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Use of complementary feeding stuff Ichtio Hexan to control Spironucleus salmonis infections in rainbow trout

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Summary

The aim of this study was to determine the efficacy of Ichtio Hexan (a complex blend of Allium sativum extract, Chelidonium majus extract, Origanum vulgare extract, carvacrol and cinnamic aldehyde) for the control of spironucleosis (hexamitosis) in rainbow trout fingerlings. For this purpose, three groups of rainbow trout naturally infected with S. salmonis were fed diets containing either no Ichtio Hexan or supplemented with Ichtio Hexan at a rate of 0.1 ml/kg bw (group 1) or 1 ml/kg bw (group 2) for 38 days. During this period mortalities were recorded and the dead fish were examined to confirm the isolations of S. salmonis. At the end of the experiment the fish were sacrificed, individually weighed and measured, their livers removed and weighed. In order to determine the prevalence and intensity of infection the intestinal contents were examined.

The results of the present study showed that Ichtio Hexan in an amount of 0.1 ml/kg bw considerably reduced mortality caused by S. salmonis in rainbow trout fingerlings. The number of S. salmonis trophozoites in the digestive tract after administration of Ichtio Hexan at this dose for 38 days was significantly reduced and limited only to the posterior part of the intestine. On the other hand, in the 1 ml/kg bw fed group none of the fish were infected and the gain in the body weight was significantly increased; however, the mortality rate was similar to the control group. The experimental groups did not differ significantly from each other with regards to the condition factor and hepatosomatic index.

Considering the above findings, Ichtio Hexan at a dose of 0.1 ml/kg bw can be successfully used in rainbow trout farms to reduce the mortality rate in S. salmonis infected fish.

Keywords: rainbow trout, Spironucleus salmonis, spironucleosis treatment, Ichtio Hexan

The flagellated protozoon from the genus *Spironucleus* causes significant losses, both in farm and in ornamental fish industry (12, 13, 15, 17, 22, 24-26, 29-31). Five species of piscine diplomonads are currently recognized: *S. barkhanus*, *S. salmonicida*, *S. salmonis*, *S. torosa* and *S. vortens* (34). *S. salmonis* typically affects young farmed rainbow trout of a standard length between 2.5-7.5 cm (32). *S. salmonis* infected fish exhibit weakness, anorexia, emaciation, lethargy, excretion of stringy feces and increased mortality. Histologically, gastrointestinal lesions ranging from no visible damage to sever enteritis may be observed (11, 31, 32, 34).

Due to the prohibition of using metronidazole in food fish in Europe (Council Regulation 613/98/EEC 1998) as well as environmental and health concerns raised by its use in the aquarium trade, the search for new agents to combat spironucleosis is crucial (29).

Although numerous studies proved that natural products can be a great source of new agents for fish parasitic disease control (10, 14, 20, 21), there is no research testing plant material against spironucleosis.

In the present study, a complex blend of herbs, Ichtio Hexan, was used for the treatment of naturally infected rainbow trout fingerlings with *S. salmonis*. Along with the estimation of the antiparasitic effect, biometric parameters were examined in order to determine treatment effects on growth performance.

Material and methods

Experimental fish. Rainbow trout (average weight 0.8 g ± 0.2 g) naturally infected with *S. salmonis* were derived from a commercial farm in Pomorskie province in Poland.

Detection of parasites. Molecular detection pair with examination of fresh intestinal contents under the light microscope randomly selected fish before the beginning of experiment, confirmed high intensity *S. salmonis* infection with the prevalence reaching 60%.

Complementary feeding stuff. Ichtio Hexan was provided by Ichtico (Orle, Poland), and its composition was shown in Tab. 1.

Tab. 1. Composition of Ichtio Hexan

Ingredient	Amount (mg/l)
Hydro-glycolic garlic (*Allium sativum*) extract	750 000
Glycolic tetterwort (*Chelidonium majus*) extract	60 000
Glycolic oregano (*Origanum vulgare*) extract	60 000
Carvacrol	20 000
Cinnamic aldehyde	20 000

Assay design. The experiments were carried out in the fish laboratory of the Department of fish Diseases and Biology, Faculty of Veterinary Medicine in Lublin, Poland. Experimental procedures were approved by II Local Ethics Committee in Lublin (application number 53/2012). 120 rainbow trout were randomly introduced into six 100-L glass aquaria equipped with a flow-through system, 20 fish for each. Water temperature was kept at 12 ± 1°C. Commercial feed (Skretting, Stavanger, Norway) was used as the basal diet for the supplementation of Ichtio Hexan in a dose recommended by Ichtico of 0.1 ml/kg body weight (bw) (group 1) and in a dose of 1 ml/kg bw (group 2). Untreated controls only received the basal diet. Each diet group was fed twice daily for a period of 38 days. The feeding rate was 3% of live body weight. Mortalities were recorded daily and the dead fish were examined to confirm the isolations of *S. salmonis*. Protection was evaluated by determining the relative percent of survival (RPS) (2) in each group using the formula:

$$RPS = \left(1 - \frac{m_t}{m_c}\right) \times 100,$$

where m_t = average cumulative mortality in treated fish, m_c = average cumulative mortality in control fish.

The fish were weighed and measured at the beginning and at the end of the experiment. Before weighing, fish were starved for 24 h, allowing the gut to be emptied. During handling, the fish were anaesthetized using a solution of tricaine methanesulphonate (MS-222; Sandoz, LTD, Basle, Switzerland) at a concentration of 0.1 g/l. At the end of the experiment surviving fish were sacrificed, their livers removed and weighed.

Quantitation of parasites. For determination of the occurrence and intensity of infection the intestine was divided into two equal parts: anterior (including pyloric region) and posterior (including anus). The intestine was carefully opened with fine scissors, and a sample of the contents were placed on a microscope slide and covered with a cover slip. The samples were examined with a light microscope (150 ×) for motile parasites. The presence of diplomonads was recorded as positive (+), and their absence as negative (–). According to Saghari-Fard (27) density was semi-quantitatively estimated, as the number of flagellates under a 22 × 22 mm cover slip (484 mm^2) (the mean of at least three countings) and expressed as follows: light infection (1+) as less than 10 flagellates/484 mm^2, moderate infection (2+) as 11-40 flagellates/484 mm^2, and heavy infection (3+) as more than 40 flagellates/484 mm^2 (27).

Biometric parameters. The condition factor (CF) of each fish was calculated using the following formula:

$$CF = \left(\frac{W}{L}\right) \times 100,$$

where W – final body weight, L – final body length.

The hepatosomatic index (HSI) was calculated with the formula:

$$HSI = \left(\frac{L}{B}\right) \times 100,$$

where L – liver weight, B – body weight.

The specific growth rates (SGR) of the different groups were calculated according to the following equation:

$$SGR = \left[\frac{(log_n w_f - log_n w_i)}{t}\right] \times 100,$$

where w_f = average weight after 38 days of Ichtio Hexan supplementation, w_i = average weight at the beginning of the study, t = time of the study (days).

Statistical analysis. All statistical tests were performed using the Statistica 8.0 package (StatSoft, Cracow, Poland). Results are given as mean ± SD. Comparisons between the experimental groups of fish were performed using ANOVA followed by NIR Fisher's test. Mortality data were analyzed with Kaplan-Meier log-rank survival analysis. Differences were considered as significant at ($p < 0.05$).

Results and discussion

The Kaplan-Meier survival curves for experimental and control fish infected with *S. salmonis* are given in Fig. 1. Log-rank tests showed differences between group 1 and the control group ($p = 0.037$). The cumulative mortalities in group 1 were significantly lower than those observed in other groups, which has given a pro-

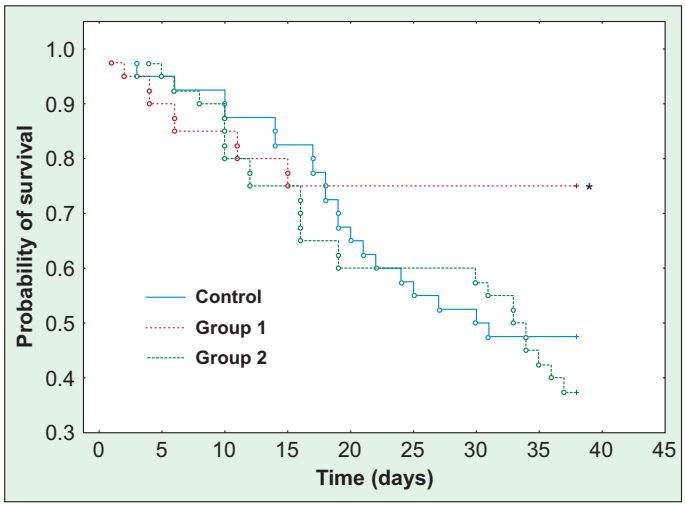

Fig. 1. Kaplan-Meier survival curve for control and tested groups

Explanation: * p = 0.037 vs. control group

Tab. 2. Protection efficacy of Ichtio Hexan administered to *S. salmonis*-infected rainbow trout for 38 days

Group	Number of fish	Number of dead fish	Cumulative mortality (%)*	RPS*
Group 1	40	10	25 ± 7.1**	56.5
Group 2	40	25	62.5 ± 3.5	–8
Control	40	23	57.5 ± 3.5	

Explanations: * values are mean ± SD of duplicate tanks; ** significantly different compared with control group ($p \leq 0.05$)

tection efficacy equal to 56.5% (Tab. 2). Furthermore after 15 days mortalities were not noticed in this group.

The necropsy examination of the surviving fish after 38 days of the experiment showed that Ichtio Hexan had a significant ($p < 0.05$) effect on the intensity and prevalence of *S. salmonis* infections. The highest prevalence of infection equal to 46.43% was in group 1. In the control group prevalence was 29.17% and in group 2 none of the fish were infected (Tab. 3). The intensity of infections was equal inside the groups and differed between groups. In case of group 1, intensity was determined as a light (1+) infection and in the control group as a heavy (3+) infection (Tab. 3).

Occurrence of *S. salmonis* along the length of the intestine differed between the groups. In case of control groups *S. salmonis* were noticed in each of the intestinal regions. In case of group 1 *S. salmonis* were noticed only in the posterior part of the intestine (Tab. 3).

The initial body weights were not significantly different between groups, but at the end of the trial body weights in group 2 were significantly higher ($p < 0.05$) when compared to the control group (Fig. 2). The values of the specific growth rate for the test group are shown in Fig. 2.

The experimental groups did not differ significantly ($p > 0.05$) from each other in respect of the condition factor and hepatosomatic index (results have not been shown).

The results of the present study confirmed that Ichtio Hexan at a dose of 0.1 ml/kg of bw considerably reduced mortality caused by *S. salmonis* in rainbow trout fingerlings. The number of parasites in the digestive tract after administrations of Ichtio Hexan at this dose for 38 days was significantly reduced and limited only to the posterior part of the intestine. This limitation of occurrence of *S. salmonis* represents the unusual microhabitat preferences of *S. salmonis* which

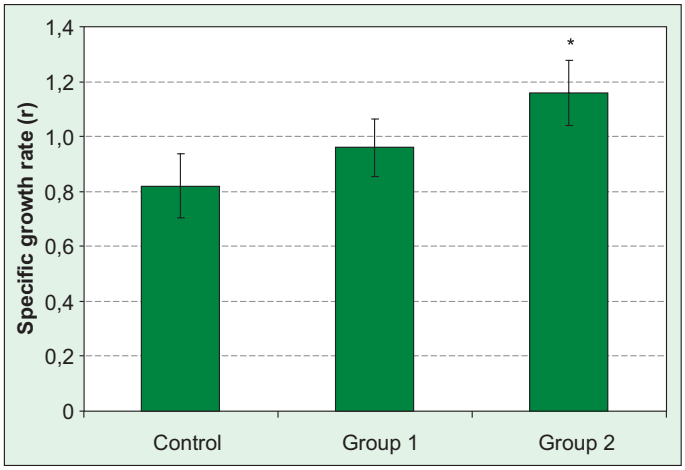

Fig. 2. Comparison of specific growth rate (r) (± SD) for control and tested groups

Explanation: * significantly different compared with control group ($p \leq 0.05$)

is normally found in the pyloric region of the intestine of fish and its occurrence in the posterior part of the intestine is associated with heavily infected fish when all 3 upstream regions of the intestine are infected (28). This can suggest that Ichtio Hexan especially make upper regions of the intestine unpleasant for *S. salmonis*. Even if the parasites were not completely eliminated, which took effect on low level reduction of prevalence at the end of the experiment in group 1 as compared to the control group, a substantial reduction of the parasite burden as shown in this study led to the recovery of the fish and paves the way for the development of protective host immunity (7). On the other hand, 1 ml/kg bw of Ichtio Hexan proved to be too high a dose for weakened fish. In the groups which were fed diets with that amount of extract none of the fish were infected but the mortality rates were similar to that of the control group.

Extracts from garlic, tetterwort and oregano as well as plant active components carvacrol and cinnamaldehyde which were used to prepare Ichtio Hexan are reputed to have excellent medicinal value. Beside antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria garlic has antiparasitic activity against such serious pathogens as *Ichthyophthirius multifiliis*, *Spironucleus vortens* and *Trichodina* sp. (5, 6, 20). Garlic also enhanced innate defense mechanisms in rainbow trout (23) which can play a role in the ability to cope with parasites. Sanguinarine, which is one of the active compounds of tetterwort, is effective against *I. multifiliis* (35). Oregano essential oils with a high content of carvacrol and thymol possess a broad spectrum of antimicrobial activities targeting bacteria (1, 3, 9), fungi (1) and parasites (4, 16, 19 http://www.sciencedirect.com/science/article/pii/S0304401705003936 - aff1).

Tab. 3. Prevalence and intensity of infections after administration of Ichtio Hexan to *S. salmonis*-infected rainbow trout for 38 days

Group	Number of survived fish	Number of *S. salmonis*-infected fish	Prevalence (%)*	Intensity of infection Part of the intestine	
				anterior	posterior
Group 1	30	14	46.43 ± 5.1**	–	+
Group 2	15	0	0 ± 0**	–	–
Control	17	6	29.17 ± 5.9	+++	+++

Explanation: as in Tab. 2

Cinnamaldehyde and carvacrol are commonly used antibacterial components of the essential oil of clove, cinnamon, oregano and thyme (8, 18, 33).

Due to the lack of effective methods for treating spironucleosis, searching for new agents for the treatment of this disease is crucial. Plant extracts are gaining increasing interest as a source of new treatment agents because of their relatively safe status, wide acceptance by consumers, and their potential for multipurpose uses. Plant extracts are also often less expensive then some other synthetic drugs, safer for fish, have less impact on the environment and can be easily incorporated into the diet.

The variety of included components in Ichtio Hexan suggest that reduced mortality of *S. salmonis* infected rainbow trout fingerlings may be attributed to both a direct cytotoxic action of its components on the parasite and an immunostimulatory effect of garlic extract (23) on the host's innate immunity resulting in parasite elimination via effective defense mechanisms (7).

The significantly reduced mortality rate in the group fed a diet supplemented with Ichtio Hexan suggests that this complementary feeding stuff may be a promising agent for the treatment of spironucleosis in farmed rainbow trout. This could be seen as a better alternative for aquaculture and the environment than the use of metronidazole, which is restricted by law in many countries.

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