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Wpływ naturalnych preparatów zawierających enzymy owocowe na organizm sterleta (*Acipenser ruthenus*)

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Praca doktorska wykonana pod kierunkiem
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Artykuł 1 (Eksperyment 1): **Wiszniewski, G.**, Jarmołowicz, S., Hassaan, M. S., Mohammady, E. Y., Soaudy, M. R., Łuczyńska, J., Tońska, E., Terech-Majewska, E., Ostaszewska, T., Kamaszewski, M., Skrobisz, M., Adamski, A., Schulz, P., Kaczorek, Siwicki, A. (2019). 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*). Aquaculture Nutrition, 25(6), 1289-1299. (IF: 2.231)

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Spis treści

1. Streszczenie

Przedstawiona rozprawa doktorska stanowi podsumowanie trzech doświadczeń, które miały na celu zbadanie wpływu enzymów owocowych na organizm młodocianego sterleta. Wybrano trzy enzymy, należące do rodziny proteaz cysteinowych: bromelainę, papainę i aktynidynę. Są to substancje wyizolowane odpowiednio z ananasa, papai oraz kiwi. Wykazują silne działanie proteolityczne, przez co wspomagają trawienie białek. Posiadają działanie bakteriobójcze i przeciwzapalne. Liczne badania wykazały ich ogromny potencjał immunostymulujący.

W niniejszej rozprawie badano wpływ dodatku 1% i 2% bromelainy, papainy i aktynidyny w paszy na tempo wzrostu, histologię wątroby i przewodu pokarmowego, aktywność wybranych enzymów trawiennych oraz najważniejsze parametry odporności nieswoistej (humoralnej i komórkowej) organizmu ryb. W przeprowadzonych eksperymentach wykorzystano młodocianego sterleta (*Acipenser ruthenus*), rybę szeroko rozpowszechnioną w akwakulturze. Ze względu na fakt, że jest to ryba odporna na manipulacje i stres oraz łatwo adoptuje się do nowej paszy, doskonale nadaje się do doświadczeń żywieniowych.

W trakcie przeprowadzonych eksperymentów zaobserwowano wpływ podawanych enzymów na analizowane parametry. Stwierdzono istotne statystycznie różnice w przyroście masy ciała we wszystkich grupach żywionych paszą z dodatkiem enzymów. Zaobserwowano istotne różnice w wybranych parametrach odporności humoralnej i komórkowej. W grupach żywionych paszą z dodatkiem enzymów odnotowano wyższe wartości ceruloplazminy, lizozymu, białka całkowitego i immunoglobulin oraz aktywność proliferacyjną limfocytów T i B.

Zastosowane substancje wpłynęły również na rozwój jelita cienkiego. Bromelaina i papaina wpłynęła na wzrost wysokości enterocytów i zwiększenie ich powierzchni chłonnej, co prawdopodobnie skutkowało zwiększeniem tempa wzrostu ryb w grupach doświadczalnych.

Analiza aktywności enzymów trawiennych wykazała wpływ bromelainy, papainy i aktynidyny na sekrecję enzymów endogennych w przewodzie pokarmowym ryb. W przypadku bromelainy zaobserwowano wzrost aktywności lipazy i zahamowanie wydzielania aminopeptydazy leucytowej, amylazy i trypsyny. Papaina wpłynęła istotnie na wzrost aktywności aminopeptydazy leucytowej i amylazy, a aktynidyna - na wzrost aktywności fosfatazy alkalicznej i fosfatazy kwaśnej.

Przeprowadzony cykl trzech eksperymentów i analiza uzyskanych wyników pozwalają stwierdzić, że zastosowane w paszy enzymy owocowe – bromelaina, papaina i aktynidyna – mają szerokie spektrum działania na organizm młodocianego sterleta. Preparaty te, są bezpieczne dla ryb i środowiska wodnego. Analizowane enzymy, mogą być wykorzystane jako dodatek funkcjonalny w paszy i stanowić element immunoprofilaktyki w akwakulturze.

2. Summary

The presented doctoral dissertation is a summary of three experiments that were aimed at examining the effect of fruit enzymes on the body of a juvenile sterlet. Three enzymes belonging to the family of cysteine proteases were selected: bromelain, papain and actinidin. These substances isolated from pineapple, papaya and kiwi. They have a strong proteolytic effect, which helps to digest proteins. They have bactericidal and anti-inflammatory properties. Numerous studies have shown their huge immunostimulatory potential.

This dissertation was conducted to evaluate the effect of a diet supplemented with 1% and 2% bromelain, papain and actinidin on growth rate, histology of the liver and digestive tract, the activity of selected digestive enzymes and the most important parameters of non-specific (humoral and cellular) immunity of the fish organism. The juvenile sterlet (*Acipenser ruthenus*), a fish widely used in aquaculture, was utilized in the experiments. Due to its resistance to manipulation and stress as well as ease of adapting to new feed, it is well suited for feeding experiments.

During the experiments, the influence of the administered enzymes on the analyzed parameters was observed. Statistically significant differences in weight gain were found in all groups fed with feed with added enzymes. Significant differences in selected parameters of humoral and cellular immunity were observed. In the groups fed with feed supplemented with enzymes, higher values of lysozyme ceruloplasmin, total protein and immunoglobulins as well as proliferative activity of T and B lymphocytes were noted.

The substances used also influenced the development of the small intestine. Bromelain and papain increased the height of enterocytes and increased their absorptive surface, which probably resulted in a higher growth rate of fish in the experimental groups.

The analysis of the activity of digestive enzymes showed an effect on the functioning of the digestive tract. In the case of bromelain, an increase in lipase activity and inhibition of the secretion of leucite aminopeptidase, amylase and trypsin were observed. Papain reduced the activity of trypsin and lipase, while the activity of leucite aminopeptidase and amylase increased. Actinidine increased alkaline and acid phosphatase activity.

The analysis of the results obtained during three experiments shows that the fruit enzymes used in the feed – bromelain, papain and actinidine – have a wide range of effects on the organism of juvenile sterlet. These preparations are safe for fish and the aquatic environment. The analyzed enzymes can be used as a functional additive in feed and support immunoprophylaxis in aquaculture.

3. Wstęp i cel pracy

Proces intensyfikacji akwakultury, obserwowany na przestrzenni ostatnich kilkunastu lat, wpłynął w znaczący sposób na wzrost zapotrzebowania na paszę, a także na nowo zdefiniował kryteria jakościowe dotyczące optymalnego i racjonalnego żywienia ryb, stanowiącego podstawę efektywności hodowli. Dostępność i maksymalne wykorzystanie składników w podawanym pokarmie, zwłaszcza białka ma ogromne znaczenie. Odpowiednio zbilansowana pasza wpływa na tempo wzrostu i stan kondycyjny, a dodatek naturalnych czy syntetycznych immunostymulatorów pozwala na poprawę wydolności układu odpornościowego ryb podchowywanych w dużych zagęszczeniach, szczególnie w hodowlach prowadzonych w systemie zamkniętych obiegów recyrkulacyjnych (RAS). Enzymy egzogenne mają istotne znaczenie dla prawidłowego rozwoju i funkcjonowania układu pokarmowego ryb, zarówno stadiów larwalnych jak i juwenilnych (Kolkovsky 2001, Dąbrowski i Glogowski 1977). O ile w środowisku naturalnym dostęp do enzymów zapewniony jest w różnorodnym pokarmie naturalnym, tak podczas hodowli w obiegach zamkniętych wskazana jest ich suplementacja. Dodanie egzogennych enzymów do paszy ryb może poprawić wykorzystanie zawartych w niej składników pokarmowych, zmniejszając w ten sposób ich straty. Udowodniono, że egzogenne enzymy poprawiają wartość odżywczą paszy i zmniejszają zanieczyszczenie środowiska w przypadku zwierząt stałocieplnych (Khattak i in. 2006). Podobny efekt uzyskano w przypadku zastosowania fitoenzymów w żywieniu ryb. Odnotowano wzrost wykorzystania białka oraz zmniejszenie ilości fosforu odprowadzanego do środowiska wodnego (Ai i in. 2007). Dodatek enzymów egzogennych do paszy otwiera zatem możliwości stosowania alternatywnych źródeł białka w żywieniu ryb, z wykorzystaniem różnorodnych (w tym nowych, nie stosowanych wcześniej) surowców (Zheng i in. 2020). Przy komponowaniu paszy z wysoką zawartością składników roślinnych, dodanie enzymu może znacząco wpłynąć na poprawę wykorzystanie białka roślinnego przez ryby (Liebert i Portz 2005, Singh i in. 2011).

W trakcie intensywnego chowu w systemach recyrkulacyjnych (RAS), organizm ryb jest bardziej podatny na choroby wirusowe, bakteryjne i grzybicze oraz inwazje pasożytnicze. Stosowanie antybiotykoterapii powoduje selekcję w kierunku rozwoju opornych bakterii, zarówno w organizmie ryb jak i w całym środowisku wodnym (Siwicki i in. 2009). Ponadto wytworzenie się lekooporności związane z nadużywaniem antybiotyków sprawia, że z czasem potencjał terapeutyczny takiej kuracji staje się mało efektywny (Anderson 1992). W takiej sytuacji profilaktyka chorób infekcyjnych powinna być oparta na ograniczeniu możliwości kontaktu z patogenemi oraz na modulacji/stymulacji nieswoistej, zarówno humoralnej jak i komórkowej odporności ryb (Kolman i in. 1998).

W żywieniu zwierząt coraz powszechniej stosuje się immunomodulatory zawierające roślinne substancje aktywne, szczególnie te, które wspomagają nieswoiste mechanizmy obronne i odporność przeciwzakaźną u zwierząt i człowieka. Głównym kryterium wyboru danego suplementu jest przede wszystkim jego bezpieczeństwo biologiczne jak i wysoka zawartość substancji czynnych. Suplementacja pokarmu substancjami aktywnymi to skuteczna metoda wspierania wrodzonej odpowiedzi immunologicznej ryb. Wiąże się ona z wykorzystaniem funkcjonalnych składników, które zastępując konwencjonalne środki lecz-

nicze, stosowane w akwakulturze, mogą łagodzić szkodliwe skutki ich oddziaływania na ekosystem, a szczególnie na wzrost lekooporności patogenów czy ich patogenności. Ograniczają lub wręcz niwelują zjawisko immunosupresji pojawiające się w momencie oddziaływania niekorzystnych warunków środowiskowych, ksenobiotyków czy czynników stresogennych na organizm ryb. Jest to element nowatorskiej i proekologicznej metody hodowli ryb (Gupta i in. 2021). Wraz z pojawieniem się probiotyków, prebiotyków, synbiotyków i roślinnych substancji aktywnych, obserwuje się wzrost zainteresowania tego typu preparatami, szczególnie pochodzenia naturalnego. Są one coraz częściej, z dobrym skutkiem, wykorzystywane nie tylko w medycynie ludzkiej i dietetyce, ale również w żywieniu zwierząt. Suplementacja pasz wyżej wymienionymi dodatkami stała się powszechnie stosowanym elementem immunoprofilaktyki w żywieniu ryb (Kobya i in. 2021). Działanie immunostymulatorów polega na przyspieszeniu odpowiedzi immunologicznej, podniesieniu jej poziomu i wydłużeniu czasu jej trwania, co uzależnione jest od statusu immunologicznego gospodarza, sposobu podania oraz dawki immunostymulatora (Rojo-Cebreros i in. 2018).

Wśród wielu substancji naturalnych, na szczególną uwagę zasługują preparaty roślinne, zwłaszcza enzymy owocowe, które posiadają szerokie spektrum możliwości. Wykazują one działanie stymulujące na układ odpornościowy, zwiększają potencjał obronny organizmu, a jednocześnie poprawiają funkcjonowanie układu pokarmowego (Nwinyi i Busola 2010, Alabi i in. 2012, Liebert i Portz 2005).

Niniejsza dysertacja doktorska została przeprowadzona w cyklu trzech doświadczeń, w których wykorzystano enzymy owocowe, należące do grupy proteaz cysteinowych: bromelainę (Eksperyment 1), papainę (Eksperyment 2) i aktynidynę (Eksperyment 3). Są to substancje pochodzące odpowiednio z ananasa, papai i kiwi. Ich właściwości są od dziesięcioleci przedmiotem badań w wielu dziedzinach, zwłaszcza w medycynie ludzkiej i weterynaryjnej oraz przemyśle spożywczym.

Bromelaina jest surowym ekstraktem wodnym łodygi i owoców ananasa (*Ananus comosus*). Jej skład stanowi mieszanina endopeptydaz tiolowych oraz substancji niebiałkowych takich jak: fosfataza, glikozydaza, peroksydaza, celulaza, glikoproteiny oraz węglowodany i inhibitory proteaz. Aktywność enzymatyczna bromelainy obejmuje szerokie spektrum w zakresie pH od 5,5 do 8,0 (Pavan i in. 2012) natomiast optimum wynosi 6,0-7,0 przy temperaturze 50-60°C (Manzoor i in. 2016). Posiada ona potencjał oddziaływania na szereg funkcji fizjologicznych, a jej właściwości lecznicze znane są od lat siedemdziesiątych ubiegłego wieku. Przede wszystkim jako enzym, ułatwia trawienie białek poprzez częściową hydrolizę cząsteczek do mniejszych peptydów, tym samym zwiększając ich dostępność w pożywieniu (Fennema 1996). Bromelaina ma także działanie immunomodulujące. Udowodniono, że enzym ten aktywuje komórki NK (Natural Killer) i moduluje aktywność immunologiczną limfocytów T i B we krwi (Engwerda i in. 2001). Bromelaina zapobiega nadmiernej lepkości płytek krwi, zmniejszając ryzyko zakrzepicy (Orsini 2006). Ponadto działa przeciwobrzękowo, przeciwnowotworowo, posiada właściwości przeciwzapalne i antybiotyczne (Reddy i in. 2013). Bromelainę wykorzystuje się także w przemyśle spożywczym m. in. przy obróbce mięsa czy w browarnictwie (Maurer 2001, Soares i in. 2012), w przemyśle tekstylnym oraz kosmetycznym (Babu i in. 2008, Ketnawa i in. 2009).

Papaina jest endolitycznym roślinnym enzymem proteazy cysteinowej, który jest izolowany z lateksu papai *Carica papaja.* Papainę o najwyższej aktywności uzyskuje się z niedojrzałych o*woco*w. Papaina wykazuje szeroką aktywność proteolityczną w stosunku do białek, peptydów krótkołańcuchowych, estrów aminokwasowych i połączeń amidowych. Rozcina wiązania peptydowe obejmujące zasadowe aminokwasy, zwłaszcza argininę, lizynę i reszty po fenyloalaninie (Menard i in. 1990). Papaina znalazła zastosowanie w przemyśle spożywczym i medycynie ludzkiej (Amri i Mamboya 2012). Enzym ten posiada właściwości przeciwzapalne, bakteriostatyczne i grzybobójcze, jest składnikiem wielu środków farmaceutycznych (Dawkins i in. 2003, Mahmood i in. 2005, Nwinyi i Busola 2010, Alabi i in. 2012, Aruljothi i in. 2014).

Aktynidyna jest proteazą cysteinową, która naturalnie występuje w owocach kiwi (*Actinidia deliciosa*). Została zidentyfikowana jako enzym, który może pomóc w hydrolizie różnych białek, w tym glutenu (Chalabi i in. 2014, Kaur i in. 2010). Badania nad owocem kiwi wykazały szereg jego właściwości leczniczych, między innymi przeciwzapalnych, regulujących poziom cukru we krwi czy antynowotworowych (Satpal i in. 2021, Richardson i in. 2018). Udowodniono również, że enzym, pozyskany z kiwi działa antybakteryjnie i przeciwutleniająco (Siddique i in. 2021). Aktynidyna wspomaga trawienie białek w żołądku i jelicie (Boeing i in. 2012, Kaur i in. 2010). Isawa i in. (2010) wykazał potencjał immunomodulujący ekstraktu z kiwi w badaniach z myszami, a szczególnie regulację produkcji wielu cytokin oraz aktywności parametrów antyoksydacyjnych.

Wpływ ww. enzymów na procesy fizjologiczne organizmów wodnych nie był do tej pory dostatecznie zbadany. Jednakże wieloletnie badania nad ich właściwościami pozwalają sądzić, iż mogą to być skuteczne środki, wspierające organizm ryb. Dlatego celem niniejszej dysertacji była ocena wpływu dodatku 1% i 2% bromelainy, papainy oraz aktynidyny w paszy na tempo wzrostu, histologię wątroby i przewodu pokarmowego, aktywność wybranych enzymów trawiennych oraz wybrane parametry nieswoistej odporności humoralnej i komórkowej ryb. Badania przeprowadzono na młodocianych formach sterleta (*Acipenser ruthenus*), ryby szeroko rozpowszechnionej w akwakulturze, która jest odporna na manipulacje i stres oraz łatwo adoptuje się do nowych warunków środowiskowych i żywieniowych (zmiany paszy).

4. Materiał i metody

4.1. Warunki podchowu

Eksperymentalny podchów jesiotrów prowadzono w Zakładzie Ichtiologii Hydrobiologii i Ekologii Wód Instytutu Rybactwa Śródlądowego – Państwowego Instytutu Badawczego (IRS - PIB) w Olsztynie. Układ doświadczalny stanowiły trzy oddzielne obiegi recyrkulacyjne (RAS), składające się z trzech basenów o pojemności 280 dm^3. Do badań wybrano sterlety, które po umieszczeniu w basenach były poddane 7-dniowej aklimatyzacji. Doświadczenie składało się z trzech wariantów żywieniowych (2 grupy doświadczalne i grupa kontrolna) w trzech powtórzeniach (n=3). W celu zapewnienia optymalnych warunków podchowu, w trakcie doświadczenia monitorowano właściwości fizykochemiczne wody tj. temperaturę, zawartość tlenu, azotu amonowego i pH. Czas trwania każdego eksperymentu wynosił 56 dni.

4.2. Przygotowanie paszy doświadczalnej

Komercyjną paszę Nutra T-2.0 (Skretting, France) o składzie podstawowym białko 54%, tłuszcz 18% i granulacji 1,9 mm suplementowano bromelainą (Sigma-Aldrich, USA) (Eksperyment 1), papainą (Sigma-Aldrich, USA) (Eksperyment 2) i aktynidyną (KiwiEnzyme.com Ltd., New Zeland) (Eksperyment 3). We wszystkich doświadczeniach dawka enzymu owocowego wynosiła odpowiednio 1 g i 2 g 100 g $paszy^{-1}$. Kontrolę (grupa C) stanowiły ryby żywione wyłącznie paszą bazową, bez dodatku enzymu. Pasze eksperymentalne przechowywano w lodówce w temperaturze 4°C. Dzienną dawkę pokarmową ustalano raz w tygodniu na podstawie biomasy ryb i tabel żywieniowych producenta paszy. Ryby żywiono za pomocą karmników automatycznych (SDK AFF04, SDK Polska) 12 razy na dobę.

4.3. Wskaźniki hodowlane

Pomiary masy (W; ± 0,01 g) i długości całkowitej ryb (TL; ± 0,1 cm) wykonano na początku i na końcu eksperymentu. Co siedem dni wykonywano pomiary biomasy ryb w celu określenia dawki paszy. Po wykonaniu pomiarów określano podstawowe wskaźniki wzrostu oraz wykorzystania paszy: dobowy przyrost masy ciała (DGR, g d^{-1}), względny przyrost masy ciała (SGR, % d^{-1}), współczynnik kondycji ryb (CF), współczynnik pokarmowy pasz (FCR), współczynnik wydajności wzrostowej białka (PER). Na zakończenie eksperymentu pobierano trzewia oraz wątrobę w celu określenia współczynnika wątrobowosomatycznego (HSI, %) i trzewiowosomatycznego (VSI, %).

4.4. Analiza aktywności enzymów trawiennych

W celu wykazania wpływu badanego suplementu na aktywność enzymatyczną, z każdej grupy badawczej, pobierano wycinki jelita przedniego i tylnego. Próby umieszczano w ciekłym azocie, a następnie przechowywano w temperaturze -80°C. Materiał przeznaczony do analiz aktywności enzymów homogenizowano w buforach, a następnie odwirowywano w temperaturze 4°C przez 15 min przy 15000×*g*. Analizowano następujące enzymy endogenne: fosfatazę alkaliczną (ALP), fosfatazę kwaśną (ACP), aminopeptydazę leucynową (LAP), amylazę, lipazę i trypsynę. Wszystkie analizy enzymów przeprowadzono w trzech powtórzeniach (n=3). Procedury analiz enzymów trawiennych przeprowadzono zgodnie z metodyką opisaną przez Kamaszewski i in. (2014a, 2014b).

4.5. Analiza histologiczna wybranych tkanek

W dniu zakończenia eksperymentu do analiz pobrano wątrobę oraz środkowy odcinek przewodu pokarmowego. Tkanki utrwalano w płynie Bouina, odwadniano w etanolu, prześwietlano w ksylenie i zatapiano w bloczki parafinowe, które następnie krojono mikrotomem rotacyjnym na skrawki o grubości 5 µm. Preparaty zostały wybarwione hematoksyliną i eozyną (HiE). Obserwacje mikroskopowe przekrojów wątroby i jelita oraz pomiary histomorfometryczne były wykonywane przy pomocy mikroskopu świetlnego (Olympus Cx31, Japan lub Nikon ECLIPSE 90i, Japan). Do obserwacji i pomiarów strukturalnych użyto programu komputerowego MultiScanBase (Computer Scanning System Ltd., Warsaw, Poland) lub NIS-Elements AR (Nikon Corporation, Japan). U każdego osobnika przeprowadzono następujące pomiary: w wątrobie – wielkość hepatocytów i ich jąder, w jelicie – grubość mięśniówki, wysokość fałdów jelitowych, wysokość enterocytów, wysokość przestrzeni nadjądrowej, wielkość jąder enterocytów (µm). Pomiary histologiczne obejmowały 50 komórek i jąder analizowanych tkanek, pobranych od każdego osobnika.

4.6. Wskaźniki immunologiczne

4.6.1. Nieswoista odpowiedź humoralna

Na zakończenie eksperymentu od 10 osobników z każdego wariantu doświadczalnego pobrano krew z żyły ogonowej. Po jej odwirowaniu (5000 *g*, 10 min) określano wybrane parametry odporności humoralnej w plazmie krwi. Analizowano poziom białka całkowitego, poziom całkowitych immunoglobulin (Ig), aktywność lizozymu i zawartość ceruloplazminy Cp (IU).

4.6.2. Nieswoista odpowiedź komórkowa

Na zakończenie eksperymentu w celu wyizolowania komórek immunokompetentnych, od ryb z każdego wariantu pobierano do analizy śledzionę. Badania immunologicznie wykonano w Zakładzie Ichtiopatologii i Ochrony Zdrowia Ryb IRS-PIB. Określano aktywność

metaboliczną makrofagów metodą spektrofotometryczną po stymulacji komórek PMA (Phorbol 12-Myristate 13-Acetate Sigma). Makrofagi izolowano ze śledziony po odwirowaniu komórek w gradiencie Gradisol G (Polfa). Aktywność fagocytarną makrofagów PKA (Potential Killing Activity) określono metodą spektrofotometryczną po stymulacji komórek przez *Aeromonas hydrophila*. Aktywność proliferacyjną limfocytów określono na podstawie odpowiedzi proliferacyjnej limfocytów T stymulowanych konkanawaliną a (ConA, Sigma) i limfocytów B stymulowanych lipopolisacharydem (LPS) przy pomocy testu MTT.

Procedury ww. analiz immunologicznych przeprowadzono zgodnie z metodyką opisaną przez Siwicki i Anderson (1993), Anderson i Siwicki (1994), Parry i in. (1965) oraz Rice i in. (1963).

4.7. Analiza statystyczna

Wyniki badań poddano analizie statystycznej za pomocą programu GraphPad Prism (Soft. Inc., USA) lub SAS (Soft Inc., USA). Wszystkie dane przetestowano pod kątem rozkładu normalnego i jednorodności wariancji. Różnice pomiędzy grupami badano jednoczynnikową analizą wariancji ANOVA. W przypadku różnic istotnych statystycznie ($P \leq 0,05$) zastosowano test post hoc Tukeya lub Duncana. Wartości przedstawiono jako średnie ± odchylenie standardowe (SD).

5. Wyniki

5.1. Wskaźniki wzrostowe i wykorzystanie składników pokarmowych

5.1.1. Wpływ bromelainy – Eksperyment 1

Dodatek bromelainy w paszy istotnie wpłynął na tempo wzrostu ryb. Wyższą końcową masę ciała odnotowano w grupie B2 w porównaniu do grupy kontrolnej (162,19 g *vs*. 147,27 g; $P < 0,05$; Tabela 1). Końcowa długość ciała była istotnie wyższa w grupach B1 i B2 ($P < 0,05$; Tabela 1). Także w tych grupach wyraźnie wzrosły dobowe (DGR) i względne (SGR) przyrosty masy ciała ($P < 0,05$; Tabela 1). Współczynnik pokarmowy pasz FCR w grupach B1 i B2 był istotnie statystycznie niższy w porównaniu do grupy kontrolnej ($P < 0,05$; Tabela 1). Nie odnotowano wpływu żywienia ryb paszą z dodatkiem bromelainy na kondycję ryb i współczynnik wydajności wzrostowej białka PER ($P < 0,05$; Tabela 1). Wartości indeksu hepatosomatycznego (HSI) i trzewiosomatycznego (VSI) były istotnie wyższe w grupach B1 i B2 w porównaniu do grupy kontrolnej ($P < 0,05$; Tabela 1). Podczas trwania eksperymentu nie odnotowano śmiertelności wśród ryb.

TABELA 1

Wpływ bromelainy (w dawkach 1% – B1 i 2% – B2) na wskaźniki hodowlane sterleta (n=20)

Wskaźniki	Warianty żywieniowe		
	C	B1	B2
Początkowa długość całkowita (cm)	26,28 ± 0,56	26,13 ± 0,23	26,21 ± 0,78
Końcowa długość całkowita (cm)	33,05 ± 0,11^{b}	33,63 ± 0,06^{a}	33,87 ± 0,64^{a}
Masa początkowa (g)	56,04 ± 3,90	54,01 ± 3,09	56,29 ± 3,72
Masa końcowa (g)	147,27 ± 10,53^{c}	156,23 ± 1,23^{b}	162,19 ± 5,75^{a}
Dobowy przyrosty masy ciała (DGR g d^{-1})	1,27 ± 0,18^{c}	1,40 ± 0,06^{b}	1,45 ± 0,12^{a}
Względny przyrost masy ciała (SGR % d^{-1})	1,34 ± 0,16^{c}	1,37 ± 0,22^{b}	1,45 ± 0,13^{a}
Początkowy współczynnik kondycji ryb (ICF)	0,31 ± 0,01	0,30 ± 0,01	0,31 ± 0,01
Końcowy współczynnik kondycji ryb (FCF)	0,41 ± 0,03	0,41 ± 0,01	0,42 ± 0,03
Współczynnik pokarmowy pasz (FCR)	1,12 ± 0,05^{a}	1,04 ± 0,06^{b}	1,04 ± 0,09^{b}
Współczynnik wydajności wzrostowej białka (PER)	1,65 ± 0,08	1,79 ± 0,11	1,79 ± 0,17
Indeks trzewiosomatyczny (VSI)	3,72 ± 0,47^{b}	4,60 ± 0,73^{a}	4,50 ± 0,57^{a}
Indeks wątrobosomatyczny (HSI)	1,05 ± 0,36^{b}	1,30 ± 0,42^{a}	1,40 ± 0,53^{a}

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich ($P < 0,05$).

5.1.2. Wpływ papainy – Eksperyment 2

Suplementacja paszy papainą istotnie wpłynęła na wskaźniki hodowlane oraz współczynniki wykorzystania paszy. Odnotowano istotne różnice końcowej masy ciała ryb w grupach P1 (107,07 g) i P2 (111,98 g) w porównaniu do grupy kontrolnej C (99,73 g) ($P < 0,05$; Tabela 2). Istotne różnice odnotowano także w przypadku dobowego (DGR)

i względnego (SGR) przyrostu masy ciała (P < 0,05; Tabela 2). Współczynnik wykorzystania paszy (FCR) był znacząco niższy w grupie P2 (1,74) w porównaniu do grupy kontrolnej C (1,94) i grupy P1 (1,95) (P < 0,05; Tabela 2). Wyższy współczynnik wydajności wzrostowej białka (PER) odnotowano w grupie P2 w porównaniu do P1 i grupy kontrolnej C (P < 0,05; Tabela 2). Współczynnik wątrobosomatyczny (HSI) w grupie P2 był istotnie wyższy w porównaniu do pozostałych wariantów żywieniowych (P < 0,05). Nie odnotowano różnic w wartościach współczynnika trzewiosomatycznego (VSI). Podczas trwania eksperymentu nie odnotowano śmiertelności wśród ryb.

TABELA 2

Wpływ papainy (w dawkach 1% - P1 i 2% - P2) na wskaźniki hodowlane sterleta (n=30)

Wskaźniki	Warianty żywieniowe		
	C	P1	P2
Początkowa długość całkowita (cm)	21,16 ± 0,51	21,74 ± 0,39	20,99 ± 0,89
Końcowa długość całkowita (cm)	29,45 ± 0,37	29,67 ± 0,53	29,56 ± 0,43
Masa początkowa (g)	36,92 ± 1,41	38,38 ± 3,05	36,22 ± 1,03
Masa końcowa (g)	99,73 ± 2,71^{c}	107,07 ± 7,66^{b}	111,98 ± 1,93^{a}
Dobowy przyrosty masy ciała (DGR g d^{-1})	1,12 ± 0,04^{c}	1,23 ± 0,08^{b}	1,35 ± 0,02^{a}
Względny przyrost masy ciała (SGR % d^{-1})	1,77 ± 0,06^{c}	1,83 ± 0,03^{b}	2,02 ± 0,02^{a}
Początkowy współczynnik kondycji ryb (ICF)	0,39 ± 0,02	0,37 ± 0,02	0,39 ± 0,04
Końcowy współczynnik kondycji ryb (FCF)	0,39 ± 0,01^{b}	0,40 ± 0,01^{a}	0,41 ± 0,01^{a}
Współczynnik pokarmowy pasz (FCR)	1,94 ± 0,07^{a}	1,95 ± 0,03^{a}	1,74 ± 0,02^{b}
Współczynnik wydajności wzrostowej białka (PER)	0,92 ± 0,03^{b}	0,91 ± 0,03^{b}	1,03 ± 0,01^{a}
Indeks trzewiosomatyczny (VSI)	5,59 ± 1,10	5,29 ± 0,91	5,51 ± 0,81
Indeks wątrobosomatyczny (HSI)	0,92 ± 0,35^{c}	1,16 ± 0,23^{b}	1,42 ± 0,50^{a}

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich (P < 0,05).

5.1.3. Wpływ aktynidyny – Eksperyment 3

Suplementacja paszy aktynidyną istotnie wpłynęła na końcowe wartości wskaźników hodowlanych ryb i wykorzystanie paszy (Tabela 3). Na zakończenie eksperymentu najwyższą masę ciała sterletów odnotowano w grupach A1 (112,88 g; P < 0,05) oraz A2 (106,40 g) i była istotnie statystycznie wyższa w porównaniu do grupy kontrolnej C (87,80 g; P < 0,05). Podobne rezultaty uzyskano w przypadku końcowej średniej długości ciała ryb TL. Widoczne różnice w końcowej masie ciała i długości całkowitej ryb korelują z dobowym (DGR) i względnym (SGR) przyrostem masy ciała. W przypadku obu wskaźników najwyższe ich wartości uzyskano także w grupach A1 i A2 (Tabela 3; P < 0,05). Suplementacja paszy aktynidyną wpłynęła także na współczynnik konwersji paszy (FCR). Najniższe wartości tego wskaźnika uzyskano w grupie A1 (0,93) oraz w grupie A2 (1,01) w porównaniu do grupy kontrolnej C (1,32; P < 0,05). W przypadku współczynnika wydajności wzrostowej białka (PER) najwyższe wartości stwierdzono również w grupach doświadczalnych A1 (1,92) i A2 (1,77) w porównaniu do grupy kontrolnej C (1,35; P < 0,05). Nie zaobserwowano różnic w przypadku indeksu wątrobowosomatycznego (HSI) i trzewio-

TABELA 3

Wpływ aktynidyny (w dawkach 1% – A1 i 2% – A2) na wskaźniki hodowlane sterleta (n=30)

Wskaźniki	Warianty żywieniowe		
	C	A1	A2
Początkowa długość całkowita (cm)	23,61 ± 0,32	23,70 ± 0,14	23,66 ± 0,27
Końcowa długość całkowita (cm)	28,60 ± 2,67^{b}	32,09 ± 0,06^{a}	31,92 ± 0,11^{a}
Początkowa masa ciała (g)	46,52 ± 0,70	45,80 ± 0,53	46,32 ± 0,16
Końcowa masa ciała (g)	87,80 ± 1,51^{c}	112,88 ± 1,20^{a}	106,40 ± 1,37^{b}
Dobowy przyrosty masy ciała (DGR g d^{-1})	0,74 ± 0,02^{c}	1,20 ± 0,03^{a}	1,07 ± 0,03^{b}
Względny przyrost masy ciała (SGR % d^{-1})	1,13 ± 0,03^{c}	1,61 ± 0,04^{a}	1,48 ± 0,03^{b}
Początkowy współczynnik kondycji ryb (ICF)	0,35 ± 0,01	0,34 ± 0,01	0,35 ± 0,01
Końcowy współczynnik kondycji ryb (FCF)	0,39 ± 0,11	0,34 ± 0,01	0,33 ± 0,01
Współczynnik pokarmowy pasz (FCR)	1,32 ± 0,03^{a}	0,93 ± 0,02^{b}	1,01 ± 0,02^{b}
Współczynnik wydajności wzrostowej białka (PER)	1,35 ± 0,03^{c}	1,92 ± 0,05^{a}	1,77 ± 0,03^{b}
Indeks trzewiosomatyczny (VSI)	5,99 ± 1,00	5,10 ± 0,86	5,35 ± 0,81
Indeks wątrobosomatyczny (HSI)	0,97 ± 0,24	1,10 ± 0,21	1,35 ± 0,42

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich (P < 0,05).

wosomatycznego (VSI). W trakcie trwania doświadczenia w żadnej badanej grupie nie odnotowano śmiertelności wśród ryb.

5.2. Wpływ enzymów owocowych na aktywność enzymów trawiennych ryb

5.2.1. Wpływ bromelainy – Eksperyment 1

Wraz z rosnącymi dawkami bromelainy w paszy obserwowano proporcjonalny spadek aktywności enzymów początkowego odcinka przewodu pokarmowego sterleta tj. aminopeptydazy leucynowej, amylazy, trypsyny i fosfatazy alkalicznej (P < 0,05, Tabela 4). Natomiast aktywność lipazy rosła i w grupie B1 była wyższa o 3,7 IU g^{-1}, a w grupie B2 o 5,2 IU g^{-1} w porównaniu do grupy kontrolnej (P < 0,05, Tabela 4).

TABELA 4

Wpływ bromelainy (w dawkach 1% – B1 i 2% – B2) na aktywność enzymów w jelicie przednim sterleta (n=5)

	Warianty żywieniowe		
	C	B1	B2
Fosfataza alkaliczna ALP (IU g^{-1})	72,72 ± 7,55^{a}	63,54 ± 10,39^{b}	51,42 ± 5,56^{c}
Fosfataza kwaśna ACP (IU g^{-1})	3,16 ± 0,64^{a}	3,26 ± 0,54^{a}	3,24 ± 0,69^{a}
Aminopeptydaza leucynowa LAP (IU g^{-1})	3,64 ± 0,40^{a}	2,79 ± 0,35^{b}	2,94 ± 0,39^{b}
Amylaza (IU g^{-1})	53,70 ± 10,81^{a}	41,98 ± 4,01^{ab}	34,00 ± 10,06^{b}
Trypsyna (IU g^{-1})	79,70 ± 2,72^{a}	37,87 ± 7,33^{c}	36,00 ± 12,58^{c}
Lipaza (IU g^{-1})	2,84 ± 0,79^{c}	6,54 ± 2,11^{b}	8,08 ± 2,46^{a}

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich (P < 0,05).

5.2.2. Wpływ papainy – Eksperyment 2

W przednim odcinku jelita wraz ze wzrostem zawartości papainy w paszy obserwowano istotny statystycznie spadek aktywności trypsyny (w grupach P1 i P2) oraz lipazy (w grupie P1; $P < 0,05$; Tabela 5). Nie stwierdzono różnic w aktywności aminopeptydazy leucynowej i amylazy. Natomiast w przypadku tylnej części jelita w grupach ryb, żywionych paszą z dodatkiem papainy wyraźnie wzrosła aktywność aminopeptydazy leucynowej (w grupie P2), amylazy (w grupie P2) i lipazy (w grupach P1 i P2) w porównaniu do grupy kontrolnej ($P < 0,05$, Tabela 5). Aktywność trypsyny nie różniła się istotnie pomiędzy grupami.

TABELA 5

Wpływ papainy (w dawkach 1% – P1 i 2% – P2) na aktywność enzymów przewodu pokarmowego sterleta (n=5)

	Warianty żywieniowe		
	C	P1	P2
Jelito przednie			
Amylaza (IU g^{-1})	35,24 ± 15,11	36,66 ± 11,96	26,77 ± 14,22
Trypsyna (IU g^{-1})	3,59 ± 1,13^{a}	1,86 ± 0,22^{b}	2,32 ± 0,45^{b}
Lipaza (IU g^{-1})	142,42 ± 63,94^{a}	47,75 ± 7,85^{b}	123,81 ± 29,40^{a}
Aminopeptydaza leucynowa LAP (IU g^{-1})	19,43 ± 4,29	20,31 ± 5,43	19,40 ± 6,24
Jelito tylne			
Amylaza (IU g^{-1})	27,04 ± 8,93^{b}	19,42 ± 9,00^{b}	47,90 ± 17,41^{a}
Trypsyna (IU g^{-1})	1,31 ± 0,66	1,85 ± 0,79	1,96 ± 1,08
Lipaza (IU g^{-1})	146,42 ± 18,23^{c}	170,63 ± 21,14^{b}	194,53 ± 13,91^{a}
Aminopeptydaza leucynowa LAP (IU g^{-1})	27,57 ± 7,53^{b}	18,60 ± 8,10^{c}	36,52 ± 10,42^{a}

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich ($P < 0,05$).

5.2.3. Wpływ aktynidyny – Eksperyment 3

Wpływ aktynidyny na wydzielanie enzymów trawiennych przedstawia tabela 6. W przedniej części jelita nie odnotowano różnic w poziomie analizowanych enzymów, choć w przypadku lipazy i trypsyny można dostrzec tendencję spadkową wraz ze wzrostem dawki aktynidyny w paszy, tym niemniej nie była ona statystycznie istotna ($P > 0,05$). W tylnej części jelita zaobserwowano znaczący wzrost fosfatazy alkalicznej (ALP) w grupie A2 (179,1 IU g^{-1}) i A1 (113,05 IU g^{-1}) w porównaniu do grupy kontrolnej C (92,4 IU g^{-1}). Z kolei poziom fosfatazy kwaśnej (ACP) był istotnie wyższy w grupie A2 (2,57 IU g^{-1}) w porównaniu do grupy A1 (1,53 IU g^{-1}). Choć poziom aktywności lipazy i trypsyny w tylnej części jelita wzrastał w grupach A1 i A2 w porównaniu do grupy kontrolnej C, tym nie mniej był on statystycznie nieistotny ($P > 0,05$; Tabela 6).

5.3. Analiza histologiczna jelita cienkiego i wątroby

5.3.1. Wpływ bromelainy – Eksperyment 1

Średnia wielkość hepatocytów ryb z trzech grup żywieniowych była podobna i kształtowała się na poziomie 15,23-15,74 µm ($P > 0,05$, Tabela 7). Nie odnotowano także różnic w średniej

TABELA 6

Wpływ aktynidyny(w dawkach 1% – A1 i 2% – A2) na aktywność enzymów przewodu pokarmowego sterleta (n=5)

	Warianty żywieniowe		
	C	A1	A2
Jelito przednie			
Fosfataza alkaliczna (ALP) (IU g^{-1})	112,51 ± 22,32	71,94 ± 49,86	103,22 ± 59,47
Fosfataza kwaśna (ACP) (IU g^{-1})	1,48 ± 0,36	1,07 ± 0,70	1,21 ± 0,72
Lipaza (IU g^{-1})	10,56 ± 4,94	8,22 ± 6,75	8,12 ± 5,61
Trypsyna (IU g^{-1})	72,82 ± 75,32	41,47 ± 51,20	43,09 ± 50,64
Jelito tylne			
Fosfataza alkaliczna (ALP) (IU g^{-1})	92,44 ± 37,71^{c}	113,05 ± 59,89^{b}	179,06 ± 83,45^{a}
Fosfataza kwaśna (ACP) (IU g^{-1})	1,96 ± 0,52^{ab}	1,53 ± 0,66^{b}	2,57 ± 0,40^{a}
Lipaza (IU g^{-1})	4,21 ± 2,25	4,63 ± 3,41	6,72 ± 5,51
Trypsyna (IU g^{-1})	6,56 ± 8,79	10,29 ± 14,99	22,14 ± 20,11

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich (P < 0,05).

TABELA 7

Analiza histologiczna wątroby i jelita sterleta żywionego paszą z dodatkiem bromelainy (w dawkach 1% – B1 i 2% – B2; n=5)

Morfometria	Warianty żywieniowe		
	C	B1	B2
Wielkość hepatocytów (µm)	15,43 ± 0,67	15,74 ± 0,49	15,23 ± 0,79
Wielkość jąder hepatocytów (µm)	4,89 ± 0,16	4,92 ± 0,23	4,68 ± 0,41
Indeks hepatonukleotyczny	0,31 ± 0,07	0,31 ± 0,03	0,30 ± 0,05
Grubość mięśniówki jelita (µm)	165,03 ± 40,51	172,45 ± 42,34	174,63 ± 33,33
Wysokość fałdów jelitowych (µm)	501,21 ± 42,56^{b}	595,82 ± 24,37^{a}	590,24 ± 48,51^{a}
Wysokość enterocytów (µm)	35,42 ± 5,21^{b}	37,56 ± 4,81^{b}	44,54 ± 3,41^{a}
Wysokość przestrzeni nadjądrowej enterocytów (µm)	13,28 ± 1,23^{b}	14,87 ± 0,61^{a}	15,24 ± 1,16^{a}
Wielkość jąder enterocytów (µm)	5,01 ± 0,45	5,20 ± 0,45	4,99 ± 0,17

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich (P < 0,05).

wielkości jąder komórek wątrobowych oraz indeksie hepatonukleotycznym (P > 0,05, Tabela 7). Średnia grubość mięśniówki jelita ryb z trzech grup żywieniowych mieściła się w przedziale 165,03 - 174,63 µm (P > 0,05, Tabela 7). Wysokość fałdów jelitowych oraz wysokość przestrzeni nadjądrowej komórek nabłonkowych były istotnie wyższe w grupach B1 i B2 (P < 0,05, Tabela 7), natomiast wysokość komórek nabłonkowych jelita była wyższa w grupie B2 w porównaniu do grupy kontrolnej C (44,54 µm *vs*. 35,42; P < 0,05, Tabela 7). Wielkość jąder enterocytów była podobna we wszystkich analizowanych grupach (P > 0,05, Tabela 7). Analiza makroskopowa jak i mikroskopowa wątroby oraz środkowego odcinka jelita nie wykazały zmian patologicznych w grupie kontrolnej i u ryb żywionych rosnącymi dawkami bromelainy.

5.3.2. Wpływ papainy – Eksperyment 2

Wyniki pomiarów histologicznych wątroby i środkowego odcinka jelita przedstawiono w tabeli 8. Nie odnotowano istotnego wpływu papainy na wielkość hepatocytów i ich jąder oraz grubość mięśniówki jelita. Istotnie wzrosła natomiast wysokość enterocytów jelita oraz wysokość powierzchni nadjądrowej enterocytów jelita w grupach P1 i P2 ($P < 0,05$, Tabela 8). Nie odnotowano także zmian patologicznych w obrazie wątroby i jelita.

TABELA 8

Analiza histologiczna wątroby i jelita sterleta żywionego paszą z dodatkiem papainy (w dawkach 1% – P1 i 2% – P2; n=7)

Morfometria	Warianty żywieniowe		
	C	P1	P2
Rozmiar hepatocytów (µm)	16,01 ± 0,82	16,23 ± 0,29	15,97 ± 0,66
Wielkość jąder hepatocytów (µm)	4,68 ± 0,28	4,85 ± 0,38	4,79 ± 0,16
Indeks hepatonukleotyczny	0,29 ± 0,08	0,30 ± 0,05	0,30 ± 0,04
Grubość mięśniówki jelita (µm)	177,11 ± 40,16	180,21 ± 52,17	186,47 ± 46,87
Wysokość fałdów jelitowych (µm)	605,89 ± 64,21	621,93 ± 39,25	614,76 ± 31,85
Wysokość enterocytów (µm)	41,12 ± 4,11^{c}	43,32 ± 3,27^{b}	47,81 ± 2,68^{a}
Wysokość przestrzeni nadjądrowej enterocytów (µm)	12,98 ± 0,89^{b}	14,27 ± 0,44^{a}	14,99 ± 0,37^{a}
Wielkość jąder enterocytów (µm)	4,81 ± 0,27	4,71 ± 0,33	4,88 ± 0,61

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich ($P < 0,05$).

5.3.3. Wpływ aktynidyny – Eksperyment 3

W tabeli 9 przedstawiono wyniki pomiarów histologicznych wątroby i jelita cienkiego. Nie odnotowano statystycznie istotnych różnic w wielkości hepatocytów i ich jąder. Suplementacja paszy aktynidyną nie miała także istotnego wpływu na wysokość fałdów jelitowych, enterocytów i przestrzeni nadjądrowej enterocytów ($P > 0,05$). W trakcie pomiarów histologicznych komórek wątroby i jelita cienkiego w żadnej analizowanej grupie nie stwierdzono zmian patologicznych.

TABELA 9

Analiza histologiczna wątroby i jelita sterleta żywionego paszą z dodatkiem aktynidyny (w dawkach 1% – A1 i 2% – A2; n=6)

Morfometria	Warianty żywieniowe		
	C	A1	A2
Powierzchnia hepatocytów (µm^2)	174,75 ± 55,62	141,10 ± 13,95	139,81 ± 8,09
Wysokość fałdów jelitowych (µm)	349,72 ± 64,35	294,81± 56,31	367,74 ± 122,24
Wysokość enterocytów (µm)	40,49 ± 14,96	29,06 ± 3,01	28,52 ± 3,91
Wysokość przestrzeni nadjądrowej enterocytów (µm)	18,98 ± 3,63	17,94 ± 2,12	17,48 ± 2,64

5.4. Parametry niespecyficznej odpowiedzi humoralnej i komórkowej

5.4.1. Wpływ bromelainy – Eksperyment 1

Na zakończenie eksperymentu w grupach B1 i B2 stwierdzono istotny wzrost parametrów nieswoistej odporności humoralnej i komórkowej. W grupie B2 odnotowano istotnie wyższą zawartość lizozymu o 3,66 mg L^{-1} w porównaniu do grupy kontrolnej C oraz białka całkowitego o 4,45 g L^{-1} i immunoglobin (Ig) o 2,80 g L^{-1} w porównaniu do grupy B1 ($P < 0,05$; Tabela 10). W grupie B2 wyższa była także aktywność proliferacyjna limfocytów T śledziony w porównaniu do grupy B1 i grupy kontrolnej C ($P < 0,05$; Tabela 10). W odniesieniu do ceruloplazminy we krwi, metabolicznej aktywności makrofagów śledziony oraz aktywności bójczej komórek fagocytarnych śledziony nie stwierdzono różnic istotnych statystycznie w porównaniu do grupy kontrolnej C ($P > 0,05$).

TABELA 10

Wpływ bromelainy (w dawkach 1% – B1 i 2% – B2) na wskaźniki immunologiczne sterleta (n=10)

Wskaźniki immunologiczne	Wariant żywieniowy		
	C	B1	B2
Nieswoista odporność humoralna			
Aktywność lizozymu (mg L^{-1})	$8,78 \pm 2,00^{b}$	$8,39 \pm 2,10^{b}$	$12,44 \pm 3,05^{a}$
Ceruloplazmina (IU)	$54,51 \pm 2,91$	$53,08 \pm 2,75$	$57,17 \pm 5,15$
Białko ogólne (g L^{-1})	$29,21 \pm 4,02^{ab}$	$26,67 \pm 2,94^{b}$	$31,12 \pm 3,54^{a}$
Immunoglobuliny (Ig) (g L^{-1})	$9,91 \pm 1,77^{ab}$	$9,05 \pm 1,90^{b}$	$11,85 \pm 2,47^{a}$
Nieswoista odporność komórkowa			
Metaboliczna aktywność makrofagów śledziony (PMA)(OD)	$0,93 \pm 0,22$	$0,71 \pm 0,25$	$0,92 \pm 0,49$
Potencjalna aktywność bójcza fagocytów śledziony (PKA)(OD)	$0,71 \pm 0,19$	$0,74 \pm 0,17$	$0,97 \pm 0,34$
Aktywność proliferacyjna limfocytów T stymulowanych konkanawaliną A (ConA)(OD)	$0,12 \pm 0,02^{b}$	$0,12 \pm 0,01^{b}$	$0,14 \pm 0,01^{a}$
Aktywność proliferacyjna limfocytów B stymulowanych lipopolisacharydem (LPS)(OD)	$0,10 \pm 0,01^{a}$	$0,08 \pm 0,01^{b}$	$0,08 \pm 0,01^{b}$

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich ($P < 0,05$).

5.4.2. Wpływ papainy – Eksperyment 2

Na zakończenie eksperymentu w dwóch grupach P1 i P2 stwierdzono istotny wzrost wybranych parametrów nieswoistej odporności humoralnej i komórkowej. Najwyższy poziom ceruloplazminy we krwi stwierdzono w grupie P2 (59,54 IU) i był on istotnie wyższy w porównaniu do grupy kontrolnej C (52,57 IU) oraz grupy P1 (54,61 IU) ($P < 0,05$; Tabela 11). W grupie P1 odnotowano istotnie wyższą zawartość immunoglobulin (16,75 g L^{-1}) w porównaniu do grupy kontrolnej C. W przypadku metabolicznej aktywności makrofagów (PMA) śledziony oraz aktywności bójczej komórek fagocytarnych (PKA) śledziony, stwierdzono istotne statystycznie różnice w grupach suplementowanych papainą w porównaniu do grupy kontrolnej ($P < 0,05$). Aktywność proliferacyjna limfocytów T i B śledziony była istotnie wyższa

TABELA 11

Wpływ papainy (w dawkach 1% – P1 i 2% – P2) na wskaźniki immunologiczne sterleta (n=10)

Wskaźniki immunologiczne	Wariant żywieniowy		
	C	P1	P2
Nieswoista odporność humoralna			
Aktywność lizozymu (mg L^{-1})	2,73 ± 1,05	2,67 ± 0,63	2,61± 0,95
Ceruloplazmina (IU)	52,57 ± 4,38^{b}	54,61±4,39^{b}	59,54±5,88^{a}
Białko ogólne (g L^{-1})	31,05± 4,01	33,43± 5,28	34,18± 6,26
Immunoglobuliny (Ig) (g L^{-1})	11,93± 3,97^{b}	16,75± 4,05^{a}	15,86± 5,06^{ab}
Nieswoista odporność komórkowa			
Metaboliczna aktywność makrofagów śledziony (PMA)(OD)	0,33±0,08^{b}	0,41±0,11^{a}	0,47±0,13^{a}
Potencjalna aktywność bójcza fagocytów śledziony (PKA)(OD)	0,33± 0,07^{b}	0,40± 0,13^{a}	0,41±0,17^{a}
Aktywność proliferacyjna limfocytów T stymulowanych konkanawaliną A(ConA)(OD)	0,09±0,01^{b}	0,09±0,01^{b}	0,12±0,01^{a}
Aktywność proliferacyjna limfocytów B stymulowanych lipopolisacharydem (LPS)(OD)	0,08± 0,06^{b}	0,07±0,002^{b}	0,11±0,01^{a}

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich (P < 0,05).

w grupie P2 w porównaniu do grupy kontrolnej C i grupy P1 (P < 0,05; Tabela 11). Nie stwierdzono istotnych różnic w aktywności lizozymu oraz aktywności białka całkowitego (P > 0,05).

5.4.3. Wpływ aktynidyny – Eksperyment 3

W przypadku parametrów niespecyficznej humoralnej odporności ryb odnotowano wzrost wartości lizozymu w grupach A1 i A2. W grupach tych istotnie statystycznie wzrosła też zawartość immunoglobulin (Ig) o odpowiednio 3,40 i 3,98 g L^{-1} w porównaniu do grupy kontrolnej (P < 0,05; Tabela 12). W przypadku niespecyficznej odpowiedzi komórkowej odnotowano wyższą aktywność metaboliczną makrofagów i aktywność bójczą fagocytów śledziony w grupach A1 i A2 (P < 0,05; Tabela 12). Nie stwierdzono natomiast wzmożonej aktywności limfocytów T i B w śledzionie (P > 0,05; Tabela12).

TABELA 12

Wpływ aktynidyny podawanej w paszy (w dawkach 1% – A1 i 2% – A2) na wskaźniki immunologiczne sterleta (średnia ± SD, n = 10)

Wskaźniki immunologiczne	Wariant żywieniowy		
	C	A1	A2
Nieswoista odporność humoralna			
Aktywność lizozymu (mg L^{-1})	3,97 ± 0,27^{b}	4,68 ± 0,25^{a}	4,71 ± 0,28^{a}
Ceruloplazmina (IU)	31,21 ± 1,66	33,68 ± 3,85	31,59 ± 3,57
Białko ogólne (g L^{-1})	25,68 ± 3,66	28,02 ± 2,71	29,13 ± 4,82
Immunoglobuliny (Ig) (g L^{-1})	11,00 ± 1,00^{b}	14,40 ± 1,30^{a}	14,98 ± 2,53^{a}
Nieswoista odporność komórkowa			
Metaboliczna aktywność makrofagów śledziony (PMA)(OD)	0,22 ± 0,04^{c}	0,32 ± 0,03^{b}	0,39 ± 0,04^{a}
Potencjalna aktywność bójcza fagocytów śledziony (PKA)(OD)	0,24 ± 0,05^{c}	0,31 ± 0,02^{b}	0,45 ± 0,06^{a}
Aktywność proliferacyjna limfocytów T stymulowanych konkanawaliną A(ConA)(OD)	0,11 ± 0,02	0,10 ± 0,01	0,12 ± 0,03
Aktywność proliferacyjna limfocytów B stymulowanych lipopolisacharydem (LPS)(OD)	0,11 ± 0,02	0,10 ± 0,00	0,11 ± 0,01

Górne indeksy literowe oznaczają różnice istotne statystycznie wartości średnich (P < 0,05).

6. Dyskusja

W przeprowadzonych doświadczeniach badano wpływ enzymów owocowych: bromelainy, papainy oraz aktynidyny dodawanych do paszy komercyjnej (w dawkach 1% i 2%) na wskaźniki hodowlane, wybrane parametry odporności humoralnej i komórkowej, aktywność enzymów trawiennych, parametry stresu oksydacyjnego oraz histologię jelita cienkiego i wątroby sterleta. Dodatek ww. enzymów w pokarmie ryb istotnie wpłynął na analizowane wskaźniki.

Intensyfikacja akwakultury i kurczące się zasoby naturalne wykorzystywane do produkcji pasz, prowadzą do poszukiwań alternatywnych źródeł białka. W związku z tym, podejmowane są próby wykorzystania rolniczych produktów ubocznych pochodzenia roślinnego do produkcji pasz (Van Doan i in. 2021). To alternatywne źródło białka, które występuje w produktach roślinnych często nie jest w pełni wykorzystywane przez organizmy wodne. Enzymy roślinne o właściwościach proteolitycznych mogą być zatem z powodzeniem wykorzystywane jako dodatek, który zwiększy dostępność białka w pokarmie (Leung-Toung i in. 2005). W trakcie przeprowadzonych doświadczeń, ryby żywione paszą z dodatkiem enzymów owocowych osiągnęły istotnie wyższe przyrosty masy ciała w porównaniu do grupy kontrolnej. Suplementacja paszy enzymami w dawkach 10 lub 20 g kg paszy^{-1} korzystnie wpłynęła na wartości wskaźników FCR, PER oraz SGR w porównaniu z grupą kontrolną C. Poprawa wartości współczynnika pokarmowego i wydajności wzrostowej białka, odnotowane na zakończenie doświadczeń mogą wskazywać na proteolityczne właściwości zastosowanych enzymów. Proteazy cysteinowe zawarte w bromelainie i papainie mają istotny wpływ na hydrolizę białek do peptydów krótkołańcuchowych, co zwiększa strawność pokarmu (Nilsang i in. 2005, Manosroi i in. 2014, Sawant i Nagendran 2014). Z kolei aktynidyna została zidentyfikowana jako enzym, wspierający proces hydrolizy wielu rodzajów białek, w tym glutenu (Chalabi i in. 2014, Boeing i in. Kaur i in. 2010). Wzrost współczynnika wydajności wzrostowej oraz względnego przyrostu masy ciała odnotowano w doświadczeniu z amurem białym *Ctenopharyngodon idella,* dodając do paszy ryb bromelainę i papainę – w dawkach 1% i 2% (Choi i in. 2016). Podobne efekty uzyskali Subandiyono i in. (2018), po zastosowaniu ekstraktu z ananasa w żywieniu *Puntius javanicus*. Jak donoszą Prabjeet i in. (2011) oraz Tewari i in. (2018), w doświadczeniach z narybkiem karpia *Cyprinus carpio*, papaina poprawiła strawność białka roślinnego, wchodzącego w skład paszy eksperymentalnej. W doświadczeniu żywieniowym z sumem rekinim *Pangasianodon hypophthalmus*, po zastosowaniu papainy oraz mieszaniny papainy i bromelainy odnotowano zwiększone przyrosty masy ciała ryb i istotny spadek wartości współczynnika pokarmowego paszy (Rostika i in. 2018). W eksperymencie, przeprowadzonym na tilapii nilowej *Oreochromis niloticus* z zastosowaniem paszy z dodatkiem mączki, pozyskanej z piór oraz papainy w dawce 4,5%, uzyskano wyższe wskaźniki strawności paszy (Munguti i in. 2014). W świetle uzyskanych wyników badań do niniejszej dysertacji oraz dostępnej literatury można stwierdzić, iż suplementacja paszy komercyjnej zaproponowanymi enzymami owocowymi istotnie przełożyła się na tempo wzrostu sterleta.

Aktynidyna pozyskiwana z owoców kiwi, papaina z papai oraz bromelaina z owoców ananasa należą do rodziny papain – wspólnej grupy proteaz cysteinowych (Ha i in. 2012). Przeprowadzone eksperymenty z dodatkiem bromelainy i papainy w paszy wykazały, iż te enzymy owocowe istotnie wpływają na aktywność lipazy w jelicie sterleta. Podobnych rezultatów spodziewano się także w odniesieniu do trzeciego, analizowanego enzymu roślinnego – aktynidyny. Jednak badania aktywności lipazy po ekspozycji na aktynidynę wykazały, iż lipaza przedniego i tylnego odcinka jelita jest mało wrażliwa lub całkowicie odporna na jej działanie. W doświadczeniu 1 z zastosowaniem bromelainy odnotowano wyraźny spadek aktywności trypsyny w przednim odcinku jelita sterleta. Zaobserwowano tutaj także wyraźną zależność pomiędzy spadkiem aktywności trypsyny a wzrostem poziomu lipazy. Takiej zależności nie stwierdzono po zastosowaniu papainy w eksperymencie 2 i aktynidyny – w eksperymencie 3. W obu tych przypadkach odnotowano spadek aktywności enzymów trawiennych w przednim odcinku jelita sterleta oraz wzrost aktywności trypsyny i lipazy w tylnym odcinku jelita. Wzrost aktywności proteaz zaobserwowano u karpia po zastosowaniu papainy w paszy w dawkach 0,75%, 1,5%, 2,25% i 3% (Rachmawati i in. 2019). Podobna zależność wystąpiła u królika *Oryctolagus cuniculus*. W tym przypadku wraz ze wzrostem dawki papainy w paszy rosła aktywność protezy oraz lipazy (El-Neney i in. 2013). W literaturze brakuje szczegółowych doniesień na temat oddziaływania enzymów owocowych na enzymy trawienne ryb. Jednakże analiza uzyskanych wyników do niniejszej dysertacji pozwala stwierdzić, iż zastosowane owocowe enzymy egzogenne mają wpływ na proces przyswajania składników pokarmowych. Mogą w sposób bezpośredni wspomagać trawienie białek lub wpływać na poziom wydzielanych enzymów endogennych.

W doświadczeniach z suplementacją paszy bromelainą i papainą odnotowano wzrost powierzchni absorpcyjnej enterocytów w grupach doświadczalnych (B1 i B2 oraz P1 i P2). Także wysokość enterocytów była istotnie wyższa w grupach ryb, żywionych paszą z dodatkiem obu enzymów egzogennych w porównaniu do grupy kontrolnej C. Wyższa powierzchnia absorpcyjna enterocytów, przekłada się na poprawę wchłaniania i wykorzystania składników pokarmowych, co także mogło wpłynąć na wyższe przyrosty masy ciała ryb w analizowanych grupach. Podobne rezultaty uzyskali Hassaan i in. (2021). Proteaza dodana do paszy tilapii nilowej (*Oreochromis niloticus*) w dawce 0,5% istotnie wpłynęła na rozwój błony śluzowej jelit ryb. Pomimo, iż aktynidyna należy do tej samej grupy proteaz cysteinowych co bromelaina i papaina, posiada prawdopodobnie inny mechanizm oddziaływania na komórki nabłonkowe jelit. W przypadku tego enzymu, przeprowadzone analizy histologiczne nie wykazały istotnych różnic w pomiarach morfometrycznych przewodu pokarmowego. Suplementacja bromelainy, papainy i aktynidyny nie wpłynęła istotnie na wielkość powierzchni hepatocytów. Także w obrazie makroskopowym i mikroskopowym, komórki wątrobowe nie odbiegały od normy.

W niniejszej dysertacji doktorskiej oceniano także wpływ bromelainy, papainy i aktynidyny na wybrane parametry niespecyficznej odporności komórkowej i humoralnej sterleta. W przypadku odporności u ryb to właśnie niespecyficzne mechanizmy obronne odgrywają kluczową rolę w obronie organizmu przed patogenami. Specyficzne mechanizmy obronne wymagają bowiem dłuższego czasu by zbudować i aktywować przeciwciała (Anderson

1992). Od wielu lat prowadzi się badania nad możliwościami wykorzystania różnych substancji, w tym preparatów roślinnych, do stymulacji odporności (Bricknell i Dalmo 2005, Dügenci i in. 2003). Liczne badania wykazały, iż enzymy z ananasa, papai i aktynidyny mogą pozytywnie oddziaływać na układ immunologiczny (Engwerda i in. 2001, Otsutki i in. 2010, Satpal i in. 2021, Richardson i in. 2018). Fitoenzymy posiadają szerokie spektrum oddziaływania na fizjologię organizmu. W badanich komórkowych *in vitro* stwierdzono że, enzymy pozyskane z ananasa i papai aktywują komórki NK (natural killers - komórki naturalnej cytotoksyczności) i modulują odpowiedź immunologiczną limfocytów T i B (Engwerda i in. 2001, Chandran i Nachimuthu 2018). Przeprowadzone doświadczenia do dysertacji doktorskiej wykazały, iż bromelaina, papaina i aktynidyna stymulują układ odpornościowy sterletów. Enzymy te podawane *per os* istotnie wpłynęły na nieswoiste komórkowe i humoralne mechanizmy obronne sterleta. Dodatek bromelainy w dawce 2% w paszy aktywował niespecyficzne parametry odporności humoralnej sterleta. Ta sama dawka bromelainy indukowała odpowiedź proliferacyjną limfocytów T. Mechanizm działania enzymu, pozyskanego z ananasa, polega na modulowaniu funkcji cząsteczek adhezyjnych w erytrocytach, śródbłonku, makrofagach oraz komórkach NK. Aktywuje on komórki odpornościowe do zwiększonej produkcji cytokin (Maurer 2001). U ryb białko całkowite surowicy odpowiada za wrodzoną odpowiedź immunologiczną i jego poziom skorelowany jest z poziomem immunoglobulin. Im jego poziom jest wyższy, tym silniejsza jest reakcja obronna organizmu (Sahu i in. 2007). Podwyższony poziom frakcji białkowej, pośrednio wskazuje na indukowany przez bromelainę, wzrost stężenia immunoglobulin we krwi sterleta. Efekt immunostymulacji zaobserwowano u amura (*Ctenopharyngodon idella*), po zastosowaniu w paszy mieszaniny 2% bromelainy i papainy. Poziom immunoglobulin i białka całkowitego w osoczu amura był istotnie wyższy w porównaniu do grupy kontrolnej (Choi i in. 2016). Lizozym z kolei jest jednym z najważniejszych parametrów odporności wrodzonej i bierze udział w niszczeniu warstwy peptydoglikanu ścian komórkowych bakterii gram-dodatnich (Alexander i Ingram 1992). Wzrost aktywności lizozymu po stymulacji bromelainą, świadczy o wzmocnieniu mechanizmów obronnych sterleta. W niniejszych badaniach, w przypadku papainy (w dawce 2%) odnotowano istotny statystycznie wzrost poziomu ceruloplazminy i aktywności proliferacyjnej limfocytów T i B, a dawka 1% enzymu w paszy ryb wpłynęła na wzrost poziomu immunoglobulin. Chandran i Nachimuthu (2018) wykazali, iż papaina stymuluje aktywność proliferacyjną limfocytów T na poziomie komórkowym, co również zaobserwowano w niniejszych badaniach, przeprowadzonych na sterlecie. Rola ceruloplazminy jest podobna do interferonu i transferyny. Hamuje bowiem rozwój bakterii, pozbawiając je niezbędnych składników odżywczych, tj. jonów miedzi (Alexander i Ingram 1992). Obie dawki papainy wpłynęły także na wzrost aktywności makrofagów śledziony stymulowanych PMA i aktywności bójczej fagocytów śledziony (PKA). Po stymulacji papainą obserwowano wzrost aktywności fagocytarnej granulocytów i makrofagów. Wyższa aktywność metaboliczna makrofagów stymulowanych PMA świadczy o tym, iż komórki fagocytujące są sprawniejsze i bardziej zdolne do efektywnego wybuchu tlenowego. Ta właściwość przekłada się na wyższą efektywność eliminacji czynnika patogennego. Stymulujące działanie enzymu ujawniło się także w zakresie zdolności fagocytów do wewnątrzkomórkowego zabijania bakterii. Po podawaniu sterletom aktyni-

dyny w paszy w dawkach 1% i 2% istotnie wzrósł poziom lizozymu i immunoglobulin (Ig). Immunoglobuliny są najważniejszymi białkami swoistej odpowiedzi odpornościowej, a ich zadaniem jest ochrona organizmu przed m. in. bakteriami i wirusami. Aktywność komórek immunokompetentnych stanowi pierwszą linię obrony, dlatego ocena ich funkcji pozwala ocenić potencjalną sprawność całego układu immunologicznego (Siwicki i in. 2009, Terech-Majewska i in. 2016). Metaboliczna aktywność makrofagów stymulowanych PMA, izolowanych ze śledziony ryb, była znacząco wyższa w grupach, którym podawano aktynidynę w paszy. Jak wspomniano, wyższe wartości aktywności makrofagów stymulowanych PMA świadczą o tym, iż komórki fagocytarne są bardziej zdolne do efektywnego wybuchu tlenowego, co oznacza sprawniejszą eliminację patogenów. W niniejszych badaniach z wykorzystaniem enzymu z kiwi wzrosła także aktywność bójcza fagocytów, pozyskanych ze śledziony. W przeciwieństwie do bromelainy i papainy, aktynidyna nie stymulowała proliferacyjnej odpowiedzi limfocytów T i B.

7. Wnioski

- Suplementacja paszy enzymami owocowymi pozyskanymi z owoców ananasa (bromelainą), papai (papainą) oraz kiwi (aktynidyną) wpłynęła na poprawę tempa wzrostu sterleta,
- Wszystkie trzy badane substancje aktywowały parametry nieswoistej odporności humoralnej i komórkowej,
  - Dodatek bromelainy istotnie podniósł poziom lizozymu, białka całkowitego i immunoglobulin oraz aktywność proliferacyjną limfocytów T i B,
  - Suplementacja papainy istotnie wpłynęła na wzrost poziomu ceruloplazminy i immunoglobulin oraz aktywność metaboliczną makrofagów oraz aktywność bójczą komórek fagocytarnych i proliferacyjną limfocytów T,
  - Aktynidyna stymulowała aktywność metaboliczną makrofagów i aktywność bójczą fagocytów oraz wpłynęła na wyższe wartości lizozymu i immunoglobulin.
- Analizowane substancje stymulowały wydzielanie wybranych enzymów trawiennych w jelicie cienkim sterleta, przez co wspomagały trawienie pokarmu.
- Bromelaina i papaina stymulowały powierzchnię absorpcyjną enterocytów, co mogło wpłynąć na poprawę wchłaniania składników odżywczych i przyspieszenie tempa wzrostu ryb.
- Zastosowane enzymy owocowe nie powodują zmian patologicznych w analizowanych narządach organizmu ryb i nie wpływają negatywnie na wybrane parametry fizyko-chemiczne wody.
- Potwierdzono wysoki potencjał bromelainy, papainy i aktynidyny jako dodatków funkcjonalnych w żywieniu ryb, a analizowane enzymy mogą być z powodzeniem suplementowane w paszy i stanowić istotny element immunoprofilaktyki nieswoistej w akwakulturze.

8. Literatura

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mój wkład w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników, zbieranie literatury i przygotowanie manuskryptu wyżej wymienionej publikacji, składającej się na rozprawę doktorską wyniósł **50%**.

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Author's contribution:

☐ concept of research, making hypotheses	☐ writing an article
☐ planning of research, selection of research methodology	☐ graphical presentation of the results
☐ conducting research	☐ collecting literature
☐ collecting data	☐ consultations
☑ statistical analysis	☑ proofreading of the manuscript before submitting it to the journal
☐ interpretation of the results	☐ obtaining funds

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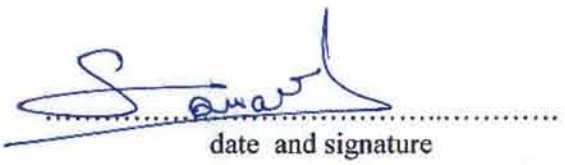

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ORIGINAL ARTICLE

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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*)

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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

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.

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my contribution was 5% of the total contribution to the final version of the above scientific publication.

Author's contribution:

☐ concept of research, making hypotheses	☐ writing an article
☐ planning of research, selection of research methodology	☐ graphical presentation of the results
☐ conducting research	☐ collecting literature
☐ collecting data	☑ consultations
☑ statistical analysis	☐ proofreading of the manuscript before submitting it to the journal
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☐ conducting research	☐ collecting literature
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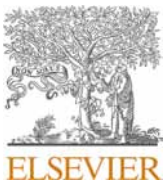

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Beneficial effects of dietary papain supplementation in juvenile sterlet (*Acipenser ruthenus*): Growth, intestinal topography, digestive enzymes, antioxidant response, immune response, and response to a challenge test

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ABSTRACT

Juvenile sterlet (*Acipenser ruthenus*) were fed commercial feed (group C) and experimental feed supplemented with papain in doses of 10 g kg^{-1} feed (P1) and 20 g kg^{-1} feed (P2) for eight weeks. Growth, digestive enzyme activity, immunity parameters, pathological changes of the liver and intestine, proximate body composition, and oxidative response were assayed. A challenge test with *Yersinia ruckeri* was also performed. The final body weights of groups P1 (107.07 ± 7.66 g) and P2 (111.98 ± 1.93 g) were significantly ($P < 0.05$) higher compared to the control group (99.73 ± 2.71 g). The height of the intestinal enterocytes was the greatest in group P2, and the highest supranuclear surface of the intestinal enterocytes was noted in groups P1 and P2. The activities of α-amylase, trypsin, lipase, and leucine aminopeptidase were significantly ($P < 0.05$) higher in the posterior intestine of the fish from group P2 than in the other dietary treatment groups. Ceruloplasmin, total immunoglobulin (Ig), the metabolic activity of splenic macrophages (PMA), and the potential killing activity of splenic phagocytes (PKA) were significantly higher in groups P1 and P2 compared with the control group. The proliferative activities of spleen T and B-lymphocytes were significantly ($P < 0.05$) higher in group P2 in comparison to the control group and group P1. Total antioxidant status (TAS), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were significantly increased in groups P1 and P2 compared with the control group. The glutathione reductase (GLURED) activities decreased significantly ($P < 0.05$) with increasing levels of papain supplementation. Furthermore, during the challenge test, fish survival was significantly higher in groups fed feed supplemented with papain. Finally, the current study indicated that supplementing feed with doses of 10 g and/or 20 g papain kg^{-1} for a period of 56-days improved growth efficiency and feed utilization and also stimulated immunity in aquaculture conditions.

1. Introduction

The intensification of aquaculture production over the past two decades has increased demand for formulated feeds (Bowyer et al., 2019; Davies et al., 2019; Hassaan et al., 2019). Scientists studying fish nutrition report that artificial feed cannot meet all nutrient requirements (Hassaan et al., 2021b; Hassaan et al., 2018; Mohammady et al., 2021; Oluwaseyi, 2016). Veterinary feed supplements, such as drugs, are added as therapeutic or growth agents and to stimulate the immune system (Rico et al., 2013). It is widely known that antibiotic treatments can cause bacterial drug resistance, which is why bioactive supplements are used increasingly in formulated feeds (Harikrishnan et al., 2010; Hassaan et al., 2018). The criteria for selecting feed supplements are that they are natural and safe with high contents of active compounds. Exogenous enzymes play significant roles in the proper development and functioning of the digestive system in larval and juvenile fishes

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(Kolkovski, 2001; Wiszniewski et al., 2019). Adding exogenous enzymes to fish feed can improve the utilization of feed components thus reducing losses. Exogenous enzymes have been confirmed to improve the nutritional value of feed (Hassaan et al., 2018). Incorporating enzymes into diets can considerably improve the utilization efficiency of plant-based proteins and decrease phosphorus loads in the aquatic environment (Ai et al., 2007; Liebert and Portz, 2005; Singh et al., 2011).

Papain is a proteolytic enzyme from the proteinases enzyme family that is extracted from different parts of papaya (*Carica papaya*) such as the leaf, unripe fruit, and fruit sap (Yogiraj et al., 2014). Papain belongs to the group of cysteine proteases that can hydrolyze proteins into short peptides (Mo et al., 2016; Pendzhiev, 2002). Papain is used in the food industry and in medicine (Amri and Mamboya, 2012). This enzyme has anti-inflammatory properties and is a component of many pharmaceuticals (Yogiraj et al., 2014). Amri and Mamboya (2012) noted that papain could break down dietary proteins into amino acids permitting fish to digest feed more easily, which improved feed efficiency. Muchlisin et al. (2016) also noted that the papain enzyme (27.5 mg kg^{-1}) increased feed utilization and growth in keureling (*Tor tambra*). In *Labeo rohita* supplementing with 10 g kg^{-1} feed is enough to increase feed efficiency (Khati et al., 2015). Furthermore, supplementing with 15 g of papain kg^{-1} feed significantly increased weight gain and feed efficiency in *C. gariepinus* (Rachmawati et al., 2019). As a feed supplement, papain increases the digestibility of protein in feeds and improves its assimilation, which affects feed utilization and growth indices advantageously (Wong et al., 1996). Based on earlier reports, the level of dietary papain supplementation required for best performance might be species specific. Papain supplementation has not been studied before in sterlet (*Acipencer ruthenus*), and to our knowledge, to date, there is no information regarding the effect of papain supplementation on non-specific immunity parameters or oxidative response in cultured fish. Therefore, the aim of this study was to examine the impacts of papain supplementation on sterlet growth, immunity parameters, enzymatic activity, the pathological structure of the liver and intestine, proximate body composition, oxidative response, and a challenge test with *Y. ruckeri*.

2. Material and methods

2.1. Rearing conditions

The 56-day feeding trial was conducted at the Stanisław Sakowicz Inland Fisheries Institute (IFI) in Olsztyn, Poland. Juvenile sterlet with an average initial body weight of 37 ± 3.59 g were selected and acclimated to experimental conditions for two weeks. During the acclimatization period, the fish were fed a commercial feed (540 g kg^{-1} crude protein, 22.6 MJ kg^{-1} gross energy) three times daily. Thirty fish were randomly distributed in each of nine tanks (tank volume 280 L) connected to a recirculating aquaculture system (RAS). The physical and chemical water parameters were measured at rearing tank outflows during the experiment and were as follows: water temperature 20 °C (± 0.2); oxygen content 6.05–7.15 mg O_2 L^{-1} (± 0.59), ammonia nitrogen (TAN = NH_4^+-N + NH_3-N) and nitrate (NO_2-N) 0.124–0.167 ± 0.098 mg TAN L^{-1} and 0.009–0.014 ± 0.004 NO_2-N L^{-1}, respectively. The water pH was within the range of 7.4–7.6.

2.2. Diet preparation

Commercial sturgeon feed was used (Nutra T-2.0, Skretting, France; 54% crude protein and 18% lipids) and was divided into three groups. The control group feed was not supplemented, while diets 2 (group P1) and 3 (group P2) were supplemented with the exogenous enzyme papain (Sigma-Aldrich, USA) at 10 g and 20 g kg^{-1} feed, respectively. Following papain supplementation, the feed was prepared according to Wiszniewski et al. (2019).

2.3. Rearing indices

The fish were weighed (W; ± 0.01 g) and their total length was measured (TL; ± 0.1 cm) at the beginning and the end of the experiment. The mean weight of the fish was measured every seven days to determine feed rations. Growth performance and feed utilization parameters (DGR, SGR, CF, FCR, PER, VSI, HIS) were calculated (Table 1) according to the following formulas described in Jarmołowicz et al. (2012).

DGR (daily growth rate, g d^{-1}) = $(W_f - W_i) \times T^{-1}$;

SGR (specific growth rate, % d^{-1}) = $100 \times [(\ln W_2 - \ln W_1) \times t^{-1}]$;

CF (condition coefficient) = $(W \times 100) \times TL^{-3}$;

FCR (feed conversion ratio) = $TFI \times (FB - IB)^{-1}$;

PER (protein efficiency ratio) = $(FB - IB) \times TFP^{-1}$.

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 (d); 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); FBP = final body protein (%); IBP = initial body protein (%); LW = liver weight (g); VW = viscera weight (g).

2.4. Body proximate composition

The proximate composition of whole fish was determined at the end of the experiment in five specimens from each of the three replicates of the dietary treatment groups according to the methods described in Wiszniewski et al. (2019) (Table 2).

2.5. Endogenous enzyme activity analysis and oxidative response

Intestine and liver samples were collected from five fish from each experimental group and stored at −80 °C until analysis. All enzyme analyses were performed in triplicate and measured with an Infinite 200 Pro device (Tecan Austria, Grödig, Austria). The digestive enzyme activity assays of α-amylase (EC 3.2.1.1), lipase (EC 3.1.1.3), trypsin (EC

Table 1
Effect of dietary papain supplementation at 10 g kg^{-1} feed (P1) and 20 g kg^{-1} feed (P2) for 56 days on sterlet rearing parameters (mean ± SD, n = 30).

	Dietary treatment		
	C	P1	P2
Initial body length (cm $fish^{-1}$)	21.16 ± 0.51	21.74 ± 0.39	20.99 ± 0.89
Final body length (cm $fish^{-1}$)	29.45 ± 0.37	29.67 ± 0.53	29.56 ± 0.43
Initial body weight (g $fish^{-1}$)	36.92 ± 1.41	38.38 ± 3.05	36.22 ± 1.03
Final body weight (g $fish^{-1}$)	99.73 ± 2.71^c	107.07 ± 7.66^b	111.98 ± 1.93^a
Daily growth rate (DGR; g day^{-1})	1.12 ± 0.04^c	1.23 ± 0.08^b	1.35 ± 0.02^a
Specific growth rate (SGR; % day^{-1})	1.77 ± 0.06^c	1.83 ± 0.03^b	2.02 ± 0.02^a
Initial condition factor	0.39 ± 0.018	0.37 ± 0.02	0.39 ± 0.04
Final condition factor	0.39 ± 0.01^b	0.40 ± 0.011^a	0.41 ± 0.01^a
Feed conversion ratio (FCR)	1.94 ± 0.07^a	1.95 ± 0.03^a	1.74 ± 0.02^b
Protein efficiency ratio (PER)	092 ± 0.03^b	0.91 ± 0.03^b	1.03 ± 0.01^a
Viscerosomatic index (VSI) (%)	5.59 ± 1.10	5.29 ± 0.91	5.51 ± 0.81
Hepatosomatic index (HSI) (%)	0.92 ± 0.35^c	1.16 ± 0.23^b	1.42 ± 0.50^a

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

Table 2
Effect of dietary papain supplementation at 10 g kg^{-1} feed (P1) and 20 g kg^{-1} feed (P2) for 56 days on sterlet proximate composition (%) (mean ± SD, n = 3).

	Dietary treatment		
	C	P 1	P 2
Whole fish proximate composition (g kg^{-1} ww)			
Protein	168.0 ± 4.8	168.4 ± 3.7	168.7 ± 2.2
Lipid	98.9 ± 3.1	100.1 ± 1.5	98.1 ± 1.7
Moisture	685.5 ± 10.0	701.3 ± 4.5	702.0 ± 8.3
Fillet proximate composition (g kg^{-1} ww)			
Protein	200.7 ± 4.3	195.01 ± 3.5	193.8 ± 4.7
Lipid	33.4 ± 2.1	29.3 ± 1.3	29.9 ± 2.2
Moisture	746.0 ± 18.1	793.7 ± 9.3	758.9 ± 3.2

Means followed by different letters in the same row are significantly different (P < 0.05).

3.4.21.4), leucine aminopeptidase (EC 3.4.11.1), to evaluate protein levels samples of sterlet were first homogenized in buffers and then centrifuged at 4 °C for 15 min at 15,000 g. The specific enzyme activities were analyzed according to procedures described for amylase (Foo and Bais, 1998), lipase (Winkler and Stuckmann, 1979), trypsin (Erlanger et al., 1961), and leucine aminopeptidase (Nagel et al., 1964). Enzyme activities were expressed as the number of micromoles of the reaction product per 1 min calculated for 1 g of protein (IU g^{-1} protein) (Table 3).

The activities of oxidative stress enzymes and total antioxidant status (TAS) were determined in liver samples. Enzymatic analyses were performed using commercially available kits for glutathione reductase (GLURED) (BioAssay Systems, cat. no. ECGR-100), superoxide dismutase (SOD) (BioAssay Systems, cat. no. ESOD-100), glutathione peroxidase (GPx) (Randox RANSEL, cat. no. RS505), and TAS (Randox TAS kit, cat. no. NX2332) following the manufacturers' instructions and methods described in Palińska-Żarska et al. (2021) and Kapusta et al. (2018) (Table 4). Analyses were performed in triplicate at 25 °C. Enzyme activities were expressed as the number of micromoles of reaction product released per 1 min calculated for 1 mg of protein (U/mg protein). TAS results were expressed as mmol Trolox equivalents per 1 mg of protein (mmol/mg) (Table 4).

Table 3
Effect of dietary papain supplementation at 10 g kg^{-1} feed (P1) and 20 g kg^{-1} feed (P2) for 56 days on sterlet digestive enzyme activity.

	Dietary treatment		
	C	P1	P2
Anterior intestine			
α-amylase[1] (IU g^{-1})	35.24 ± 15.11	36.66 ± 11.96	26.77 ± 14.22
Trypsin[1] (IU g^{-1})	3.59 ± 1.13[a]	1.86 ± 0.22[b]	2.32 ± 0.45[b]
Lipase[1] (IU g^{-1})	142.42 ± 63.94[a]	47.75 ± 7.85[b]	123.81 ± 29.40[a]
Leucine aminopeptidase LAP[1] (IU g^{-1})	19.43 ± 4.29	20.31 ± 5.43	19.40 ± 6.24
Posterior intestine			
α-amylase[1] (IU g^{-1})	27.04 ± 8.93[b]	19.42 ± 9.00[b]	47.90 ± 17.41[a]
Trypsin[1] (IU g^{-1})	1.31 ± 0.66	1.85 ± 0.79	1.96 ± 1.08
Lipase[1] (IU g^{-1})	146.42 ± 18.23[c]	170.63 ± 21.14[b]	194.53 ± 13.91[a]
Leucine aminopeptidase LAP[1] (IU g^{-1})	27.57 ± 7.53[b]	18.60 ± 8.10[c]	36.52 ± 10.42[a]

Means followed by different letters in the same row are significantly different (P < 0.05).

Table 4
Effect of dietary papain supplementation at 10 g kg^{-1} feed (P1) and 20 g kg^{-1} feed (P2) for 56 days on sterlet oxidative stress activity.

	Dietary treatment		
	C	P1	P2
Total antioxidant status TAS (mmol g^{-1})	4.02 ± 2.64[a]	2.15 ± 0.586[b]	1.93 ± 0.77[b]
Superoxide dismutase SOD (IU g^{-1})	198.11 ± 71.092[c]	686.93 ± 589.40[a]	648.35 ± 452.34[b]
Glutathione peroxidase GPX (IU g^{-1})	62.08 ± 32.67[c]	65.95 ± 24.06[b]	101.91 ± 30.50[a]
Glutathione reductase GLURED (IU g^{-1})	16.70 ± 8.38[a]	12.13 ± 3.627[b]	13.35 ± 5.55[b]

Means followed by different letters in the same row are significantly different (P < 0.05)

2.6. Histological analysis

On the last day of the experiment, the livers and midguts were removed from seven specimens from each dietary treatment group and subjected to histological analysis (Zawistowski, 1986). The histological samples were prepared and analyzed with the methods described in Wiszniewski et al. (2019) (Table 5).

2.7. Immunological indices

At the end of the experiment, blood was drawn from the caudal veins of ten specimens from each dietary treatment group to analyze non-specific humoral parameters. Immunocompetent cells were isolated from spleens that had been removed from ten specimens from each dietary treatment group to analyze cellular immunity. Non-specific humoral and cellular immunity analyses were performed according to the method described in Wiszniewski et al. (2019) (Table 6).

2.8. Challenge test

At the end of the experiment, a challenge test was performed based on the method described in Siwicki et al. (1994). Briefly, 45 fish from the three groups were each given a single intraperitoneal injection of *Yersinia ruckeri* (0.2 mL; 1×10^7). *Y. ruckeri* serotype O1, biotype 2 isolated from fatal cases at a rainbow trout fish farm in Poland was used. Before experimental infection, *Y. ruckeri* was cultured on nutrient agar supplemented with 5% horse blood and tryptone soya agar (TSA, Oxoid) at 25 °C 1 °C for 24 h. Abnormal behavior, clinical symptoms, and daily mortality were monitored in the challenged fish twice daily for seven days. Dead fish were removed. Three replicates and control groups were used for each dietary treatment. Throughout the challenge period, the

Table 5
Histological morphometrics of liver and gut samples of sterlet fed diets supplemented with papain at 10 g kg^{-1} feed (P1) and 20 g kg^{-1} feed (P2) (mean ± SD, n = 7).

Morphometric data	Dietary treatment		
	C	P1	P2
Hepatocyte size (μm)	16.01 ± 0.82	16.23 ± 0.29	15.97 ± 0.66
Nucleus size (μm)	4.68 ± 0.28	4.85 ± 0.38	4.79 ± 0.16
Hepatonuclei index	0.29 ± 0.08	0.30 ± 0.05	0.30 ± 0.04
Muscularis thickness (μm)	177.11 ± 40.16	180.21 ± 52.17	186.47 ± 46.87
Mucosal fold height (μm)	605.89 ± 64.21	621.93 ± 39.25	614.76 ± 31.85
Enterocyte height (μm)	41.12 ± 4.11[c]	43.32 ± 3.27[b]	47.81 ± 2.68[a]
Supranuclear zone height (μm)	12.98 ± 0.89[b]	14.27 ± 0.44[a]	14.99 ± 0.37[a]
Nucleus size (μm)	4.81 ± 0.27	4.71 ± 0.33	4.88 ± 0.61

Means followed by different letters in the same row are significantly different (P < 0.05).

Table 6
Effect of dietary papain supplementation at 10 g kg^{-1} feed (P1) and 20 g kg^{-1} feed (P2) for 56 days on sterlet on non-specific cellular and humoral defense mechanisms (mean ± SD, n = 10).

	Dietary treatment		
	C	P1	P2
Non-specific humoral immunity			
Lysozyme activity (mg L^{-1})	2.73 ± 1.05	2.67 ± 0.63	2.61 ± 0.95
Ceruloplasmin (IU)	52.57 ± 4.38^{b}	54.61 ± 4.39^{b}	59.54 ± 5.88^{a}
Total protein level (g L^{-1})	31.05 ± 4.01	33.43 ± 5.28	34.18 ± 6.26
Total immunoglobulin (Ig) level (g L^{-1})	11.93 ± 3.97^{b}	16.75 ± 4.05^{a}	15.86 ± 5.06^{ab}
Non-specific cellular immunity			
Metabolic activity of splenic macrophages (PMA)	0.33 ± 0.08^{b}	0.41 ± 0.11^{a}	0.47 ± 0.13^{a}
Potential killing activity of splenic phagocytes (PKA)	0.33 ± 0.07^{b}	0.40 ± 0.13^{a}	0.41 ± 0.17^{a}
Proliferative response of lymphocytes T stimulated with mitogen concanavaline A (ConA)	0.09 ± 0.01^{b}	0.09 ± 0.01^{b}	0.12 ± 0.01^{a}
Proliferative response of lymphocytes B stimulated with lipopolysaccharide (LPS)	0.08 ± 0.06^{b}	0.07 ± 0.002^{b}	0.11 ± 0.01^{a}

Means followed by different letters in the same row are significantly different (P < 0.05).

fish were fed their respective diets.

2.9. Statistical analysis

All data were tested for normal distribution and homogeneity of variance using Bartlett's tests. The data were subjected to one-way ANOVA to show the effects of the different levels of papain enzyme supplementation. Duncan's multiple range test was applied as a *post-hoc* test using the SAS ANOVA procedure (SAS, version 6.03, Soft Inc., Tulsa, OK, USA, 1993). Differences were considered significant at P < 0.05. Values are presented as means ± standard deviation (SD).

3. Results

3.1. Growth performance and nutrient utilization

Supplementing feed with different papain levels significantly (P < 0.05) influenced performance indices and feed utilization coefficients. Significant differences were noted in the final body weights in groups P1 (107.07 ± 7.66 g) and P2 (111.98 ± 1.93 g) in comparison to the control group (99.73 ± 2.71 g) (P < 0.05; Table 1). Significant (P < 0.05) differences were also noted in DGR and SGR values (Table 1). The FCR was significantly lower in group P2 (1.74) in comparison to the control group (1.94 ± 0.07) and to group P1 (1.95 ± 0.03) (Table 1). Higher protein efficiency ratio (PER) values were noted in group P2 in comparison to P1 and the control group (P < 0.05; Table 1). The HSI value in group P2 was significantly higher than that in the other dietary treatment groups. No differences were noted in VSI values. No fish mortality was noted in any of the groups during the experiment.

3.2. Body composition

The analyses of the proximate compositions of whole fish and fillets are presented in Table 2. No significant (P > 0.05) differences were noted in the content of crude protein, lipid, or moisture in whole fish or in filets among the dietary treatments groups.

3.3. Digestive enzymes

Digestive enzymes in the anterior and posterior intestines of fish fed different levels of papain are presented in Table 3. No significant (P > 0.05) differences in α-amylase or leucine aminopeptidase in the anterior intestine of fish fed different levels of papain were noted. However, trypsin activities in the anterior intestine of the fish decreased with increasing papain levels. The activity of lipase in the anterior intestine in group P2 was significantly (P < 0.05) higher than in the other dietary treatment groups. The activities of α-amylase, trypsin, lipase, and leucine aminopeptidase were significantly higher in the posterior intestine of the fish from group P2 than in those from the other dietary treatment groups.

3.4. Oxidative stress

The effect of papain on the activity of antioxidant enzymes is presented in Table 4. The activities of TAS, SOD, and GPx increased significantly (P < 0.05) in groups P1 and P2 compared with the control group. The highest TAS and GPx activities were noted in group P2, while the highest SOD level was observed in group P2. GLURED activity decreased with increasing papain supplementation levels.

3.5. Histological analysis of the gastrointestinal tract

The results of the liver and midgut histological measurements are presented in Table 5. Papain had no significant (P > 0.05) effect on the size of hepatocytes or their nuclei, the intestinal muscle thickness, or the intestinal mucosa size. However, the height of the intestinal enterocytes was the greatest in group P2, and the highest supranuclear surface of the intestinal enterocytes was noted in groups P1 and P2. There were also no pathological changes in either the liver or the intestines (Fig. 1).

3.6. Non-specific humoral immunity

The non-specific humoral immunity indices are presented in Table 6. No significant differences were noted in lysozyme or total protein levels among the dietary treatment groups, but significant (P < 0.05) increases in ceruloplasmin and total immunoglobulin (Ig) were confirmed in groups P1 and P2 compared to the control group. The highest level of blood ceruloplasmin was noted in group P2 (59.54 ± 5.88 IU), and it was significantly higher in comparison to the control group (52.57 ± 4.38 IU) and group P1 (54.61 ± 4.39 IU) (P < 0.05; Table 6). Significantly higher values of immunoglobulin (16.75 ± 4.05 g L^{-1}) were noted in group P1 in comparison to the other groups.

3.7. Non-specific cellular immunity and disease resistance

Table 6 presents the non-specific humoral immunity for fish fed different papain levels. Statistically (P < 0.05) significant differences in the metabolic activities of splenic macrophages (PMA) and splenic phagocyte potential killing activity (PKA) were noted in the groups fed feed supplemented with papain in comparison to the control group. The proliferative activity of spleen T lymphocytes stimulated by mitogen concanavaline A (ConA) and B lymphocytes stimulated by lipopolysaccharide (LPS) were significantly (P < 0.05) higher in group P2 in comparison to the control group and group P1.

3.8. Challenge test

At the end of the challenge trial, the survival of fish fed the diet supplemented with 20 g papain kg^{-1} feed was significantly higher (*P* < 0.05) than that of fish fed the other diets (Fig. 2).

4. Discussion

In the current experiment, papain supplementation at 10 g or 20 g kg^{-1} feed positively affected sterlet growth performance and feed utilization. The fish fed feed supplemented with papain enzyme had

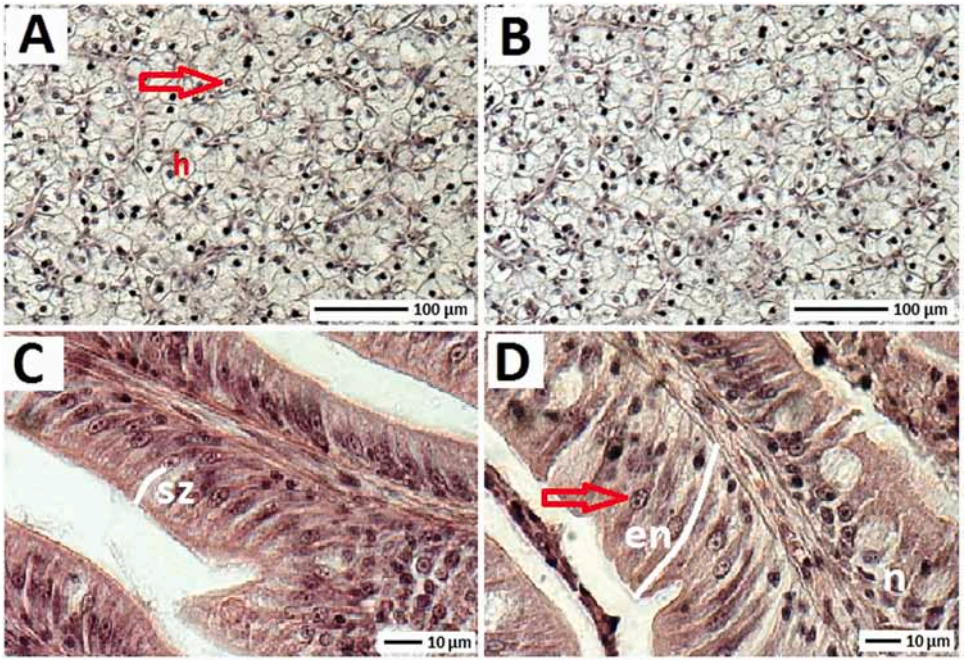

Fig. 1. Histological structure of the: sterlet liver in control group (A) and fed dietary papain levels 20 g kg^{-1} (P2; B); sterlet anterior of intenstine in control group (C) and fed dietary papain levels 20 g kg^{-1} for 8 weeks (P2; D). Haematoxylin and eosin stain; 20 x magnification (A, B); 100 x magnification (C, D); Letters: h – hepatocyte; sz – supranuclear zone of enterocyte; en – enterocyte; Arrows: nucleus.

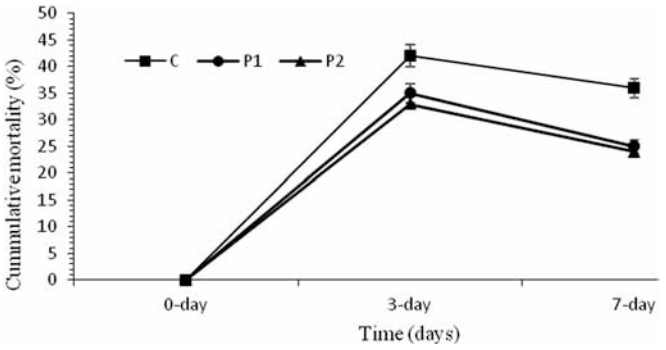

Fig. 2. Effect of dietary papain levels of 10 g kg^{-1} (P1) and 20 g kg^{-1} (P2) on the cumulative mortality of sterlet after the challenge test with *Yersinia ruckeri* at a dose of 0.2 mL; 1×10^7.

higher weight gain and SGR values than the control group. Particularly, significant ($P < 0.05$) differences were noted in the group fed feed supplemented with 20 g papain kg^{-1} feed. The addition of papain or a mixture of papain and bromelain to the diets of iridescent shark (*Pangasianodon hypophthalmus*) significantly increased weight gain and improved FCR values (Rostika et al., 2018). Enzymes extracted from pineapple waste significantly improved SGR and PER values in *Labeo rohita* in comparison to the control, and their application could be suitable for reducing production costs (Deka et al., 2003). Weight gain and SGR and FCR values of juvenile sterlet (*Acipenser ruthenus*) also improved significantly in comparison to the control group when their feed was supplemented with 10 or 20 g of exogenous bromelain kg^{-1} feed (Wiszniewski et al., 2019). The proteolytic properties of papain significantly influence the hydrolysis of proteins into short-chain peptides, which increases feed digestibility (Manosroi et al., 2014; Nilsang et al., 2005; Sawant and Nagendran, 2014) and subsequently improves fish growth performance. Furthermore, supplementing Nile tilapia diets containing feather meal with papain significantly improved digestibility (Munguti et al., 2014). When tilapia diets containing a high level of palm kernel meal were pre-treated with enzyme, net protein utilization improved in these fish (Boonyaratpalin et al., 2000). Consequently, enzyme feed supplementation could decrease or eliminate anti-nutritional factors thus improving nutrient efficiency (Cowieson et al., 2005; Hassaan et al., 2021a). This positively influences fish feed utilization, improves feed digestibility, and positively influences fish growth rates. According to Singh et al. (2011), the improved performance of fish fed diets supplemented with papain could stem from the mitigation of anti-nutritional factors of plant protein ingredients.

The digestion of feeds in fish is mainly influenced by the secretion of digestive enzymes by the intestines, the intestinal topography, and fish feeding habits (Hassaan et al., 2021b; Ribeiro et al., 2008). The results of the present study showed that papain supplementation did not have a significant effect on intestinal α-amylase or trypsin activity, but it increased pepsin and lipase activities significantly. Furthermore, papain supplementation significantly improved leucine aminopeptidase (LAP) activity in the posterior intestine, but it had no significant effect on LAP enzyme in the anterior intestine. The confirmation of decreased trypsin activity in the fish fed experimental diets could have resulted from the proteolytic activity of papain on the proteins contained in the feed at the preparation stage; thus, this could have resulted in reduced requirements for protein digestion (Wiszniewski et al., 2019). Kamaszewski et al. (2014) indicated that LAP activity can be used as a marker of enterocyte development. Digestive enzymes in animals are present in the gastrointestinal lumen and are associated with the intestinal epithelial cell brush-border membrane (Bakke et al., 2010). Fish digestive and metabolic functions are clearly correlated with digestive and brush-border enzyme activities (Hakim et al., 2006). Rachmawati et al. (2019) evaluated the effects of supplementing *C. gariepinus* diets with papain on endogenous protease activity, and their study revealed higher intestinal protease activity with papain supplementation. Compared with the control diet, amylase activity increased significantly in Nile tilapia fed a diet supplemented with mixed enzymes (protease neutral, ß-glucan, and xylanase) (Lin et al., 2007). In contrast, Liu et al. (2018) noted that protease activity in Gibel carp (*Carassius auratus gibelio*) was not affected by protease enzyme supplementation. Higher pepsin values were noted in snakehead (*Channa argus*) fed a diet supplemented with a

mixture of enzymes as compared to fish fed the control diet (Dai et al., 2019). Gut tissue morphometrics improved significantly with the addition of papain. The improvement in intestinal morphology revealed by the current study could have resulted from the complementary enhancement of increased digestion rates and assimilation after ingesting the diets. In the present study, the enterocyte absorptive area of the sterlet in this study fed diets supplemented with papain was larger than that in the fish fed the control diet, which resulted in higher nutrient utilization in the fish fed diets supplemented with 10 or 20 g papain kg^{-1} feed. Similarly, Dang et al. (2018) noted that the intestinal mucosal topography of grass carp (*Ctenopharyngodon idella*) was improved by the addition of protease to feed. Smirnov et al. (2004) and Hassaan et al. (2021a) reported improvement in intestinal barrier function, mucosal function, and nutrient absorption. The examination of the sizes of the hepatocytes and nuclei and the hepatonuclei index indicated no significant changes in the fish fed diets supplemented with papain; thus, this confirmed that papain supplementation of 10 or 20 g kg^{-1} feed had no pathological influence on hepatic tissues.

Antioxidant enzymes such as SOD can accelerate the decomposition of reactive oxygen species to H_2O_2, and they are considered indicators of defense mechanisms of aquatic animals against oxidative stress (Ruas et al., 2008). The performance of SOD antioxidant enzymes depends on their collaboration with the other antioxidant agents such as CAT and Gpx (Burgos-Aceves et al., 2018; Faggio et al., 2016). In the present study, sterlet fed the two experimental diets displayed higher SOD and GPx activities than those fed the control diet, which indicated that papain could improve enzymatic antioxidant protection in this fish. The effects of papain on fish antioxidant enzymes are poorly understood to date. Enzymatic antioxidant GPx and SOD play key roles in primary antioxidant protection against free radicals in organisms (Yang et al., 2019b, 2019a). Fish have evolved systems to protect cells from these highly toxic radicals. The production of detoxifying SOD and catalase enzymes, which decompose superoxide and peroxide radicals, respectively, are reported to contribute to the disposal of many pathogens (Lefebre and Valvano, 2001; Lynch and Kuramitsu, 2000; Uzzau et al., 2002). Dietary protease improved the immune system in shrimp (Song et al., 2017). Protease supplementation also increased free radical scavenging in white shrimp, *Litopenaeus vannamei*, and tilapia, *Oreochromis niloticus* × *O. aureus* (Li et al., 2016). Xu et al. (2016) and Wu et al. (2020) showed that the digestibility of dietary protein in grass carp and tilapia diets was increased by the addition of protease, which indicated that optimally digestible dietary protein might protect fish from intestinal damage.

Fish survival following a pathogenic bacteria challenge test is a key indicator of fish health status (Ringø et al., 2010). Few reports are available that focus on the effects of dietary enzyme supplementation on fish resistance to disease. The present study revealed that the 56-day course of papain dietary supplementation increased sterlet survival following the challenge test. The current study also indicated that feeding sterlet juveniles diets supplemented with papain for 56 days increased survival after the challenge test. Dietary exogenous protease also increased survival in Nile tilapia from 51.11% (PE0) to 64.44% (PE5) following a challenge test with *S. agalactiae* (Wu et al., 2020). Previous studies have demonstrated that papaya enzyme might have a positive impact on the immune system (Otsuki et al., 2010). Non-specific immunity in fish plays a significant role in body defense mechanisms (Anderson, 1992), and studies have investigated the possibilities of using various substances, including plant-based enzyme preparations, to stimulate immune responses. These studies showed that plant-based enzymes have a wide spectrum of activity on body physiology (Bricknell and Dalmo, 2005; Dügenci et al., 2003), and the present study revealed that dietary papain supplementation stimulated the sterlet immune system. Natural killer cells and the immune responses of T and B-lymphocytes in the blood are activated by exogenous enzyme supplementation (Chandran and Nachimuthu, 2018). Supplementing feed with 20 g papain kg^{-1} feed led to statistically significantly increased ceruloplasmin levels and proliferative activity of T and B-lymphocytes in sterlet, while supplementing feed with 1% of the enzyme resulted in increased immunoglobulin levels. Furthermore, both the 10 and 20 g papain kg^{-1} feed supplementation levels applied in the present study significantly increased the metabolic activities of PMA and PKA in sterlet. The role of ceruloplasmin is similar to those of interferon and transferrin in that is inhibits the growth of bacteria by depriving them of essential nutrients, i.e., copper ions (Alexander and Ingram, 1992), and concentrations of blood ceruloplasmin increase with fish growth and liver weight (Kolman, 2001). In the current study, the distinctly higher HSI values noted in the groups of fish stimulated with papain confirmed this dependency. Chandran and Nachimuthu (2018) demonstrated that papain stimulated the proliferative activity of T lymphocytes, which was also observed in the sterlet in the present study. Following stimulation with papain, the phagocytic activity of granulocytes and macrophages increased. Higher values of PMA indicated that the phagocytic cells were more efficient and were capable of more effective respiratory burst, which meant that the elimination of pathogenic factors was more effective. The stimulatory effects of the enzyme were also apparent in the ability of phagocytes to kill bacteria intracellularly.

5. Conclusions

The current study indicated that supplementing feed with doses of 10 g and/or 20 g papain kg^{-1} for a 56-day period improved growth efficiency and feed utilization and stimulated the immunity of juvenile sterlet in aquaculture conditions. Additionally, this method was safe for both the fish and the natural environment. Few studies have investigated the effects of papain supplementation on fish, and this provides the impetus to conduct further experiments focused specifically on its immunostimulatory effects.

CRediT authorship contribution statement

Grzegorz Wiszniewski: Experiment design, Collecting data statistical analyses, Drafting the paper. **Mohamed S. Hassaan Mohamed:** Experimental design, Drafting the paper. **Mohamed R. Soaudy:** Experimental design, Drafting the paper. **Maciej Kamaszewski:** Enzymes analysis, Drafting enzymes parameters section. **Hubert Szudrowicz:** Enzymes analysis, Drafting enzymes parameters section. **Elżbieta Terech-Majewska:** Immunity parameters analysis, Drafting enzymes parameters section. **Joanna Pajdak-Czaus Joanna:** Immunity parameters analysis, Drafting enzymes parameters section. **Wiktoria Wiechetek:** Oxidative stress parameters analysis, Drafting oxidative stress parameters section. **Andrzej Krzysztof Siwicki:** Immunity parameters analysis, Drafting enzymes parameters section.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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OŚWIADCZENIE AUTORA O UDZIALE W PUBLIKACJI

Ja, niżej podpisany, niniejszym oświadczam, że w artykule:

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mój wkład w opracowanie koncepcji, wykonanie części eksperymentalnej, opracowanie i interpretację wyników, zbieranie literatury i przygotowanie manuskryptu wyżej wymienionej publikacji, składającej się na rozprawę doktorską wyniósł **50%**.

Mgr inż. Grzegorz Wiszniewski

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AUTHOR'S DECLARATION

I, the undersigned co-author, declare that in the article:

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my contribution was 5% of the total contribution to the final version of above scientific publication.

Author's contribution:

☐	concept of research, making hypotheses	☐	writing an article
☐	planning of research, selection of research methodology	☐	graphical presentation of the results
☐	conducting research	☐	collecting literature
☐	collecting data	☐	consultations
☑	statistical analysis	☑	proofreading of the manuscript before submitting it to the journal
☐	interpretation of the results	☐	obtaining funds

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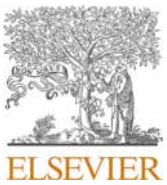

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Dietary effect of actinidin enzyme on growth, digestive enzymes activity, immunity, liver and intestine histology of juvenile sterlet sturgeon (*Acipenser ruthenus*)

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ABSTRACT

This 56-day experiment examined the effect of actinidin added to commercial feed at concentrations of 0 g (control group C), 10 g (A1 group) and 20 g (A2 group) kg^{-1} diet on growth, feed utilization, intestinal morphology, digestive enzyme and immune response of juvenile sterlet (*Acipenser ruthenus*). A total of 270 juvenile sterlet with an average body weight of (46.21 ± 0.37 g) were divided into nine tanks (280 L for each), with 30 fish per tank (three replicates each) under a recirculating aquaculture system (RAS). At the end of the experiment, distinct differences in the mean body weights of the fish were observed, with the greatest differences ($P < 0.05$) noted in A1 group (112.88 ± 1.20 g). The mean body weight in group A2 (106.40 ± 1.37 g) was statistically significantly ($P < 0.05$) higher compared with control group C (87.80 ± 1.51 g). A significant decrease in the activity of alkaline phosphatase (ALP) was noted in the liver in A2 group (57.72 IU g^{-1}) compared with the control group (127.38 IU g^{-1}). In the hindgut part of the intestine, a significant increase in the activity of alkaline phosphatase (ALP) was noted in A2 group (179.06 IU g^{-1}) compared with the control group (92.44 IU g^{-1}). An analysis of the immunity response showed an increase in the lysozyme value in A1 and A2 groups. In these groups, the immunoglobulin (Ig) content increased statistically significantly compared with the control group. A significant increase was noted in the phagocytic killing activity in the pronephros of fish fed feed supplemented with actinidin. A higher metabolic activity of macrophages and a higher phagocytic killing activity were also noted in the spleen in A1 and A2 groups. Based on the experiment, it can be concluded that the actinidin has a positive effect on Sterlet growth and stimulates its immune system.

1. Introduction

Feed supplementation with active substances is an effective method to support the innate immune response in fish as well as mitigate the harmful effects of conventional drugs used in aquaculture (Ghodrati et al., 2021a; Hassaan et al., 2021). They also reduce the immunosuppressive effects of unfavorable environmental conditions or stress factors. Furthermore, such an approach serves as part of a responsible and ecological method of fish farming (Gupta et al., 2021). With the emergence of probiotics, prebiotics, synbiotics and plant-derived active substances, interest in such preparations, especially of natural origin, is growing. They are being used successfully, with increasing frequency, not only in human medicine, but also in animal nutrition. Supplementation with these agents has become a commonly used element of

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immune prevention in fish nutrition (Hassaan et al., 2020; Ghodrati et al., 2021b; Kobya et al., 2021; Shekarabi et al., 2022). The action of immunostimulants involves the acceleration of the immune response, raising its level and prolonging its duration. Among the many substances that stimulate the immune system, special attention should be paid to plant preparations, which offer a wide spectrum of possibilities. In this group, the enzymes that support the functioning of the gastrointestinal tract are particularly noteworthy. The addition of exogenous enzymes opens up possibilities for the use of alternative protein sources in fish nutrition, thus enabling the use of raw materials, particularly those of plant origin, that have not been applied to date (Zheng et al., 2020). In a series of studies examining the potential use of fruit enzymes in fish nutrition, researchers showed their immunomodulatory potential (Wiszniewski et al., 2019, 2022). The experiments proved that bromelain and papain, isolated from the pineapple *Ananas comosus* and papaya *Carica papaya* (L.) fruits have a positive effect both on fish immunity and physiology of digestion. Histological analysis showed the stimulation of the growth of enterocytes and the enterocyte supranuclear region in the experimental groups of fish fed feed supplemented with fruit enzymes. This translated into an increase in the absorptive surface area and thus into an increase in the growth and nutrient use rates. The results prompted the authors to continue research in this direction. This experiment investigated the effect of actinidin on fish growth, the histology of the liver and gastrointestinal tract, the activity of selected digestive enzymes and the major parameters of non-specific cellular and humoral immunity of the sterlet (*Acipenser ruthenus*). Actinidin is a cysteine protease found naturally in kiwifruit (*Actinidia deliciosa*). It was identified as an enzyme that can assist in the hydrolysis of various proteins, including gluten (Chalabi et al., 2014; Kaur et al., 2010a, 2010b). Numerous studies on kiwifruit demonstrated its numerous medicinal properties, including anti-inflammatory, anti-diabetic and anti-carcinogenic properties (Satpal et al., 2021; Richardson et al., 2018). Over the years, kiwifruit has also proven to exhibit antibacterial and antioxidant properties (Siddique et al., 2021). Actinidin, found in kiwifruit, supports protein digestion in the stomach and intestine (Boeing et al., 2012; Kaur et al., 2010a, 2010b). To date, no research has been carried out into the use of actinidin in fish nutrition. Based on the available knowledge, it can be assumed that the enzyme can have a multi-directional effect and positively affect growth rates and fish immunity.

2. Material and methods

2.1. Rearing conditions

The experiment was conducted at the Institute of Inland Fisheries in Olsztyn. For the experiment, 270 juvenile sterlet individuals with a mean body weight of 46.21 ± 0.37 g were selected. The fish were then placed under a recirculating aquaculture system (RAS) into nine tanks with a capacity of 280 L, with 30 fish per tank. The fish were allowed to acclimate to the new conditions for two weeks. During this period, they were fed commercial feed (540 g kg^{-1} crude protein, 22.6 MJ kg^{-1} gross energy) three times a day. The scheduled duration of the experiment was 56 days. During this time, the physicochemical parameters of the water were monitored at the outflow of the tanks, and they were maintained at the following levels: water temperature of 20 °C (± 0.2); oxygen content of 6.45–7.33 mg O_2 L^{-1} (± 0.42); ammonium nitrogen (TAN = NH_4^+-N + NH_3-N) content of 0.146 ± 0.082 mg TAN dm^{-3}; nitrite nitrogen (NO_2-N) content of 0.011 ± 0.003 NO_2-N dm^{-3}; the pH value of 7.35–7.62. At the end of the experiment the fish were anesthetized with etomidate (Propiscin) at a concentration of 1.5 ml L^{-1}.

2.2. Diet preparation

During the experiment, the fish were fed commercial feed (Nutra T-2.0, Skretting, France; 54 % crude protein and 18 % lipids; Table 1), which was subjected to the procedure according to Wiszniewski et al. (2019). The feed for experimental groups (A1, A2) was supplemented with actinidin (KiwiEnzyme.com Ltd., New Zealand) in amounts of 10 and 20 g kg^{-1}, respectively. The feed for the control group was not supplemented. Fish were fed 12 times per day using an automatic feeder at a feeding rate of 1.5 % of biomass.

Table 1
Proximate compositions (g kg^{-1} of dry weight) of experimental diets containing 1 % of actinidin (A1) and 2 % of actinidin (A2).

	Diets		
	C	A1	A2
Crude protein	560.0	560.0	560.0
Crude lipid	150.0	150.0	150.0
Crude fiber	10.0	10.0	10.0
Crude ash	114.0	114.0	114.0
NFE[a]	166.0	166.0	166.0
Gross energy[b] (MJ kg^{-1})	22.1	22.1	22.1
Actinidin	0	10	20

[a] Nitrogen free extracts = 100 − (crude protein + crude lipid + crude fiber + crude ash) (Shearer, 1994).
[b] Gross energy was calculated from the chemical composition using the following energy conversion factors: 24 kJ g^{-1} proteins, 39 kJ g^{-1} lipids and 17 kJ g^{-1} NFE (Jobling, 1994).

2.3. Rearing indices

The measurements of the weight (W; 0.01 g) and the total length of the fish (n = 30) were taken at the beginning and the end of the experiment (TL; ± 0.1 cm). Every seven days, measurements of the mean weight were taken in order to determine the feed dose. On their basis, the primary indicators of growth and feed conversion were determined (WG, DGR, SGR, CF, FCR, PER, VSI, HSI) according to the method provided by Jarmołowicz et al. (2012) (Table 2). At the end of the experiment, five fish from each replicate (n = 5) were randomly collected and the viscera and liver were weighed to determine the values of the HSI and VSI indexes.

WG (weight gain, g $fish^{-1}$) = (Wf − Wi)

DGR (daily growth rate, g d^{-1}) = $(W_f - W_i) \times T^{-1}$;

Table 2
Effect of dietary actinidin supplementation at 10 g kg^{-1} feed (A1) and 20 g kg^{-1} feed (A2) for 56 days on sterlet sturgeon (*Acipenser ruthenus*) rearing parameters (mean ± SD, n = 3).

	Dietary treatment		
	C	A1	A2
Initial body weight (g $fish^{-1}$)	46,52 ± 0,70	45,80 ± 0,53	46,32 ± 0,16
Final body weight (g $fish^{-1}$)	87,80 ± 1,51[c]	112,88 ± 1,20[a]	106,40 ± 1,37[b]
Weight gain (g $fish^{-1}$)	41.28 ± 1.54[c]	67.08 ± 1.32[a]	60.08 ± 1.12[b]
Daily growth rate (DGR; g day^{-1})	0,74 ± 0,02[c]	1,20 ± 0,03[a]	1,07 ± 0,03[b]
Specific growth rate (SGR; % day^{-1})	1,13 ± 0,03[c]	1,61 ± 0,04[a]	1,48 ± 0,03[b]
Initial condition factor	0,35 ± 0,01	0,34 ± 0,01	0,35 ± 0,01
Final condition factor	0,39 ± 0,11	0,34 ± 0,01	0,33 ± 0,01
Feed conversion ratio (FCR)	1,32 ± 0,03[a]	0,93 ± 0,02[b]	1,01 ± 0,02[b]
Protein efficiency ratio (PER)	1,35 ± 0,03[c]	1,92 ± 0,05[a]	1,77 ± 0,03[b]
Viscerosomatic index (VSI) (%)	5,99 ± 1,00	5,10 ± 0,86	5,35 ± 0,81
Hepatosomatic index (HSI) (%)	0,97 ± 0,24	1,10 ± 0,21	1,35 ± 0,42

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

SGR (specific growth rate, % d^{-1}) = $100 \times [(\ln W_2 - \ln W_1) \times t^{-1}]$;

CF (condition coefficient) = $(W \times 100) \times TL^{-3}$;

FCR (feed conversion ratio) = $TFI \times (FB - IB)^{-1}$;

PER (protein efficiency ratio) = $(FB - IB) \times TFP^{-1}$;

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 (d); 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); FBP = final body protein (%); IBP = initial body protein (%); LW = liver weight (g); VW = viscera weight (g).

2.4. Endogenous enzyme activity analysis and oxidative response

To investigate the effect of the investigated nutritional supplement on enzymatic activity, samples of liver, midgut and hindgut with spiral valve were collected from five specimens from each experimental group (n = 5). The material was then homogenized in deionized water at a temperature of 4 °C, and centrifuged at the same temperature for 10 min at 14,000 *g*. The supernatant was collected and diluted for analysis. Both after the collection and the centrifugation, the material was fixed in liquid nitrogen and kept at − 80 °C. The enzymatic activity was determined according to the methodologies described by Kamaszewski et al. (2014a, 2014b) and Palińska-Żarska et al. (2021). Analyses of the activity of the following enzymes were conducted using kits manufactured by Spinreact (Spain): alkaline phosphatase (Kaplan, 1972) and acid phosphatase (Abbott, 1984); using the kits manufactured by Randox (Great Britain): superoxide dismutase (Wooliams et al., 1983), and substrates manufactured by Sigma-Aldrich (USA): lipase (Winkler and Stuckmann, 1979). The activity results were divided by the protein concentration in the sample and expressed as U/g, and protein was determined using the methodology by Lowry et al. (1951). The determinations were conducted following the previous adaptation of the indicated methodologies to 96-well plates. The reactions were performed at 37 °C using a Tecan microplate spectrophotometer (Infinite 200 PRO), with the samples measured in triplicates.

2.5. Histological and immunohistochemical analyses

In order to conduct the histological and immunohistochemical analyses, the liver and the foregut specimens were fixed in Bouin's fluid, dehydrated, and embedded in paraffin. The embedded tissues were cut with a Leica RM 2265 microtome (Leica Microsystems, Germany) into 5 μm thick sections and stained with hematoxylin-eosin (H&E). The microscopic observations of the cross-sections through the liver and intestine, as well as histomorphometric measurements, were conducted using a Nikon ECLIPSE 90i microscope connected to a Nikon DS5-U1 camera and the computer image analysis system NIS-Elements AR (Nikon Corporation, Japan). Histometric measurements of the hepatocyte surface area in the liver (100 measurements per individual, six individuals) were performed. The following measurements were conducted in the intestine: intestinal fold height, enterocyte height, enterocyte supranuclear region height, brush border height, and the middle lamella width (each parameter – 50 measurements per individual, six individuals). The proliferating hepatocyte nuclei in the liver were determined using antibodies of the anti-proliferating cell nuclear antigen (PCNA) in accordance with the methodology described by Kamaszewski et al. (2014a, 2014b, 2020). The hepatocyte proliferation index in the liver was expressed as the number of PCNA-positive cells per 1 mm^2 of the liver cross-section. The observations were carried out for six individuals per experimental group, on three fields per individual.

2.6. Immunological indices

The assessment of the non-specific cellular and humoral immunity parameters was conducted according to the method described by Wiszniewski et al. (2019). In order to determine the examined parameters, at the end of the experiment, blood was collected from the tail vein, spleen, and kidneys of 10 individuals from each experimental variant. Selected biochemical parameters and non-specific humoral immunity parameters were compared by spectrophotometric methods: the levels of total protein, gamma-globulins, lysozyme activity, and the serum ceruloplasmin activity. Determinations and comparisons of the following selected non-specific cellular immunity parameters were conducted by spectrophotometric methods: proliferative activity of the T-lymphocytes stimulated with concanavalin A (ConA, Sigma) and B-lymphocytes stimulated with lipopolysaccharide (LPS) using the MTT test. Moreover, the metabolic activity of the macrophages after cell stimulation with PMA (Phorbol Myristate Acetate) and the phagocytic activity of macrophage PKA (Potential Killing Activity) after cell stimulation by *Aeromonas hydrophila* (35654 ATCC strain) were determined according to the method described by Siwicki and Anderson (1993).

2.7. Statistical analysis

The study results were statistically analyzed using the program GraphPad Prism (Soft. Inc. Avenida de la Playa la Jolla, CA, USA). First, the Shapiro-Wilk normality test was applied, then the Levene's test was used to assess the equality of variances. The differences between the groups were examined using the one - factor variance analysis ANOVA. For statistically significant differences ($P \leq 0.05$), the post hoc Tukey test was used. For the analysis of PCNA, a non-parametric Kruskal-Wallis test was used.

3. Results

3.1. Growth performance and nutrient utilization

Actinidin had a significant effect on growth performance and feed conversion by the fish (Table 2). At the end of the experiment, distinct differences in the mean final body weight of the fish were observed, with the greatest difference noted in A1 group (112.88 ± 1.20 g; $P < 0.05$). The mean body weight in A2 group (106.40 ± 1.37 g) was statistically significantly higher compared with the control group C (87.80 ± 1.51 g; $P < 0.05$). The visible differences in the mean body weight correlate with the WG, DGR and relative (SGR) growth rates. The highest values for these indicators were also obtained in experimental A1 and A2 groups (Table 2 ; $P < 0.05$). The feed supplementation with actinidin had a significant effect on the feed conversion ratio (FCR). The lowest values of this index were obtained in A1 group, with a value of 0.93 (± 0.02) and in A2 group, with a value of 1.01 (± 0.02), while in the control group (C), its value amounted to 1.32 (± 0.03; $P < 0.05$). As regards the protein efficiency ratio (PER), the highest values were also noted in experimental A1 (1.92 ± 0.05) and A2 (1.77 ± 0.03) groups compared with control group C (1.35 ± 0.03). No differences between the groups were observed for the hepatosomatic index (HSI) or viscerosomatic index (VSI). No fish mortality was noted in any group studied during the experiment.

3.2. Digestive enzymes and oxidative stress

The effect of actinidin on the secretion of digestive enzymes and the oxidative stress parameters is shown in Table 3. A significant decrease in the activity of alkaline phosphatase (ALP) was noted in the liver in A2 group (57.72 IU g^{-1}) compared with the control group (127.38 IU g^{-1}) ($P < 0.05$; Table 3). In the case of ACP acid phosphatase, a decrease in its

Table 3
Effect of dietary actinidin supplementation at 10 g kg^{-1} (A1) and 20 g kg^{-1} (A2) for 56 days on digestive enzyme activity and oxidative response of sterlet sturgeon (*Acipenser ruthenus*).

	Dietary treatment		
	C	A1	A2
Liver			
Alkaline phosphatase (ALP) (IU g^{-1})	127.38 ± 45.15[a]	75.15 ± 45.60[ab]	57.72 ± 37.69[b]
Acid phosphatase (ACP) (IU g^{-1})	1.82 ± 0.74	1.34 ± 0.64	1.35 ± 0.81
Superoxide dismutase SOD (IU g^{-1})	8.59 ± 3.97	5.89 ± 3.39	5.40 ± 3.78
Midgut			
Alkaline phosphatase (ALP) (IU g^{-1})	112.51 ± 22.32	71.94 ± 49.86	103.22 ± 59.47
Acid phosphatase (ACP) (IU g^{-1})	1.48 ± 0.36	1.07 ± 0.70	1.21 ± 0.72
Lipase (IU g^{-1})	10.56 ± 4.94	8.22 ± 6.75	8.12 ± 5.61
Trypsin (IU g^{-1})	72.82 ± 75.32	41.47 ± 51.20	43.09 ± 50.64
Hindgut			
Alkaline phosphatase (ALP) (IU g^{-1})	92.44 ± 37.71[c]	113.05 ± 59.89[b]	179.06 ± 83.45[a]
Acid phosphatase (ACP) (IU g^{-1})	1.96 ± 0.52[ab]	1.53 ± 0.66[b]	2.57 ± 0.40[a]
Lipase (IU g^{-1})	4.21 ± 2.25	4.63 ± 3.41	6.72 ± 5.51
Trypsin (IU g^{-1})	6.56 ± 8.79	10.29 ± 14.99	22.14 ± 20.11

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

activity was also observed with an increase in actinidin in the feed, but it was not statistically significant ($P > 0.05$). As for superoxide dismutase (SOD), i.e. the oxidative stress parameter, no statistically significant differences were noted. No differences in the lipase activity levels were noted in the midgut, although a decreasing trend can be noted as the actinidin dose in the feed increases, yet it was not significant ($P > 0.05$). In the hindgut, a significant increase in the activity of alkaline phosphatase (ALP) was observed in A2 group (179.06 IU g^{-1}) compared with the control group (92.44 IU g^{-1}). However, the activity of acid phosphatase (ACP) was significantly higher in group A2 (2.57 IU g^{-1}) compared with A1 group (1.53 IU g^{-1}). Although the lipase activity level in the hindgut was higher in A1 and A2 groups compared with the control group, it was statistically insignificant ($P > 0.05$; Table 3). In both the midgut and hindgut, great variation in the lipolytic activity was noted between individuals in all the studied experimental groups (Table 3).

3.3. Histological and immunohistochemical analyses of the gastrointestinal tract and liver

Table 4 presents the results of histological measurements of the liver and foregut. No statistically significant differences in the hepatocyte surface area were noted. The supplementation of feed with actinidin had no significant effect on the height of intestinal folds, enterocytes or the enterocyte supranuclear regions ($P > 0.05$). Morphometric analysis of the *lamina propria* width and the brush border height only showed significant differences for the *lamina propria*, which was significantly narrower in A1 group (5.75 μm) compared with the control group (8.87 μm). During the histopathological measurements of the liver and enterocytes in foregut, no pathological lesions were found in any of the analyzed groups (Fig. 2; Fig. 3).

Immunohistochemical analysis of the liver parenchyma of the studied fish showed a statistically significantly greater number of proliferating cell nuclei (PCNA-positive) in the livers of the fish from A1 and A2 groups, compared with the livers of the fish from the control group (Fig. 1; Fig. 3).

Table 4
Histological morphometrics of liver and intestine of sterlet sturgeon (*Acipenser ruthenus*) fed dietary actinidin levels of 10 g kg^{-1} (A1) and 20 g kg^{-1} (A2) (mean ± SD, n = 3) for 56 days.

Morphometric data	Dietary treatments		
	C	A1	A2
Size of hepatocyte (μm^2)	174.75 ± 55.62	141.10 ± 13.95	139.81 ± 8.09
Height of mucosal fold (μm)	349.72 ± 64.35	294.81± 56.31	367.74 ± 122.24
Height of enterocytes (μm)	40.49 ± 14.96	29.06 ± 3.01	28.52 ± 3.91
Height of supranuclear zone (μm)	18.98 ± 3.63	17.94 ± 2.12	17.48 ± 2.64
Width of *lamina propria* (μm)	8.87 ± 2.95[b]	5.75 ± 0.71[a]	6,44 ± 0,73[ab]
Height of brush border (μm)	4.65 ± 2.97	2.47 ± 0.57	3.05 ± 0.35

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

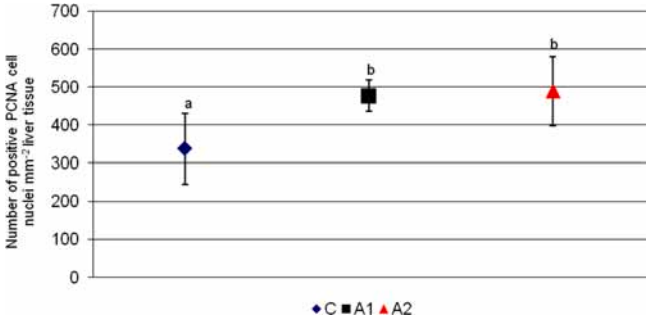

Fig. 1. Number of proliferating cell nuclear antigen-positive cell nuclei per mm^2 of liver tissue in sterlet sturgeon (*Acipenser ruthenus*) fed a diet supplemented with actinidin in the amount of 10 g kg^{-1} (A1) and 20 g kg^{-1} (A2) for 56 days.

3.4. Non-specific humoral and cellular immunity

As regards the non-specific humoral immunity parameters, an increase in the lysozyme value was noted in A1 and A2 groups. In these groups, the immunoglobulin (Ig) content increased statistically significantly by 3.40 and 3.98 g L^{-1}, respectively, compared with the control group ($P < 0.05$; Table 5). As regards the non-specific cellular response in the pronephros, a significant increase was noted in the phagocytic killing activity in the groups of fish fed feed supplemented with actinidin ($P < 0.05$; Table 5). Higher metabolic activity of macrophages and higher phagocytic killing activity were then noted in A1 and A2 groups ($P < 0.05$; Table 5). No increased activity of the T and B-lymphocytes was found in the kidney or the spleen ($P > 0.05$; Table 5).

4. Discussion

Utilization of the by-product of agricultural such as fruits and vegetables in aquatic feed industry not only increase the income of farmers but also, achieved the aquaculture sustainability (Wiszniewski et al., 2019; Van Doan et al., 2021). Cysteine proteases extracted from plants such as papaya, pineapple, fig and kiwifruit (Salas et al., 2008; Nieuwenhuizen et al., 2012), are used as phytochemical treatments (Leung-Toung et al., 2005). Accordingly, Actinidin is a member of the plant cysteine protease family (Chalabi et al., 2014), and no reports have been published on the proteolytic activities of actinidin on fish performance or health status. Therefore, the present study evaluated the effects of kiwifruit powder on growth performance, immune response and disease resistance. The present study showed that higher weight gain and growth rates were detected in fish fed a diet supplemented with 10 g actinidin kg^{-1}. The supplementation of either 10 or 20 g of actinidin per

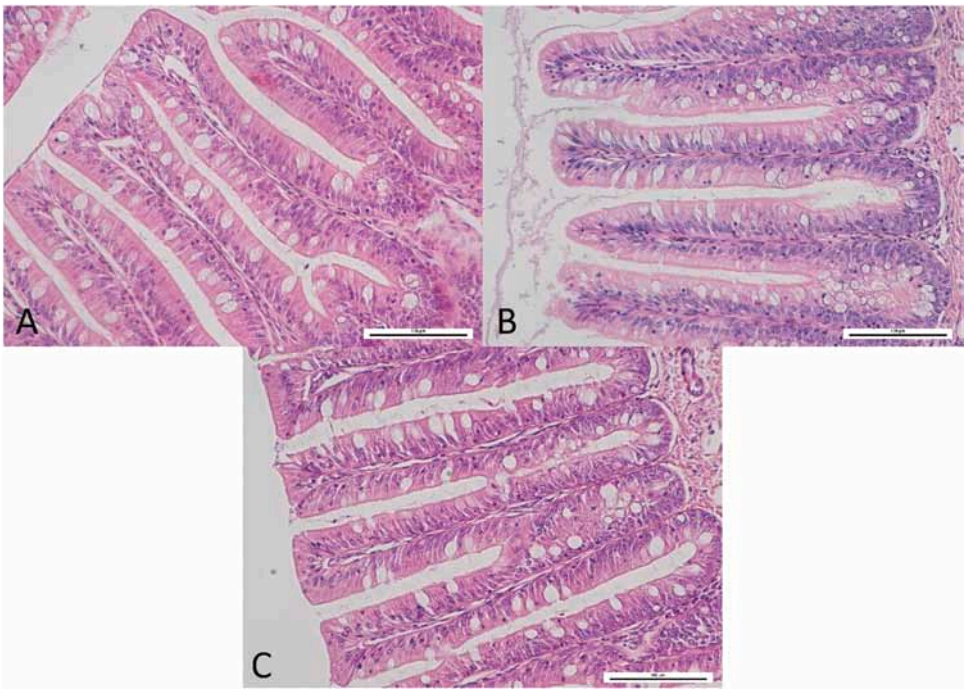

Fig. 2. Cross-section through the foregut of sterlet sturgeon (*Acipenser ruthenus*) from: A) control group, B) group A1, C) group A2. Staining with hematoxylin/eosin. Scale bar 100 μm.

kg of feed significantly improved FCR and PER values compared with the control diet. The higher feed utilization in the present study could stem from the proteases in actinidin derived from *Actinidia chinensis,* increasing protein solubility (Rutherfurd et al., 2011). These findings are in line with Wiszniewski et al. (2019, 2022), who found that sterlet fish fed a diet supplemented with bromelain or papain at 10 g kg^{-1} produced significantly ($P < 0.05$) higher growth performance and nutrient utilization than a control diet. Furthermore, the inclusion of pineapple for eight weeks resulted in a significantly ($P < 0.05$) improved growth performance of Nile tilapia (Yuangsoi et al., 2018; Van Doan et al., 2021). In this context, a dietary mixture of bromelain and papain could achieve significantly higher apparent net protein utilization and the best FCR for grass carp and grey mullet compared with a control mullet diet (Choi et al., 2016). Furthermore, Divakaran and Velasco (1999) showed that protein digestibility was significantly higher in Pacific white shrimp, *Litopenaeus vannamei*, fed a diet supplemented with bromelain than those fed a control diet. The growth performance of *Labeo rohita* was significantly higher when fed a diet supplemented with pineapple waste than a control diet (Deka et al., 2003). Generally, using cysteine proteases as feed additives could increase the digestibility, acceptable flavor and palatability of ingredients (Grzonka et al., 2007; Hassaan et al., 2019).

Actinidin derived from the kiwifruit, papain from papaya latex, and bromelain from the pineapple belong to papains, i.e. a common family of cysteine proteases (Ha et al., 2012). Numerous studies show that actinidin supports the protein digestion process (Kaur et al., 2010a, 2010b; Kaur and Boland, 2013; Rutherfurd et al., 2011).

Studies with feed supplementation with bromelain and papain found an increase in the intestinal absorption area in the experimental groups. The enterocyte height was significantly greater in the fish groups receiving the exogenous enzyme compared with the control groups (Wiszniewski et al., 2019, 2022). Although actinidin belongs to the same group of cysteine proteases as bromelain and papain, it probably has a different mechanism of action on the intestinal epithelial cells. Histological analyses showed no significant differences in the structure of the gastrointestinal tract. Cross-sections through the foregut showed no excessive infiltration of the epithelium by the lymphocyte cells, and the histomorphological analysis of the *lamina propria* width showed (in the fish from groups A1 and A2) a width that was simply statistically smaller than that in the fish from the control group. These parameters, as reported by Ostaszewska et al. (2013), may indicate the formation of inflammation in the gastrointestinal tract. Moreover, the presence of changes such as: a reduction in the surface area of absorption in the intestine through reduced intestinal fold height; the absence of vacuoles in the supranuclear areas of enterocytes indicating a process of impaired transport of digestive products into the blood; infiltration of inflammatory cells into lamina propria; may indicate the development of enteropathy or enteritis (Lilleeng et al., 2009). Therefore, based on an analysis of the morphology of the intestine of the sturgeons under study, it can be concluded that feed supplementation with actinidin does not cause inflammation in the gastrointestinal tract or disturb the homeostasis of the body.

Nutrient digestion and absorption is determined by the activity of digestive enzymes, in particular, those located in the intestinal brush border segment which are responsible for the final stages of food breakdown and assimilation (Tibaldi et al., 2006). The dominant brush border enzyme is alkaline phosphatase which is mainly found in cell membranes where active transport takes place. The enzyme is used as a marker for nutrient absorption and normal intestinal function (Wahnon et al., 1992; Silva et al., 2010). As noted by Lallès (2020), the highest ALP activity was noted in the midgut, while the lowest activity was in the hindgut. Higher protein content in the feed also has an effect on the increase in ALP activity. In the study, the statistically significant increase in ALP activity in hindgut in groups A1 and A2, compared with the control, suggests an increase in protein digestion efficiency. The action of actinidin in the midgut part of the gastrointestinal tract resulted in an increase in its supply in the hindgut part. During the experiment, differences were observed in acid phosphatase activity in the hindgut segment of the intestine between groups A1 and A2. Acid phosphatase in the gastrointestinal mucosa may be involved in the mechanism of superficial cell exfoliation and in protection against harmful agents. It is also correlated with metabolite absorption and transport (Faccioli et al., 2016). The experiments on feeding sterlet, a diet supplemented with bromelain and papain showed that these

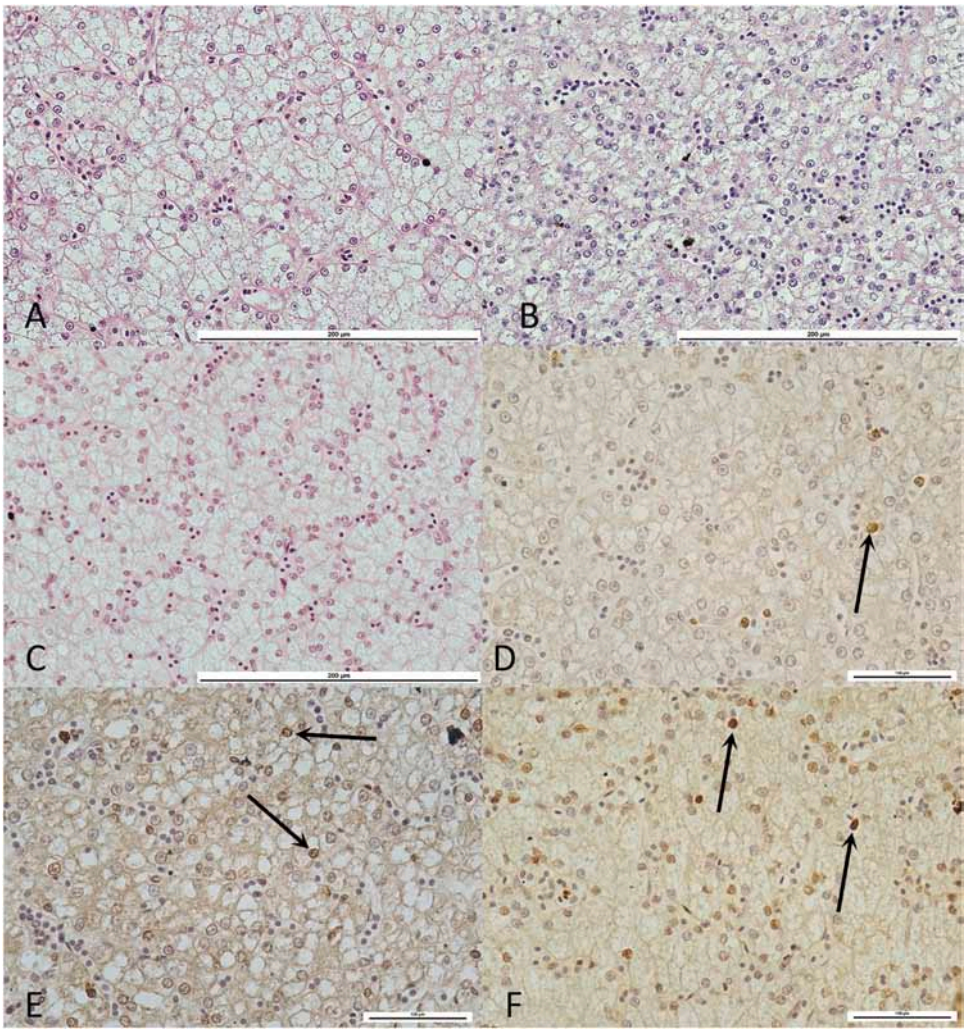

Fig. 3. Cross-section through the liver of sterlet sturgeon (*Acipenser ruthenus*) from: A) and D) control group, B) and E) group A1, C) and F) group A2. The arrows indicate proliferating (PCNA-positive) hepatocyte nuclei. Photographs A–C – staining with hematoxylin/eosin, scale bar 200 μm. Photographs D–F – PCNA staining, scale bar 100 μm.

enzymes affect the lipase activity in the midgut (Wiszniewski et al., 2019, 2022). This suggests that actinidin will exhibit similar properties. However, a study into the activity of lipase after exposure to actinidin showed that lipase was not very sensitive or completely resistant to its action. However, the effect of bromelain and papain on its activity was observed (Martin et al., 2017). In the experiment concerned, the slight decrease in lipase activity ($P > 0.05$; Table 3) in the midgut and its slight increase in the hindgut segment confirm the limited effect of actinidin on this enzyme.

No pathological lesions were observed in the liver morphology, and a similar hepatocyte surface area was noted in all groups. Moreover, the supplementation of feed with 10 and 20 g kg^{-1} actinidin caused no pathological lesions in the liver and intestine. However, in the fish fed a diet supplemented with actinidin, an increase in the number of proliferating nuclei in the liver parenchyma was found for both concentrations. Cell proliferation is the main mechanism that maintains homeostasis in the liver and is involved in the regeneration of this organ under pathological situations (Chen et al., 2020). The use of various supplements in fish feed may result in hepatocyte *steatosis* leading to cell damage, which can consequently impair the cell renewal process, and affect the proliferation processes (Chung et al., 2001; Ostaszewska et al., 2010). The results obtained in that experiment indicate that the increase in hepatocyte proliferation in the fish fed a diet supplemented with actinidin, compared with the fish from the control group, can be associated with a statistically significantly higher fish growth rate, which also translates into the HSI value in the study groups. This increase in hepatocyte proliferation is neither the response of the body associated with an adverse effect of the feed ingredients or with the stimulation of cells involved in cell renewal processes in the liver.

Moreover, the supplementation with actinidin had a significant effect on the difference in the alkaline phosphatase activity in the liver. Its level is regarded as a diagnostic index in the case of inflammation and damage to this organ (Banaee, 2020). The feed used in aquaculture was aimed at ensuring the fastest possible growth of fish. Despite its excellent balance, it cannot replace natural food. An inadequate lipid content can lead to liver damage, impaired health status and suboptimal growth (Meyer et al., 2016). The statistically significant decrease in the ALP activity in group A2 (2 % actinidin) may indicate a beneficial effect on liver function and the feed nutrient conversion rate.

Fish, like all aerobic organisms, are susceptible to the action of reactive oxygen species. Part of the protection is an antioxidant defence system in the form of a number of enzymes with such properties. The lack of balance between the oxidation process and the antioxidant defense system results in oxidative stress, which leads to tissue damage and impaired body function (Trenzado et al., 2006; Halliwell and Gutteridge, 2015). Superoxide dismutase (SOD) is the first enzyme that responds to oxygen radicals and the enzyme that provides the greatest response to oxidative stress (Winston and Di Giulio, 1991). The results of

Table 5
Effect of dietary actinidin supplementation at 10 g kg^{-1} (A1) and 20 g kg^{-1} (A2) for 56 days on the humoral and non-specific cellular defense mechanisms sterlet sturgeon (*Acipenser ruthenus*) (mean ± SD, n = 3).

	Dietary treatment		
	C	A1	A2
Non-specific humoral immunity			
Lysozyme activity (mg L^{-1})	3.97 ± 0.27[b]	4.68 ± 0.25[a]	4.71 ± 0.28[a]
Ceruloplasmin (IU)	31.21 ± 1.66	33.68 ± 3.85	31.59 ± 3.57
Total protein level (g L^{-1})	25.68 ± 3.66	28.02 ± 2.71	29.13 ± 4.82
Total immunoglobulin (Ig) level (g L^{-1})	11.00 ± 1.00[b]	14.40 ± 1.30[a]	14.98 ± 2.53[a]
Non-specific cellular immunity of pronephros			
NBT	0.24 ± 0.04	0.25 ± 0.02	0.24 ± 0.02
Metabolic activity of macrophages (PMA) (OD)	0.27 ± 0.03	0.31 ± 0.06	0.27 ± 0.03
Potential killing activity of phagocytes (PKA) (OD)	0.26 ± 0.03[b]	0.30 ± 0.03[a]	0.30 ± 0.03[a]
Proliferative response of lymphocytes T stimulated by mitogen concanavalin A (ConA) (OD)	0.09 ± 0.00	0.10 ± 0.02	0.10 ± 0.02
Proliferative response of lymphocytes B stimulated by lipopolysaccharide (LPS) (OD)	0.09 ± 0.00	0.08 ± 0.01	0.09 ± 0.01
Non-specific cellular immunity of spleen			
NBT	0.18 ± 0.03[b]	0.24 ± 0.02[a]	0.33 ± 0.05[a]
Metabolic activity of macrophages (PMA) (OD)	0.22 ± 0.04[c]	0.32 ± 0.03[b]	0.39 ± 0.04[a]
Potential killing activity of phagocytes (PKA) (OD)	0.24 ± 0.05[c]	0.31 ± 0.02[b]	0.45 ± 0.06[a]
Proliferative response of lymphocytes T stimulated by mitogen concanavalin A (ConA) (OD)	0.11 ± 0.02	0.10 ± 0.01	0.12 ± 0.03
Proliferative response of lymphocytes B stimulated by lipopolysaccharide (LPS) (OD)	0.11 ± 0.02	0.10 ± 0.00	0.11 ± 0.01

SOD activity measurements showed no statistically significant differences in the activity of this enzyme after the use of actinidin, which indicates the lack of stimulation of the antioxidant response associated with the diet used in the experimental groups.

As regards immunity in fish, it is the non-specific defense mechanisms that play a key role in defending the body against pathogens, as the specific defense mechanisms require a longer time to produce and activate antibodies (Anderson, 1992). In this paper, the parameters of the non-specific humoral immunity of fish clearly increased. The lysozyme and immunoglobulin (Ig) levels increased in the groups of fish fed with 10 and 20 g actinidin per kg of feed. Both doses stimulated the analyzed parameters. In the present results, the enhancement of immune response associated with dietary exogenous enzymes supplementation could be due to their inhibitory effects against the pathogenic microorganisms throughout the gastrointestinal tract. Hassaan et al. (2021) exogenous protease enzyme increased immunoglobulin M (IgM), phagocytic and lysozymes activity of tilapia. Similar results were observed by Wiszniewski et al. (2022) who introduced papain to feed at the same doses. Immunoglobulins are the most important proteins of the specific immune response, and their function is to protect the body against such threats as bacteria and viruses.

Due to the fact that the activity of immunocompetent cells is the first line of defense, an assessment of their functions (PMA, PKA) enables determining the potential efficiency of the entire immune system (Siwicki et al., 2009; Terech-Majewska et al., 2016). The metabolic activity of macrophages (PMA) isolated from blood-forming organs, in this case the spleen, significantly stimulated in the groups of fish fed actinidin with the diet. Similarly, Wiszniewski et al. (2022) noted an increase in splenic PMA after providing juvenile sterlet with papain in feed. The higher PMA values indicate that the phagocytic cells are more efficient and capable of an effective oxidative burst, which implies a more efficient elimination of pathogenic agents. In this study, the killing activity of the phagocytes derived from the kidney and the spleen increased as well. Wiszniewski et al. (2022) also observed an increase in splenic PKA in juvenile sterlet. The higher PKA value is associated with a better ability of the phagocytes to intracellularly kill pathogenic bacteria. In contrast to bromelain (Wiszniewski et al., 2019) and papain (Wiszniewski et al., 2022), actinidin did not stimulate the proliferative response of T and B-lymphocytes.

5. Conclusions

This study indicated that supplementing feed with doses of 10 g and/or 20 g actinidin kg^{-1} for a period of 56-day improved growth efficiency and feed utilization, and stimulated the immunity of juvenile sterlet in aquaculture conditions. Additionally, this method was safe for both the fish and the natural environment. Few studies investigated cleared the effects of enzyme actinidin addition on fish, which provides the impetus to conduct further experiments specifically focused on its immunostimulatory effects.

CRediT authorship contribution statement

Grzegorz Wiszniewski: Experiment design, collecting data, statistical analyses, drafting the paper. **Sylwia Jarmołowicz:** Experimental design, drafting the paper. **Mohamed S. Hassaan:** Experimental design, drafting the paper. **Maciej Kamaszewski:** Enzymes analyses, drafting enzymes parameters section. **Hubert Szudrowicz:** Enzymes analyses, drafting enzymes parameters section. **Elżbieta Terech-Majewska:** Immunity parameters analyses, drafting enzymes parameters section. **Kacper Kawalski:** Histological and immunohistochemical analyses. **Jakub Martynow:** Histological and immunohistochemical analyses. **Adrian Szczepański:** Oxidative stress parameters analyses. **Andrzej Krzysztof Siwicki:** Immunity parameters analyses, drafting enzymes parameters section

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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