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Pike-perch larvae growth in response to administration of lactobacilli-enriched inert feed during first feeding

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ABSTRACT

This study evaluated whether inert feed enriched with *Lb. paracasei* subsp. *paracasei* BGHN14 may be used as a weaning diet for first feeding pike-perch larvae. Three experimental groups were weaned from the start of exogenous feeding: two groups were given inert feed enriched with BGHN14 either via 12 h incubation with live BGHN14 cells or via coating with homogenized BGHN14 cells and one group was supplemented non-enriched inert feed. In all three groups *Artemia* was co-fed with inert feed during weaning. Control group larvae were fed *Artemia* exclusively during the treatment period. Treatment lasted fourteen days, starting from the 6th day post-hatch (DPH). Larval sampling was performed on the 20th DPH for gene expression and enzyme activity analysis. Larvae were also sampled on the 32nd DPH for morphometric and body composition analysis. Our results showed that weaning of first feeding pike-perch larvae was associated with an increase of fish condition (0.72 ± 0.12–0.77 ± 0.11 versus 0.67 ± 0.11 in controls), but it suppressed skeleton development, according to *Col1* mRNA expression (1 ± 0.51–1.06 ± 0.36 versus 2.07 ± 0.53 in controls) and reduced fat deposition (1.25 ± 0.23–1.49 ± 0.33 versus 1.84 ± 0.31% in controls). This presumably reflected lower availability of soluble proteins in microdiet as opposed to live food, along with high leaching rate of amino acids from solid feed particles, as reported in our previous studies. However, skeleton differentiation was not impaired in group weaned on BGHN14 homogenate coated feed (*Col1* mRNA expression: 2.68 ± 0.72), which was enriched in skeleton building and taste stimulating amino acids. These larvae were also presented with substantially higher length (15.28 ± 2.55 versus 13.93 ± 2.31 mm in controls) and weight (26.56 ± 13.83 versus 21.03 ± 11.25 mg in controls), which correlated with lower trypsin activity (1.06 ± 0.13 versus 1.43 ± 0.26 mU/mg of proteins in controls) and an increase of PLA2 to trypsin activity ratio (453.12 ± 109.36 versus 264.84 ± 69.03 in controls). Present study suggests that weaning of first feeding pike-perch larvae using BGHN14 homogenate coated microdiet supports skeleton development and improves fish growth.

1. Introduction

Indoor pike-perch production is considered a promising branch in fast growing fish farming sector. As for many other fish species, larviculture is the bottleneck in the rearing process. It is associated with substantial mortalities, mainly because of the stress due to fish crowding and handling (Rehman et al., 2017). Rotifers (*Brachionus* spp.) are preferable first diet choice for cultured carnivorous fish, since their composition closely resembles the composition of plankton, which is the first feed in fish's natural environment (Dhont et al., 2013). However, due to production difficulties, rotifers are commonly replaced by brine shrimp (*Artemia* spp.), which does not have satisfactory nutritional profile for some fish and has inconsistent biochemical composition, resulting in unpredictable outcome in terms of fish growth and survival

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(Person-Le Ruyet, 1989; van Stappen, 1996). This is why early weaning via replacement of brine shrimp with artificial microdiet has been successfully used in several fish species (Liu et al., 2012; Pinto et al., 2018) and was used in pike-perch as well (Szkudlarek and Zakęś, 2007). Microdiet has not only a more stable biochemical composition, but also its use is more profitable as its administration to fish is far less laborious in comparison to live food (Person-Le Ruyet, 1989). Research dealing with weaning of larval pike-perch demonstrated that pike-perch may be successfully weaned from 15th DPH onwards, with co-administration of live food (Hamza et al., 2007; Kestemont et al., 2007; Ljubobratović et al., 2015). Aside from aforementioned financial benefits, earlier weaning of pike-perch may suppress cannibalistic behavior (Kestemont et al., 2007). Research performed by our group has also shown that the later the weaning, the higher the incidence of cannibalism in pike-perch culture (Ljubobratović et al., 2015).

Though larval diet is made from the highest quality ingredients and can be successfully accepted by fish larvae from mouth opening, it has low amount of food attractants, which stimulate food intake by larvae (Kolkovski et al., 1997). Food attractants are water soluble molecules, mostly soluble proteins and free amino acids (e.g. glycine, alanine, arginine). Though free amino acid content of larval microdiet is close to the content in *Artemia* (~1.5 mg/g per dry mass in delipidated samples, according to our results with OTOHIME B1), soluble proteins are poorly represented in inert feed (~1.5 in OTOHIME B1 and ~ 6.5 mg/g per dry mass in *Artemia*) (Ljubobratovic et al., 2020). Furthermore, both free amino acids and proteins are prone to high leaching rate from solid feed particles (almost 70 and 50% for OTOHIME B1 for amino acids and soluble proteins, respectively) (Lukić et al., under review). Simple enrichment of inert feed with soluble molecules may be ineffective, because of their high leaching rate (Kolkovski, 2013). Our group has made significant progress in evaluation of novel feed manipulation techniques which could increase the availability of nutrients in microdiet by treatment with probiotic lactobacilli, mostly concerning the reduction of triglyceride content (Lukic et al., 2019), since triglycerides, when present in excess, may potentially impede larval growth (Morais et al., 2007). This is far simpler and a cost-effective manner of triglyceride level manipulation than application of purified enzyme (Hermansyah et al., 2015). In this respect, introduction of microdiet treated with lactobacilli to pike-perch from 18th DPH improved fish growth and skeleton development (Ljubobratovic et al., 2020). Aside from modulation of lipid content, our recent *in vitro* study demonstrated that *Lactobacillus paracasei* subsp. *paracasei* BGHN14 cells coated onto the feed particles, do not only serve as a source of free amino acids, but also act as encapsulating agents, increasing the retention of soluble proteins after simulated leaching test (Lukić et al., under review).

In the present research, we aimed to analyze whether modulation of microdiet nutritive content by strain BGHN14 may improve the response of first feeding pike-perch larvae to weaning. As stated above, biochemical composition of microdiet was modulated in two directions: towards an increase of leaching-resistant taste stimulating and collagen-building amino acids, and towards the reduction of triglyceride amount (Lukić et al., under review). Results presented here provide an evidence of successful weaning of first feeding pike-perch larvae by using probiotic lactobacilli. Potential mechanisms of treatment efficacy are discussed.

2. Materials & methods

2.1. Feed treatment

OTOHIME B1 larval feed (Marubeni Nishin Feed Co., Ltd., Tokyo, Japan) was stored unopened at −20 °C for three months before use in the experiment. Feed was treated with *Lactobacillus paracasei* subsp. *paracasei* BGHN14 as explained in Lukić et al. (under review). Treatment was performed in glass 200 × 30 mm Petri dishes by pouring the bacterial suspension into the Petri dish and then slowly adding the feed with continuous shaking of dish by hand. The volume of bacterial suspension used was 2.6 x (per dry feed weight). The amount of feed treated per one Petri dish was 8 g. Bacterial suspension included either live BGHN14 cells or BGHN14 homogenate. Live BGHN14 cells were prepared by adding saline at 1.3 × volume and glycerol at 0.3 × volume (per wet pellet weight) to the pellet from exponential phase (10 h) BGHN14 culture. BGHN14 homogenate was prepared by adding saline at 1.7 × volume (per wet pellet weight) to pulverized pellets of stationary phase (16 h) BGHN14 culture exposed to 1 M NaCl as described in Lukić et al. (manuscript under review). Both live and homogenate suspensions were stored at −80 °C until feed treatment. In the case of feed mixing with live BGHN14 suspension, after mixing the bacterial suspension with feed, Petri dishes were incubated aerobically for 12 h at 37 °C. Afterwards, feed was dried at 50–55 °C for 4 h. In the case of feed mixing with BGHN14 homogenate, feed was immediately placed at 50–55 °C for 4 h drying. After drying, feed was stored at +4 °C until *in vivo* trial. Strain BGHN14 is the part of bacterial collection of the Laboratory for Molecular Microbiology (LMM), Institute of Molecular Genetics and Genetic Engineering (IMGGE) and has been deposited in Belgian coordinated collections of micro-organisms (BCCM™). Cultivation conditions for BGHN14 have been described in (Lukic et al., 2019).

2.2. In vivo study design

Animal trial was performed in Recirculating aquaculture system (RAS) unit of Research Center of Fisheries and Aquaculture, Hungarian University of Agriculture and Life Sciences (MATE HAKI), Szarvas, Hungary. Fish manipulation was performed according to Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guideline (Kilkenny et al., 2010) and regulations of the Animal Ethical Panel of the Center, which was established according to Hungarian State law (10/1999.I.27). Pike-perch larvae (5th day post-hatch, DPH) were distributed in 12 tanks (250 L) (10,000 larvae per tank). Food administration started on 6th DPH, which corresponds to the moment prior to the onset of exogenous feeding of pike-perch larvae reared at 15 ± 2 °C, as reported by Xu et al. (2017). Likewise, based on five-year experiences with our larviculture protocol, this is the optimal moment to start feeding using freshly hatcher *Artemia franciscana* nauplii in terms of high rate (>80%) of feeding larvae on the given temperature. Trial included four experimental groups (three tanks per group):

1. W = group weaned on 6th DPH administered 10 g OTOHIME B1/day and 100–200 *Artemia franciscana* nauplii/larva/day;
2. WLB = group weaned on 6th DPH administered 10 g live BGHN14 treated OTOHIME B1/day (from 6th to 19th DPH) and 100–200 *A. franciscana* naupli/larva/day;
3. WHB = group weaned on 6th DPH administered 10 g BGHN14 homogenate coated OTOHIME B1/day (from 6th to 19th DPH) and 100–200 *A. franciscana* naupli/larva/day;
4. C = control non-weaned group administered 100–500 *A. franciscana* nauplii/larva/day.

It was assured that the amount of nauplii provided in group 4 (100–500-larva-day) and the amount of OTOHIME B1 provided in groups 1, 2 and 3 (10 g/day) were in excess of the amount consumed by larvae. Larvae from groups WLB and WHB were administered an increasing percent of feed modified by BGHN14, from 6th to 19th DPH: 20, 30, 40, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50, 50%. Starting from 20th DPH, larvae from all experimental groups were daily given 15 g of (non-modified) OTOHIME B1 and B2 grades mixed in equal proportion, while the amount of *Artemia* was gradually reduced from 21st DPH for 20%/day. Starting from 25th DPH larvae were fed OTOHIME B2 exclusively until the end of the trial.

Feed was supplied continuously each 3 min during day via automatic feeder for 14 h per day, while *Artemia* was supplied manually five times a day at 3–4 h intervals. Debris from tanks, including feces, uneaten feed

and dead fish, was cleaned twice on a daily basis via end valve located at the bottom of the tanks. Photo-period was kept on 14:10 light:dark. Wide sprayer was used as a surface cleaning device to increase swim bladder inflation rate (Fazekas et al., 2021). Temperature in tanks was maintained at 15.8 ± 0.2 °C and oxygen concentration in the range of 8.7–10.8 mg/L measured at the tank's outflow. Water flow was upwelling with an exchange rate in the tanks set at 33% hour^{-1} upon stocking and was gradually increased until 75%/h until the end of trial. Water quality parameters (pH, electroconductivity, ammonium-ion, nitrate-ion and nitrite-ion) were estimated in the water samples from tank overflow outflow chambers from one tank per group (arbitrary selection) in four time points: 8th, 12th, 13th and 19th DPH.

Fish sampling was performed after overnight fasting, at two time points:

1. On the 20th DPH - fish samples were frozen at −80 °C and used for digestive enzyme analysis and RNA isolation; for each analysis 32 larvae per tank were sampled;
2. On the 32nd DPH - all fish were harvested from each tank and graded based on the presence of inflated swim bladder (Steenfeldt, 2015). Further on, only the fish with inflated swim bladder (SBI) were counted and their number was used for evaluation of larval survival. Body weight, total length and skeletal deformities (scoliosis, lordosis and jaw deformities) were assessed on 32 larvae per tank and samples were frozen at −20 °C for body composition analysis (64 larvae per tank); prior to weight measurement fish were dubbed on paper towel.

2.3. Digestive enzyme activity analyses

Larval samples were homogenized as described in Ljubobratovic et al. (2020). Before homogenization, 12 larvae (four per each tank from the same group) were pooled together, giving totally eight pooled samples per group. Activities of trypsin, chymotrypsin, lipase and phospholipase A2 (PLA2) were analyzed as detailed in Ljubobratovic et al. (2017). Samples were homogenized in 50 mM Tris-HCl, pH 7, 2 mM mannitol, using dounce tissue grinder, centrifuged at 15500 ×*g*, 15 min, +4 °C and supernatants were stored at −80 °C until analysis. Different substrates were added to prepared supernatants during assay procedures: Nα-benzoyl-DL-arginine-ρ-nitroanilidehydrochloride (BAPNA) for trypsin; succinyl-(ala)2-pro-phe-ρ-nitroanilide (SAPNA) for chymotrypsin and ρ-nitrophenyl palmitate (ρNPP) for lipase assay. Samples were incubated at 37 °C and absorbance at 410 nm was measured for the following 10 min after substrate addition. Differences in absorbances at 10 and 0 min were used for calculation of enzyme activities, using relevant equations (Ljubobratovic et al., 2017). Phospholipase A2 (PLA2) activity was determined using EnzChek™ Phospholipase A2 Assay Kit, ThermoFisher Scientific, Waltham, US, following instructions provide in the manual. Bradford reagent was used for protein quantification.

2.4. Gene expression analyses

Isolation of RNA from larval samples was performed as described in Ljubobratovic et al. (2017), after pooling the samples (12 larvae, four per each tank from the same group) as above. Quantitative PCR (qPCR) was performed as described in Ljubobratovic et al. (2020). Expression of genes at mRNA level coding for products related to (Table 1): muscle development and function (contractile proteins: *TnI* and *TMY4a* and structural protein *MYHC*) (Ochiai and Ozawa, 2020), fish nutritional (*IGFBP*) (Opazo et al., 2017) and energetic status (mitochondrial enzymes: *SDH*, *CS* and *COX*) (Ibarz et al., 2010; Lucassen et al., 2003; Yang et al., 2016), metamorphosis (*TSH*) (Campinho, 2019), growth regulation (*GH* and *GHR*) (Abdolahnejad et al., 2015) and skeleton differentiation (*Col2*, *Col1*, *osteonectin*) (Ljubobratovic et al., 2020) was analyzed. Additionally, levels of 16S rRNA specific for common fish bacterial pathogens *Aeromonas*, *Vibrio* and *Mycobacterium* spp. were quantified (Sudheesh et al., 2012). Primers used in the experiment were either designed for the purpose of this study, using sequences from National Center for Biotechnology Information (NCBI) (Table 1) or were designed in our previous studies (Ljubobratovic et al., 2017, 2020). Weaned group (W) was used as calibrator in calculations of relative gene expressions.

Table 1
Primers used in the study.

Primer name	5′-3′ sequence	Reference
Troponin I (fast skeletal muscle) (TnI)	CATGGAAGCCTGCAAGAAGC	This study
	CAGCTTTGCCCACTTTGGAC	This study
Tropomyosin 4a (cardiac) (TMY4a)	CTGGTGGAGGAGGAGTTGGA	This study
	CTCGTCTGCGGCCTTCTC	This study
Myosin heavy chain (fast skeletal muscles) (MYHC)	AAGTTCAAGCAGAAGCAGCG	This study
	CCCAGCAAGTAAGCAACCTT	This study
Thyroid stimulating hormone (TSH)	ACACAACCATCTGCATGGGA	This study
	CTGGATAAGGAAGCGTGGGC	This study
Succinate dehydrogenase (SDH)	TTCAACACTGCCTGCGTCA	This study
	AGAGCTGCATTGATCCCACC	This study
Insulin growth factor binding protein (IGFBP)	AGATGGAGAACGCAGCCAT	This study
	CCTCGGGTCGTAGGGTTTACT	This study
Growth hormone (GH)	ATGGAAAGAGCCGTCCTCCT	This study
	CGATGGAGAACAGACGCTGG	This study
Growth hormone receptor (GHR)	CCTGCACCCAAAATTAAAGGCA	This study
	ACCTCCACCACTCAGGATGA	This study
Citrate synthase (CS)	CTGGGTGTCCAAAGAGTGGG	This study
	GGGTGAAGGTTTGTGGGGAA	This study
Cytochrome oxidase (COX)	TACGGCACATGCCTTCGTAA	This study
	GGGCACCGATCATAAGTGGA	This study
Type II collagen (Col2)	GGGCAAGACAGTGATCGAAT	Ljubobratovic et al. (2020)
	CTCCTGGTCTGTTCCTCCAA	Ljubobratovic et al. (2020)
Type I collagen (Col1)	CAAGACATCGAAAACATCTCG	Ljubobratovic et al. (2020)
	TTCAAGGCCAAACTCTTGGT	Ljubobratovic et al. (2020)
Osteonectin	CTGAGAATGCCTGCCTGAAC	Ljubobratovic et al. (2020)
	GGTCCTGGCACACACACAT	Ljubobratovic et al. (2020)
Aeromonas spp.	CCTGGACAAAGACTGACGCT	Ljubobratovic et al. (2017)
	GAAGCCACGTCTCAAGGACA	Ljubobratovic et al. (2017)
Vibrio spp.	AGGGAGACTGCCGGTGATAA	Ljubobratovic et al. (2017)
	GTATGCGCCATTGTAGCACG	Ljubobratovic et al. (2017)
Mycobacterium spp.	TACTGCAGGGGAGACTGGAA	Ljubobratovic et al. (2017)
	CAGTTACTGCCCAGAGACCC	Ljubobratovic et al. (2017)

2.5. Body composition analysis

Pooled samples were processed for biochemical analysis (three fish for lipid analysis, six fish for free water analysis and 20 mg of fish on a dry weight basis for nitrogen content analysis). Lipid content was estimated gravimetrically according to Folch et al. (1957). Briefly, larvae were homogenized with 20 parts of 2:1 chloroform/methanol mixture. Distilled water was then added to achieve final ratio of 8:4:3 chloroform : methanol : water. After shaking, the mixture was centrifuged, the entire upper phase was removed and lower phase was evaporated to dryness. Lipid percent was expressed on a dry weight basis, taking into account the content of free water in the samples. Free water content was determined gravimetrically after drying the samples at 60 °C for 48 h according to Kjørsvik et al. (2009). Protein percent on a dry weight basis was calculated by multiplying the nitrogen content by 6.25 as

conversion factor. The content of nitrogen in dried samples was estimated using CHNS analyzer (the Vario El cube, Elementar, Germany).

2.6. Statistical data analyses

Statistical analysis was performed using Kruskal-Wallis test with Nemeny post-hoc. Survival was analyzed using Analysis of Variance (ANOVA) with Tukey-HSD post-hoc, due to the small number of samples per group (three tanks per group). The significance (alpha) level was set to 0.05. Condition factor (Kn) was estimated using the equation (Ayo-Olalusi, 2014):

$$Kn = 100\ W/L^b$$

W – individual fish weight (g); L – individual fish total length (cm); b – slope of log transformed individual weight – log transformed individual total length linear regression.

Fish energy density on a dry weight basis was calculated using formula (Breck, 2014):

$$Ed\ (kJ/g) = 23.6\ x\ P + 36.2 \times L$$

P – protein weight (g) on a dry weight basis (g); L – lipid weight (g) on a dry weight basis (g)

Analysis of water quality parameters was performed using Analysis of covariance (ANCOVA) and Tukey-HSD post-hoc, with time points entered as co-variates. The significance (alpha) level was set to 0.05. Statistical analysis and graph drawing was performed using Real Statistics Resource Pack software (Release 7.2), Copyright (2013−2020) Charles Zaiontz, www.real-statistics.com.

3. Results

3.1. Digestive enzyme activities at 20th DPH

Assessment of digestive enzyme activity was performed in order to evaluate nutrient utilization capacity at 20th DPH. Enzyme activities are expressed as units of activity per total protein amount. The results (Table 2) showed that group weaned using homogenized BGHN14 coated feed (WHB) was presented with higher PLA2 to trypsin activity ratio ($p = 0.007$) and lower trypsin activity ($p = 0.049$) in comparison to control (C). Group weaned using live BGHN14 treated feed (WLB) was also presented with an increase of PLA2 to trypsin activity ratio in comparison to C, but the difference was not significant ($p = 0.095$, statistical trend). Similarly, the same group was presented with statistical trend ($p = 0.084$) towards decrease of PLA2 activity in comparison to C. On the other hand, WLB larvae had higher lipase activity in comparison to weaned larvae (W) ($p = 0.012$), as well as lower PLA2 to lipase activity ratio in comparison to C ($p = 0.02$).

3.2. RNA gene expression analysis

Skeleton development, growth and energy generation related gene expression analysis at mRNA level is shown in Fig. 1. Reduced *Col1* mRNA expression was observed in WLB ($p = 0.024$) and W ($p = 0.041$) groups in comparison to C group, along with higher expression in WHB in comparison to W group ($p = 0.002$). In addition, WLB group was presented with non-significant ($p = 0.058$, trend) reduction of *GHR* mRNA expression and also non-significant stimulation of *Col2* expression ($p = 0.081$, trend) in comparison to C larvae. Evaluation of bacterial 16S rRNA expression demonstrated a reduction of *Aeromonas* to *Vibrio* spp. ratio in WHB in comparison to both W ($p = 0.024$) and C ($p = 0.018$) groups.

3.3. Biochemical and morphometric analyses at 32nd DPH

Estimation of body composition and morphometric measurements were performed in order to evaluate larval growth and condition. Total number of fish with inflated swim bladder was counted in each tank to estimate fish survival. Results (Table 3) indicated lower lipid percentage in WLB group in comparison to C larvae ($p = 0.002$) and higher protein percent in comparison to W group ($p = 0.013$). W group was, however, presented with non-significant ($p = 0.089$, statistical trend) decrease of lipid percent in comparison to C. Length and weight of WHB fish were significantly higher in comparison to C ($p < 0.001$ for length and $p = 0.004$ for weight). For WLB larvae only non-significant increase of length ($p = 0.084$) and weight ($p = 0.07$) in comparison to C was observed. On the other hand, condition factors (Kn) in both WHB and WLB, as well as W group, were significantly higher in comparison to C ($p < 0.001$ for WHB and WLB, $p = 0.008$ for W). WHB was furthermore presented with increased Kn in comparison to W ($p < 0.001$). No differences in skeletal deformities and the number of surviving swim bladder inflated (SBI) fish were observed between treatment groups.

3.4. Water physico-chemical quality

Physical and chemical evaluation of water quality was performed at 7th, 11th, 14th and 18th DPH. The results (Table 4) indicated that nitrate levels were significantly lower in WLB ($p = 0.026$) and WHB ($p = 0.017$) in comparison to C group. Non-significant ($p = 0.056$) decrease of nitrate level was also seen in W in comparison to C. There were no differences in other analyzed parameters.

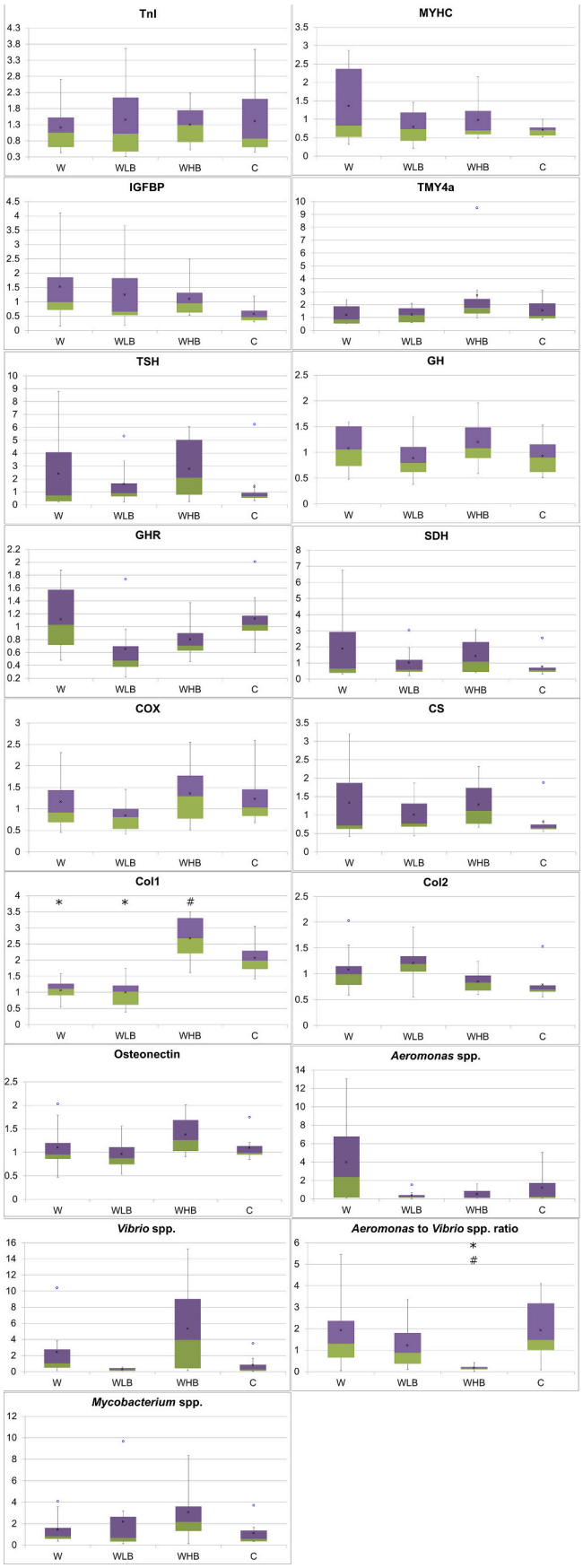
4. Discussion

Probiotic use in aquaculture is receiving a growing attention both from scientific community and industrial sector (Sahu et al., 2008). Use of probiotics as larval feed modifiers in pike-perch has been investigated

Table 2
Enzyme activities at 20th DPH (mean ± standard deviation, $n = 8$).

	W	WLB	WHB	C	Kruskal-Wallis test *p*-value	Nemenyi post-hoc test p-value
Trypsin (mU/mg of proteins)	1.13 ± 0.41	1.25 ± 0.58	1.06 ± 0.13	1.43 ± 0.26	**0.048**	0.049 (WHB/C)
Phospholipase A2 (PLA2) (mU/mg of proteins)	414.66 ± 69.68	459.98 ± 80.82	473.37 ± 88.61	366.21 ± 63.38	0.061	0.084 (WLB/C)
PLA2 to trypsin activity ratio	405.17 ± 156.52	429.51 ± 213.24	453.12 ± 109.36	264.84 ± 69.03	**0.011**	0.095 (WLB/C), 0.007 (WHB/C)
Lipase (mU/mg of proteins)	1.35 ± 0.32	1.76 ± 0.32	1.29 ± 0.14	1.40 ± 0.17	**0.004**	0.012 (W/WLB), 0.008 (WHB/WLB)
PLA2 to lipase activity ratio	321.25 ± 86.91	265.32 ± 54.47	368.12 ± 71.90	262.89 ± 38.35	**0.009**	0.020 (C/WHB), 0.030 (WLB/WHB)
Chymotrypsin (mU/mg of proteins)	0.18 ± 0.06	0.22 ± 0.14	0.19 ± 0.07	0.18 ± 0.08	0.974	
Trypsin to chymotrypsin activity ratio	6.82 ± 2.79	7.62 ± 4.96	6.95 ± 3.84	9.44 ± 4.31	0.492	

W = weaned larvae, WLB = weaned larvae administered live BGHN14 treated feed, WHB = weaned larvae administered BGHN14 homogenate coated feed, C = control (non-weaned) larvae.
DHP = day post-hatch.



(caption on next column)

Fig. 1. Box-plots showing mRNA expression at 20th day post-hatch (DPH) (W = weaned larvae, WLB = weaned larvae administered live BGHN14 treated feed, WHB = weaned larvae administered BGHN14 homogenate coated feed, C = control (non-weaned) larvae); * indicates significant ($p < 0.05$) difference relative to C; # indicates significant difference relative to W; ° indicates an outlier value; TnI = Troponin I (fast skeletal muscle), MYHC = Myosin heavy chain (fast skeletal muscles), TMY4a = Tropomyosin 4a (cardiac), TSH = Thyroid stimulating hormone, IGFBP = Insulin growth factor binding protein, GH = Growth hormone, GHR = Growth hormone receptor, SDH = Succinate dehydrogenase, COX = Cytochrome oxidase, CS = Citrate synthase, Col1 = Type I collagen, Col2 = Type II collagen.

by our group (Ljubobratovic et al., 2020; Lukic et al., 2019). Current research was focused on examination of *in vivo* effects of larval feed stably enriched with water soluble molecules or depleted from triglycerides via lactobacilli treatment. Fish were weaned from mouth opening using either regular non-enriched larval feed or feed treated with live or homogenized *Lb. paracasei* subsp. *paracasei* BGHN14. Weaned fish in present research were presented with poorer osteoblast differentiation in comparison to non-weaned control fish. This was evident from the mRNA expression of osteoblast (*Col1*) marker, though, according to osteonectin expression, the process of skeleton mineralization was not impaired due to weaning (Rosset and Bradshaw, 2016; Toh et al., 2009). Early weaning was also associated with lower fat deposition. These effects were probably caused by lower amount of soluble proteins and amino acids in microdiet as opposed to *Artemia* (Ljubobratovic et al., 2020). Furthermore, the lack of digestive enzyme activity in larval fish, along with lower appealing of microdiet (Kolkovski et al., 1997), presumably reduced the accessibility of nutrients from solid feed particles and/or reduced feed consumption (Lahnsteiner, 2017; Rathore et al., 2016). In line with this, larvae administered live BGHN14 treated OTOHIME B1, which has more than twice the leaching rate of free amino acids in comparison to non-treated feed (Lukić et al., under review), were presented with the most emphasized fat level reduction and skeleton differentiation impairment, according to chondrocyte (*Col2*) marker mRNA expression. Furthermore, these larvae demonstrated a trend ($p < 0.1$) towards growth hormone receptor (*GHR*) mRNA level reduction. This indicates cellular GH desensitization (Vélez et al., 2016) and slower growth, presumably as the result of mild energy deficiency during treatment period. On the other hand, larvae administered BGHN14 homogenate coated feed did not suffer any retardation in skeleton development in comparison to non-weaned fish. OTOHIME B1 coated with BGHN14 homogenate was presented with almost ten times lower leaching rate of soluble proteins, higher digestible protein level and almost the twice the level of free and protein-bound Gly and Pro, respectively (Lukić et al., under review). Gly and Pro are two most important amino acids for skeleton build-up (Li and Wu, 2018). This probably compensated for the protein deficiency present in inert feed, improving skeleton ossification and fat deposition.

Aside from acting as collagen building component, Gly is a well-known feed attractant, along with soluble proteins, which amount was also increased in BGHN14 homogenate coated OTOHIME B1 in comparison to control feed, both before (~6 vs. ~4 μg/mg of dried feed) and after leaching (~2 vs. ~1 μg/mg of dried feed (Dong et al., 2016; Kasumyan and Doving, 2003; Lukić et al., under review). It is thus possible that the amount of artificial feed consumption in larvae fed BGHN14 homogenate coated feed was the highest among the weaned groups. In line with this, a decrease of trypsin activity was observed in larvae administered BGHN14 homogenate coated feed. Compound feed is assumed to contain anti-nutritional factors, probably plant derived additives as feed binders, e.g. starch, which may suppress trypsin activity (Tiamiyu and Solomon, 2012). Suppression of trypsin activity may explain the highest morphometric growth in these fish, in comparison to other treatment groups. Savoie et al. (2011) reported stimulating effects of trypsin inhibition on larval fish growth, and this was ascribed to an over-release of pancreatic enzymes via activation of cholecystokinin

Table 3
Body composition and morphometric indices, 32nd DPH (mean ± standard deviation, $n = 8$).

	W	WLB	WHB	C	Kruskal-Wallis test p-value	Nemenyi post-hoc test p-value
Protein percent on a dry weight basis (%)	65.13 ± 5.56	68.41 ± 0.82	67.58 ± 0.72	67.38 ± 0.99	**0.01**	0.013 (W/WLB)
Lipid percent on a dry weight basis (%)	1.42 ± 0.2	1.25 ± 0.23	1.49 ± 0.33	1.84 ± 0.31	**0.005**	0.089 (W/C), 0.002 (WLB/C)
Free water percent (%)	76.27 ± 2.89	75.82 ± 3.1	75.94 ± 1.62	74.89 ± 2.77	0.795	
Energy density on a dry weight basis (kJ/g)	15.88 ± 1.31	16.6 ± 0.2	16.49 ± 0.27	16.57 ± 0.22	0.12	
Length (mm)	14.67 ± 2.31	14.8 ± 2.19	15.28 ± 2.55	13.93 ± 2.31	**0.002**	<0.001 (WHB/C) 0.084 (WLB/C)
Weight (mg)	23.95 ± 11.43	24.58 ± 11.25	26.56 ± 13.83	21.03 ± 11.25	**0.006**	0.004 (WHB/C) 0.07 (WLB/C)
Condition factor (Kn)[#]	0.72 ± 0.12	0.74 ± 0.12	0.77 ± 0.11	0.67 ± 0.11	**<0.001**	<0.001 (WLB/C), <0.001 (WHB/C) 0.008 (W/C) <0.001 (WHB/W) 0.064 (WHB/WLB)
SBI fish count/tank[*]	1960 ± 196	1663 ± 535	1528 ± 430	1678 ± 477	0.671[*]	

W = weaned larvae, WLB = weaned larvae administered live BGHN14 treated feed, WHB = weaned larvae administered BGHN14 homogenate coated feed, C = control (non-weaned) larvae.
DPH = day post-hatch.
SBI = swim bladder inflated.
[*] Since there were three measurements per group (for each tank), ANOVA with Tukey-HSD post-hoc was used instead of Kruskal-Wallis test.
[#] Slope of weight-length regression were: W = 2.96, WLB = 2.92, WHB = 2.81, C = 3.2.

(CCK). CCK is also known to slow down gastric emptying, potentially improving absorption of nutrients in the foregut and contributing to better growth. Furthermore, CCK increases the motility of hindgut, improving evacuation of gut content (Le et al., 2019). In line with this, in fish fed BGHN14 homogenate coated feed the ratio of *Vibrio* to *Aeromonas* spp. was increased, which was reported to positively correlate with gut motility, according to studies in zebrafish Wiles et al. (2016). Additional research is needed to reveal the function of CCK in fish administered BGHN14 homogenate coated feed, as well as the potential role of trypsin inhibition.

Although earlier transition to inert feed slowed ossification and fat reserve deposition, weaned larvae were in better condition, according to condition factor (Kn) values. Early introduction of inert feed was related to faster gut maturation in *Senegalese sole* larvae, as evidenced by alkaline phosphatase activity, a marker of enterocyte differentiation (Engrola et al., 2009; Hinnebusch et al., 2004). Though estimation of gut maturation index was beyond the scope of this study, it can be assumed that slight feed restriction from 6th to 19th DPH in weaned groups increased enterocyte absorbing capacity. This might have led to better energy extraction from ingested feed and improvement of fish condition observed at 32nd DPH. Additional molecular analysis, e.g. alkaline phosphatase activity, should be performed to confirm this assumption.

In contrast to Kn, which could not be clearly correlated with feed utilization efficiency, fish growth seemed to be associated with higher phospholipase A2 (PLA2) to trypsin activity ratio. This was demonstrated in groups administered live and homogenized BGHN14 treated feed, though the growth improvement was more emphasized in the latter group. Phospholipids are crucial components of cellular membranes and are of utmost importance for growth (Chowdhury et al., 2019). It seems that phospholipids can compensate for negative effects of trypsin activation on nutrient availability, as assumed above, probably by overcoming for a defect in nutrient absorption potentially caused by suppression of CCK activity. We note here that findings discussed above are in contrast to the results reported by Hamza et al. (2007), demonstrating growth suppression in pike-perch larvae weaned at 9th DPH. There might be two possible reasons for this contradictory outcome. Different larval starters were found to be of different suitability for both pike-perch (Kestemont et al., 2007) and walleye (*Sander vitreus*) (Johnson et al., 2008). On the other hand, it is possible that lower rearing temperature used in this study (~16 °C vs. ~19 °C in the above study) may have affected the outcome of early weaning. Though lower rearing temperature positively affects skeleton development (Boglione et al., 2013), growth of pike-perch is suppressed in these conditions (Geay and Kestemont, 2015). Slower growing fish consume less energy, which potentially makes artificial feed satisfactory energy source for early stage larvae.

Although OTOHIME B1 feed treated with live BGHN14 had approximately four times lower neutral lipid content in comparison to control feed after feed leaching test (~18 vs. ~75 μg/mg of dried feed) (Lukić et al., under review), there was no deterioration of growth of larvae consuming BGHN14 treated feed. However, fish body fat percent was reduced, which may decrease the adaptive potential of fish in the case of limited food supply (Gisbert et al., 2008). Nevertheless, this did not affect body energy density. It could be that other energy sources, most notably yolk and oil globule derived lipids, were still available to larvae during the treatment, given the slower yolk resorption rate in fish reared at lower temperatures (Kamler, 2008). Indeed, Xu et al. (2017), demonstrated that complete oil globule absorption occurs at 14th DPH in pike-perch larvae reared at 15 °C. It is also interesting that, in spite of reduction of lipid amount in live BGHN14 treated feed, lipase and PLA2 activities were elevated in fish from this group. Similar observation was already reported for sea bass (Morais et al., 2004). Short-term reduction of total lipid amount in diet seems to "pre-condition" larval digestive tract for more efficient lipid utilization capacity. Though higher neutral lipid assimilation capacity may potentially interfere with fish growth (Morais et al., 2007), as long as the appropriate phospho- to neutral lipid digestion ratio is preserved, this approach may beneficially affect fish growth, primarily via PLA2 to trypsin activity ratio modulation, as detailed above.

Two important parameters related to the success of fish larviculture, which were not addressed in this study, deserve more attention in future research: swim bladder inflation rate and cannibalism. These variables are expressed in evaluated survival which included only the fish with inflated swim bladder and, as such, is within the range of recently published pike-perch larviculture studies, which took place in pilot type experimental RAS designs with larvae originating from domesticated breeders (Colchen et al., 2020; Ljubobratović et al., 2019; Ljubobratovic et al., 2020). However, both of mentioned culture variables may have been affected by feed coating/treatment with lactobacilli. Not only the cannibalism may be directly suppressed by an improvement of feed quality by BGHN14 homogenate (Lappalainen et al., 2006), but also the

Table 4
Water quality parameters (individual measurements).

	W	WLB	WHB	C	ANCOVA *p*-value	Tukey-HSD/Kramer post-hoc test p-value
pH (7th DPH)	8.58	8.57	8.58	8.58	0.988	
pH (11th DPH)	8.50	8.51	8.52	8.52		
pH (14th DP)	8.35	8.35	8.38	8.37		
pH (18th DPH)	8.47	8.45	8.46	8.46		
Electroconductivity at 20 °C (7th DPH) (μS/cm)	647	648	645	645		
Electroconductivity at 20 °C (11th DPH) (μS/cm)	1160	1180	1180	1160		
Electroconductivity at 20 °C (14th DPH) (μS/cm)	1450	1450	1450	1440		
Electroconductivity at 20 °C (18th DPH) (μS/cm)	1470	1470	1470	1470		
Ammonium ion (7th DPH) (ppm)	<0.13*	<0.13*	<0.13*	<0.13*	0.256	
Ammonium ion (11th DPH) (ppm)	0.13	0.13	<0.13*	0.53		
Ammonium ion (14th DPH) (ppm)	<0.13*	<0.13*	<0.13*	0.18		
Ammonium ion (18th DPH) (ppm)	<0.13*	<0.13*	<0.13*	<0.13*		
Nitrate ion (7th DPH) (ppm)	1.30	1.30	1.11	2.57	**0.013**	0.056 (W/C), 0.026 (WLB/C), 0.017 (WHB/C)
Nitrate ion (11th DPH) (ppm)	3.09	3.06	3.01	3.14		
Nitrate ion (14th DPH) (ppm)	4.31	4.47	4.49	5.53		
Nitrate ion (18th DPH) (ppm)	5.62	4.98	4.93	6.37		
Nitrite ion (7th DPH) (ppm)	<0.07*	<0.07*	<0.07*	<0.07*	N/A	
Nitrite ion (11th DPH) (ppm)	<0.07*	<0.07*	<0.07*	<0.07*		
Nitrite ion (14th DPH) (ppm)	<0.07*	<0.07*	<0.07*	<0.07*		
Nitrite ion (18th DPH) (ppm)	<0.07*	<0.07*	<0.07*	<0.07*		

W = weaned larvae, WLB = weaned larvae administered live BGHN14 treated feed, WHB = weaned larvae administered BGHN14 homogenate coated feed, C = control (non-weaned) larvae.
N/A = non-applicable.
DHP = day post-hatch.
* Measurements below detection threshold were entered as threshold values for ANCOVA.

introduction of bacterial cells into rearing water may alter light diffusion in tanks and indirectly affect gas bladder inflation and cannibalism rate (Król and Zakęś, 2016; Rieger and Summerfelt, 1997). Information on these factors will be of critical importance for an improvement of feed coating/treatment mode (e.g. lactobacilli concentration and water percent in feed coating mixture).

This study demonstrated that stimulation of growth of larval pikeperch reared at lower temperature can be achieved by using starter feed coated with homogenized lactobacilli. In addition to direct provision of skeleton building amino acids by lactobacilli coated feed, beneficial effects on larval growth seem to correlate with up-regulation of PLA2 to trypsin activity ratio, indicating mutually dependent role of phospholipids and proteins in fish development. Aside from providing the evidence for a new way of exploitation of lactobacilli homogenates for fish larviculture, this research opens the way for additional enzyme targeting approach for improvement of larval fish growth. In this respect, we suggest that the effect of modulation of gut enzyme activity on development of early stage larval fish, particularly trypsin and trypsin-regulated hormone activity, such as CCK, be examined more thoroughly in future studies.

Data availability statement

Raw data obtained in the study is publicly available as Linked data to this publication from ResearchGate .

CRediT authorship contribution statement

Uroš Ljubobratović: Funding acquisition, Project administration, Conceptualization, Methodology, Investigation, Writing - review & editing. **Georgina Fazekas**: Investigation, Writing - review & editing. **Alan Koljukaj**: Investigation, Writing - review & editing. **Tijana Ristović**: Investigation, Writing - review & editing. **Vivien Vass**: Investigation, Writing - review & editing. **László Ardó**: Conceptualization, Methodology, Investigation, Writing - review & editing. **Nemanja Stanisavljević**: Investigation, Writing - review & editing. **Goran Vukotić**: Investigation, Writing - review & editing. **Mirjana Pešić**: Conceptualization, Methodology, Investigation, Writing - review & editing. **Danijel Milinčić**: Investigation, Writing - review & editing. **Aleksandar Kostić**: Investigation, Writing - review & editing. **Jovanka Lukić**: Project administration, Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

There were no conflicts of interest associated with this research.

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