C. A., Machado, C. S., Silveira, E., & Tambourgi, E. B. (2015). Use of artificial neural networks to predict aqueous two-phases system optimal conditions on Bromelain's purification. *Chemical Engineering Transactions*, *43*, 1417–1422.

Deka, A., Sahu, N. P., & Jain, K. K. (2003). Utilization of fruit processing wastes in the diet of *Labeo rohita* fingerling. *Asian-Australasian Journal of Animal Sciences*, *16*, 1661–1665. https://doi.org/10.5713/ajas.2003.1661

Divakaran, S., & Velasco, M. (1999). Effect of proteolytic enzyme addition to a practical feed on growth of the Pacific white shrimp, *Litopenaeus vannamei* (Boone). *Aquaculture Research*, *30*, 335–339. https://doi.org/10.1046/j.1365-2109.1999.00333.x

Engwerda, C. R., Andrew, D., Ladhams, A., & Mynott, T. L. (2001). Bromelain modulates T cell and B cell immune responses in vitro and in vivo. *Cellular Immunology*, *210*, 66–75. https://doi.org/10.1006/cimm.2001.1807

Erlanger, B. F., Kokowsky, N., & Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics*, *95*, 271–278. https://doi.org/10.1016/0003-9861(61)90145-x

Fennema, O. R. (1996). *Food chemistry*, 3rd edn. New York: University of Wisconsin. Marcel Dekker, Inc.

Foo, A., & Bais, R. (1998). Amylase measurement with 2-chloro-4-nitrophenyl maltotrioside as substrate. *Clinica Chimica Acta*, *272*, 137–147. https://doi.org/10.1016/s0009-8981(98)00009-6

Grzonka, Z., Kasprzykowski, F., & Wiczk, W. (2007). Cysteine proteases. In *Industrial enzymes* (pp. 181–195). Dordrecht, The Netherlands: Springer.

Hakim, Y., Uni, Z., Hulata, G., & Harpaz, S. (2006). Relationship between intestinal brush border enzymatic activity and growth rate in tilapias fed diets containing 30% or 48% protein. *Aquaculture*, *257*, 420–428. https://doi.org/10.1016/j.aquaculture.2006.02.034

Hale, L. P., Greer, P. K., Trinh, C. T., & James, C. L. (2005). Proteinase activity and stability of natural bromelain preparations. *International Immunopharmacology*, *5*, 783–793. https://doi.org/10.1016/j.intimp.2004.12.007

Hassaan, M. S., El-Sayed, A., Soltan, M. A., Iraqi, M. M., Goda, A. M., Davies, S. J., … Ramadan, H. A. (2019). Partial dietary fish meal replacement with cotton seed meal and supplementation with exogenous protease alters growth, feed performance, hematological indices and associated gene expression markers (GH, IGF-I) for Nile tilapia, *Oreochromis niloticus*. *Aquaculture*, *503*, 282–292. https://doi.org/10.1016/j.aquaculture.2019.01.009

Hassaan, M. S., Mahmoud, S. A., Jarmolowicz, S., El-Haroun, E. R., Mohammady, E. Y., & Davies, S. J. (2018). Effects of dietary baker's yeast extract on the growth, blood indices and histology of Nile tilapia (Oreochromis niloticus L.) fingerlings. *Aquaculture Nutrition*, *24*(6), 1709–1717.

Hassaan, M. S., & Soltan, M. A. (2016). Evaluation of essential oil of fennel and garlic separately or combined with *Bacillus licheniformis* on the growth, feeding behaviour, hemato-biochemical indices of *Oreochromis niloticus* (L.) fry. *Journal of Aquaculture Research & Development*, *7*, 422–429. https://doi.org/10.4172/2155-9546.1000422

Hassaan, M. S., Soltan, M. A., & Ghonemy, M. M. R. (2014). Effect of synbiotics between *Bacillus licheniformis* and yeast extract on growth, hematological and biochemical indices of the Nile tilapia (*Oreochromis niloticus*). *The Egyptian Journal of Aquatic Research*, *40*(2), 199–208. https://doi.org/10.1016/j.ejar.2014.04.001

He, T., Xiao, Z., Liu, Q., Ma, D., Xu, S., Xiao, Y., & Li, J. (2012). Ontogeny of the digestive tract and enzymes in rock bream *Oplegnathus fasciatus* (Temminck et Schlegel 1844) larvae. *Fish Physiology and Biochemistry*, *38*, 297–308. https://doi.org/10.1007/s10695-011-9507-y

Inaolaji, O. W. (2011). *Growth performance and digestibility of Nile Tilapia, Oreochromis niloticus fed pineapple (Ananascomosus) Peel Meal-Based Diets*. [Essay]. Department of Aquaculture and Fishery management, University of Agriculture Abeokuta, Abeokuta, Ogun State, 45.

Jobling, M. (1994). *Fish Bioenergetics Chapman and Hall London Google Scholar*.

Johansson, M. E. V., Ambort, D., Pelaseyed, T., Schütte, A., Gustafsson, J. K., Ermund, A., … Hansson, G. C. (2011). Composition and functional role of the mucus layers in the intestine. *Cellular and Molecular Life Sciences*, *68*, 3635–3641. https://doi.org/10.1007/s00018-011-0822-3

Ketnawa, S., Chaiwut, P., & Rawdkuen, S. (2012). Pineapple wastes: A potential source for bromelain extraction. *Food and Bioproducts Processing*, *90*, 385–391. https://doi.org/10.1016/j.fbp.2011.12.006

Ketnawa, S., Sai-Ut, S., Theppakorn, T., Chaiwut, P., & Rawdkuen, S. (2009). Partitioning of bromelain from pineapple peel (Nang Laecultv.) by aqueous two-phase system. *Asian Journal of Food and Agro-Industry*, *2*, 457–468.

Kolman, R., Kolman, H., & Siwicki, A. K. (1998). Effect of some immunomodulators on the growth rate of sturgeon fry (*Acipenseridae*). *Archives of Polish Fisheries*, *6*, 383–390.

Kvåle, A., Mangor-Jensen, A., Moren, M., Espe, M., & Hamre, K. (2007). Development and characterisation of some intestinal enzymes in Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. *Aquaculture*, *264*, 457–468. https://doi.org/10.1016/j.aquaculture.2006.12.024

Leung-Toung, R., Li, W., Tam, T., & Kaarimian, K. (2002). Thiol-Dependent Enzymes and Their Inhibitors: A Review. *Current Medicinal Chemistry*, *9*, 979–1002. https://doi.org/10.2174/0929867024606704

Liebert, F., & Portz, L. (2005). Nutrient utilization of Nile tilapia *Oreochromis niloticus* fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. *Aquaculture*, *248*, 111–119. https://doi.org/10.1016/j.aquaculture.2005.04.009

Lima, F. C., Simões, A. J. A., Vieira, I. M. M., Silva, D. P., & Ruzene, D. S. (2018). An overview of applications in pineapple agroindustrial residues. *Acta Agriculturae Slovenica*, *111*, 445–462. https://doi.org/10.14720/aas.2018.111.2.18

Lin, S., Mai, K., & Tan, B. (2007). Effects of exogenous enzyme supplementation in diets on growth and feed utilization in tilapia, *Oreochromis niloticus* x *O. aureus*. *Aquaculture Research*, *38*, 1645–1653. https://doi.org/10.1111/j.1365-2109.2007.01825.x

Liu, W., Wu, J., Li, Z., Duan, Z., & Wen, H. (2018). Effects of dietary coated protease on growth performance, feed utilization, nutrient apparent digestibility, intestinal and hepatopancreas structure in juvenile Gibel carp (*Carassius auratus gibelio*). *Aquaculture Nutrition*, *24*, 47–55. https://doi.org/10.1111/anu.12531

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Manosroi, A., Chankhampan, C., Pattamapun, K., Manosroi, W., & Manosroi, J. (2014). Antioxidant and gelatinolytic activities of papain from papaya latex and bromelain from pineapple fruits. *Chiang Mai Journal of Science*, *41*, 635–648.

Manzoor, Z., Nawaz, A., Mukhtar, H., & Haq, I. (2016). Bromelain: methods of extraction, purification and therapeutic applications. *Brazilian Archives of Biology and Technology*, *59*, 1590–1670. http://dx.doi.org/10.1590/1678-4324-2016150010

Martins, B. C., Rescolino, R., Coelho, D. F., Zanchetta, B., Tambourgi, E. B., & Silveira, E. (2014). Characterization of bromelain from *ananas comosus* agroindustrial residues purified by ethanol factional precipitation. *Chemical Engineering Transactions*, *37*, 781–786. https://doi.org/10.3303/CET1437131

Mattsson, S., & Swartling, P. (1954). *Determination of calcium and phosphorus in cheese – Report No. 43*. Dairy Department of the Alnarp Institute, Sweden, Malmö: 1–8.

Maurer, H. (2001). Bromelain: Biochemistry, pharmacology and medical use. *Cellular and Molecular Life Sciences*, *58*, 1234–1245. https://doi.org/10.1007/pl00000936

Nagel, W., Willig, F., & Schmidt, F. H. (1964). On amino acid arylamidase (so-called leucine aminopeptidase) activity in the human serum. *KlinischeWochenschrift*, *42*, 447–449.

Nilsang, S., Lertsiri, S., Suphantharika, M., & Assavanig, A. (2005). Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. *Journal of Food Engineering*, *70*, 571–578. https://doi.org/10.1016/j.jfoodeng.2004.10.011

Novaes, L. C., Ebinuma, V. D., Mazzola, P. G., & Júnior, A. P. (2013). Polymer-based alternative method to extract bromelain from pineapple peel waste. *Biotechnology and Applied Biochemistry*, *60*, 527–535. https://doi.org/10.1002/bab.1121

Orsini, R. A. (2006). Bromelain. *Plastic and Reconstructive Surgery*, *118*, 1640–1644. https://doi.org/10.1097/01.prs.0000242503.50548.ee

Padma, P. S., Jayakumar, K., Mathai, V., Chintu, S., & Sarath, B. K. (2012). Immobilization and kinetic studies of bromelain: A plant cysteine protease from pineapple (*Ananas comosus*) plant parts. *International Journal Medical Health Science*, *3*, 10–14.

Parry, R. M. Jr, Chandan, R. C., & Shahani, K. M. (1965). A rapid and sensitive assay of muramidase. *Proceedings of the Society for Experimental Biology and Medicine*, *119*, 384–386. https://doi.org/10.3181%2F00379727-119-30188

Pavan, R., Jain, S., Shraddha, , & Kumar, A. (2012). Properties and therapeutic application of bromelain: A review. *Biotechnology Research International*, *2012*, 1–6. https://doi.org/10.1155/2012/976203

Rathnavelu, V., Alitheen, N. B., Sohila, S., Kanagesan, S., & Ramesh, R. (2016). Potential role of bromelain in clinical and therapeutic applications. *Biomedical Reports*, *5*, 283–288. https://doi.org/10.3892/br.2016.720

Reddy, K. K., Grossman, L., & Rogers, G. S. (2013). Common complementary and alternative therapies with potential use in dermatologic surgery: Risks and benefits. *Journal of the American Academy of Dermatology*, *68*, 127–135. https://doi.org/10.1016/j.jaad.2011.06.030

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture*, *433*, 50–61. https://doi.org/10.1016/j.aquaculture.2014.05.048

Ribeiro, L., Couto, A., Olmedo, M., Álvarez-Blázquez, B., Linares, F., & Valente, L. M. (2008). Digestive enzyme activity at different developmental stages of blackspot seabream, *Pagellus bogaraveo* (Brunnich 1768). *Aquaculture Research*, *39*, 339–346. https://doi.org/10.1111/j.1365-2109.2007.01684.x

Rice, E. W., Wagman, E., & Takenaka, Y. (1963). Ceruloplasmin assay in serum: Standardization of ceruloplasmin activity in terms of international enzyme units. *Standard Methods of Clinical Chemistry*, *4*, 39–46.

Rico, A., Phu, T. M., Satapornvanit, K., Min, J., Shahabuddin, A., Henriksson, P. J., … Van den Brink, P. J. (2013). Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. *Aquaculture*, *412*, 231–243. https://doi.org/10.1016/j.aquaculture.2013.07.028

Rutkowska, U. (1981). *Selected methods of study of composition and nutritional value of food – PZWL, Warsaw (in Polish)*.

Sahu, S., Das, B. K., Mishra, B. K., Pradhan, J., & Sarangi, N. (2007). Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Journal of Applied Ichthyology*, *23*, 80–86. https://doi.org/10.1111/j.1439-0426.2006.00785.x

Sawant, R., & Nagendran, S. (2014). Protease: An enzyme with multiple industrial applications. *World Journal of Pharmacy and Pharmaceutical Sciences*, *3*, 568–579.

Shearer, K. D. (1994). Factors affecting the proximate composition of cultured fishes with emphasis on salmonids. *Aquaculture*, *119*, 63–88. https://doi.org/10.1016/0044-8486(94)90444-8

Singh, P., Maqsood, S., Samoon, M. H., Phulia, V., Danish, M., & Chalal, R. S. (2011). Exogenous supplementation of papain as growth promoter in diet of fingerlings of *Cyprinus carpio*. *International Aquatic Research*, *3*, 1–9.

Siwicki, A. K., & Anderson, D. P. (1993). Immunostimulation in fish: Measuring the effects of stimulants by serological and immunological methods. *US Fish Wildl Service-IFI*, *1*, 1–17.

Smirnov, A., Sklan, D., & Uni, Z. (2004). Mucin dynamics in the chick small intestine are altered by starvation. *The Journal of Nutrition*, *134*, 736–742. https://doi.org/10.1093/jn/134.4.736

Smith-Marshall, J., & Golden, K. D. (2012). Characterization of Bromelain from *Morinda citrifolia* (Noni). *Journal of Scientific Research*, *4*, 445–456. https://doi.org/10.3329/jsr.v4i2.8125

Soares, P. A., Vaz, A. F., Correia, M. T., Pessoa, A. Jr, & Carneiro-da-Cunha, M. G. (2012). Purification of bromelain from pineapple wastes by ethanol precipitation. *Separation and Purification Technology*, *98*, 389–395. https://doi.org/10.1016/j.seppur.2012.06.042

Song, H. L., Tan, B. P., Chi, S. Y., Liu, Y., Chowdhury, M. A., & Dong, X. H. (2017). The effects of a dietary protease-complex on performance, digestive and immune enzyme activity, and disease resistance of *Litopenaeus vannamei* fed high plant protein diets. *Aquaculture Research*, *48*, 2550–2560. https://doi.org/10.1111/are.13091

Van Beckhoven, R. F., Zenting, H. M., Maurer, K. H., Van Solingen, P., & Weiss, A. (1995). *Bacillus cellulases* and its application for detergents and textile treatment. *European Patent*, *739*, 982.

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, *74*, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Wenger, C., Kaplan, A., Rubaltelli, F. F., & Hammerman, C. (1984). Alkaline phosphatase. In *Clinical Chemistry* (pp. 1094–1098). St Louis, Toronto, Princeton: The C.V. Mosby Co.

Whiteside, P. J., & Miner, B. (1984). *Pye Unicam Atomic Absorption Data Book*. Cambridge, UK: PyeUnicamLtd.

Winkler, U. K., & Stuckmann, M. (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *Journal of Bacteriology*, *138*, 663–670.

Zawistowski, S. (1986). *Histology technique. Histology and the foundations of histopathology -. PZWL, Warsaw*, 548, pp. (in Polish).

How to cite this article: Wiszniewski G, Jarmołowicz S, Hassaan MS, et al. The use of bromelain as a feed additive in fish diets: Growth performance, intestinal morphology, digestive enzyme and immune response of juvenile Sterlet (*Acipenser ruthenus*). *Aquacult Nutr.* 2019;00:1–11. https://doi.org/10.1111/anu.12949