FISHERIES & AQUATIC LIFE (2023) 31: 105 - 111
Archives of Polish Fisheries
DOI 10.2478/aopf-2023-0012

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RESEARCH ARTICLE

Improved innate immunity in juvenile vimba bream (*Vimba vimba*) fed a dry diet with an additive of hydrochloric acid (HCl)

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Received – 26 July 2023/Accepted – 29 August 2023. Published online: 30 September 2023;
Citation: Kamiński, R., Kazuń, B., Małaczewska, J., Sikorska, J., Grabowski, R., Jędroszka, N., Hassaan, M. S., Wolnicki, J. (2023). Improved innate immunity in juvenile vimba bream (*Vimba vimba)* fed a dry diet with an additive of hydrochloric acid (HCl). Fisheries & Aquatic Life 31, 105-111.

Abstract. A few reports indicate the beneficial effects of organic acids and their salts on anti-infective immunity in fish species. In the role of immunostimulants, inorganic acids may prove to be a much cheaper alternative to their organic equivalents. However, no report has described the effect of using inorganic acid as a feed additive on fish immunity. This study is the first attempt to evaluate the effect of hydrochloric acid (HCl) as a fish feed supplement on cellular immunity in the stomachless fish, cypriniform vimba bream, *Vimba vimba* (L.). Two groups, three replicates each, of juvenile vimba were fed a commercial dry fish diet or its variant containing a 1.5% additive of HCl for 55 days. The experiment was conducted in a recirculating aquaculture system at 25°C. Mortality, growth, condition factor, feed conversion ratio, respiratory burst activity (RBA), potential killing activity (PKA), and the proliferative response of head kidney lymphocytes were determined for both groups at the end of the experiment and compared. The PKA was higher in the group fed the diet with HCl. For all other parameters studied, there were no significant differences between the experimental groups. The results of the current study prove that the dietary additive of inorganic hydrochloric acid can substantially improve immune response to bacterial infections in juvenile vimba bream.

Keywords: growth, inorganic acid, potential killing activity, respiratory burst activity, stomachless fish

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Introduction

In recent decades, there has been increasing interest in the possibility of intensive rearing of larval and juvenile fish under controlled conditions in recirculating aquaculture systems (RAS). Among the key advantages of this technology is the possibility of implementing effective biosecurity measures to minimize exposure and susceptibility to pathogens and reduce economic losses from mortalities. However,

the lack of contact with pathogens limits the opportunity to develop the immune system's ability to respond to future infections. Therefore, effective methods to increase fish immunity in RAS are highly encouraged. Among them, very promising results have been obtained for fish feed additives based on organic acids and their salts. They were first introduced to aquaculture as growth promoters, replacing antibiotics in this role (Lückstädt 2008a, 2008b, Ng and Koh 2017). However, a few reports also indicate the beneficial effects of organic acids and their salts on anti-infective immunity in several fish species (Ramli et al. 2005, Lim et al. 2010, Park et al. 2011, Abu Elala and Ragaa 2015, Reda et al. 2016). The mechanism behind this phenomenon remains unclear. The most common explanation is that when used with dry feed organic acids inhibit the growth of pathogenic bacteria in the gastrointestinal tract, while favoring an increase of beneficial bacteria characterized by a high degree of resistance to low intestinal pH. Abu Elala and Ragaa (2015) found a significant increase in lactic acid bacteria in Nile tilapia *Oreochromis niloticus* (L.) supplemented with potassium diformate and noted an increase in phagocytic activity and lysozyme activity.

Despite their effectiveness, additives for fish feed based on organic acids and their salts have not found widespread use in practice, probably because of their high price. However, recent preliminary findings show that inorganic hydrochloric acid could be a much cheaper alternative. In stomachless fish, like crucian carp, *Carassius carassius* (L.), a 1.5% additive of this acid to two different commercial fish feeds resulted in an increase in juvenile fish growth and considerably lowered incidence of body deformities (Kamiński et al. 2021). Other preliminary observations carried out on juvenile tench, *Tinca tinca* (L.), indicated that a 1.5% supplement of hydrochloric acid to the dry feed was close to optimum as it gave better results compared to supplements of 0.75% or 3.0% in terms of fish growth, mineral nutrition, and the occurrence of body deformities (Kamiński et al., unpublished data). An HCl additive to fish feed can also alter fish gonadogenesis (Hliwa et al. 2018) and improve their resistance to bacterial infections (Sikorska et al. 2022). So far, however, no evidence has been found of the effects of an inorganic acid as a feed additive on fish immunity. Therefore, the present pilot study was conceived as the first attempt to evaluate the effects of hydrochloric acid as an additive to dry feed on the immunity in the stomachless cypriniform vimba bream, *Vimba vimba* (L.).

Materials and Methods

Fish

The juvenile vimba bream used in the experiment were the pooled progeny of four females and five males. They were obtained from the experimental hatchery in Żabieniec of the National Inland Fisheries Research Institute (Poland). The fish were prepared for the experiment under controlled conditions in the same hatchery. The experiment began when the fish were six months old and their mean (± SD) body weight (BW) and total length (TL) were 0.47 ± 0.10 g and 43.0 ± 29.0 mm, respectively. Prior to stocking in the experimental aquaria, the fish were checked for the absence of body deformities and graded according to their BW to unify their initial size distribution in all aquaria.

Experimental design and conditions

The fish were stocked in six flow-through glass aquaria (V = 20 dm^3), 30 in each. The aquaria were continuously supplied with filtered, heated, aerated water from a RAS at approximately 0.25 dm^3 min^{-1}. The water temperature and dissolved oxygen concentration in the water were measured twice daily. The mean water temperature (± SD) was 25.0 ± 0.1°C. The water in the aquaria was continuously aerated with airstones to maintain an oxygen concentration above 70% saturation. Other water quality parameters were monitored weekly in one aquarium per group. The concentrations of total ammonia and nitrites were below 0.7 mg dm^{-3} and 0.1 mg dm^{-3}, respectively. Water conductivity was 407–426 μS cm^{-1},

and pH was 7.87–8.03. Aquaria were illuminated from 08:00 to 21:00 by fluorescent tube lights at about 600 lx at the water surface.

Commercial starter feed Aller Futura EX GR 0.5–1.0 mm (Aller Aqua, Denmark) was used as a control diet in the experiment (group FO). According to data from the producer, the proximate composition of the feed was 60.0% crude protein, 12.6% ash, 15.0% total lipids, 5.7% nitrogen-free extract, 0.7% fiber, 1.4% phosphorus, 2.5% calcium, and 21.2 MJ kg^{-1} gross energy, and the pH was 6.67. The experimental diet (group FM) consisted of the feed used in the FO group with a 1.5% additive of hydrochloric acid (HCl), and the pH of this diet was 5.01. The inclusion level of the additive was based on the pilot study by Kamiński et al. (2021). Thirty-six per cent food grade HCl was used (Kemikals, Gdynia, Poland). The appropriate weights of the HCl were dissolved in 100 cm^3 distilled water first and then sprayed homogeneously onto 1 kg of the commercial pellets. Afterward, they were, in sequence, mixed, air-dried at 40°C for 48 h, placed in plastic bags, and stored in a refrigerator until use. The feed was delivered manually in equal portions five times daily every 3 h between 08:00 and 20:00. The fish were fed to apparent satiation. The daily food ration per aquarium was initially 0.56 g, which constituted 4.0% of fish biomass. It was increased on day 15 to 0.9 g (3.8% of fish biomass), and on day 29 to 1.45 g (3.7% of fish biomass) to adjust for the increase in fish biomass during the experiment. The experiment lasted 55 days.

Calculations for condition factor, growth performance, and feed conversion

Fish growth performance and feed conversion indices were calculated according to the following equations, where BW is expressed in grams (g) and TL in millimeters (mm):

- condition factor: $K = 10^5 \times BW \times TL^{-3}$;
- relative growth rate (% d^{-1}): $RGR = 100 \times (e^G - 1)$,

where $G = (\ln BW_f - \ln BW_i) \times n^{-1}$; BW_i and BW_f are the initial and final body weight, respectively; n is the number of feeding days;

- daily increment in total length (mm day^{-1}): $ITL = (TL_f - TL_i) \times n^{-1}$,

where TL_f and TL_i are the final and initial mean TL of fish, respectively; n is the number of feeding days;

- feed conversion ratio (FCR) = weight of consumed feed × fish weight $gain^{-1}$.

Sample collection

All fish from each experimental aquarium were euthanized by immersion with an overdose (250 mg dm^{-3}) of MS-222 (Sigma-Aldrich). Then, their individual BW and TL were measured, and head kidney samples were taken.

Isolation of fish immune cells

Fish head kidneys were pooled within the groups (organs from all individuals kept in the same aquarium, three aquaria per group, n = 3). Head kidney immune cells were isolated using Histopaque 1077 (Sigma-Aldrich) density gradient centrifugation, suspended at a concentration of 1×10^6 cells cm^{-3} in RPMI-1640 medium supplemented with 10% fetal calf serum and 1% antibiotic–antimycotic solution (both reagents from Sigma-Aldrich), and cultured/incubated as described before (Kazuń et al. 2020). Isolated cells were then used for assays of respiratory burst activity, potential killing activity, and proliferative response of lymphocytes. Samples obtained from each pool were tested in duplicate.

Respiratory burst activity (RBA) and potential killing activity (PKA) tests

The intracellular respiratory burst and potential killing activities of phagocytes were determined as previously described by Kazuń et al. (2018). In short, the adherent immune cells were incubated in a fresh medium containing 0.1% nitroblue tetrazolium (NBT, Sigma-Aldrich) and 1 μg cm^{-3} phorbol myristate acetate (PMA, Sigma-Aldrich) or *Aeromonas hydrophila* (1×108 cells cm^{-3}) for 60 min at 22°C. Once the

supernatant was removed, the cells were fixed with absolute ethanol and the reduced NBT was extracted using potassium hydroxide (KOH, Chempur, Poland) and dimethylsulphoxide (DMSO, POCh, Poland). The optical density (OD) of the samples was measured colorimetrically at 620 nm. The results were expressed as a stimulation index (SI), which was calculated by dividing the mean OD of PMA (RBA test) or bacteria-stimulated cells (PKA test) by the OD of the unstimulated control cells.

Proliferative response of lymphocytes – An MTT reduction assay

The mitogenic response of vimba lymphocytes was determined using the 3-(4,5 dimethylthiazol--2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) colorimetric assay as described by Kazuń et al. (2018). The head kidney immune cells were cultured in the presence of mitogens: concanavalin A (ConA) as a T-cell mitogen or lipopolysaccharide from *Escherichia coli* (LPS) as a B-cell mitogen (both mitogens were purchased from Sigma-Aldrich and used at concentrations of 10 $\mu g\ cm^{-3}$), for 72 h at 22°C. Unstimulated control cells were maintained in a medium without mitogens. Following incubation, 10 mm^3 of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) solution (10 $mg\ cm^{-3}$) was added to each well, and the plate was incubated for 3 h. After the supernatant was removed, the reduced MTT was dissolved in DMSO, and the optical density was measured at a wavelength of 570 nm, using 640 nm as the reference wavelength. The results were expressed as a stimulation index (SI), which was calculated by dividing the mean OD of the mitogen-stimulated cells by the OD of the unstimulated control cells.

Statistical analysis

The data obtained were submitted to the Kolmogorov-Smirnov test to assess whether the data distribution was within the normality curve and Levene's test to verify homoscedasticity. The data obtained that met the prerequisites of normality and homoscedasticity were submitted to Student's t-test.

Results

No mortality occurred during the experiment. Potential killing activity (PKA) was significantly higher in fish fed the FM diet as compared to those fed the FO diet (Table 1). No significant difference between the experimental groups was found for fish growth parameters, feed conversion ratio, RBA and proliferative response of head kidney lymphocytes stimulated by ConA (T lymphocytes), or LPS (B lymphocytes).

Discussion

Innate immunity plays a dominant role in fish disease resistance since acquired immunity mechanisms are much slower and less efficient in fish than in mammals (Magnadóttir 2006). Thus, it was not surprising that in the present experiment, there was no effect of the experimental factor on the proliferative response of lymphocytes (Table 1). However, higher potential killing activity (PKA) in fish fed dry feed with the HCl additive indicated increased innate cellular resistance of the fish immune system against bacterial infections with no effect on growth parameters or the feed conversion ratio (Table 1). No significant differences in RBA suggested that the overall potential of fish phagocytes to perform respiratory bursts was similar in both groups. A much stronger response, obtained in the PKA test in group FM, showed that in this group immune potential was considerably more efficiently activated in contact with the bacterial pathogen *Aeromonas hydrophila*. This explained the findings of Sikorska et al. (2022), who in RAS observed no signs of infection in barbel *Barbus barbus* (L.) females fed the commercial dry diet with a 1.5% additive of HCl, while 27% of fish fed the same feed without the additive showed bacterial infection of the genital papillae.

Table 1
Fish size, condition, growth, feed conversion, and immune parameters of juvenile vimba bream fed a commercial dry diet (FO) or the same diet with a 1.5% additive of HCl (FM)

Parameter	FO	FM	P-value
Initial parameters			
Initial body weight, g	0.475 ± 0.001	0.476 ± 0.001	0.299
Initial total length, mm	42.93 ± 0.14	43.10 ± 0.12	0.170
Initial condition factor K	1.095 ± 0.001	1.095 ± 0.007	0.977
Final parameters			
Final body weight, g	3.107 ± 0.087	3.022 ± 0.017	0.177
Final total length, mm	74.53 ± 0.76	73.9 ± 0.24	0.248
Final condition factor K	0.746 ± 0.003	0.743 ± 0.009	0.576
Relative growth rate, % / d	3.473 ± 0.056	3.418 ± 0.011	0.171
Daily increment in total length, mm / d	0.575 ± 0.012	0.560 ± 0.002	0.103
Feed conversion ratio	0.821 ± 0.028	0.848 ± 0.006	0.175
Respiratory burst activity of head kidney phagocytes (SI)	2.116 ± 0.145	1.867 ± 0.294	0.259
Potential killing activity of head kidney phagocytes (SI)	1.209 ± 0.061	1.613 ± 0.120	0.007
Proliferative response of head kidney lymphocytes stimulated by ConA (SI)	2.195 ± 0.153	1.855 ± 0.193	0.075
Proliferative response of head kidney lymphocytes stimulated by LPS (SI)	1.395 ± 0.170	1.192 ± 0.083	0.137

Data are means ± SD; n = 3; unpaired Student's t-test.

Feed additives lowering feed pH are expected to positively influence growth conditions for beneficial bacteria inhabiting the alimentary tract of fish, e.g., lactic acid bacteria. It seems that fish immunity might be enhanced by acidifying the diet in this way. Perhaps hydrochloric acid, by directly acidifying the diet, also causes a lowering of pH in the intestinal environment and also inhibits the growth of bacteria sensitive to low pH, thereby regulating the composition of the microflora of the fish's digestive tract. Balcázar et al. (2007) confirmed, in a study on rainbow trout, *Oncorhynchus mykiss* (Walbaum), that modification of the intestinal flora had beneficial effects on the non-specific humoral and cellular immune response by increasing phagocytic cell activity. However, rainbow trout has a functional stomach, where pH can range from 2.7 to 4.9 (Bucking and Wood 2009). In contrast, in stomachless cypriniform fish, the natural reaction inside the alimentary tract does not deviate much from neutrality (Solovyev et al. 2018). Thus, acidifying the feed of stomachless fish could potentially be even more important because they do not possess the ability to considerably lower diet pH in their digestive tract. However, acidifying the alimentary tract of stomachless fish is not a natural state for these animals, therefore such a procedure can also potentially disturb their natural digestive processes. The results of the current experiment do not support this hypothesis because the dietary additive of HCl did not have a significant effect on fish growth or the feed conversion ratio.

Determining the mechanisms behind HCl as an additive to dry fish feed undoubtedly requires further extensive research. Nevertheless, the results obtained in the current pilot study confirmed earlier assumptions that a dietary additive of HCl can substantially improve the immune response to bacterial infections in stomachless fishes.

Ethics statement. The research was in compliance with Polish animal welfare regulations.

Author contributions. R.K. designed the work, investigation, organized, analyzed, and interpreted the data, wrote the original work, methodology,; B.K. organized,

analyzed, and interpreted the data, wrote the original work, methodology; J.M. organized, analyzed, and interpreted the data, wrote the original work, methodology, validation; J.S. investigation, validation, resources; R.G. investigation; N.J. investigation; M.S.H. analyzed and interpreted the data, validation; J.W. supervised the work on the experiment, investigated. All authors - reviewed, edited, and approved the manuscript for publication.

Declaration of competing interest. The authors confirm they have no known conflicts of interest.

Data availability. Data will be made available upon request.

Acknowledgements. The study was financed by the IMMUNOVICTU project, supported by the Polish Operational Programme "PO RYBY 2014–2020" within the European Maritime and Fisheries Fund (00004-65-21.1-OR1400002/22/23).

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