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Short communication

Effect of immunostimulatory feed supplements on the development of acquired immunity in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Immunostimulatory feed supplements are an increasingly common feature of aquaculture management and their benefit has been confirmed for a wide area of products. However, these investigations have often focused on the benefit of these supplements on the innate immune system. In the current project, we investigated a mixture of two commercial feed supplements (Biotronic® Top 3 and Levabon® Aquagrow E) with a known protective effect against bacterial infections. The effect of the supplemented diet on antibody titters of *Oncorhynchus mykiss* vaccinated against *Yersinia ruckeri* was determined by ELISA. Furthermore, an infection trial was performed to confirm the effect of the supplements on the survival of the fish. Finally, their effects on the growth parameters of the fish were also determined. The results from this study found no significant effect on the general antibody titters. However, when considering only the titters of specific anti-*Y ruckeri* antibodies, the supplemented feed was associated with an improved response to the vaccine, significantly better than in the fish that had received the control feed.

Feed supplements have been an increasingly popular management technique in fish farming and multiple supplements have proven efficacious at improving the fish immune response or controlling the severity of disease outbreaks [1,2]. For example, Biotronic® Top 3 is a commercially available feed supplement composed of a mix of weak organic acids, extracts of cinnamaldehyde (known to interfere with the quorum sensing apparatus [3] and to have anti-microbial [4] and anti-inflammatory properties [5,6]) as well as a proprietary substance designed to permeabilize the bacterial cell membranes and enhance the activity of the other compounds [2]. Similarly, Levabon® Aquagrow E is composed of autolyzed *Saccharomyces cerevisiae* which is well established as probiotic and prebiotic feed supplement [7] and is known to have an stimulatory effect on the immune system of fish and other organisms [8]. Notably, investigation in the mechanisms through which these supplements contribute to the protection of fish have been limited and have mostly focused on the innate immune system [9]. However, a few studies have suggested that immunostimulation could be associated with elevated immunoglobulin levels [10] and it is plausible that an improved non-specific immune response could also improve the uptake and recognition of antigens and the development of the specific immunity.

In the present experiment, fish with no prior history of *Yersinia ruckeri* infection were allocated in 16 aquaria, fifteen fish each (Table 1). The fish were fed twice daily with either unsupplemented feed or an identical feed supplemented with a mixture of Levabon and Top3. Weighing of the fish was performed at the start of the experiment as well as at week 5, 10, 12 and at the end of the experiment (week 15).

At week 9, one week before vaccination, and at week 13, 100 µl of blood was sampled from the caudal vein of 8 fish per treatment the sera were extracted and stored at −80 °C until being used in two enzyme-linked immunosorbent assays (ELISA).

Vaccination was performed at week 10 on half of the aquaria from both treatments using the commercial Aquavac®ERM immersion vaccine (Merck, Kenilworth, United States) according to the manufacturer's instructions. The protection of the vaccine was estimated by calculating the relative percent survival according to the formula by Amend (82), as quoted by Hoare et al. [11,12].

A first ELISA was performed on the sera samples using an Immunoglobulin ELISA kit (MyBioSource, San Diego, United States) according to the manufacturer's instructions (data not shown). When this first ELISA failed to produce significant results, an indirect ELISA was performed by coating 96 well plates with an overnight culture of *Y.*

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Table 1
Fish allocation during the experiment.

Treatments		# aquaria	Vaccinated	Challenge Mock Infected/infected	# of fish/aquarium
1	Control non vaccinated	4	No	2/2	15
2	Control vaccinated	4	Yes	2/2	15
3	TOP3 + Levabon	4	Yes	2/2	15
4	TOP3 + Levabon	4	No	2/2	15

ruckeri isolate CSF007–82 and using mouse anti-rainbow trout Immunoglobulin M secondary antibodies (Aquatic diagnostic; UK). For both ELISAs, a colorimetric test was performed using a Tecan Sunrise™ (Männedorf, Switzerland) Absorbance Microplate Reader and the values were tested for equality of variance using a Levene's test then, because it was found that variances were not equal, compared using a non-parametric test (Dunett T3) as this does not assume equality of variances.

As mentioned, a second blood sampling as performed at week 13. The challenge was performed immediately afterwards on week 13: *Yersinia ruckeri* isolate CSF007–82 (an isolate obtained from the National Centre for Cool and Cold Water Aquaculture, Kearneysville, West Virginia, USA) was cultivated at 25 °C with 125 rpm shaking until reaching a theoretical concentration of 2.10^9 CFU/ml (assessed by spectrometry). The bacterial solution was added to half of the aquaria to a final concentration of 2.10^6 CFU/ml and the fish were exposed for 2 h to this solution. Following infection, fish were closely monitored over a period of two weeks and fish that were obviously severely compromised were removed and humanely euthanatized. Bacteria were re-isolated from the kidney to confirm infection by *Y. ruckeri*. At the end of the experiment, a survival curve was constructed and the survival numbers were compared between feeding groups.

The final weights were similar between feeding groups ($P = 0.35$) and the vaccine proved highly protective: none of the vaccinated fish required euthanasia or developed advanced signs of the disease while an average of 45% of the unvaccinated fish had to be euthanatized (relative percent survival 100%; $P < 0.05$). Among the non-vaccinated, infected fish, 18 of the 30 fish that had received the unsupplemented feed were euthanatized while only 9 out of the 30 fish that had received the supplemented feed were euthanatized (Fig. 1) (Relative protection 2.0; $P < 0.05$). Previous experiments had previously shown that both Levabon and Top3 had protective effects for the fish [2], so this result was not unexpected.

Vaccination resulted in a significantly higher average vaccine titters in the fish ($P < 0.05$). When investigating the general overall antibody titers, the feed supplements were not associated with antibody titters significantly different from the controls ($P > 0.05$). However, when using indirect ELISA to only consider antibodies specific against *Y. ruckeri*, a modest but significant difference was observed between the fish fed the control feed and the fish fed the supplemented feed ($P < 0.05$). Nevertheless, because the supplements were more protective than the difference in antibody titters would suggest, it is very likely that most of the immunostimulatory properties of either supplement relied on the stimulation of the innate immunity. Taken together, these results suggest that immunostimulation could be integrated in vaccination strategies in order to optimize antigen uptake and the development of specific antibodies.

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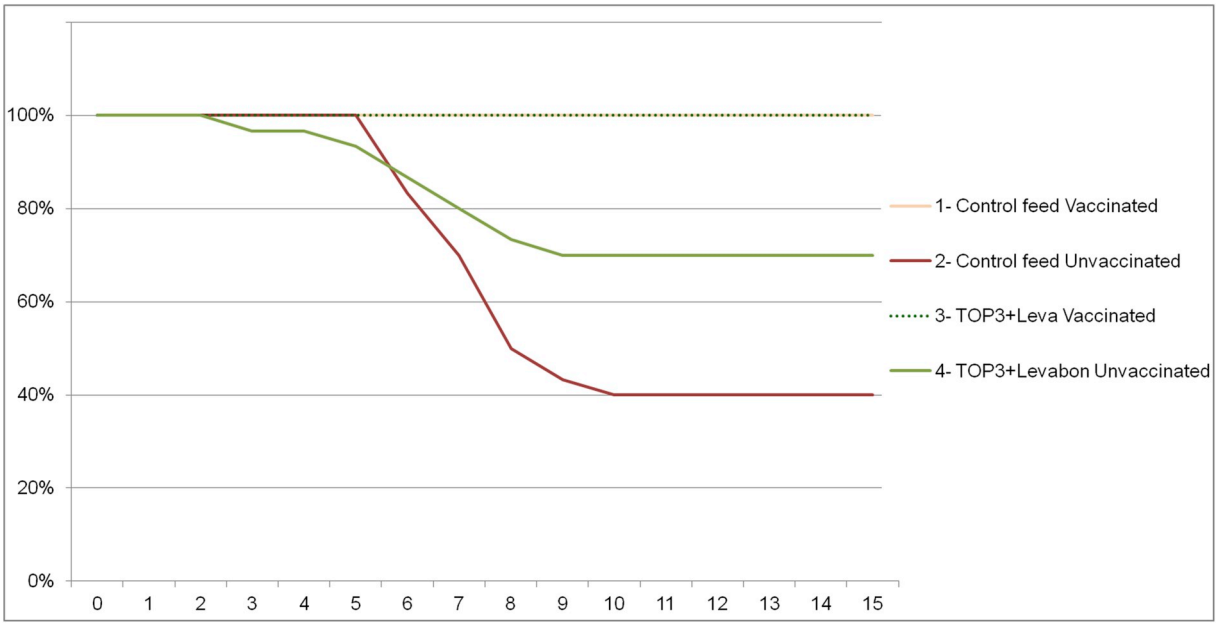

Fig. 1. Change in fish survival following infection by immersion in a solution of *Yersinia ruckeri* isolate CSF007–82.

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