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Dietary Supplementation Strategies to Improve Performance of Rainbow Trout *Oncorhynchus mykiss* Fed Plant-based Diets

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Abstract：Higher inclusion levels of plant-based feedstuffs in diets for rainbow trout necessitate identification of methods to mitigate the negative effects on fish growth and health. Thus, an increasing number of novel and re-purposed dietary supplements are becoming commercially available to counteract the anti-nutritional effects of plant-based ingredients. Specifically, probiotic bacteria, which have previously been primarily investigated for their anti-pathogenic effects in fish, are now promoted to aid in the utilization of plant-based diets and digestive tract health. Additionally, dietary supplementation of commercially produced anti-inflammatory antibodies used to reduce gastrointestinal tract inflammation in terrestrial animals may also have potential to improve digestive tract health and performance of aquatic animals fed plant-based diets. The present paper describes two studies conducted at the Hagerman Fish Culture Experiment Station that investigated the ability of dietary probiotics and anti-phospholipase A_2 antibody to improve performance of rainbow trout fed soybean meal-based diets.

Key words：rainbow trout, soybean meal, probiotics, anti-phospholipase A_2

Increased utilization of plant protein meals has been embraced as a sustainable alternative to fish meal (Barrows *et al.,* 2008; Gatlin *et al.*, 2007), with soybean meal being the most viable plant protein supplement widely available and competitively priced (Nordrum *et al.*, 2000). However, inclusion rates of soybean meal in salmonid diets are currently kept at less than 20% (Hardy, 2002). At levels of >20% reduced weight gain and feed efficiency (Olli and Krogdahl, 1995; Olli *et al.,* 1995) and pathomorphological changes in the distal intestinal epithelium with diarrhea (Rumsey *et al.*, 1994) are observed. Identification of new methods to mitigate the negative effects of soybean meal on salmonid growth and health are needed. Because the hindgut of teleost fish is known to play an important immunogenic role, and strong immune and inflammatory responses are achieved by delivering antigens to this site (Ellis, 1995) methods that target this location are of particular interest. Two such methods include dietary supplementation of probiotics or anti-inflammatory antibodies.

Probiotics have been used to treat both infectious and noninfectious enteritis in humans and other terrestrial animals (Marteau *et al.*, 2002; Marteau *et al.*, 2001) and may represent a feasible method for increasing soy utilization in soy-sensitive aquatic species. Although the majority of previous probiotics research in aquatic animals has focused on reducing disease outbreaks (reviewed by Kesarcodi-Watson *et al.*, 2008; Verschuere *et al.*, 2000), it is difficult to attribute probiotic effects to suppression of a pathogen or other beneficial effects (Kesarcodi-Watson *et al.*, 2008; Gatesoupe,

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2007). The presence and endocytosis of bacteria by enterocytes in the hindgut of larvae (Hansen and Olafsen, 1999) links the gut and its microbial flora to the development of immune responses in fish. These linkages suggest that probiotics may have the potential to alter dietary soybean antigen processing in the hindgut of first feeding fish thus altering soy sensitivity in carnivorous fish (Bakke-McKellep *et al.*, 2000)

Dietary supplementation of commercially produced anti-inflammatory antibodies used to reduce gastrointestinal tract inflammation in terrestrial animals may also have potential to improve digestive tract health and performance of aquatic animals fed plant-based diets. Recently, a commercial source of anti-phospholipase A_2 antibody has become available (Aova Technology, Madison, Wisconsin, USA) and is marketed as a dietary supplement that decreases the inflammatory response observed in the gut following ingestion of a meal (Barry and Yang, 2008). Phospholipase A_2 enzyme catalyzes the hydrolysis of the fatty acid at the sn-2 position of glycerophospholipids, in response to agonist stimulation, to generate free arachidonic acid for eicosanoid biosynthesis (Balsinde *et al.*, 2006). Research in rats suggests involvement of this cascade and specifically arachadonic acid levels on the induction heat shock proteins (Gosslau and Rensing, 2000). Heat shock proteins (HSPs) are considered to be a cell survival factor and have been shown to improve cell function in multiple models of cell injury including soybean meal-induced enteritis in salmonids. Thus alteration of phospholipase A_2 activity may provide an opportunity to alter heat shock protein expression and reduce gut inflammation in rainbow trout fed diets containing high levels of soybean meal.

Materials and methods

Probiotics study

To test the hypothesis that probiotics incorporation in rainbow trout starter diets can induce immune-mediated soybean tolerance, a two-phase experimental design was employed. In the starter phase (first feeding (0.13 ± 0.01) to 6.5 ± 0.32g fish^{-1}), a practical-type diet was formulated to contain 48% crude protein and 20% crude fat containing either 0 , 10 or 20% soybean meal and supplemented with or without a commercially available probiotic in a 3 X 2 factorial design. Diets were fed to four replicate tanks of fish per treatment (300 fish tank^{-1}; House Creek strain) for eight weeks. Trout were reared in 150L tanks supplied with 4L min^{-1} of constant temperature (14.8℃) flow-through spring water. Potentially soy tolerant rainbow trout produced by feeding probiotics and increased levels of soybean meal in starter diets as described above were then fed the industry standard level 15% soybean meal or a diet with a challenge level of 43% soybean meal during a 12 week grow-out trial. Histopathological examination was performed on the distal intestine of three randomly selected fish from each tank at the end of both the starter and grow-out trial.

Antibody study

To examine the effect of a commercially available anti-phospholipase A_2 supplement on performance of rainbow trout fed high levels of soybean meal, rainbow trout were fed a practical-type control diet formulated to contain 45% protein and 15% fat with 0, 17.5 or 35% soybean meal with and without the commercially available anti-phospholipase A_2 supplement at an inclusion level of 0.3%. Diets were fed to three replicate tanks of fish per treatment (20 fish/tank, House Creek strain) for nine weeks. Trout were reared in 80L tanks supplied with 4L/min of 14.8℃ flow-through spring water. Total RNA was isolated from liver and intestinal tissues (n=3) to detect alterations in HSP27, HSP70 and HSP90 gene expression by real time quantitative PCR; protein expression was examined by Western blot. Histopathological examination was performed on the distal intestine of three randomly selected fish from each tank.

Results and discussion

Probiotics study

The use of bacterial preparations to adapt fish to new types of nutrients represents a novel probiotic application. A beneficial effect of probiotic supplementation on growth of fish fed 20% soybean meal was observed at the end of the starter trial (Table 1). Stimulation of the immune system is considered an important mechanism to support probiotic activity (Sealey, 2000). However, no detectable benefit of probiotic supplementation on gut health or immune status was observed in fish fed 20% soybean meal. The practical application of probiotics to improve soy utilization in trout fry diets at the levels examined in starter phase are limited given that most salmonid fry diets currently do not contain soybean meal. More important, is whether the inclusion of probiotics and/or soybean meal in the diet of first feeding trout altered soy sensitivity in a manner that would increase utilization when these fish are fed higher levels during the grow-out portion of the production trial.

Table 1. Growth performance [*1], of rainbow trout fed diets containing 0, 10 or 20% soybean meal (SBM) with or without probiotics from first feeding for 8 weeks during the starter trial.

Diet	Weight gain[*2] (% increase)	FCR[*3] (g feed g gain^{-1})	Survival (%)
Without Probiotics			
0% SBM	5383[a]	1.00	98.5
10% SBM	5277[a]	1.00	97.5
20% SBM	4714[b]	0.99	97.3
With Probiotics			
0% SBM	4938[a]	1.03	97.8
10% SBM	4959[a]	1.02	97.1
20% SBM	5069[a]	0.99	96.8
Pooled SE	123	0.02	1.01
ANOVA, Pr > F[*4]	0.0142	0.8603	
Probiotics	0.1927	0.5749	0.5088
Soy	0.0401	0.5451	0.5045
Probiotics x Soy	0.0095	0.8679	0.9923

[*1]Means of four replicate tanks (300 fish tank^{-1}).
[*2](Final weight –initial weight)/initial weight x100.
[*3]FCR = feed conversion ratio; (g dry feed g wet gain^{-1}).
[*4]Significance probability associated with the *F*-statistic.

Table 2. Growth performance *1 of rainbow trout fed probiotics then diets containing 15 or 43% soybean meal (SBM) for 12 weeks during grow-out.

Starter Diet	Grow-out Diet	Weight gain*2 (% increase)	FCR*3 (g feed g gain^{-1})	Survival (%)
Without Probiotics				
0% SBM	15% SBM	1515^{A}	1.17	99
	43% SBM	1213^{B}	1.28	98
10% SBM	15% SBM	1373^{A}	1.21	97
	43% SBM	1341^{A}	1.17	98
20% SBM	15% SBM	1216^{Bb}	1.26	90^{yB}
	43% SBM	1237^{Bb}	1.26	94^{yA}
With Probiotics				
0% SBM	15% SBM	1387^{A}	1.10	99
	43% SBM	1261^{B}	1.19	98
10% SBM	15% SBM	1333^{A}	1.18	99
	43% SBM	1367^{A}	1.19	99
20% SBM	15% SBM	1408^{aA}	1.19	97^{x}
	43% SBM	1253^{bB}	1.28	99^{x}
Pooled SE		41	0.03	0.8
*ANOVA, Pr > F**4		0.0029	0.0126	<0.0001
Probiotics		0.4884	0.0535	0.0001 W>W/O
Soy		0.0658	0.0165 0=10<20	<0.0001 0=10>20
Probiotics x Soy		0.0969	0.1711	0.0010
Grow-out Diet		0.0021 15>43	0.0248 15>43	0.1188
Soy x Grow-out Diet		0.0110	0.0593	0.0249
Probiotics x Grow-out Diet		0.6956	0.2733	0.3415
Soy x Probiotics x Grow-out Diet		0.0404	0.4707	0.8705

*1Means of three replicate tanks (150 fish tank^{-1}).
*2Final weight –initial weight/initial weight *100.
*3FCR = feed conversion ratio; (g dry feed g wet gain^{-1}).
*4Significance probability associated with the *F*-statistic.

Fig. 1. Mucosal epithelium (ME) of control fish fed 0% soybean meal (SBM) during the starter trial contains numerous, finely granular absorptive vacuoles (AV's). AV's are lacking in some ME cells in villi of fish fed 20% SBM during the starter trial (arrows). Note large AV's devoid of finely granular material denoted by the arrow head. Leucocytic inflammation of the lamina propria is also apparent (L) Bars equals 50 microns.

Growth performance of trout during grow-out in the same study supports a role of dietary history in soy sensitivity; both soy and probiotic inclusion in starter-diets affected fish performance in grow-out (Table 2). The beneficial probiotic effects observed during growout, however, may simply indicate an inability of fish fed 20% soybean meal in their starter diet to overcome existing tissue damage (Fig. 1). The existing damage could then result in a failure to thrive and decreased survival regardless of soybean level in grow-out diets rather than a persistent beneficial effect of probiotics that would be indicative of colonization.

Antibody study

Research by Barry and Yang (2008) examined the effects of anti-phospholipase A_2 on the growth and feed efficiency of rainbow trout fed a commercial "steelhead" diet (45% crude protein and 16% crude lipid) top-coated with 0.3% of anti-phospholipase A_2 and observed improvements compared to those fish fed the un-supplemented diet. Commercial "steelhead" diets generally contain high levels of fish meal, terrestrial animal proteins, and high quality plant protein concentrates. These diets generally include low levels of non-concentrated plant proteins to minimize the detrimental effects of plant-endogenous anti-nutritional factors; soybean meal levels, specifically, are kept below 15%. In the current study, decreased growth was observed for fish fed 35% soybean meal regardless of anti-phospholipase A_2 supplementation (Table 3). Fish fed 35% soybean meal had significantly higher feed conversion ratios than fish fed 0 or 17.5% soybean meal. Histology of the distal intestine revealed significant pathology in fish fed 35% soybean meal diets (with and without anti-phospholipase A_2) similar to that previously observed (Sealey *et al.*, 2009; Rumsey *et al.*, 1994). HSP27 and HSP90 mRNA expression was elevated in fish fed 35% soybean meal. A significant interaction was observed for HSP90 mRNA expression in that fish fed 35% soybean meal with anti-phospholipase A_2 had lower HSP90 mRNA expression that fish without supplement (Fig. 2). These data indicate feeding high levels of soybean meal alters the expression and regulation of various HSP mRNA of rainbow trout in a HSP specific manner.

Conclusions

Feeding diets with increased levels of plant protein and reduced levels of fish meal has many benefits. Soybean meal is readily available and

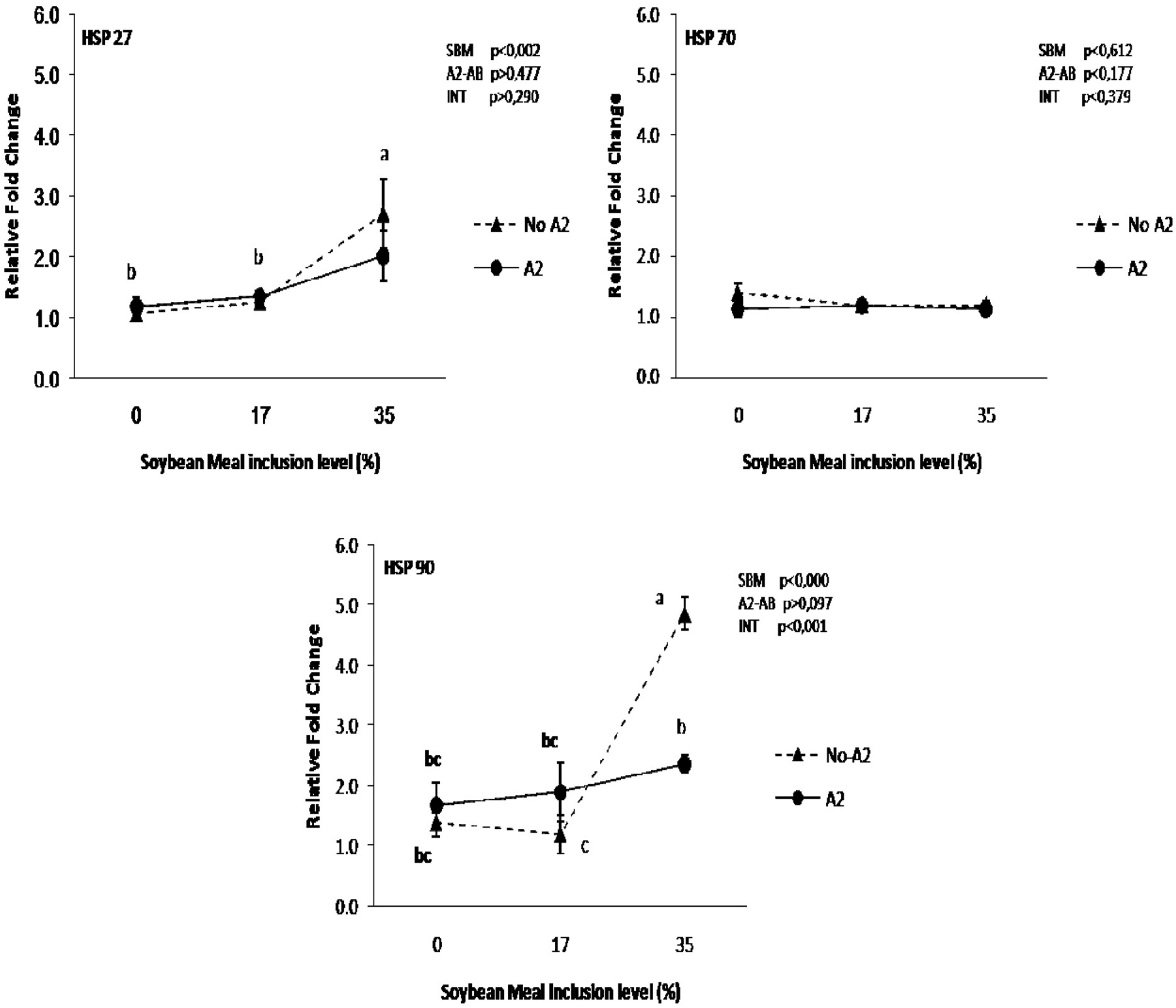

Fig. 2. Relative fold changes in the expression levels of HSP 27 - HSP 70 - HSP 90 in the small intestine of rainbow trout fed increasing levels of soybean meal (SBM) with or without the inclusion of anti-phopholipase A_2 antibody. A significance level of $P < 0.05$ was used and Fisher' s LSD tests were used to assess significant differences among treatments. All values are reported as means (n=3) ± SEM unless otherwise indicated.

cost competitive, but dietary levels are limited due to deleterious effects on growth. Results from the current studies demonstrate the potential of two different dietary strategies to improve performance of rainbow trout fed high levels of soybean meal. Specifically, the results of the probiotics study suggest that the amount of dietary soybean meal tolerated by rainbow trout can be increased in the starter phase of production by feeding probiotics. Dietary soybean meal levels may also be increased in grow-out diets by inclusion of low levels of soybean meal in the starter diets. Additionally, although dietary inclusion of anti-phospholipase A_2 at the investigated levels had only minimal effects on the pathology associated with high levels of soybean meal in rainbow trout, the beneficial effects on HSP expression suggest additional research is needed before this supplementation strategy can be completely dismissed.

Table 3. Growth performance [*1] of rainbow trout fed diets containing 0, 17.5 or 35% soybean meal (SBM) with or without anti-phospholipase A_2 for nine weeks.

Diet	Weight gain[*2] (% increase)	FCR[*3] (g feed g gain^{-1})	Survival (%)
Without antibody			
0% SBM	740[a]	0.98[by]	93
17.5% SBM	737[a]	1.00[by]	100
35% SBM	666[b]	1.08[ay]	98
With antibody			
0% SBM	731[a]	1.03[bz]	100
17.5% SBM	752[a]	1.04[bz]	100
35% SBM	682[b]	1.10[az]	98
Pooled SE	123	0.02	2.89
ANOVA, Pr > F[*4]	0.0295	0.0093	0.5705
BigFish	0.8505	0.0477	0.5314
Soy	0.0040	0.0025	0.3644
BigFish x Soy	0.8325	0.7898	0.4365

[*1]Means of three replicate tanks (20 fish/tank).

[*2](Final average fish weight –initial average fish weight)/initial average fish weight x100.

[*3]FCR = feed conversion ratio; (g dry feed fed per g wet gain^{-1}).

[*4]Significance probability associated with the *F*-statistic.

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Bakke-McKellep A.M., Penn, M.H., Salas, P.M., Refstie, S., Sperstad, S., Landsverk, T., Ringo, E., and Krogdahl, A., 2007: Effects of dietary soybean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the telost Atlantic salmon (*Salmo salar* L.) *Br. J. Nutr.*, **97**, 699–713.

The authors explore the role of the intestinal microbiota of salmon in the pathogenesis of soybean meal-induced enteritis. A comprehensive approach is described involving identification of both descriptive and functional indices of gut health. Detailed methodology for the varied indices is provided allowing for more thorough evaluation of the effects of alternative protein sources in salmonids as well as less studied species. Results of the study indicate that soybean meal-induced enteritis is accompanied by induction of distal intestine epithelial cell responses and changes in microbiota. These results strengthen the putative linkage between gut health and microbiota and indicate that further research in this area is warranted.

Gatesoupe F.J., 2007: Live yeasts in the gut: Natural occurrence, dietary introduction and their effects on fish health and development. *Aquaculture*. **267**, 20–30.

The author summarises the present state of knowledge regarding the importance of yeast in the fish gut. The putative role of yeast is discussed in regards to both the natural occurrence and the use of "industrial" yeast in aquaculture. Functions discussed include pathogencity, colonization and competition, modulation of immune responses and alteration of host (fish) metabolism. Species, and yeast subcomponents of importance (i.e. glucans) are identified and supporting references are included.

Kesarcodi-Watson A., Kaspar, H., Lategan, M. J., and Gibson, L., 2008: Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture*, **274**, 1–14.

The authors review recent and historic literature regarding the use of probiotics in aquaculture. Special emphasis is placed on differences between the use of probiotics in mammalian and aquatic species. These differences illustrate and the authors described why the definition of probiotic as described by Fuller (1989), "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" must be expanded for aquatic species.

Definitions which more accurately reflect the mode of actions observed for probiotics in aquatic species are proposed. Modes of action including improving the use of feed or enhancing the nutritional value are discussed.