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The effect of live and inert feed treatment with lactobacilli on weaning success in intensively reared pike-perch larvae

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ABSTRACT

Weaning to inert diet in intensively reared pike-perch larvae is confronted with significant fish loss, which prevents successful commercialization of pike-perch production. Achievement of satisfactory feed quality and effective assimilation of nutrients by larval fish is the major challenge in larval production process. Aim of this study was to evaluate whether treatment of live and inert feed with lactobacilli could alleviate growth retardation associated with early weaning of pike-perch reared in recirculating system. Weaning started on 18th day post-hatching (DPH) either as sudden weaning (SW) or by co-administration of *Artemia* for six days (gradual weaning, GW). Prior to administration to fish, *Artemia* was treated with *Lactobacillus salivarius* BGHO1/*Lb. reuteri* BGGO6-55, while inert feed was treated with *Lb. paracasei* subsp. *paracasei* BGHN14/Lb. *rhamnosus* BGT10. Treatment with lactobacilli slightly raised neutral lipid level in *Artemia* nauplii, but significantly reduced their content in dry feed. Fish were sampled on the 24th DPH. Survival, morphometric indices, skeleton differentiation, digestive enzyme activity and opportunistic pathogenic bacteria level were assessed in whole fish specimens. GW fish were presented with better survival, body growth and phospholipase A2 (PLA2) activity. Alongside, *Vibrio* spp. growth was suppressed in these fish and skeleton development was improved, according to Alizarin Red staining and *Col1A1/Sparc* mRNA expression data. Lactobacilli application in GW fish correlated with an increase of survival, condition factor and growth rate, according to trypsin and chymotrypsin activities, indicating better utilization of dietary proteins for muscle building. In SW fish, lactobacilli elevated chymotrypsin activity, PLA2 to lipase activity ratio and improved survival and ossification, as evident from Alizarin Red staining and *Col1A1/Sparc* mRNA expression. This indicated improved fatty acid absorption and control of metamorphosis process. Furthermore, lactobacilli suppressed *Vibrio* spp. growth in SW fish. Aside from demonstrating the ability of lactobacilli to aid weaning in pike-perch larvae, this study indicated that different types of food treatment may direct fish growth in a predictable manner, allowing further cost-effective improvements of larval pike-perch rearing in intensive system.

1. Introduction

Weaning presents the most critical moment in fish production, because of high demand of larval fish for soluble proteins and fatty acids, which cannot be obtained from formulated diet (Rønnestad et al., 2013). This especially refers to carnivorous fish larvae, because of their huge demands for lipids and proteins, both for energy generation and for body building (Andersen et al., 2016). Due to poor efficiency of phospholipid and unsaturated fat synthesis in carnivorous larval fish, high consumption and uninterrupted absorption of these nutrients is required for sustainment of larval growth (Sargent et al., 2003; Twining et al., 2016). Though live food has optimal nutritive composition, costs associated with live food provision impose the need for early transition to commercially manufactured feed. However, artificial diet, mostly based on fishmeal for carnivorous fish, has lower amounts of soluble proteins in comparison to live food. This prevents its complete assimilation by fish larvae due to their poor capacity for protein degradation (Rønnestad et al., 2013; Samuelsen and Oterhals, 2016; Vikas et al.,

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2014). Marine products (fish- and krill) used for the preparation of inert feed for carnivorous fish may contain similar amounts of phospho- and neutral lipids as live food; however, technological processes related to dry feed preparation and subsequent feed storage substantially alter their nutritional value. Phospholipid amount and digestibility may be reduced due to oxidation and formation of complexes with starch and proteins (Pandey, 2018; Rokey et al., 2010). Additionally, feed preparation usually involves the final step of lipid coating (Ambalkar, 2018; Goeritz et al., 2014). As the mechanical feed digestion in larval fish is not effective enough, because of poor stomach development, surface layer of granular feed is most likely to be attacked by larval digestive enzymes (Langdon and Barrows, 2011; Zambonino Infante et al., 2008). This significantly increases the level of neutral lipids available to larval fish in dry feed. High amount of neutral lipids leads to accumulation of fat globules in enterocytes (Morais et al., 2007; Zambonino Infante and Cahu, 2010). This may impede absorption of phospholipids (Morais et al., 2007), which are essential both for transport of fatty acids to the circulation and for skeleton growth (Tocher et al., 2008).

Principal weaning strategy used in hatcheries includes co-feeding of live food and inert feed for several days, in order to gradually increase the acceptability of dry feed (Ljubobratović et al., 2015; Rosenlund et al., 1997). Another weaning strategy, termed sudden weaning, which includes direct transition to inert feed, has been tested in several fish species, but with limited success (Herath and Atapaththu, 2013). So far, several approaches, mainly related to manipulation of feed nutritive value, were evaluated to overcome the growth retardation in early weaned fish. Kestemont et al. (2007) reported an improvement of fish growth and survival after administration of *Artemia* enriched with polyunsaturated fatty acids and vitamin C during gradual weaning in pike-perch. Hamza et al. (2008) showed significant benefits of inclusion of phospholipids into the formulated diet of pike-perch during and after gradual weaning. Similar result was reported for sea bass larvae by Cahu et al. (2003). Inclusion of protein hydrolyzate into the formulated diet was linked to better growth and survival of both suddenly weaned European sea bass larvae (Kotzamanis et al., 2007) and gradually weaned Asian seabass larvae (Srichanun et al., 2014).

Pike-perch (*Sander lucioperca*) is a freshwater fish species which has been recognized as a promising candidate for intensive rearing in aquaculture (Blecha et al., 2016). Exogenous feeding of pike-perch begins, depending on the water temperature, approximately on the 4th-6th day post-hatching (DPH). Intensive bone development starts after swim bladder inflation (approximately 12th-17th DPH). Larval development ends up at around 34th DPH (Ostaszewska et al., 2005; Ott et al., 2012). Though rainbow trout and common carp currently represent dominant freshwater species reared in captivity, aquaculture production of pike-perch is expected to rise in next decades. This is mainly because of high quality and tasteful meat of pike-perch, as well as a huge potential for rearing in closed system (Bostock et al., 2016). This is particularly important, given the fact that recirculating system represents the only ecologically acceptable manner of fish production (Pyanov et al., 2014). Although commercial pike-perch cultivation in recirculating system has been achieved in several European countries (Pyanov et al., 2014), more refinements are needed to attain maximal production efficiency. Aggressive nature, along with high stress sensitivity in this species, significantly hampers successful larval rearing, especially the process of weaning (Szczepkowski et al., 2011; Swirplies et al., 2019). This is further aggravated by the fact that digestive tract of early-stage pike-perch larvae is poorly differentiated and the development of stomach and efficient proteolytic digestion commences only after 15th DPH (Ostaszewska et al., 2005). First feeding larvae are mostly specialized for lipid rich diet and are recommended to be fed live food at the start of exogenous feeding (Lahnsteiner, 2017). Hence, optimization of weaning process would significantly aid the economic sustainability of pike-perch production and upscale its production throughout the Europe and on the global level.

Although enrichment with proteins and lipids has been proved to affect the efficacy of fish weaning, the expenses linked to above procedures might surpass the profits of fish production. Probiotics, as more economically viable alternative, have been successfully applied in fish larviculture. Enrichment of *Artemia* live food with probiotics was demonstrated to stimulate metamorpohosis of gradually weaned *Senegalese sole* larvae (Lobo et al., 2014). Due to their qualified presumption of safety (QPS) status, lactobacilli represent the most promising candidates for use in fish larviculture (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). Lactobacilli have been demonstrated to stimulate the development of sea bass when supplemented via live food (Silvi et al., 2008). Furthermore, they were reported to modify protein solubility in formulated animal feed (Amadou et al., 2011). However, data concerning their potential to modulate other relevant nutrient level, as well as the data on the correlation of these modifications with *in vivo* effects, are scarce. In our previous research, we evaluated whether lactobacilli could aid early gradual weaning of intensively reared pike-perch (Ljubobratovic et al., 2017). Treatment of *Artemia* live prey with *Lactobacillus salivarius* BGHO1 and *Lb. reuteri* BGGO6-55 and dry feed with *Lb. paracasei* subsp. *paracasei* BGHN14 and *Lb. rhamnosus* BGT10, prior to administration to fish, stimulated fish growth, skeleton development and protein digestion. Also, research performed in our laboratory has shown that BGHO1 and BGGO6-55 may alter nutritive profile of *Artemia* nauplii, depending on incubation conditions and bacterial concentration (unpublished data). Similar study demonstrated that BGHN14 and BGT10 substantially affected neutral lipid composition of larval formulated feed (Lukic et al., 2019). Based on these results, in present research, we aimed to test whether *Artemia*/dry feed treatment with same lactobacilli strains may also aid the process of sudden weaning in pike-perch, and to additionally make the correlation between nutritional profile of treated feed and *in vivo* outcomes. Furthermore, in order to simulate the application of probiotics in real terms in fish hatcheries, cryopreserved, instead of fresh lactobacilli cultures were used for feed treatment. Results obtained in this research provide novel data on lactobacilli application for pike-perch weaning, as well as the relationship between feed composition and fish response.

2. Materials & methods

2.1. Preparation of bacterial cultures

Four bacterial strains were used in the research: *Lactobacillus salivarius* BGHO1 and *Lactobacillus reuteri* BGGO6-55 for *Artemia* treatment, and *Lactobacillus paracasei* subsp. *paracasei* BGHN14 and *Lactobacillus rhamnosus* BGT10 for dry feed treatment. All strains are part of the collection of Laboratory for Molecular Microbiology (LMM), IMGGE, Serbia. Bacterial strains were cultivated as described in Ljubobratovic et al. (2017) and frozen as follows:

BGHO1 and BGGO6-55 - Log phase cultures (both strains containing 2×10^8 of cells/mg of wet pellet) were centrifuged; after pellet weighing saline and glycerol were added using formulas:

Saline (in μL) = 4 x pellet weight (in mg); Glycerol (in μL) = 0.88 x pellet weight (in mg).

BGHN14 and BGT10 - Log phase cultures (BGHN14 culture containing 5×10^8 and BGT10 culture containing 3×10^8 of cells/mg of wet pellet) were centrifuged; after pellet weighing saline and glycerol were added using formulas:

Saline (in μL) = 1.3 x pellet weight (in mg); Glycerol (in μL) = 0.4 x pellet weight (in mg).

After preparation, cultures were frozen at −80 °C for long-term storage.

2.2. Cultivation and treatment of Artemia

Cultivation of *Artemia* was performed as described in Ljubobratovic et al. (2017). Cysts were suspended in water at a concentration of 1 g of cysts per 1 L of water with 30 parts per thousand (‰) NaCl and incubated at 28 °C exposed to light with continuous aeration. Protocol for the treatment of *Artemia franciscana* with fresh BGHO1/BGGO6-55 cultures, which led to an increase of soluble protein and phospholipid level in *Artemia* nauplii, was selected in our previous research (unpublished data). It included 6 h incubation of lactobacilli with hatched *Artemia* nauplii, with 75:25 BGHO1:BGGO6-55 ratio and total bacterial mass of 250 mg per g of dry *Artemia* cysts. Same combination was used in this research, except that frozen lactobacilli cultures were used. The biochemical and microbiological profile of *Artemia* nauplii treated with frozen BGHO1/BGGO6-55 cultures in above selected combination was checked at a small scale prior to *in vivo* treatment. Frozen BGHO1 and BGGO6-55 suspensions (totally 735 µL, containing 125 mg of bacterial wet mass) were mixed in inverted 1 L bottle in a ratio 75:25 BGHO1:BGGO6-55 with 0.5 L of hatched (18 h) *Artemia* culture. Incubation lasted 6 h. Afterwards, *Artemia* culture was filtered (mesh size 80 µm) and share of freshly hatched nauplii was immediately used for biochemical and microbiological analyses. Same conditions for *Artemia* cultivation were applied for *in vivo* fish feeding experiment, except that 80 L conical hatching jars were used with same concentration of *Artemia* nauplii as above (1 g/L). The amount of *Artemia* hatched throughout 24 h period during fish feeding experiment is given in Table 1.

Since, in fish hatcheries, *Artemia* is commonly hatched up to twice per day, then stored and provided to fish larvae in the next few feeding rounds until the next tour of *Artemia* hatching (Evjemo et al., 2001), we simulated these conditions in our study. *Artemia* was hatched every 12 h. Freshly hatched nauplii were given immediately to fish larvae (at 0 h), while the other part was resuspended in 30‰ NaCl (approximately 10 fold less medium as compared to initial volume), cooled at +4 °C and stored in dark at ambiental temperature with aeration. Stored (starved) nauplii were fed to fish in the next two feeding tours (4 and 8 h). At 12th h, freshly hatched nauplii were supplied again. As with fresh nauplii, we assessed the biochemical and microbiological profile of *Artemia* nauplii stored for 12 h, which corresponds to maximal expected period of nauplii starvation until being eaten by fish larvae (8 h storage before feeding and up to 4 h in fish tanks before being eaten by fish larvae). All *in vitro* small-scale experiments for assessment of fresh and starved *Artemia* nauplii biochemical and microbiological profiles were done in triplicate.

2.3. Treatment of commercial feed with frozen BGHN14/BGT10 cultures

In order to prevent the agglomeration and allow maximal preservation of integrity of feed granules (OTOHIME B1 larval feed, Marubeni Nishin Feed Co., Ltd., Tokyo, Japan, size ~360 µm) throughout the incubation, solid state treatment of feed with lactobacilli cultures was performed (Lukic et al., 2019). Feed granules were spread in thin layer on Petri dish (diameter 10 cm) surface. Lactobacilli suspension in saline was added in sufficiently small amount to avoid the immersion of granules. Suspension was mixed homogenously with feed particles using inoculation loop. According to our previous study (Lukic et al., 2019), two regimens of treatment of OTOHIME B1 dry feed with BGHN14/BGT10 substantially lowered neutral lipid level in dry feed, without inducing undesirable bacterial growth: 75:25 and 50:50 BGHN14:BGT10 ratio, 200 mg of bacteria/g of dry feed, 260% moisture and 12 h incubation at 37 °C. These two combinations were used for our current study, except that frozen lactobacilli cultures were applied. Prior to *in vivo* fish feeding experiment, small-scale *in vitro* evaluation of the effects of frozen lactobacilli cultures was performed. For this purpose, frozen BGHN14 and BGT10 suspensions (totally 27 µL, containing 10 mg of bacteria, wet weight) at a ratio of 75:25 or 50:50 BGHN14:BGT10, were diluted with saline to a final volume of 0.13 mL and mixed with 50 mg of OTOHIME B1 (260% of moisture) as explained above. Petri dishes were wrapped in foil to minimize evaporation and incubated at 37 °C for 12 h, as written above. Feed was air-dried for 4 h at 50 °C. Dried feed was taken for biochemical analyses. Furthermore, samples of feed before 4 h drying were taken for microbiological analyses. For *in vivo* fish feeding experiment, OTOHIME B1 was treated as above, except that 2 g of feed per Petri dish were mixed with bacteria (totally 1080 µL of frozen lactobacilli suspension per one Petri dish). The amount of feed treated each day is given in Table 1.

OTOHIME B1 was incubated separately with above two combinations and equal amounts of both treatment types were administered to treated fish. *In vitro* small-scale feed treatments and analyses were performed in triplicate.

2.4. Analysis of feed composition

Prior to biochemical analyses, *Artemia* nauplii were filtered through

Table 1
Feeding protocols in lactobacilli modified feed (LMF) treated fish; corresponding controls received the same amount of non-treated *Artemia* and OTOHIME B1.

	Sudden weaning (SW)				Gradual weaning (GW)			
DPH (Day post-hatching)	*Artemia* (g of cysts)/tank	Percentage of BGHO1/BGGO6-55 treated *Artemia*	OTOHIME B1 (g)/tank	Percentage of BGHN14/BGT10 treated OTOHIME B1	*Artemia* (g of cysts)/tank	Percentage of BGHO1/BGGO6-55 treated *Artemia*	OTOHIME B1 (g)/tank	Percentage of BGHN14/BGT10 treated OTOHIME B1
6	3	0			3	0		
7	3	0			3	0		
8	4	0			4	0		
9	5	10			5	10		
10	6	20			6	20		
11	6	30			6	30		
12	8	40			8	40		
13	9	50			9	50		
14	9	60			9	60		
15	9	70			9	70		
16	9	80			9	80		
17	10	90			10	90		
18	0		30	100	10	100	30	100
19	0		30	85	8	100	30	85
20	0		30	70	6	100	30	70
21	0		30	55	4	100	30	55
22	0		30	40	2	100	30	40
23	0		30	25	0		30	25

Artemia was supplied 6 times a day at 4 h intervals, while OTOHIME B1 was supplied continuously via belt feeder.

80 µm mesh and collected in methanol. Afterwards, nauplii were transferred to Petri dish and dried at room temperature (RT) for 2 h. Dried nauplii were homogenized using mortar and pestle. Dry feed granules were not homogenized since we aimed to estimate the composition of surface portion of granules, which was most likely altered by lactobacilli treatment. Furthermore, this portion is less cohesive than the inner part of the granules and is expected to be easily assimilated by larval fish (Lukic et al., 2019). Nauplii and dry feed samples were defatted using 2:1 chloroform: methanol (Folch extraction) (Jeyasanta and Patterson, 2013). Mixture was incubated for 1 h at RT with shaking. After centrifugation, pellets and supernatants were vacuum desiccated. Pellets were used for peptide and protein assessment, while desiccated lipid mass from supernatants was used for phospho- (PL), neutral (NL) and unsaturated lipid (UL) quantification. Pellets were resuspended in distilled water and incubated at RT for 10 min with occasional shaking. Mixture was centrifuged and supernatants were used for analysis of soluble protein (SPR) content, using commercial Bradford reagent. The rest of supernatant was precipitated with trichloroacetic acid (TCA) for free amino acid and short peptide (FAA/SP) assay (Mcsweeney and Fox, 1997). After TCA precipitation, remaining supernatant was mixed with 0.1 M sodium phosphate buffer pH 8.0 and 0.1% TNBS dissolved in the same buffer. After 45 min of incubation at 60 °C in the dark, 0.25 M HCl was added to stop the reaction and the absorbance at 420 nm was measured.

For PL measurement, lipid mass obtained after defatting was resuspended in chloroform, while for NL and UL measurements, lipid mass was resuspended in 20% ethanol. Quantification of PL was performed using ammonium thiocyanate method (Jeyasanta and Patterson, 2013). Ammonium thiocyanate reagent (0.27 g of $FeCl_3$ x $6H_2O$, 0.3 g of NH_4SCN, 10 mL of distilled water) was mixed with prepared samples and the mixture was centrifuged. Lower phase was transferred to clean tubes, vacuum desiccated and resuspended in 96% of ethanol. Absorbance was measured at 488 nm. NL were measured using Nile Red assay (Higgins et al., 2014). Samples were mixed with Nile Red reagent in dark microplates for fluorescence detection. Measurement was performed for 20 min at 37 °C each 60 s (excitation at 480 nm and emission wavelength 570–580 nm). Differences between latest and first measurement points were calculated. UL quantification was performed by colorimetric sulfo-phospho-vanillin (SPV) assay (Cheng et al., 2011), which is specific for unsaturated fatty acids (Byreddy et al., 2016). Samples were mixed with sulfuric acid, boiled for 10 min, cooled and mixed with phospho-vanillin (PV) reagent (60 mg of vanillin/L of water and 85% phosphoric acid in a ratio 1:4). Mixture was incubated at 37 °C for 15 min and then at RT for additional 45 min in dark. Absorbance was measured at 530 nm.

For microbiological analysis, sterile saline was added to weighed wet nauplii or feed (prior to drying) and appropriate dilutions were made. Aliquots of dilutions were spotted onto the surface of universal Luria Agar (LA) medium and on the surface of lactobacilli-specific De Man, Rogosa and Sharpe (MRS) agar. Plates were incubated for 48 h at 37 °C aerobically. Colony number was counted.

2.5. In vivo study design

In vivo trial was performed in Recirculating aquaculture system (RAS) unit of National Agricultural Research and Innovation Centre, Research Institute for Fisheries, Aquaculture and Irrigation (NAIK-HAKI), Szarvas, Hungary. Fish larvae were obtained from the pre-seasonal artificial propagation of domesticated broodstock. At the day of hatching, larvae were stocked into 12 tanks (volume 250 L/tank) with 10 000 larvae/tank (40 larvae/L). Tanks were supplied with surface sprayer to enhance the inflation of swim bladder. Tanks were part of the RAS equipped with ozone, biological and mechanical filter. Light intensity above the water surface ranged from 5 to 15 lx. Tanks were cleaned on a daily basis and the water temperature was maintained at 16.3 ± 1.0 °C. Tanks were equipped with 0.2 L cleaning cups positioned at the very bottom of the cylinder, which enabled minimally invasive extraction of unused food, feces and dead fish. Twice per day independent tap was used to drain the sediment on the cup's bottom. Detailed description of the tank design and larviculture protocol is described in Ljubobratović et al. (2019a and b). Water quality parameters (oxygen saturation, conductivity, nitrogen compounds and pH) were monitored at regular intervals and kept within the range acceptable for pike-perch larviculture - oxygen saturation 115.2 ± 8.0%, conductivity 2.4 ± 0.8 mS/cm ammonium nitrogen 0.19 ± 0.05 mg/L, nitrite-nitrogen 0.03 ± 0.01 mg/L, nitrate-nitrogen 1.5 ± 0.0 mg/L and pH 7.6 ± 0.1. Flow rate was set at 30% exchange per h at the beginning of larviculture and was gradually increased with fish growth to 100% per h until the end of the experiment. Fish handling 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 Institute, which was established according to Hungarian State law (10/1999.I.27).

Artemia was hatched and treated as described above. Nauplii were administered to fish six times a day at 4 h intervals. After hatching and treatment, share of nauplii were administered to fish as fresh culture and the rest was stored as detailed above, and was applied in the next two feedings. Prior to fish feeding, nauplii were filtered through 80 µm mesh. Commercial OTOHIME B1 feed was administered continuously via automatic belt feeder. Based on observations of the stomach color in this and previous experiments from our laboratory, ingestion of dry feed starts at the very first day of application of dry feed. In case of proper feeding regimen (feeding every 3–5 min with small portion of feed) the ingestion is rather quick and the signs of feed content in the stomach are visible already several hours post-application. There were totally four experimental groups, with three tanks per each group:

1. Suddenly weaned (SW) control group - received non-treated *Artemia* nauplii from 6th-17th day post-hatching (DPH) and non-treated OTOHIME B1 from 18th-23rd DPH;
2. SW lactobacilli modified feed (LMF) treated group – same as 1 but *Artemia* nauplii were treated with BGHO1/BGGO6-55 from 9th-17th DPH and OTOHIME B1 was treated with BGHN14/BGT10 from 18th-23rd DPH;
3. Gradually weaned (GW) control group - received non-treated *Artemia* nauplii from 6th-17th DPH and non-treated *Artemia* nauplii plus non-treated OTOHIME B1 from 18th-23rd DPH;
4. GW LMF treated group – same as 3 but *Artemia* nauplii were treated with BGHO1/BGGO6-55 from 9th-23rd DPH and OTOHIME B1 was treated with BGHN14/BGT10 from 18th-23rd DPH.

Detailed treatment schedule is provided in Table 1. On the 24th DPH fish were graded based on the presence of inflated swim bladder as explained by Steenfeldt (2015). The grading of fish took place into the solution of salt and phenoxy ethanol, so anaesthetized floating fish were randomly sampled, preventing us from sampling only weak fish which swim slower. Only fish with inflated swim bladder were counted for the evaluation of the survival and further analyses. Additionally, samples of fish taken for counting were euthanized for estimation of morphometric indices (50 fish per tank), biochemical (36 fish per tank) and gene expression analyses (36 fish per tank). To avoid artifacts in weight measurement, larvae were gently dabbed on paper towel prior to measurement. For biochemical and gene expression analyses, whole fish samples were preserved at −80 °C. Furthermore, 10-15 fish per tank were preserved in formalin for estimation of skeletal development by Alcian blue/Alizarin red staining.

2.6. Biochemical analyses

Biochemical assays were done as described in Ljubobratovic et al. (2017). Twelve fish specimens from three tanks belonging to same treatment group (4 fish per each tank) were pooled together before

homogenization, giving totally 9 homogenized samples per treatment group. Homogenates were prepared in 50 mM Tris-HCl, pH 7, 2 mM mannitol, RT, using dounce tissue grinder set (Sigma), with 40 strokes with pestle A and 10 strokes with pestle B. Homogenates were centrifuged at 15 500 × *g*, 15 min, +4 °C, and supernatants were mixed with appropriate substrate: Nα-benzoyl-DL-arginine-ρ-nitroanilidehydrochloride (BAPNA) for trypsin assay; succinyl-(ala)2-pro-phe-ρ-nitroanilide (SAPNA) for chymotrypsin assay and ρ-nitrophenyl palmitate (ρNPP) for lipase assay. Absorbances at 410 nm were measured immediately after mixing the homogenates with the substrates and each minute during next 10 min at 37 °C. Enzyme activities were calculated from differences in absorbances between values obtained in close to the plateau phase and values obtained in zero time point, using relevant extinction coefficient. Phospholipase A2 (PLA2) activity was determined using commercially available kit, according to manufacturer instructions (EnzChek™ Phospholipase A2 Assay Kit, ThermoFisher Scientific, Waltham, US).

2.7. RNA isolation and quantitative PCR (qPCR)

Isolation of RNA and qPCR were done as described in Lukic et al. (2013). The same as with biochemical assays, 12 fish were pooled together before homogenization, resulting in 9 isolated RNA samples per treatment group. Samples were homogenized using liquid nitrogen, then guanidine denaturing solution was added and acid phenol extraction was performed. Reverse transcriptions were performed using RevertAid reverse transcriptase (ThermoFisher Scientific). QPCR reactions were performed using Fast SYBR™ Green Master Mix (ThermoFisher Scientific) and 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, US). Expression of genes coding for Secreted Protein Acidic And Cysteine Rich (Sparc), Collagen type II alpha 1 chain (Col2A1), Collagen type I alpha 1 chain (Col1A1), as well as genes coding for 16S rRNA specific for *Vibrio* spp., *Aeromonas* spp. and *Mycobacterium* spp. was assessed. Pike-perch elongation factor (EF) mRNA was used as an endogenous control. Primers used in the study were either those used in Ljubobratovic et al. (2017) or novel primers were designed (Table 2) using pike-perch transcriptome available at National Center for Biotechnology Information (NCBI) (Han et al., 2016). Furthermore, Insulin-like growth factor II (IGF-II), IGF-I, Myogenic Factor 5 (Myf5), Myogenic Differentiation (MyoD1), Myogenin (MyoG) and Myocyte Enhancer Factor 2C (Mef2c) expression was assessed. However, according to qPCR assay, mRNA transcripts coding for these proteins were expressed in very low amounts, presumably because of low proportion of muscle tissue in total body mass of larval fish. Hence, quantification of these transcripts could not be performed.

2.8. Alcian blue (AB)/Alizarin red (AR) staining

For double AB/AR staining (Rigueur and Lyons, 2014), fish were fixed in 4% phosphate buffered formalin for 2 days at RT. After that, fish specimens were rinsed with fresh water, moved to 50% ethanol for 2 days, then moved to 96% ethanol for 2 days and after that stained with AB. For AB staining, fish were immersed in Solution A (20 mg Alcian blue, 30 mL acetic acid and 70 mL absolute ethanol) for 1 day and then transferred to saturated sodium borate for half a day. Afterwards, specimens were immersed in bleaching solution (15 mL of 3% hydrogen peroxide, 85 mL of 1% KOH) for 40 min and then digested with trypsin (1 g trypsin, 25 mL saturated sodium borate, 65 mL distilled water) for 7 days at RT. Then, samples were transferred to bone staining solution (0.001 g/mL Alizarin red in 1% KOH) for 24 h at RT. Destaining was again done in trypsin solution for 2 days. Preservation of stained specimens was done in increasing gradient of glycerol (with KOH) solution until finally reaching 100% glycerol. After that, fish were mounted on microscopic slides for visualization and scoring. Semi-quantitative scoring was performed to assess the number of fish with AR staining (mineralized bone, red) of vertebral column, which is the part of skeleton that first undergoes ossification (Darias et al., 2010) (versus AB staining (cartilage, blue)). Only fish samples which were completely stained were analyzed.

2.9. Statistical analysis

Statistical evaluation of effects of lactobacilli treatments in *Artemia* and dry feed was done by *t*-test comparisons. For *Artemia* nauplii, comparisons were made among treated and respective control (fresh or starved) nauplii and among fresh and starved control nauplii. The latter comparisons were performed by paired *t*-test. For dry feed, comparisons were made between treated samples and both dry and wet controls and between dry and wet control samples.

For *in vivo* study, Student test was also used for comparisons of morphometric indices, enzyme activities and mRNA expression in fish samples among treatment groups and relevant controls. Data were log transformed where needed, if the normality assumption was not satisfied. Data were considered to be normally distributed if Shapiro-Wilk test p value was higher than 0.05. Statistical comparisons were made among control and LMF treated fish in both weaning protocols and among SW and GW control fish. Outliers were removed by Grubbs' test ($p < 0.05$) and covariate matrix principal component analysis (PCA) based anomaly detection (Deepthi and Rao, 2014). Since for morphometric analysis 50 fish per tank were sampled, average values of 5 fish were calculated giving in total 30 data per group (to avoid statistical bias due to large sample size) for statistical comparisons of morphometric differences. Assuming that average survival of fish per tank was ~3 000, sampling of above specified amounts of fish per tank (36 for

Table 2
Primers used in the study.

Primer name	Primer sequence (5′-3′)	Reference
Col2a1_fw	GGGCAAGACAGTGATCGAAT	This study
Col2a1_rev	CTCCTGGTCTGTTCCTCCAA	This study
Sparc_fw	CTGAGAATGCCTGCCTGAAC	This study
Sparc_rev	GGTCCTGGCACACACACAT	This study
Col1a1_fw	CAAGACATCGAAAACATCTCG	This study
Col1a1_rev	TTCAAGGCCAAACTCTTGGT	This study
Vibrio_fw	AGGGAGACTGCCGGTGATAA	Ljubobratovic et al. (2017)
Vibrio_rev	GTATGCGCCATTGTAGCACG	Ljubobratovic et al. (2017)
Aeromonas_fw	CCTGGACAAAGACTGACGCT	Ljubobratovic et al. (2017)
Aeromonas_rev	GAAGCCACGTCTCAAGGACA	Ljubobratovic et al. (2017)
Mycobacterium_fw	TACTGCAGGGGAGACTGGAA	Ljubobratovic et al. (2017)
Mycobacterium_rev	CAGTTACTGCCCAGAGACCC	Ljubobratovic et al. (2017)
EF_fw	AGTGCGGAGGAATCGACAAG	Ljubobratovic et al. (2017)
EF_rev	CCCAGGCGTACTTGAAGGAA	Ljubobratovic et al. (2017)

biochemical and gene expression analyses and 50 for morphometric analyses) gave approximately 15% margin of error with 95% confidence interval.

Condition factor was calculated according to the formula (Doyon et al., 1988):

$(100 \times W)/L^b$ (W = individual weight (g), L = individual body length (cm), b = allometric coefficient).

Allometric coefficients were calculated as the slopes of linear regressions fitting the logarithmic weight-length relationships (WLR) in each group. Calculations were made only in the case that linear model representing WLR was statistically valid (according to coefficient of determination value, R^2). Determination of allometric growth phase was performed according to Datta et al. (2013). Growth rate was estimated from the linear relationship between trypsin (T) activity and trypsin to chymotrypsin (T/C) activity ratio (Rungruangsak-Torrissen, 2016). Comparisons of fish survival and semi-quantitative AB/AR scoring results were performed by Pearson's chi-squared test. Survival rate was estimated by subtraction of the number of swim bladder inflated fish on the 24th DPH from the initial number of fish in each tank.

Differences between groups were considered to be statistically significant if t- or chi-square test p value was below 0.05. The exception was fish survival where the threshold p value was 0.0001, because of the sensitivity of chi-square test to sample size (Bergh, 2015). Pearson's chi-square test was performed using Chi-Square Test Calculator (https://www.socscistatistics.com/tests/chisquare2/Default2.aspx). Statistics for other analyses were performed using SPSS 20.0 for Windows.

3. Results

3.1. Nutritive profile of Artemia nauplii treated with frozen BGHO1/BGGO6-55 cultures

Mean values of biochemical measurements in *Artemia fransciscana* nauplii treated with BGHO1/BGGO6-55 cultures are presented as means ± standard deviations (SD) in Table 3A. Results are expressed relatively to the mean value of fresh control nauplii group. According to *t*-test, BGHO1/BGGO6-5 treatment significantly reduced SPR level in fresh nauplii ($p = 0.026$) and significantly increased NL level in starved nauplii ($p = 0.029$).

Microbiological analysis revealed substantial growth of bacteria in starved nauplii (Table 3A). This growth was more pronounced in control nauplii. Statistical comparisons for bacterial count could not be made due to significant deviations from normality. Lactobacilli were not observed neither in freshly hatched nor in starved nauplii.

3.2. Nutritive profile of OTOHIME B1 treated with frozen BGHN14/BGT10 cultures

Surface profile of OTOHIME B1 granules was analyzed by using whole, non-homogenized granules for lipid and soluble protein extraction processes. The granules preserved the integrity after defatting and subsequent procedures for protein level assessment, enabling the estimation of their surface portion. Biochemical profile (average values) of surface portion of OTOHIME B1 granules treated with BGHN14/BGT10 is presented in Table 3B. Results are expressed relatively to the mean value of control dry feed. According to *t*-test, in BGHN14:BGT10 treated feed, PL level was decreased relative to dry control ($p = 0.04$ and 0.045, respectively), while NL level was highly reduced relative to both dry ($p = 0.011$ and 0.1, respectively) and wet controls ($p = 0.011$ and 0.007, respectively).

According to microbiological inspection on universal LA medium, wet control feed samples were presented with growth of non-lactobacilli like bacteria (Table 3B). No non-lactobacilli like colonies were detected in BGHN14/BGT10 treated samples. There was no bacterial growth in dry control feed. Because of significant deviation from normality, statistical comparisons for bacterial count were not performed.

3.3. Fish growth indices

Results of assessment of fish growth indices and survival on the 24th DPH are given in Table 4. According to *t*-test, GW fish were longer

Table 3
Biochemical and microbiological profiles of *A. franciscana* nauplii (A) and surface portion of OTOHIME B1 (B) treated with frozen BGHO1/BGGO6-55 and BGHN14/BGT10 cultures, respectively; values of FAA/SP, SPR, PL, NL and UL are calibrated to the mean values of fresh control nauplii (A) or control dry feed (B); bacterial count shows the number of non-lactobacilli like colonies obtained on universal Luria agar (LA) medium, expressed per μg of naupliar wet weight (A) or mg of OTOHIME B1 wet weight (B); bacterial count is expressed as colony forming units (CFU) (lowest sensitivity threshold for microbiological assay was 10 CFU); statistical comparisons for bacterial count were not made because of significant deviations from normality.

	Means ± standard deviations (SD)					
	FAA/SP	SPR	PL	NL	UL	Bacterial count
A						
Fresh control nauplii	1 ± 0.42	1 ± 0.11	1 ± 0.11	1 ± 0.4	1 ± 0.29	10 ± 17.32
Fresh 75:25 BGHO1:BGGO6-55 treated nauplii	0.87 ± 0.16	***0.64 ± 0.14 (p = 0.026)**	1.03 ± 0.18	1.23 ± 0.3	0.9 ± 0.25	0 ± 0
Starved control nauplii	0.95 ± 0.21	0.68 ± 0.29	0.92 ± 0.01	0.78 ± 0.2	1.74 ± 0.77	1 073 ± 1 669
Starved 75:25 BGHO1:BGGO6-55 treated nauplii	0.87 ± 0.4	0.83 ± 0.26	0.94 ± 0.02	**#1.17 ± 0.04 (p = 0.029)**	1.21 ± 0.31	174 ± 282
B						
Control dry feed	1 ± 0.29	1 ± 0.11	1 ± 0.08	1 ± 0.3	1 ± 0.17	0 ± 0
Control wet feed	0.95 ± 0.07	1.12 ± 0.18	0.95 ± 0.12	0.56 ± 0.12	1.17 ± 0.32	357 ± 471
75:25 BGHN14:BGT10 treated feed	0.84 ± 0.12	1 ± 0.14	***0.8 ± 0.08 (p = 0.04)**	***#0.2 ± 0.08 (p = *0.011, # 0. 011)**	0.84 ± 0.37	0 ± 0
50:50 BGHN14:BGT10 treated feed	1.58 ± 1.53	0.92 ± 0.11	***0.8 ± 0.09 (p = 0.045)**	***#0.21 ± 0.04 (p = *0.01, # 0.007)**	0.8 ± 0.13	0 ± 0

*indicates significant difference in comparison to fresh nauplii (A) or dry feed control (B); # indicates significant difference in comparison to control nauplii (A) or wet feed control (B); in 3A statistical comparisons were made between treated and respective control (fresh or starved) nauplii and between fresh and starved control nauplii; in 3B statistical comparisons were made between treated feed and both dry and wet controls as well as between dry and wet controls; FAA/SP – free amino acids/short peptides, SPR – soluble proteins, PL – phospholipids, NL – neutral lipids, UL – unsaturated lipids.

Table 4
Growth-related indices on the 24th DPH. Fish survival is presented as the mean number of surviving fish per tank ± standard deviation (SD) at the 24th DPH; individual weights, body lengths and condition factors are presented as mean ± SD values; allometric coefficients are presented as the slopes of linearly fitted model of $\log_{10}$weight - $\log_{10}$ length relationship (WLR) and are marked in bold in WLR function; coefficients of determination (R^2) of WLR linear fit are provided in separate column with significance of model fit (p) given in parenthesis; Pearson correlation between trypsin activity and trypsin to chymotrypsin activity ratio is shown as an indicator of fish growth rate with significance (p) of correlation factors given in parenthesis. Skeletal development is shown as the percentage of analyzed fish showing Alizarin Red (AR) staining of vertebral column.

Treatment groups	Number of surviveing fish per tank (n = 3)(mean ± SD)	Individual weight (mg) (mean ± SD)	Body length (mm) (mean ± SD)	WLR function	WLR coefficient of determination, R^2	Condition factor (mean ± SD)	T activity to T/C activity ratio Pearson correlation	Percentage of analyzed fish with dominant AR staining (%)
SW control fish	2176 ± 488	12.91 ± 2.585	11.661 ± 0.916	$W = 0.089 L^{2.024}$	0.65 ($p < 0.0001$)		0.313 (p = 0.495)	40
SW LMF treated fish	***2561 ± 175 ($p < 0.0001$)**	***14.3 ± 2.042 (p = 0.024)**	11.853 ± 0.849	$W = 0.37 L^{1.476}$	0.517 ($p < 0.0001$)		0.275 (p = 0.474)	***78 (p = 0.01)**
GW control fish	***2481 ± 721 ($p < 0.0001$)**	***18.836 ± 5.047 ($p < 0.0001$)**	***13.357 ± 1.397($p < 0.0001$)**	$W = 0.024 L^{2.565}$	0.883 ($p < 0.0001$)	0.88 ± 0.084	0.339 (p = 0.371)	***95 (p = 0.0002)**
GW LMF treated fish	**#2743 ± 411 ($p < 0.0001$)**	19.094 ± 4.003	13.186 ± 1.148	$W = 0.036 L^{2.427}$	0.919 ($p < 0.0001$)	**#0.964 ± 0.062 ($p < 0.0001$)**	**0.833 (p = 0.005)**	90

* denotes statistically significant difference relative to SW control; # denotes significant difference relative to GW control (statistical comparisons were made between LMF treated groups and respective controls (SW or GW) and between SW and GW controls); condition factors for SW fish were not calculated because, according to R^2, WLR do not fit linear regression. SW - sudden weaning, GW - gradual weaning, LMF - lactobacilli modified feed, DPH - day post-hatching, T - trypsin; C- chymotrypsin.

($p < 0.0001$) and heavier ($p < 0.0001$) in comparison to SW fish. In addition, weights of SW LMF treated fish were higher ($p = 0.024$) from SW controls. According to the values of R^2, logarithmic WLR relationship fitted into the linear regression only in GW fish (0.883 and 0.919, in control and LMF treated GW fish, respectively). Hence, allometric coefficients and condition factors were estimated only in GW fish. According to these results, both GW control and LMF treated fish were in negative allometric growth phase, with GW LMF treated fish having higher condition factor than GW control fish ($p < 0.0001$). According to Pearson correlation factor values, GW LMF treated fish were presented with significant positive T to T/C relationship ($p = 0.005$). This suggests positive growth rate in this group. No significant correlation between T and T/C was observed in other treatment groups, indicating steady growth phases.

Fish survival on the 24th DPH was estimated by comparison of sums of fish numbers in all three tanks per group by Pearson's chi-square test (initial count of fish in all three tanks was 30 000). According to chi-square test results, fish survival was significantly increased in control GW fish as compared to control SW fish and in both SW and GW LMF treated fish as compared to respective controls.

3.4. Digestive enzyme activity

Results of enzymatic activities in whole fish samples on the 24th DPH are presented as mean values ± SD in Table 5. GW was associated with higher PLA2 specific activity ($p = 0.0004$) when compared to SW fish. There was not a difference in lipase activity among SW and GW fish. Furthermore, inspection of T and T/C activity ratios revealed no differences among treatment groups or among different weaning regimes. However, there was a significant increase in chymotrypsin specific activity ($p = 0.04$) and PLA2 to lipase activity ratio ($p = 0.009$) in SW LMF treated fish, as compared to SW control group.

3.5. Skeleton differentiation

Results of mRNA expression of skeleton development-related genes in whole fish samples on the 24th DPH are presented as mean values with SD in Table 6. EF served as endogenous control. EF-scaled target gene expression values were calibrated to the mean value of SW control group. According to *t*-test, expression levels of *Sparc* and *Col1A1* mRNA were lower in GW as compared to SW fish ($p < 0.0001$ and $= 0.012$, respectively) (Table 6). Furthermore, *Sparc* and *Col1A1* mRNA levels were lower in SW LMF treated fish as compared to SW controls ($p = 0.0003$ and 0.039, respectively). These results correlated with the results of AB/AR staining, which showed higher percentage of fish with AR stained vertebral column in GW control and SW LMF treated fish in comparison to SW control ($p = 0.0002$ and 0.01, respectively) (Table 4). No differences were observed in expression of *Col2A1* mRNA among treatment groups (Table 6).

3.6. Bacterial growth

Levels of *Vibrio*, *Aeromonas* and *Mycobacterium* spp. in whole fish specimens are presented as mean values ± SD in Table 7. Student t-test revealed significant decrease in relative *Vibrio* spp. level ($p = 0.021$) in GW fish as compared to SW fish, as well a decrease of *Vibrio* spp. count in SW LMF treated fish as compared to SW controls ($p = 0.031$). *Aeromonas* spp. levels followed the similar trend as *Vibrio* spp. but no statistical significance was reached. No differences in *Mycobacterium* spp. amount were observed among treatment groups.

4. Discussion

Early weaning is linked to significant growth suppression, mostly because of the lack of phospholipid biosynthesis pathways (Sargent et al., 2003; Twining et al., 2016) and poor efficacy of protein digestion

Table 5
Trypsin, chymotrypsin, lipase and phospholipase A2 (PLA2) activities in whole fish samples on the 24th DPH.

	Means ± standard deviations (SD)					
	Phospholipase A2 (PLA2) specific activity (mU/mg of protein)	Lipase specific activity (mU/mg of protein)	Trypsin specific activity (mU/mg of protein)	Chymotrypsin specific activity (mU/mg of protein)	PLA2 to lipase activity ratio	Trypsin to chymotrypsin activity ratio
SW control fish	623 ± 158	0.0035 ± 0.001	0.039 ± 0.005	0.006 ± 0.001	149025 ± 28574	6.8 ± 1.4
SW LMF treated fish	767 ± 294	0.0036 ± 0.001	0.041 ± 0.005	***0.01 ± 0.005 (p = 0.04)**	***214 555 ± 50 598 (p = 0.009)**	5.9 ± 4.4
GW control fish	***1 157 ± 302 (p = 0.0004)**	0.0044 ± 0.0028	0.04 ± 0.005	0.006 ± 0.002	286717 ± 168730	7.4 ± 2.4
GW LMF treated fish	1 028 ± 266	0.0048 ± 0.0009	0.041 ± 0.008	0.007 ± 0.003	225171 ± 80935	7 ± 3.4

* indicates significant difference relative to SW control fish (statistical comparisons were made between LMF treated groups and respective controls (SW or GW) and between SW and GW controls); SW - sudden weaning, GW - gradual weaning; LMF - lactobacilli modified feed.

Table 6
Col1A1, *Col2A1* and *Sparc* mRNA expression in whole fish samples on the 24th DPH relative to elongation factor (EF) mRNA level.

	Means ± standard deviations (SD)		
	Sparc mRNA	*Col1A1* mRNA	*Col2A1* mRNA
SW control fish	1 ± 0.19	1 ± 0.36	1 ± 0.26
SW LMF treated fish	***0.42 ± 0.27 (p = 0.0003)**	***0.62 ± 0.26 (p = 0.039)**	0.9 ± 0.23
GW control fish	***0.3 ± 0.22 (p < 0.0001)**	***0.46 ± 0.35 (p = 0.012)**	1.18 ± 0.69
GW LMF treated fish	0.2 ± 0.14	0.59 ± 0.37	0.75 ± 0.62

* indicates significant difference relative to SW control fish (statistical comparisons were made between LMF treated groups and respective controls (SW or GW) and between SW and GW controls); SW - sudden weaning, GW - gradual weaning; LMF - lactobacilli modified feed.

(Rønnestad et al., 2013) in larval fish. This is particularly pronounced in larvae that suddenly wean to inert feed (Ljubobratović et al., 2015). In current research, GW fish had better survival, higher allometric coefficient, larger individual length and weight, indicating faster morphogenesis and growth than SW fish (Pepin, 1995). In support of this, GW fish were presented with better skeleton differentiation, as evident from *Col1A1/Sparc* expression and Alizarin Red staining. Col1A1 functions as a key regulator of and its expression decreases with bone development ossification (Aceto et al., 2015; Gistelinck et al., 2016). Sparc plays crucial role in bone mineralization (Renn et al., 2006) and decreases in mature bones (Mundlos et al., 1992; Rosset and Bradshaw, 2016). Though skeletal development was shown to be positively influenced by proteins, especially peptides, phospholipids and unsaturated fatty acids have been defined as key nutrients supporting skeletal growth (Cahu et al., 2003). However, availability of fats is affected not only by their dietary level but also by the efficacy of their utilization inside the gut (Morais et al., 2005a). In this respect, phospholipids aid the transport of fatty acids from enterocytes to circulation and their presence in the feed increases feed palatability (Cahu et al., 2009). On the other hand, excess neutral lipid level may impede fatty acid absorption (Tocher et al., 2008). It could therefore be assumed that better development of skeleton in GW fish is presumably the result of better lipid quality in live food in comparison to inert feed, at the first place higher phospho-to neutral lipid ratio. This might have affected both lipid utilization efficacy as well as feed palatability. This is supported by higher PLA2 activity in GW fish, eventually indicating higher feed consumption rate in GW fish. Similarly, trypsin and chymotrypsin activities reflect feed consumption and protein availability in the diet (Rungruangsak-Torrissen, 2012). However, their activity seems also to be dependent on the growth status of fish. Chymotrypsin was reported to increase in fish with better feed consumption if fish are in steady or reducing growth phase, while trypsin activity was shown to increase in fish with better feed consumption if fish are in rapid growth phase (Rungruangsak-Torrissen et al., 2006). Although both SW and GW fish were at the steady growth phase, no difference in chymotrypsin activity was observed among them, which was expected according to PLA2 activity. This presumably reflects the tendency of alkaline protease activities to decrease with maturation of digestive tract and stomach development, as reported by Savona et al. (2011). Though growth rate was the same in SW and GW fish at the moment of fish sampling (6 days after start of the weaning), GW fish had larger body size. It is possible that SW fish had reduced growth immediately after the beginning of weaning (18th DPH), which caused lag in growth observed on the 24th DPH.

After transition to exogenous diet and complete yolk resorption, specific density of fish increases and this coincides with the swim bladder inflation period (approximately 8-18th DPH in pike-perch, depending on the water temperature) (Szkudlarek and Zakęś, 2007).

Table 7
Aeromonas, Mycobacterium and *Vibrio* spp. 16S rRNA expression in whole fish samples on the 24th DPH relative to pike-perch elongation factor (EF) mRNA level.

	Means ± standard deviations (SD)		
	Vibrio spp. 16S rRNA	*Aeromonas* spp. 16S rRNA	*Mycobacterium* spp. 16S rRNA
SW control fish	1 ± 0.6	1 ± 0.66	1 ± 0.94
SW LMF treated fish	***0.41 ± 0.32 (p = 0.031)**	0.57 ± 0.43	0.69 ± 0.4
GW control fish	***0.42 ± 0.27 (p = 0.021)**	0.59 ± 0.41	0.96 ± 0.63
GW LMF treated fish	0.29 ± 0.28	0.26 ± 0.26	0.58 ± 0.19

* indicates significant difference relative to SW control fish (statistical comparisons were made between LMF treated groups and respective controls (SW or GW) and between SW and GW controls); SW - sudden weaning, GW - gradual weaning; LMF - lactobacilli modified feed.

Fish without swim bladder at this period has high energetic demands for feeding and maintenance of their position in water, which leads to high fish mortality (Summerfelt, 2013; Szkudlarek and Zakęś, 2007). This is of special relevance if we consider the fact that, in hatcheries, *Artemia* is commonly starved after hatching (Evjemo et al., 2001). This starvation may significantly reduce the caloric content of *Artemia*, including both protein and lipid content (Evjemo et al., 2001). In this research, 12 h starved live food treated with BGHO1/BGGO6-55 combination was presented with higher neutral lipid amount when compared to control 12 h starved *Artemia.* Though neutral lipid quantity was not significantly changed in freshly hatched *Artemia* after treatment with BGHO1/BGGO6-55, it could be assumed that overall amount of neutral lipids ingested by fish via *Artemia* nauplii during 12 h period was slightly higher in fish administered lactobacilli treated nauplii. In line with this, both SW and GW fish administered lactobacilli treated *Artemia* were presented with better survival as compared to control fish. Although other factors later during weaning might have influenced survival, the input of energy-rich nauplii could have eventually contributed to the reduction of larval mortality at the critical period around swim bladder inflation, as explained above.

There is a plenty of research data reporting correlation between neutral lipid reduction and improvement of fish growth (Gisbert et al., 2005; Kjørsvik et al., 2009; Morais et al., 2007). This was ascribed to improvement of fatty acid absorption (Morais et al., 2007), which are essential for proper skeleton growth (Cahu et al., 2003). In line with this, SW LMF treated fish, which were weaned only on lactobacilli treated dry feed containing lower neutral lipid level than non-treated feed, showed better skeleton ossification when compared to SW controls. In line with this, PLA2 to lipase activity ratio was higher in SW LMF treated fish as compared to SW controls, eventually reflecting higher phospho-to neutral lipid ratio in consumed feed. In support of the results obtained with Alizarin Red staining and *Col1A1/Sparc* expression, chymotrypsin activity was shown to be increased in SW LMF treated fish when compared to SW control fish. This was presumably the result of better feed palatability, due to lower neutral lipid level in treated dry feed. It is known that chymotrypsin increases the pool of free aromatic amino acids (AAA) (Rungruangsak-Torrissen et al., 2006), which serve as precursors for thyroid hormone synthesis. Since thyroid hormones control skeletal development (Pinto et al., 2009; Shkil et al., 2012), it is possible that higher chymotrypsin activity boosted skeleton growth in SW LMF treated fish.

Though GW fish were also administered dry feed during weaning, the impact of dry feed nutritional profile on GW fish growth is expected to be lower than in SW fish, due to simultaneous administration of live food. Studies testing ingestion rate in seabass have shown that ingestion of microdiet is lower in the presence of *Artemia* nauplii (Kolkovski et al., 1997). Interestingly, *Artemia* was shown to aid dry feed assimilation efficacy in the same study. It was hypothesized that polar lipids present in *Artemia* may improve fatty acid absorption, alleviating potential adverse effects of higher neutral lipid dose present in dry feed. Hence, neutral lipid reduction in dry feed after lactobacilli treatment presumably did not produce a considerable difference in fatty acid absorption efficacy in GW fish. This may explain the lack of difference in skeleton differentiation between GW LMF treated and GW control fish. However, GW LMF fish had higher condition factor as compared to GW control. Higher condition factor may indicate an increase in fat and/or muscle deposition (Barnham and Baxter, 2003; Pazianoto et al., 2016). Since growth rate in these fish was higher, and growth is primarily the result of muscle mass increase (Ambariyanto et al., 2013), we assume that musculature development was improved in GW LMF treated fish. In line with this, early reduction of neutral lipids in fish diet was shown to stimulate muscle growth and amino acid absorption (Alami-Durante et al., 2014; Morais et al., 2005b). In comparison to SW fish, GW fish were supplied with higher amount of soluble proteins originating from live food. It could thus be assumed that the presence of sufficient amount of amino acids, which are essential for muscle development (Rønnestad et al., 1999), along with reduction of neutral lipid level, which potentially improved amino acid absorption efficacy, increased muscle buildup in GW LMF treated fish.

Opportunistic pathogenic bacteria are commonly present in fish tanks. This is related to water quality and type of administered food (Corsin et al., 2009). Bacterial growth in tank water inversely correlates with the degree of utilization of feed by fish, including both feed ingestion and feed assimilation rate. This is because uneaten and poorly digested feed remaining in tank water serves as a substrate for bacterial growth (Wold et al., 2014). This allows the accumulation of potential fish pathogens in tank water which for the large part determines the microbiota of fish skin and intestine, especially in fish larvae with poorly developed digestive tract (Cahill, 1990; Giatsis et al., 2015). In line with this, SW fish had higher amount of potentially pathogenic bacteria, especially *Vibrio* spp., when compared to GW fish. This may be related to dry feed assimilation rate, which was shown to be higher in the presence of *Artemia* in GW fish (Kolkovski et al., 1997). Furthermore, SW LMF fish were presented with lower *Vibrio* spp. as compared to SW control fish, presumably as the result of improved absorption of nutrients derived from lactobacilli treated dry feed. Additionally, fish associated *Vibrio* species are known to be highly lipolytic (Egerton et al., 2018; Henderson and Millar, 1998). It is thus possible that reduction of neutral lipid level in lactobacilli treated dry feed further reduced *Vibrio* spp. growth. Though also GW LMF fish were assumedly presented with improved nutrient utilization, at least when it comes to proteins, there was no significant difference in *Vibrio* spp. count between LMF and control GW fish. However, statistical tendency towards *Aeromonas* spp. decrease in LMF GW fish was seen ($p = 0.058$). This eventually reflects differences in lipid and protein utilization potential by *Vibrio* and *Aeromonas* spp. (Abd-El-Malek, 2017).

The above results suggest that modulation of fish feed composition by lactobacilli may affect the success of pike-perch weaning. Reduction of excess neutral lipid amount in dry feed correlates with better skeleton ossification in SW fish, while in GW fish, which are administered sufficient amount of soluble proteins via *Artemia* live food, this correlates with improved musculature growth. On the other hand, slight increase of neutral lipids in live food may eventually be linked to improved survival of both SW and GW fish, due to potential improvement of fish energetic status prior to swim bladder inflation. This is the first study to demonstrate that moderate elevation of triglyceride amount in

Artemia nauplii may beneficially affect larval development, which allows for further manipulation of *Artemia* composition in the same direction in order to achieve maximal benefits for growing fish. In this respect, use of other BGHO1/BGGO6-55 combinations and doses for *Artemia* treatment, which correlated with pronounced increase of neutral, along with phospho- and unsaturated lipid amount (unpublished data) may be considered as an option for future studies. However, optimizations regarding the application of cryopreserved lactobacilli cultures would be needed. This is because BGHO1/BGGO6-55 combination applied in present study, which increased soluble protein and phospholipid amount in newly hatched *Artemia* nauplii when fresh cultures were used (unpublished data), did not produce the same effect when frozen cultures were applied. Frozen bacteria are known to produce cold shock proteins (CSP), which suppress transcription and translation processes (Hofweber et al., 2005) and might have slowed metabolic processes in hatched nauplii. This was evident from reduction of neutral lipid utilization in starved BGHO1/BGGO6-55 treated nauplii. Metabolic changes in cryopreserved bacterial cells seemed also to affect bacterial survival during incubation in *Artemia* hatching medium, since no viable nauplii-associated lactobacilli were detected after incubation. Contrary to *Artemia*, composition of dry feed treated with frozen lactobacilli cultures was not significantly different from the composition obtained with fresh cultures. In both cases, significant neutral lipid drop was observed after incubation with BGHN14/BGT10, presumably as the result of activation of endogenous feed-derived lipases (Lukic et al., 2019).The exception was phospholipid amount, which was significantly decreased after treatment with frozen lactobacilli cultures both with 75:25 and 50:50 combinations. This may eventually result from emulsifying properties of glycerol, which was used as cryo-protectant and may have promoted phospholipid degradation by internal phospholipases (Norn, 2015).

Presented correlations of feed nutritive content with fish growth should serve as a backbone for the design of further studies aiming to achieve maximal fish growth. Use of lactobacilli for stimulation of harmonious skeleton and musculature growth in larval pike-perch during weaning would aid economically and ecologically sustainable pike-perch production in recirculating system and would finally increase overall contribution of aquaculture to global food production.

Declaration of competing interest

Authors declare no conflicts of interest.

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