An investigation into the effect of two diets on the phagocytotic index of rainbow trout (*O. mykiss*) macrophages

Introduction

One aspect of immunostimulants is they can increase the phagocytotic ability of phagocytic cells such as macrophages. This often occurs with increases of other components of the innate immune system such as compliment and lysozyme levels.

In this trial two diets were provided by New Era Aquaculture ltd. Designated diet 1 and diet 2 these diets were provided blind to the research group and the aim of this work was to investigate if these diets increased the phagocytotic index of rainbow trout (*O. mykiss*) macrophages and liver lysozyme levels of fish fed on these diets for 4 weeks

Material and Methods

Diets

Diet 1 and diet 2 these diets were provided by New Era Aquaculture ltd. The diets were blinded to the research group (i.e. the researchers do not know if the diets contained an immunostimulant, were a placebo, or what type of immunostimulant they contained.) The following amount of each diet, 30.87g of diet 1 and 31.04g of diet two, was supplied by New Era Aquaculture ltd.

Fish and feeding regime.

60 5g (meat wt 5.12±0.62g) rainbow trout (FRS Marine research production unit Aultbea) were randomly placed into 3 0.5m^3 aquariums by means of a dice roll (1-2 tank 1, 3-4 tank 2, 5-6 tank 3) until each tank contained 20 animals. The diets were also randomly assigned by means of a dice roll and tank 1 received the control diet tank, 2 received diet 1, tank 3 received diet 2.

The test diets was fed at a rate of 1% body weight per day (each tank received 1g of experimental or control diet) tank 1 fish received an extra ration of trout pellets at the same time the test diets were fed to tanks 2 & 3. The experimental diets were fed by hand as the first meal of the day the remaining ration consisting of a further 2% bw per day of trout pellets (EWOS) was fed via an automatic feeder system.

The experimental animals were maintained under this regime for 28 days the temperature during this period was maintained at 14$\pm$1^{o}C which is considered optimum for rainbow trout immune function.

Sampling regime

After 28 days the experiment was terminated by euthanizing the animals with an overdose of anaesthetic (MS222). Each animal was weighed and the pronephros of each animal was removed and placed in L15 tissue culture medium on ice for subsequent extraction of macrophages. The liver was also removed and placed in individual cryo-vials (Nunc) and stored at -80^{o}C for later determination of lysozyme levels.

Determination of phagocytotic index of rainbow trout macrophages

The preparation of macrophages from the head kidney was performed according to Secombes (1990) using a 51-34 Percoll gradient (*G.E*. Bioscience). Percoll was diluted with Hanks balanced salt solution (HBSS; Gibco) to the correct dilutions. Into each 15ml Falcon-tube 5ml of 34 % Percoll was layered carefully on top of 5ml of 51 % Percoll. The Pronephros of each animal was disassociated by passing through 100 µm cell dissociation kit (Sigma) with L-15 medium acting as a diluent. This cell suspension was layered on a prepared 51-34 Percoll gradient and centrifuged at 400 x g for 30min at 4°C. The band at the gradient interface was collected washed in L-15 medium then centrifuged again at 200 x g for 5 min at 4°C. The cells were re-suspended in 3 ml of L-15 medium and counted in a haemocytometer with trypan blue staining to determine cell viability. Cells were adjusted to a concentration of 1 x 10^6 cells/ml and seeded into 96-well plates (Nunc) at a density of 3 x 10^5 cells/cm^2. After incubation at 14°C for 120 min to allow macrophage adhesion, the cells were washed with L-15 medium and 200µl of L-15 medium added to each well.

After 24 hours L-15 medium was removed and replaced with L-15 media containing 1x10^7 cells/ml of washed formalin killed yeast cells (*Saccharomyces cerevisiae*), these 96 well plates were incubated for a further 4 hours at 14°C, the washed in HBSS and fixed in 70% methanol and stained by with Giemsa stain and examined under an inverted microscope (Olympus). 100 macrophages per fish were examined and the number of yeast cells ingested by each macrophage recorded.

Determination of liver lysozyme levels

Each liver sample was removed from -80^{o}C storage thawed and homogenised using a Griffith's grinder in 1ml of phosphate buffered saline. Each sample was then centrifuged at 500xg for 30 minutes to remove debris. The total protein levels of the liver homogenates was determined by the BioRad total protein kit following the manufactures instructions, each sample was then adjusted to 1mg/ml total protein.

The method for measuring serum lysozyme was described for rainbow trout by Ellis (1990), It is based on the lysis of the gram positive bacterium *Micrococcus*

lysodeikticus. The lysis of the bacterium can be detected by a decrease in optical density of a solution *M. lysodeikticus* at 530 nm in a spectrophotometer.

In a 1m cuvette 950µl *M. lysodeikticus* solution (Sigma; *M. lysodeikticus* = 0.5 mg/ml in 0.05M sodium phosphate buffer (pH 6.2)) were pipetted into the cuvette and the OD recorded, followed by the addition of 50 µL of kidney homgentate. Absorption was measured after 0, 0.5, 1, 2, 3, 4 and 5 min, and lysozyme activity was expressed as decrease of optical density (ΔOD) per min.

Results

Phagocytotic index

Not surprisingly the control diet gave the lowest number of yeast cells per macrophage with a mean value of 3.82±2.36. Both Diet 1 and Diet two induced a higher PI than the control diet of 6.74±3.91 and 7.82±5.03 respectively (seeTable 1). Statistically both diets 1 & 2 gave a significantly higher PI when compared to the control diets (p=0.05 Student's t-test). However, there was no significant difference between the PI induced by Diet 1 and Diet 2 (p=0.38 Student's t-test). The raw data of mean individual responses can be found in Figure 1 and the processed data in figure 2.

Table 1 mean Phagocytotic index (PI) for the three treatment groups

	Control Diet	Diet 1	Diet 2
Phagocytotic index	5.13	7.70	8.14
Standard deviation	1.55	3.83	4.91

Liver lysozyme assay

Again the control diet gave the lowest level Lysozyme Units/mg liver with a mean value of 0.07±0.04 Lysozyme Units/mg tissue. Both Diet 1 & 2 induced a higher Lysozyme Units/mg tissue than the control diet of 0.18±0.05 (p=≤0.001) and 0.10±0.05 (p=≤0.03 Student's t-test; see Table 2). However, there was also a significant difference between the Lysozyme Units/mg tissue induced by Diet 1 and Diet 2 (p=≤0.001; Student's t-test), with diet 1 inducing a much higher lysozyme level than diet 2.

The raw data of mean individual responses can be found in Figure 3 and the processed data in Figure 4.

Table 2 mean Lysozyme Units/mg for the three treatment groups

	Control Diet	Diet 1	Diet 2
Lysozyme Units/mg	0.07	0.18	0.10
Standard deviation	0.04	0.05	0.05

Fish growth

No significant difference in fish growth was observed between the diets (P=0.112; Anovo) as shown in table 3

Table 3 weight of the three groups at the start and end of the experiment

	Control Diet	Diet 1	Diet 2
Weight (start)	5.12±0.62g	5.12±0.62g	5.12±0.62g
Weight (end)	9.7±1.2g	10.01±0.87g	9.4±1.3g

Figure 1 Plot of the raw data showing the three treatment groups and the mean number of yeast cells each macrophage contained (Blue diamond control; red square diet 1; green triangle diet 2)

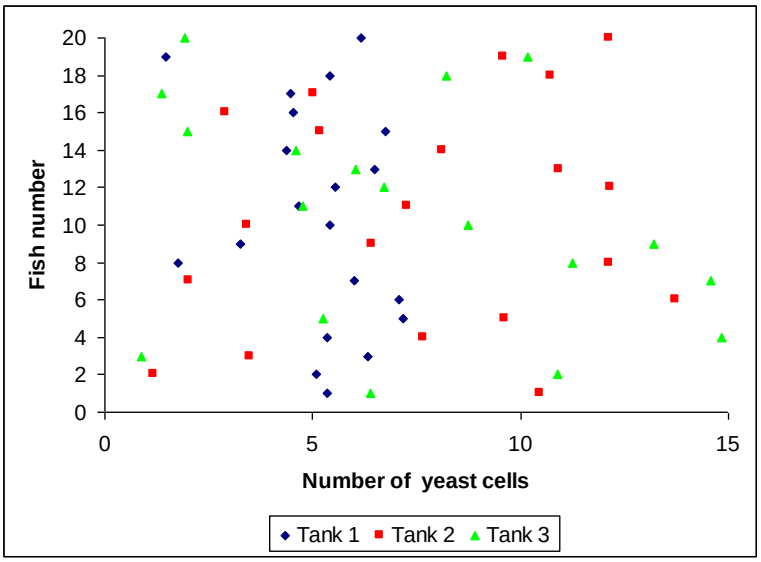


Figure 2 Mean PI for diets 1, 2 & 3 (mean values + standard deviation)

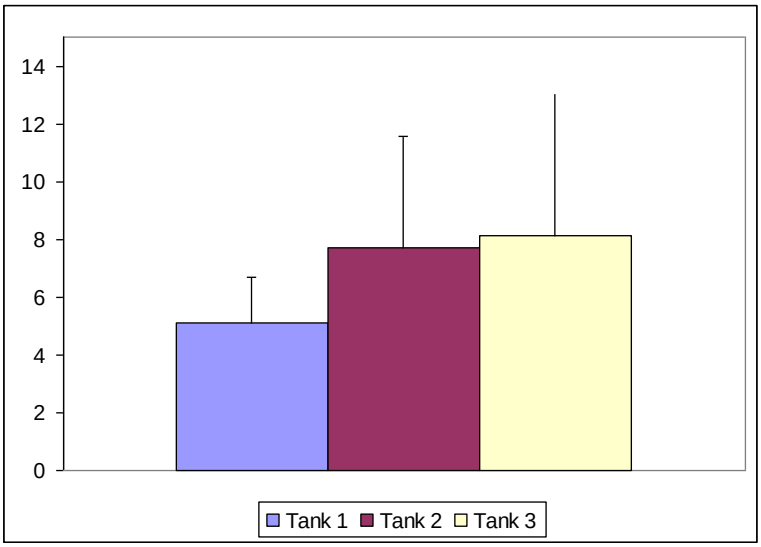


Figure 3 Plot of the raw data showing the three treatment groups and the mean Lysozyme Units/mg liver tissue (Blue diamond control; red square diet 1; green triangle diet 2)

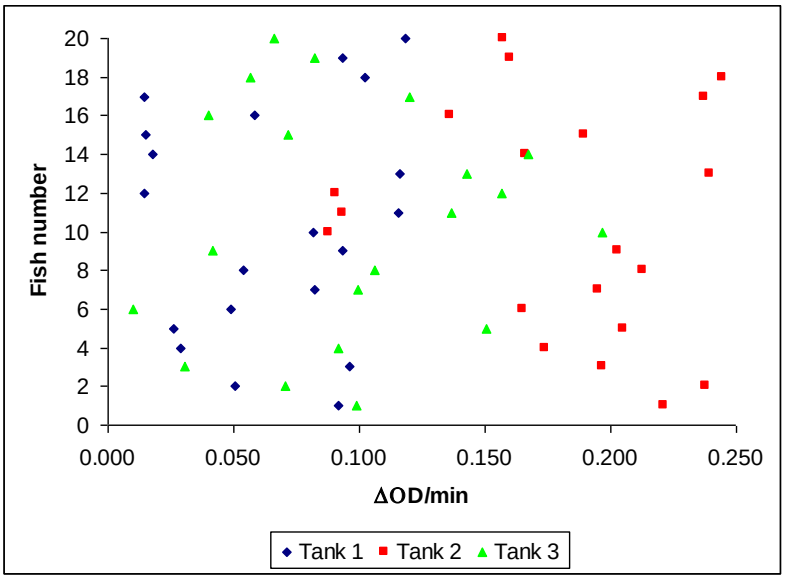
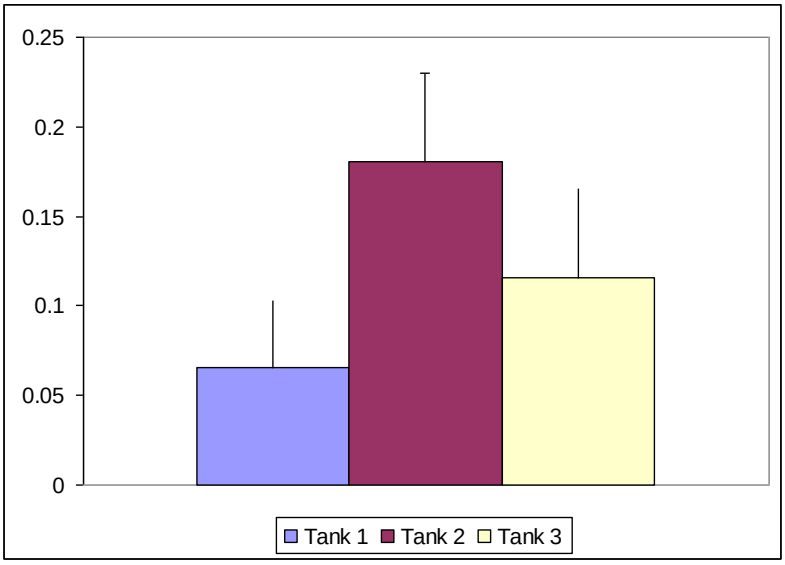


Figure 4 Mean Lysozyme Units/mg liver tissue for diets 1, 2 & 3 (mean values + standard deviation)



Discussion

Both diets 1 & 2 demonstrated a significant enhancement of both Phagocytotic index and lysozyme levels when compared to the controls which is an exceedingly encouraging result clearly showing that both diets enhance non-specific immune responses without the presence of a pathogen. Theoretically this would put the animals in a much better position to defend against a pathogenic insult should one occur while their immune system is in this state.

There was one interesting observations between the two immunostimulant diets and although the Phagocytotic index's were not significantly different between the two diets the liver lysozyme levels were significantly different. Here diet 1 inducing a significantly higher lysozyme levels than diet 2. What this means in unclear, at its simplest it can be concluded that diet 1 is simply a better inductor of lysozyme activity than diet two. There are several explanations for this, it is activating different Toll-like receptors or it is simply a more biologically active molecule, the diets formulation is more effective than diet 2, it may be act on a different immune cascade than diet 1 or it is triggering the degranulation of granulocytes directly. It is simply impossible to tell from this study and answering this question would involve undertaking some specialised cell assays both *in vivo* and *in vitro*.

Neither diet had any adverse affect on growth when compared to the standard trout pellets the controls were offered. Although it was reported by the technician looking after the fish that during the first 5 days of being offered the diet, diet 1 was not readily eaten (this did not occur with diet 2 which was readily accepted by the fish). The flake was taken in by the animals and then spat out and it was clear that for the first week the animals fed diet 1 were probably rejecting 80% of the ration. However, they did accept the food well from week two of the experiment. Ultimately this did not have an effect on either the immunostimulatory effect of the diet or the growth of the fish (although they may have compensated by eating more of the standard pellet diet). This observation may be of concern if this diet is used on fastidious animals as they may refuse to eat this formulation and hence no benefit will be induced in that population.

Future work

- To determine how long the immunomodualtory effect lasts for to enable the timing between courses each diet to be correctly established.
- To determine the delay, if any, in mortality of populations fed these diets when challenged with pathogenic organisms
- To establish which arms of the immune systems are modulated by these diets
- To establish the effect of temperature on the efficacy of the diets