Potency Testing of ß-Glucan Immunostimulating Effect in Food for Ornamental Fish

Daniela Türnau[1], H.Schmidt[2], H. Kürzinger[2] and K.H.Böhm[1]

[1]Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany. [2]Tetra Werke, Herrenteich 78, 49304 Melle

Abstract

The object of the investigation was to test, whether a significant reduction of mortality might occur in glucan-treated fish compared to controls consisting of fish fed a glucan-free diet. Five different diets were tested combined with six pathogen/fish species variations. Mortality was compared to matching controls and the results were statistically analysed. Enhanced resistance to challenge with *A. hydrophila* or *Ps. fluorescens* was found in the glucan-fed ornamentals.

Introduction

The use of ß-glucans as an immunostimulant for fish has been investigated for about ten years but experiments have concerned commercially farmed fish like Atlantic salmon or carp rather than small ornamental fish from tropical regions. Several authors (*Yano et al.*, 1991, Wang *et al.*, 1993, Nikl *et al.*, 1993, Wang *et al.*,1996, Efthimiou 1996) noticed an encouraging enhancement in resistance against different bacterial pathogens in various fish species. Enthusiasm though was reduced by the observation that oral application of the immunostimulants often resulted in enhanced activity of nonspecific defence in vitro, but protection of the fish from infection did not always occur (Ogier de Baulny *et al.*, 1996, Duncan and Klesius 1996). An overview of this aspect has recently been given by Robertsen (1999).

Our observations indicate there is an immunostimulating effect in orally applied ß-glucans in tropical ornamentals, and the possibilities opened by this food-additive to the breeder and aquarist are discussed.

Materials and Methods

The ornamental fish used in the experiments were swordtails (*Xiphophorus helleri*), rosy barbs (*Barbus conchonius*) and black tetras (*Gymnocorymbus ternetzi*), all of them popular ornamental tropical fish. The fish were kept at 24 °C. The fish were challenged at groups of 26 with two replicate groups per diet.

The bacterial pathogens chosen for these trials were *Aeromonas (A.) hydrophila* and *Pseudomonas (P.) fluorescens*. They were cultured on Columbia agar for 24 h at 25 °C.

Diets	Description
Control diet K:	synthetic glucan-free diet
V1	synthetic glucan-free diet, + 0.1 % Schizophyllan
V2	synthetic glucan-free diet, + 1.0 % Schizophyllan
V3	commercial diet, based on the yeast *Torula utilis*, naturally containing glucans
V4	V3 + 0.1 % Schizophyllan
V5	V3 + 1.0 % Schizophyllan

Table 1. Control and glucan-supplemented diets

The diets were developed by Tetra. Four different diets containing industrially produced schizophyllan (VST produced by Taito Co., Japan) at different concentrations were tested, two of them based on a synthetic glucan-free food and two based on a commercial standard food also naturally containing glucans. A diet consisting of the synthetic diet and the commercial standard food without artificial glucan addition was included as controls. A description of the diets is given in table 1.

A reproductive infection model was developed. Intensive pre-studies revealed intraperitoneal injection as bacteria application method of choice. LD_{60-90} was evaluated for each experimental combination of pathogen/fish species (see table 2). Development of infection, symptoms and post mortem status were annotated.

The five diets and a glucan-free control diet were fed to the test fish (n = 52) for 21 days. The fish received the diets twice a day at libitum. On day 22 the fish were challenged with either *A. hydrophila* or *Ps. fluorescens* at groups of 26 with two replicate groups per diet. For the infection one colony of either *A. hydrophila* or *Ps. fluorescens* was suspended in 50 ml broth, incubated at 25 °C for one h and diluted to the infection dose with physiological saline. The infection dose is shown in table 2. Fish were injected intraperitoneally with 0,1 ml of the bacterial suspension after being narcotized with benzocaine.

A second test was performed with a lower infection dose of 9×10^5 cfu/ml. Only rosy barbs were challenged with *A. hydrophila* and the diets tested were V1, V2 and K as control. Mortalities were evaluated and the results analysed by means of SAS one-tailed chi-square-test.

Fish	Infection dose for *A. hydrophila*	Infection dose for *Ps. fluorescens*
Black tetras	2.0×10^6 cfu/ml	1.0×10^8 cfu/ml
Rosy barbs	2.0×10^6 cfu/ml	1.0×10^8 cfu/ml
Swordtails	1.0×10^6 cfu/ml	2.0×10^8 cfu/ml

Table 2. Challenge doses for each of the fish species

Results

Infected fish showed nonspecific clinical symptoms like anorexia and dark colour, low swimming activities, depression, exophthalmia and when infected with *Ps. fluorescens* sometimes discoordinated movements.

Mortalities occured in fish infected with *A. hydrophila* during the first 48 h after exposure to the bacteria. *Ps. fluorescens* caused mortality within the first three days post infection. No fish died after this time. Observation period was ten days. All deaths were due to *A. hydrophila* or *Ps. fluorescens* as each was reisolated in pure culture from liver, kidney, gill and skin.

Pathologic and anatomic examination revealed that all dead and moribund fish showed the typical picture of haemorrhagic septicaemia. All fish showed a typical inflammation in the place of injection and skin lesions in the abdominal region. They showed a distended abdomen with seroanguinous fluid and a swollen intestine. Kidney and spleen were enlarged. Particularly, the rosy barbs showed a strong spreading of scales. Infection with *Ps. fluorescens* in some cases led to inflammation of the swim bladder.

Results are shown in table 3. The average mortality in the control groups was 76.9 %. Reduction of mortality compared to the control groups was observed in 27 (90 %) out of 30 experimental groups and reached between 1.9 and 21.2 %. In one third of the tested groups significant levels of reduction of mortality were achieved.

It was of note that the diet V1, containing 0.1 % schizophyllan, reduced mortality compared to the control by 11.2 %, about twice as much as diet V3, a food naturally containing glucan, which reduced mortality by an average of 6.7 %(V2 = 9.6%; V4 = 6.4%; V5 = 8.7%). However, a significant difference between diets containing technologically modified ß-glucan and diets consisting of ingredients naturally containing glucan could not be statistically proven.

In the second test with the lower infection dose the mortality in the controls fed the synthetic diet K reached 26.7 % and was reduced in the glucan-containing diet V2 fed group to 13.5 %.

Diet	Mortality Swordtails/ *A. hydrophila*	Mortality Swordtails/ *Ps. fluorescens*	Mortality Rosy barbs/ *A. hydrophila*	Mortality Rosy barbs/ *Ps. fluorescens*	Mortality Black tetra/ *A. hydrophila*	Mortality Black tetra/ *Ps. fluorescens*
K	41(78.8)	45(86.5)	36(69.2)	43(82.7)	38 (73.1)	37(71.2)
V1	35(67.3)	36(69.2)	31(59.6)	42(80.8)	33(63.5)	28(53.8)
V2	40(76.9)	36(69.2)	25(48.1)	37(71.2)	34(65.4)	38(73.1)
V3	38(73.1)	35(67.3)	27(51.9)	40(76.9)	45(86.5)	34(65.4)
V4	37(71.2)	37(71.2)	36(69.2)	41(78.8)	35(67.3)	34(65.4)
V5	40(76.9)	38(73.1)	30(57.7)	39(75.0)	29(55.8)	37(71.2)

Table 3. Number of mortalities of each species following each infection. Numbers in parentheses indicate percentage mortality.

Discussion

A.hydrophila and *Ps. fluorescens* were chosen for the examination since they belong to a group of bacterial pathogens which are often isolated from ornamental fish. Motile aeromonads represented 49.8 % of all strains isolated from ornamental fish in our laboratory (Siesenop and Böhm 1998). The main aim was to develop and test a food for ornamental fish, which increases the resistance of the fish towards these bacterial pathogens. Our results show that it is possible to increase the resistance of ornamental fish to bacterial infection using ß-glucan as a food additive. However, the reduction of mortality obtained were much lower than those described by other authors (Yano *et al*., 1991, Nikl *et al*., 1992, Wang *et al*.,1993, 1996, Anderson and Siwicki 1994). A possible explanation may be the method of infection by injecting the bacterial suspension. However, in previous examinations, it was not possible to reproduce a constant mortality by waterborne infection. It is possible, that the protection offered by the glucan could not be fully measured, since the bacteria were injected intraperitoneally, and did not have to pass the first line of defence, i.e. skin, mucus and gills. Another important point could be the high infectious pressure necessary for a control group mortality of 80 %. We thus ran a second test with a lower mortality in the control groups and a significant reduction of mortality in the groups fed glucan-containing diets was observed.

We conclude that in our examinations, ß-glucan added to ornamental fish food did induce an immunostimulation. Comparisons with the results of other authors indicate that intensity of the immunostimulating effect is dependent on different factors, which seem to be differently relevant. Priority lies in the type of ß-glucan, the application of the glucan and the pathogen and the infectious pressure (Ogier de Baulny *et al.,* 1996, Anderson 1997, Dalmo *et al*.,1997). The type of fish used in the trials is obviously of less importance, as very similar results in different species implicate (Nikl *et al*., 1993, Yano *et al*., 1991, Samuel *et al*., 1996). Oral application of ß-glucan seems to be less effective than injection of the glucan (Duncan and Klesius 1996, Yoshida *et al*., 1995).

ß-glucans as a food-additive for tropical, ornamental fish do present a possibility to support resistance against unspecific bacterious infection and are promising immunostimulants. However, we regard our results as promising for a practical use of schizophyllan as a food-additive for ornamental fish.

References

Anderson, D .P. (1997)Adjuvants and immonostimulants for enhancing vaccine potency in fish. Dev. Biol. Stand. 90: 257-265

Anderson, D. P. and Siwicki, A. K. (1994) Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. Prog.Fish Cult., 56, 258-261

Dalmo, R.A., K. Ingebringtsen and J. Bøgwald (1997) Non-specific defence mechanisms in fish, with particular reference to the endothelial system (RES).J. Fish. Dis. 20: 241-273

Duncan, P.L. and Klesius, P.H. (1996) Dietary immunostimulants enhance nonspecific immune responses in channel catfish but not resistance to *Edwardsiella ictaluri*. J. Aquat. Anim. Health., 8: 241-248,

Efthimiou, S. (1996) Dietary intake of beta-1,3/1,6 glucans in juvenile dentex (*Dentex dentex*), Sparidae: effects on growth performance, mortalities and non-specific defense mechanisms. J. Appl. Ichthyol. 12:1-7

Nikl, L., Albright, L.J. and Evelyn, T.P.T. (1992) Immunostimulants hold promise in furunculosis prevention. Bulletin Aquacult. Ass. Canada. 1: 49-52

Nikl, L., Evelyn, T.P.T. and Albright, L.J. (1993) Trials with an orally and immersion-administered beta-1,3 glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org. 17: 191-196

Ogier De Baulny, M., Quentel, C., Fournier, V., Lamour, F. and Le Gouvello, R. (1996) Effect of long-term oral administration of beta-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot *Scophthalmus maximus*. Dis. Aquat. Org., 26: 139-147

Robertsen, B. (1999) Modulation of non-specific defence of fish by structurally conserved microbial polymers. Fish. Shellfish. Immunol., 9, 269-290

Samuel, M., Lam, T.J. and Sin, Y.M. (1996) Effect of Laminaran [beta(1,3)-D-Glucan] on the protective immunity of blue gourami, *Trichogaster trichopterus* against *Aeromonas hydrophila*. Fish. Shellfish. Immunol. 6: 443-454

Siesenop, U. and Böhm, K. H. (1998) Antibiotikaresistenzen pathogener Bakterien bei Zierfischen - Situation und Entwicklung Kleintierprax. 43: 361-372

Wang, W.S., Wang, D.H., Lee, J.S., Chien, M.S. and Liu, C.I. (1993) Polysaccharide-induced protection of tilapia, *Tilapia aureus* P., against bacterial infections *in vivo*. Taiwan J. Vet. Med. Anim., 62: 21-28

Wang, W.S. and Wang, D.H. (1996)Use of glycans to increase resistance of bighead carp, *Aristichthys nobilis*, and milkfish, *Chanos chanos*, to bacterial infections. Taiwan J. Vet. Med. Anim. 66: 83-91

Yano, T., Matsuyama, H. and Mangindaan, R. E. P. (1991) Polysaccharide-induced protection of carp, *Cyprinus carpio* L., against bacterial infection. J. Fish. Dis. 14: 577-582

Yoshida, T., Kruger, R. and Inglis, V. (1995) Augmentation of non-specific protection in African catfish, *Clarias gariepinus* (Burchell), by the long-term oral administration of immunostimulants.J. Fish. Dis. 18: 195-198