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AUTOPHAGIC FUNCTION OF THE LIVER OF VACCINATED RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) FOLLOWING *YERSINIA RUCKERI* INFECTION

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The liver plays a critical role in maintaining metabolic homeostasis and immune defence in fish, particularly in response to bacterial infections. Autophagy, a conserved cellular process essential for homeostasis and pathogen clearance, has been implicated in host defence mechanisms. However, the role of autophagy in the liver of vaccinated fish following pathogen exposure remains largely unexplored. Yersinia ruckeri, the causative agent of enteric redmouth disease (ERM), poses a significant threat to rainbow trout (Oncorhynchus mykiss Walbaum) aquaculture, primarily affecting the liver, spleen and kidneys. Vaccination is a widely used preventive strategy, but its effect on autophagic activity during infection is not well understood. The aim of this study was to evaluate the autophagic response in the liver of vaccinated rainbow trout following Y. ruckeri infection by assessing the activity of four lysosomal enzymes: alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG). Rainbow trout were divided into experimental groups: unvaccinated control, vaccinated uninfected, unvaccinated infected and vaccinated infected. The fish were orally immunised with a Y. ruckeri vaccine and challenged with a virulent strain of Y. ruckeri. The results showed significant differences in lysosomal enzyme activity between groups, indicating that vaccination modulated the hepatic autophagic response during bacterial infection. AAP and LAP activity peaked in unvaccinated infected fish, whereas vaccinated fish exhibited a blunted enzymatic response, suggesting that vaccination attenuated excessive autophagic activation. Similarly, AcP and NAG activity patterns indicated an infection-induced autophagic response that was partially attenuated in vaccinated fish. These results suggest that vaccination influences autophagy-related enzymatic activity in the liver of rainbow trout, potentially enhancing pathogen clearance while preventing excessive cellular stress. Understanding the interplay between vaccination, infection and autophagy may provide valuable insights to optimise vaccination strategies and improve disease management in aquaculture.

Keywords: autophagy, liver, rainbow trout, *Yersinia ruckeri*, lysosomal enzymes, vaccination, bacterial infection, metabolic homeostasis

АУТОФАГІЧНА ФУНКЦІЯ ПЕЧІНКИ ВАКЦИНОВАНОЇ РАЙДУЖНОЇ ФОРЕЛІ (*ONCORHYNCHUS MYKISS* WALBAUM) ПІСЛЯ ІНФЕКУВАННЯ ШТАМОМ *YERSINIA RUCKERI*

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Печінка відіграє важливу роль у підтриманні метаболічної гомеостазу та імунного захисту у риб, особливо у відповідь на бактеріальні інфекції. Аутофагія, консервативний клітинний процес, необхідний для гомеостазу та очищення від патогенів, пов'язана з механізмами захисту організму. Однак роль аутофагії у печінці вакцинованих риб після впливу патогенів залишається в основному не дослідженою. Yersinia ruckeri, збудник кишкового єрсиніозу, становить серйозну загрозу для розведення райдужної форелі (Oncorhynchus mykiss Walbaum), впливаючи насамперед на печінку, селезінку та нирки. Вакцинація є широко використовуваною профілактичною стратегією, але її вплив на аутофагічну активність під час інфекції не до кінця зрозумілий. Метою цього дослідження було оцінити аутофагічну відповідь у печінці вакцинованої райдужної форелі після інфекції Y. ruckeri, оцінюючи активність чотирьох лізосомальних ферментів: аланіламіноептидази (AAP), лейциламіноептидази (LAP), кислотної фосфатази (AcP) та β-N-ацетилглюкозамінідази (NAG). Райдужну форель поділили на експериментальні групи: невакциновану контрольну, вакциновану неінфековану, невакциновану заражену та вакциновану заражену. Рибу орально імунізували вакциною від Y. ruckeri та інфікували штамом Y. ruckeri. Результати показали значні відмінності в активності лізосомальних ферментів між групами, що вказує на те, що вакцинація модулює аутофагічну відповідь печінки під час бактеріальної інфекції. Активність AAP і LAP досягла піку у невакцинованих заражених риб, в той час як вакциновані риби показали знижену ензиматичну відповідь, що вказує на те, що вакцинація послабила надмірну аутофагічну активацію. Аналогічно, патерни активності AcP і NAG вказують на аутофагічну відповідь, індуковану інфекцією, яка частково була ослаблена у вакцинованих риб. Ці результати свідчать про те, що вакцинація впливає на активність аутофагії у печінці райдужної форелі, потенційно покращуючи очищення від патогенів і запобігаючи надмірному клітинному стресу. Розуміння взаємодії між вакцинацією, інфекцією та аутофагією може надати важливі відомості для оптимізації стратегій вакцинації та покращення управління захворюваннями у аквакультурі.

Ключові слова: аутофагія, печінка, райдужна форель, *Yersinia ruckeri*, лізосомальні ферменти, вакцинація, бактеріальна інфекція, метаболічний гомеостаз.

Introduction. The liver plays a crucial role in maintaining homeostasis and immune defence in fish, acting as a central metabolic organ and a key component of the innate immune system (Mokhtar D.M. et al., 2023). Autophagy, a highly conserved cellular degradation and recycling process, is essential for cellular homeostasis, pathogen clearance and immune regulation (Chun Y. and Kim J., 2018; Gómez-Virgilio L. et al., 2022). In teleost fish, the autophagic process has been implicated in response to various stressors, including infectious diseases (Johnstone C. and Chaves-Pozo E., 2022; Zhou Z. et al., 2022). However, the precise role of autophagy in the liver of vaccinated fish following pathogen exposure remains largely unexplored.

Yersinia ruckeri, the causative agent of enteric redmouth disease (ERM), is a major bacterial pathogen affecting rainbow trout (*Oncorhynchus mykiss* Walbaum) aquaculture worldwide (Kumar G. et al., 2015). This Gram-negative bacterium primarily affects the liver, spleen and kidneys, resulting in severe economic losses in aquaculture (Wrobel A. et al., 2019). Vaccination has been widely used as a preventive strategy against ERM, significantly reducing mortality and improving disease resistance (Villumsen K.R. et al., 2014; Wangkahart E. et al., 2019). However, the effect of vaccination on cellular processes such as autophagy in the liver during *Y. ruckeri* infection remains poorly understood.

Recent studies suggest that autophagy plays a dual role in bacterial infections, acting both as a host defence mechanism and as a potential pathway for pathogen survival (Desai M. et al., 2015; Zhou Z. et al., 2022). In mammals, autophagy has been shown to eliminate intracellular pathogens, regulate inflammation and modulate immune responses (Deretic V., 2021; Gan T. et al., 2023). Similar mechanisms are thought to exist in fish, but empirical evidence remains limited (Zhou Z. et al., 2022). Understanding how vaccination affects the autophagic response in infected fish may provide valuable insights into the protective mechanisms underlying vaccine efficacy.

The liver, as a major immune and metabolic organ, is particularly sensitive to bacterial infection (Tarasenko T.N. and McGuire P.J., 2017). During *Y. ruckeri* infection, hepatocytes undergo significant cellular stress, which may induce autophagy as an adaptive response. Activation of autophagy-related genes and proteins in response to bacterial invasion may enhance the host's ability to eliminate pathogens and mitigate tissue damage (Kroemer G. et al., 2010; Rahman M.A. et al., 2024). However, excessive or dysregulated autophagy can also contribute to cellular dysfunction and a balanced response is required for optimal liver function (Ke P.Y., 2019).

Studies in model organisms have shown that vaccination can modulate autophagic pathways, potentially enhancing pathogen clearance and immune responses (Jang Y.J. et al., 2019). In fish, the role of autophagy in vaccinated individuals remains an emerging area of research (Kurhaluk N. and Tkachenko H., 2021). Investigating autophagic activity in the liver of vaccinated and infected rainbow trout may provide new insights into how vaccination affects cellular defence mechanisms against bacterial pathogens.

This study aims to assess the autophagic function of the liver in vaccinated rainbow trout following infection with *Y. ruckeri*. By evaluating the activity of four lysosomal enzymes – alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG) – we aim to determine whether vaccination enhances or suppresses autophagic responses during bacterial challenge. A deeper understanding of this process may contribute to the development of improved vaccination strategies and disease management practices in aquaculture. Elucidating the interplay between vaccination, infection and autophagy in rainbow trout may have wider implications for fish health and the sustainability of aquaculture.

Materials and methods.

Fish. Rainbow trout (*Oncorhynchus mykiss* Walbaum) with an average weight of 310-320 g were used in the experiment. The study was conducted at the Salmonid Research Department of the Stanislaw Sakowicz Institute of Inland Fisheries in Olsztyn, Poland. The fish were kept under controlled environmental conditions to ensure stable water parameters: temperature of 14.5 ± 0.5°C, pH of 7.5 and dissolved oxygen of approximately 12 ppm. Supplemental oxygen was provided by aeration at a rate of 25 litres per minute under a 12 h light/12 h dark photoperiod. Fish were fed a commercial pelleted diet using 12-hour fish belt feeders to ensure optimal food intake. Daily food allowances were calculated according to established dietary guidelines.

All enzymatic assays were performed at the Department of Zoology and Department of Animal Physiology, Institute of Biology, University of Pomerania, Słupsk, Poland, using standardised protocols to ensure consistency and reliability of results.

Study groups. Fish were divided into two experimental groups: (I) an unvaccinated control group and (II) a group orally vaccinated against *Yersinia ruckeri*. The fish were housed in 1000 L square tanks, with 150 fish per tank, under identical environmental conditions. The vaccine was developed at the Department of Fish Diseases, National Veterinary Research Institute in Puławy, Poland, according to a patented process (Patent No. P.428259). The oral vaccine contained *Y. ruckeri* at a concentration of 1×10^9 cells/ml, which was incorporated into the feed and administered over three consecutive days. After vaccination, the fish were maintained in water at 14.5 ± 0.5°C and pH 7.5 for 60 days. The challenge test was performed on day 61 after vaccination.

For the challenge test, 40 fish were divided into four subgroups (10 fish per group): (I) uninfected control, (II) infected control, (III) vaccinated uninfected and (IV) vaccinated infected. Experimental infection was induced with *Y. ruckeri* serotype O1, biotype 2, isolated from an outbreak on a rainbow trout farm. Prior to infection, *Y. ruckeri* was cultured on tryptone soy agar supplemented with 5% horse blood (Oxoid®) at 25 ± 1°C for 24 h. Fish were infected intraperitoneally with *Y. ruckeri* at a dose of 1×10^7 CFU/mL suspension.

The experiment lasted 10 days, during which time fish were observed three times daily for behavioural changes, clinical signs and mortality. Cumulative survival was assessed based on mortality counts. Swabs from the head kidney of deceased fish were analysed to confirm *Y. ruckeri* as the cause of death. Mortality rates were expressed as percentages, with n = 10 considered as 100%.

Sampling procedures. Fish were euthanised 10 days after challenge. The liver was excised *in situ*. For lysosomal enzyme assays, liver tissue was rinsed with 0.15 M KCl cold isolation buffer to remove blood before homogenisation on ice using a Potter-Elvehjem glass homogeniser with a Teflon motorised pestle. The isolation buffer contained 0.25 M sucrose and 2 mM EDTA, adjusted to pH 7.0 with KOH. Homogenates (20% w/v) were prepared for differential centrifugation according to the method of DeMartino and Goldberg (1978). After centrifugation, the supernatant fractions were resuspended in 50 mM acetic acid/sodium acetate buffer (pH 5.0) before storage and further analysis. Isolated fractions were subjected to two freeze-thaw cycles to ensure enzyme activation. Protein concentration was determined by the Bradford method using bovine serum albumin as standard. The absorbance was measured at 595 nm. All assays were performed in duplicate at 22 ± 0.5°C, with enzymatic reactions initiated by the addition of tissue supernatant.

Lysosomal enzyme assay. The activities of alanyl aminopeptidase and leucyl aminopeptidase were determined spectrophotometrically according to the method of

DeMartino and Goldberg (1978). The reaction was initiated by incubating 50 μL of sample with 500 μL substrate incubation medium containing DMF (Serva, Germany) at 37°C, pH 6.0, for 60 min. The reaction was terminated by the addition of 500 μL stop buffer containing Fast Blue BB salt dissolved in 2% Tween 20 (Sigma, USA). The absorbance was measured at 540 nm. Alanyl aminopeptidase activity was determined using L-alanyl-2-naphthylamine in 0.1 M PBS buffer, while leucyl aminopeptidase activity was measured using L-leucyl-2-naphthylamine in 0.1 M PBS buffer (pH 7.0).

Acid phosphatase and β-N-acetylglucosaminidase activities were measured at 420 nm using 4-nitrophenyl derivatives as substrates according to the method of Barrett and Heath (1977). Enzyme activities were expressed as nmol per hour per mg protein.

Statistical analysis. Results are expressed as mean ± S.D. Each data set was processed separately using Statistica 13.3 (TIBCO Software Inc., USA). Normality of data was assessed using the Kolmogorov-Smirnov test ($p > 0.05$), while homogeneity of variance was assessed using Levene's test. Significant differences within and between groups were determined using unequal sample size one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Correlation and regression analyses were also performed to assess relationships between parameters. Differences were considered statistically significant at $p < 0.05$.

Results. The activity of key lysosomal enzymes in the hepatic tissue of rainbow trout of rainbow trout orally immunised against *Y. ruckeri* and challenged with *Y. ruckeri* is shown in Fig. 1.

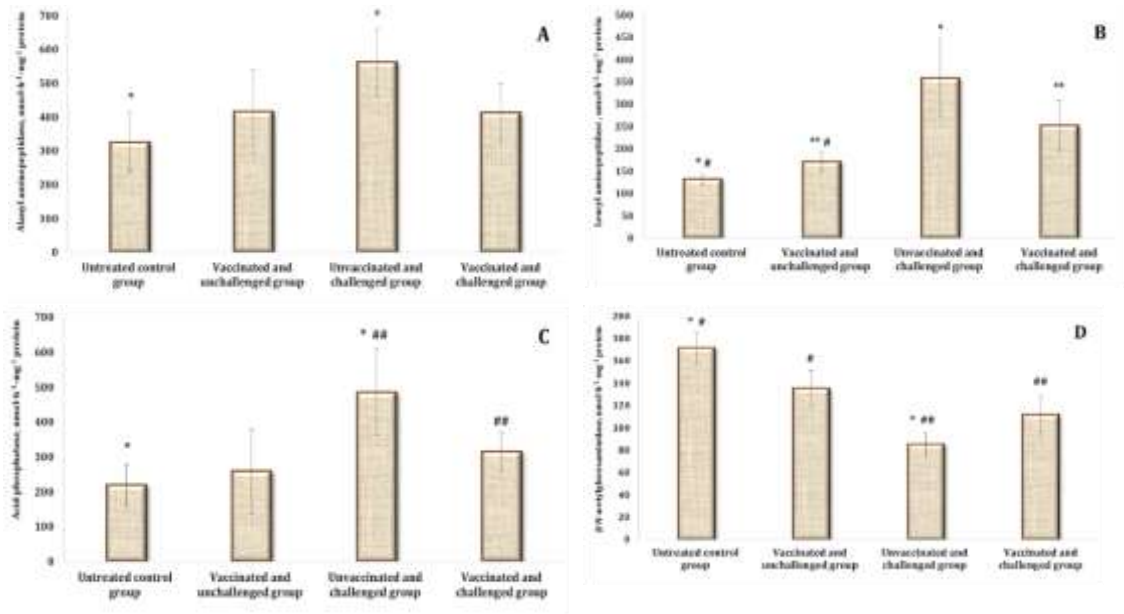

Fig. 1. **The activity of key lysosomal enzymes [alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG)] in the hepatic tissues of rainbow trout orally immunised against *Y. ruckeri* and challenged with *Y. ruckeri*.**

Data are presented as means ± S.D. (n = 10).
** Significant differences ($p < 0.05$) between the untreated control group and the group challenged with Y. ruckeri;*
*** Significant differences ($p < 0.05$) between the vaccinated group subjected to the Y. ruckeri challenge and the vaccinated group;*
 # Significant differences ($p < 0.05$) between the untreated control group and the vaccinated group;
Significant differences ($p < 0.05$) between the vaccinated group subjected to the Y. ruckeri challenge and the group challenged with Y. ruckeri.

The activity of four lysosomal enzymes – alanyl aminopeptidase (AAP), leucyl aminopeptidase (LAP), acid phosphatase (AcP) and β-N-acetylglucosaminidase (NAG) – varied significantly among the experimental groups, reflecting the influence of vaccination and *Y. ruckeri* infection on liver function in rainbow trout.

AAP activity had the highest mean value in the unvaccinated and challenged group (562.11 ± 98.78 nmol·h^{-1}·mg^{-1} protein), indicating a pronounced enzymatic response to bacterial infection. The vaccinated and unchallenged group showed increased AAP activity (415.54 ± 124.1 nmol·h^{-1}·mg^{-1} protein) compared to the untreated control group (325.21 ± 87.11 nmol·h^{-1}·mg^{-1} protein), indicating a possible vaccine-induced priming effect. However, the vaccinated and challenged group showed AAP activity (411.89 ± 85.47 nmol·h^{-1}·mg^{-1} protein) comparable to the vaccinated and unchallenged group, suggesting that vaccination attenuated the infection-induced increase in AAP activity (Fig. 1A).

LAP activity followed a similar trend, with the highest value observed in the unvaccinated and challenged group (358.01 ± 87.58 nmol·h^{-1}·mg^{-1} protein). This significant increase suggests that infection resulted in an enhanced proteolytic response. The vaccinated and challenged group had a lower LAP activity (252.12 ± 56.04 nmol·h^{-1}·mg^{-1} protein) than the unvaccinated and challenged group, but it remained higher than in the vaccinated and unchallenged group (170.69 ± 22.11 nmol·h^{-1}·mg^{-1} protein). The untreated control group had the lowest LAP activity (132.33 ± 10.2 nmol·h^{-1}·mg^{-1} protein), highlighting the baseline enzyme levels in unstressed fish (Fig. 1B).

AcP activity was significantly increased in the unvaccinated and challenged group (485.47 ± 124.1 nmol·h^{-1}·mg^{-1} protein), indicating increased lysosomal degradation processes in response to infection. The vaccinated and challenged group had intermediate AcP activity (314.25 ± 56.2 nmol·h^{-1}·mg^{-1} protein), suggesting that vaccination attenuated the infection-induced increase in lysosomal activity. The vaccinated and unchallenged group had slightly higher AcP activity (258.21 ± 121.2 nmol·h^{-1}·mg^{-1} protein) than the untreated control group (219.45 ± 58.33 nmol·h^{-1}·mg^{-1} protein), possibly due to the immune stimulation associated with vaccination (Fig. 1C).

In contrast to the other enzymes, NAG activity showed an inverse trend, with the lowest activity observed in the unvaccinated and challenged group (85.16 ± 10.2 nmol·h^{-1}·mg^{-1} protein). This decrease suggests a possible exhaustion of lysosomal function under intense infection stress. The vaccinated and challenged group showed higher NAG activity (111.5 ± 16.45 nmol·h^{-1}·mg^{-1} protein), suggesting a partial restoration of enzymatic function by vaccination. The highest NAG activity was observed in the untreated control group (171.25 ± 14.22 nmol·h^{-1}·mg^{-1} protein), followed by the vaccinated and unchallenged group (135.2 ± 16.25 nmol·h^{-1}·mg^{-1} protein), suggesting that both infection and vaccination affect NAG activity differently from the other lysosomal enzymes (Fig. 1D).

Collectively, these results indicate that bacterial infection significantly alters lysosomal enzyme activity in the liver of rainbow trout, with vaccination playing a modulatory role in attenuating infection-induced enzymatic changes. The observed differences in enzyme activity between groups highlight the potential impact of vaccination in regulating lysosomal function during pathogenic challenges.

Discussion. The observed variation in lysosomal enzyme activity between experimental groups highlights the complex interplay between bacterial infection and vaccination in modulating liver function in rainbow trout. The significant increase in AAP, LAP and AcP activities in the unvaccinated and challenged groups is consistent with previous studies indicating that bacterial infection triggers lysosomal activation as part of the immune response (van der Vaart M. et al., 2012; Pérez-Stuardo D. et al., 2020;

Chiang Y.R. et al., 2022). The marked elevation of these enzymes suggests an enhanced proteolytic and degradative response to *Y. ruckeri* infection, likely associated with the breakdown of damaged cellular components and pathogen clearance (Menanteau-Ledouble S. et al., 2020).

Vaccination appeared to attenuate the infection-induced increase in lysosomal enzyme activity. The vaccinated and challenged group had lower AAP, LAP and AcP activities than the unvaccinated and challenged group, suggesting that prior immunisation reduced the inflammatory response and cellular damage caused by the pathogen. This finding is consistent with reports indicating that effective vaccination limits excessive lysosomal activation by enhancing adaptive immune mechanisms, thereby reducing reliance on lysosomal degradation pathways (Zwack E.E. et al., 2015; Osterloh A., 2022).

Interestingly, NAG activity followed a distinct pattern, with the lowest levels observed in the unvaccinated and challenged groups (Fig. 1D). This decrease suggests potential lysosomal dysfunction under severe infection stress, possibly due to depletion of cellular resources or enzyme inactivation caused by prolonged immune activation. The partial restoration of NAG activity in the vaccinated and challenged groups further supports the protective role of vaccination in maintaining lysosomal function. These results corroborate previous findings highlighting the immunomodulatory effects of vaccination in fish species, where vaccine-induced immune priming prevents excessive metabolic stress on lysosomal pathways (Du et al., 2022).

The moderate increase in AAP, LAP and AcP activities in the vaccinated and unvaccinated groups compared to the untreated control group suggests that vaccination alone affects lysosomal function, probably through the stimulation of immune-related metabolic processes. Such effects have been documented in other teleost species, where activation of the immune system following vaccination leads to transient metabolic adjustments in hepatocytes (Aluru N. and Vijayan M.M., 2009; Mussap M. et al., 2024).

In our previous study (Kurhaluk N. et al., 2024), we investigated oxidative stress biomarkers, antioxidant and lysosomal enzyme activity, and biochemical parameters in the liver of rainbow trout vaccinated against enteric redmouth disease and challenged with *Y. ruckeri*. The results showed that in unvaccinated fish, *Y. ruckeri* infection disrupted the oxidative balance, increasing lipid peroxidation, oxidative protein modification and lysosomal enzyme activity, while reducing total antioxidant capacity. In contrast, vaccinated fish showed increased glutathione-related enzyme activity, reduced lipid peroxidation and lower lysosomal enzyme activity after infection compared to unvaccinated and challenged fish. These results suggest that vaccination mitigates oxidative damage and modulates enzymatic responses in fish exposed to *Y. ruckeri* (Kurhaluk N. et al., 2024).

We also evaluated the time-dependent effects of *Y. ruckeri* vaccination on oxidative mechanisms by assessing key biomarkers of lipid peroxidation [2-thiobarbituric acid reactive substances (TBARS)] and protein oxidation [aldehyde and ketone derivatives of oxidatively modified proteins (OMP)], antioxidant defences [superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), total antioxidant capacity (TAC)] in rainbow trout liver tissue (Tkaczenko H. et al., 2023). A concentrated *Y. ruckeri* vaccine was incorporated into the fish feed and administered three times at two-day intervals. Fish were euthanised at 31, 61 and 181 days post vaccination for liver tissue analysis. Vaccination against *Y. ruckeri* did not significantly alter TBARS levels, but reduced aldehyde and ketonic OMP derivatives, particularly in the first and second months after immunisation. Glutathione-dependent enzyme activity increased, particularly at one and six months post-vaccination, with the highest TAC levels observed at two and six months. The results suggest that vaccination-

induced oxidative stress in liver tissue triggers adaptive responses through transient activation of antioxidant and lysosomal enzymes. In addition, vaccination affected lysosomal membrane permeability, particularly for carbohydrate cleavage, following activation of the immune system against *Y. ruckeri*. Antioxidant defences were generally preserved, as evidenced by the maintenance or increase in CAT, GR and GPx activity after vaccination. These results highlight the role of oxidative mechanisms in the immune response and the potential of vaccination to modulate lysosomal and antioxidant enzyme activity in rainbow trout (Tkaczenko H. et al., 2023).

Collectively, these findings highlight the critical role of lysosomal enzymes in the immune response to bacterial infection and provide further evidence for the beneficial effects of vaccination in modulating lysosomal activity. Further research is needed to elucidate the long-term effects of vaccination on lysosomal function and to explore the potential metabolic trade-offs associated with immune stimulation in fish.

Conclusions. The present study demonstrates that *Y. ruckeri* infection significantly alters lysosomal enzyme activity in the liver of rainbow trout, reflecting an enhanced proteolytic and degradative response to bacterial challenge. Vaccination played a modulatory role by attenuating the infection-induced increase in AAP, LAP and AcP activities, while partially restoring NAG activity, suggesting its protective effect in maintaining lysosomal function. The moderate increase in lysosomal enzyme activity in the vaccinated and unvaccinated groups further suggests that vaccination alone affects metabolic and immune processes in hepatocytes.

These findings highlight the importance of lysosomal enzymes as potential biomarkers for assessing infection severity and vaccine efficacy in fish. Future research should investigate the long-term metabolic consequences of vaccination and its impact on immune homeostasis in aquaculture species. Understanding these mechanisms could help to optimise vaccination strategies and improve disease resistance in farmed fish populations.

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