Journal of Aquaculture Feed Science and Nutrition 7 (1-3): 6-15, 2015
ISSN: 2070-1667

Effects of a Fermented Soybean Meal Diet on Rainbow Trout Mortality and Immune Function During a Disease Outbreak

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Abstract: This experiment evaluated the response of McConaughy strain rainbow trout *Oncorhynchus mykiss* fed isonitrogenous, isocaloric diets containing either 40% fish meal and no fermented soybean meal or 15% fish meal and 35% fermented meal, prior to and during a disease outbreak in a production hatchery. The trout were initially reared for 30 days in indoor circular tanks with negligible mortality. They were then moved to rectangular raceways and maintained on the same treatment diets. Mortality started 18 days later and continued for the next 7 weeks. Overall mortality was not significantly different between the groups fed either of the two diets and ranged from 2.16-8.11%. Although, visible and histopathological indications of Bacterial Coldwater Disease were observed, neither bacteriological culture nor molecular methods confirmed the presence of *Flavobacterium psychrophilum*. There were no significant differences in qualititative health assessments, viscerosomatic index or hepatosomatic index in fish fed either diet. Distal intestine inflammation was not observed in any of the fish and qualitative rankings of distal intestine morphology were not significantly different between the diets. Immunological sampling 25 days following the transfer to raceway cages indicated no significant differences in spleen somatic index, macrophage activity, respiratory burst or plasma lysozyme activity in fish fed either diet. These results indicate that feeding a diet with fermented soybean meal as the primary protein source will not lead to increased mortality during a disease event in rainbow trout, even if fish growth is negatively affected.

Key words: Rainbow trout, *Oncorhynchus mykiss*, disease, fermented soybean meal, fish

INTRODUCTION

Because fish meal supplies are limited, plant proteins are increasingly used in salmonid diets (Tacon and Metian, 2008; Hardy, 2010). In particular, Fermented Soybean Meal (FSBM) has been shown to be able to replace much of the fish meal ingredient in rainbow trout *Oncorhynchus mykiss* diets with minimal adverse effects on growth or feed conversion (Yamamoto *et al*., 2010; Barnes *et al*., 2012, 2013). However, little is known about the effect of FSBM on rainbow trout disease resistance and immune function.

The results from studies evaluating the impacts of non-fermented, defatted soybean meal on fish health are mixed. Burrells *et al*. (1999) reported that high levels of dietary soybean meal depressed non-specific immunity in rainbow trout. Dietary soybean meal also had negative effects on *Atlantic salmon* salmosalar immune function (Bakke-McKellep *et al*., 2007; Krogdahl *et al*., 2000) and disease resistance (Krogdahl *et al*., 2000). However, there have also been positive immune responses to plant-based diets. Sitja-Bobadilla *et al*. (2005) replaced fish meal with a mixture of plant protein sources and observed some positive changes in innate immunity as did Barros *et al*. (2002) when soybean meal was replaced with solvent-extracted cottonseed meal in channel catfish *Ictalurus punctatus* diets. Bioprocessing, such as fermentation, may reduce antigenicity and immuno reactivity agents found in soybean meal. It is also possible that as a result of the fermentation process, FSBM may contain immunomo dulatory components that enhance non-specific immune responses (Sachindra and Bhaskar, 2008; Kim *et al*., 2009, 2010).

Flavobacterium psychrophilum is a ubiquitous bacterium in the aquatic environment, particularly in freshwater. As the etiological agent of Bacterial Coldwater

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Disease (BCWD), it is a serious fish pathogen causing substantial economic losses and rearing difficulties to both commercial and conservation aquaculture. *F. psychrophilum* strongly suppresses the nonspecific humoral defense mechanisms of infected fish and juvenile rainbow trout are particularly susceptible. Because this pathogen typically enters the fish through damaged tissue (Decostere *et al*., 2000; Krogdahl *et al*., 2000; Madetoja *et al*., 2002; Miwa and Nakayasu, 2005), intestinal enteritis such as that observed with higher levels of dietary soybean meal (van den Ingh *et al*., 1991; Burrells *et al*., 1999; Nordrum *et al*., 2000; Buttle *et al*., 2001; Sealey *et al*., 2013) could potentially make infection more virulent.

Nutrition appears to play a role in BCWD mortality. listed malnutrition as a probable factor that influences BCWD susceptibility BCWD and Daskalov observed a direct link between a diet with highly oxidized lipid concentrations and decreased mortality after a *F. psychrophilum* challenge. Dietary soybean meal also has been linked to increased susceptibility to *Aeromonas salmonicida*, the bacterium which causes furunculosis (Krogdahl *et al*., 2000). The susceptibility of rainbow trout fed FSBM to BCWD is unknown.

The objective of this study was to determine the effect of high concentrations of dietary FSBM on rainbow trout immune function and mortality during an outbreak of BCWD.

MATERIALS AND METHODS

This experiment occurred at McNenny State Fish Hatchery, Spearfish, South Dakota. Rainbow trout reared at McNenny experience consistent BCWD outbreaks, confirmed by both bacterial culture and molecular methods, 3-4 weeks after the fish are moved from the hatchery tankroom to external raceways. Hence, the experimental design for this study attempted to model the typical rainbow trout rearing strategy employed at McNenny. The protocol used in this experiment was approved by the South Dakota State University Institutional Animal Care and Use Committee, Approval Number 13-006A.

Two isonitrogenous and isocaloric diets formulated to meet all of the nutritional needs of rainbow trout (NRC, 1993) were used in this experiment. One diet contained 40% fish meal with no FSBM while the other diet contained 35% FSBM (PepSoyGen®; Nutraferma Inc., North Sioux City, South Dakota) and 15% fish meal (Table 1). Large particle ingredients were milled with a Fitzpatrick comminutor (Fitzpatrick Company, Elmhurst, Illinois). Dry diet blends were then mixed for 20 min using a V-10 mixer with an intensifier bar (Vanguard Pharmaceutical Machinery, Inc., Spring, Texas) and the blended diets were transferred to a Hobart HL200 mixer (Hobart Corporation, Troy, Ohio) where oils and extruding water were homogenized. Feeds were then cold-pressed using a Hobart 4146 grinder with a 3.5 mm die. Variable frequency controls on the screw hopper and cutting head at the die plate provided consistent pellet size. Pellets were then dried under cool, forced-air conditions. Following drying, feeds were placed in frozen storage at -20°C until fed.

Feeds were analyzed according to AOAC (2009) Method 2001.11 for protein, method 2003.5 (modified by substituting petroleum ether for diethyl ether) for crude lipid and ash content by AACC (2000) Method 08-03. Isoperibol bomb calorimetry was used toobtain total gross energy of each diet.

From a common pool of 3,200 fish, 400 McConaughy strain rainbow trout (4.3±0.2 g, 74±1 mm, mean±SE) were placed into each of eight, 1.8 m diameter fiberglass circular

Table 1: Percent composition and chemical analysis of the diets used in the trial

Fermented soybean meal (%)	Diet 1 (0)	Diet 2 (35)
Ingredients		
Menhaden meal[a]	40.00	15.00
PepSoyGen[b]	0.00	35.00
Whole wheat[c]	15.00	6.00
Yellow corn gluten[d]	20.00	18.00
Menhaden oil[e]	13.70	14.90
CMC[f]	7.70	6.80
Vitamin premix[g]	1.50	1.50
Mineral premix[h]	1.50	1.50
Vitamin C (Stay-C)[i]	0.50	0.50
Yeast[j]	0.10	0.10
L-Methionine[k]	0.00	0.20
Sodium chloride	0.00	0.50
Potassium chloride	0.00	0.50
Calcium phosphate	0.00	0.50
Chemical analysis (% dry basis)[l]		
Crude protein	43.80	45.40
Crude lipid	15.60	14.10
Crude fiber	0.79	1.72
Ash	13.20	11.20
Gross energy (kJ g^{-1})	16.50	16.30

[a]IPC 740, Scoular, Minneapolis, Minnesota, USA. [b]Nutra-flo Protein and Biotech Products, Sioux City, Iowa, USA. [c]Bob's Red Mill Natural Foods, Milwaukie, Oregon, USA. [d]Consumers Supply Distributing, Sioux City, Iowa, USA. [e]Omega Protein, Inc., Houston, Texas, USA. [f]Carboxymethyl cellulose, USB Corporation, Cleveland, Ohio, USA. [g]ARS 702, Barrows *et al*., 2008, Nelson and Sons, Inc., Murray, Utah, USA. [h]ARS 640, Barrows *et al*. (2008), Nelson and Sons, Inc., Murray, Utah, USA. [i]DSM Nutritional Products France SAS, Village-Neuf, France. [j]Diamond V, Cedar Rapids, Iowa, USA. [k]PureBulk, Roseburg, Oregon, USA. [l]Analysis conducted on post-manufacturing pellets

tanks (1.8 m diameter, 0.8 m deep) in the hatchery tank room on May 27, 2013. Each group of 400 fish was maintained discretely throughout the experiment, first during rearing for 30 days in indoor circular tanks and after subsequent transfer to exterior cages. The cages were constructed of rectangular cuboid wire mesh (1.2 m long, 0.6 m wide, 0.6 m high; ~880 fish/m^3) and placed in the inflow area end of separate covered raceways (30.5 m long, 2.4 m wide, 0.5 m deep). The same well water (11 C; total hardness at $CaCO_3$, 360 mg L^{-1}; alkalinity as $CaCO_3$, 210 mg L^{-1}; pH 7.6; total dissolved solids, 390 mg L^{-1}) was used in all rearing units throughout the experiment. One of the two diets was randomly assigned to each of the eight tanks with four replicate tanks receiving the same diet. Each tank (group) of fish received the same diet for the entirety of the experiment.

Feeding amounts were the same for the all of the groups of fish, both during tank room and raceway cage rearing and were determined by the Hatchery Constant (HC) Method (Buterbaugh and Willoughby, 1967) with a planned feed conversion ratio of 1.1 and a maximum growth rate of 0.061 cm day^{-1}. Feeding levels were at or above satiation for all of the groups, based on observed residual feed. Fish were fed by hand once per day. All feed fed and fish mortalities were recorded daily. Percent mortality was determined by dividing the number of fish that died by the total number of fish initially present in each tank.

Tank room feeding commenced on May 28, 2013 and continued for 30 days. At the beginning and end of the tank room rearing phase of the trial, total tank weights were measured to the nearest 1.0 g with weight gain calculated by subtracting the initial weight from the final weight for each tank. Feed conversion ratio for each tank was conservatively estimated by dividing the total amount of feed fed by the total weight gain. In addition to total tank measurements at the end of tank room rearing, five fish were randomly selected from each tank and euthanized in 250 mg L^{-1} MS-222. These fish were then individually weighed to the nearest 0.1 g and measured (total length) to the nearest 1.0 mm. Also at the end of tank room rearing, fish health profiles, based on a modification by Goede and Barton (1990), Adams *et al.* (1993) and Barton *et al.* (2002) were completed using the score sheet described in Table 2. Liver weights were recorded to the nearest 1.0 mg and the Hepatosomatic Index (HSI) calculated using the formula: HSI (%) = 100× (Liver weight/Whole fish weight) (Strange, 1996). Viscera weights (minus digestive contents) were also recorded to the nearest 1.0 mg and the Viscerosomatic Index (VSI) determined using the formula: VSI (%) = 100×(Viscera weight/Whole fish weight).

Table 2: Criteria used at the end of the study for fish health observations (based on Goede and Barton (1990), Adams *et al.* (1993) and Barton *et al.* (2002))

Structure or tissues	Rating criteria	Numeric rating
Eyes	Normal	0
	Abnormal	1
Fat	None	0
	<50% of gut covered	1
	>50% of gut covered	2
	100% of gut covered	3
Fins	No erosion	0
	Light erosion	1
	Moderate erosion	2
	Severe erosion	3
Gills	Normal	0
	Clubbed, frayed or discolored	1
Gut	Normal	0
	Slight inflammation	1
	Moderate inflammation	2
	Severe inflammation	3
Kidney	Normal	0
	Abnormal	1
Liver	Normal	0
	Abnormal	1
Pseudobranchs	Normal	0
	Abnormal	1
Opercles	Normal	0
	Short	1
Spleen	Normal	0
	Cysts or enlarged	1

After 30 days of tank room rearing, all but 20 fish per tank were moved to wire mesh cages in the raceways and continued to receive the same diet. The experiment continued in raceway cages for the next 72 days, ending 5 days after the cessation of any mortality. At the end of the experiment, five fish per cage were individually weighed to the nearest 0.1 g and measured (total length) to the nearest 1.0 mm. In addition, to assess any possible soy-induced changes in distal intestine morphology (van den Ingh *et al.*, 1991; Burrells *et al.*, 1999; Nordrum *et al.*, 2000; Buttle *et al.*, 2001; Sealey *et al.*, 2013), distal intestine samples were also collected from five fish per raceway cage at the end of the trial for histological examination. A 2 mm long section of distal intestine was removed from each fish, fixed in 10% buffered formalin and stained with hematoxylin and eosin using standard histological techniques (Bureau *et al.*, 1998; Burrells *et al.*, 1999). Intestinal morphology was assessed using an ordinal scoring system on lamina propria thickness and cellularity, submucosal connective tissue width and the number of large vacuoles (Knudsen *et al.*, 2007; Colburn *et al.*, 2012; Sealey *et al.*, 2013). Table 3 describes the ranking criteria.

Samples to determine the presence of *Flavobacterium psychrophilum* and other possible microbialpathogens were collected immediately prior to fish movement from the tank room to the raceway cages as well as on moribund or dead fish from the raceway cages. At the end of tank room rearing, five fish per tank

Table 3: Histological Scoring System (modified by Knudsen *et al*., 2007; Colburn *et al*., 2012; Sealey *et al*., 2013)

Scores	Appearance
Lamina propria of simple folds	Thin and delicate core of connective tissue in all simple folds
	Lamina propria slightly more distinct and robust in some of the folds
	Clear increase in lamina propria in most of the simple folds
	Thick lamina propria in many folds
	Very thick lamina propria in many folds
Connective tissue between base of folds and stratum compactum	Very thin layer of connective tissue between base of folds and stratum compactum
	Slightly increased amount of connective tissue beneath some of the mucosal folds
	Clear increase of connective tissue beneath most of the mucosal folds
	Thick layer of connective tissue beneath many folds
	Extremely thick layer of connective tissue beneath some of the folds
Vacuoles	Large vacuoles absent
	Very few large vacuoles present
	Increased number of large vacuoles
	Large vacuoles are numerous
	Large vacuoles are abundant in present in most epithelial cells

were euthanizedin 250 mg L^{-1} MS-222 and swabs were taken from the viscera, calvarium and oral cavity (including the gills). The swabs from five fish were pooled, plated on TYE agar, blood agar and MacConkey agar and incubated at 20°C for 6 days. Bacterial colonies were then isolated and specific colony morphology was examined. Conventional biochemical tests were performed as needed and isolates were also identified using MALDI-TOF (Matrix-assisted Laser Desorption/ Ionization with a Time-of-Flight mass spectrometer). Gross and microscopic examinations of fresh samples were also performed. During the raceway cage rearing phase of the experiment, fish samples were either frozen or stored in 10% buffered formalin. Bacteriology procedures on 60 of the frozen fish were the same as with the fish sampled at the end of tank room rearing. In addition, Polymerase Chain Reaction (PCR) for *Flavobacterium psychrophilum* detection (Toyama *et al*., 1994; Crumlish *et al*., 2007) was conducted on the abdominal viscera. Additional raceway cage mortalities were stored in 10% buffered formalin for subsequent histopathology.

Fish sampling for immunological metrics occurred 25 days after fish were moved to the raceway cages which was 7 days following onset of mortality. Five fish were collected from each raceway cage and from each of the indoor rearing tanks (which each contained twenty trout which were not moved). The fish were euthanized with 250 mg L^{-1} MS-222. Measurements of length, weight and spleen weight were taken at the time of necropsy. Spleen weights were recorded to the nearest mg and Spleen Somatic Index (SSI) was calculated using the following formula: SSI = 100×(Spleen weight/Total body weight). Head kidney macrophages were extracted using methods adapted by Mustafa *et al*. (2008). Blood was collected and pooled from five fish per treatment by severing the caudal vein and capillary drawin heparinized Vacutainer tubes (BD Corporation, Franklin Lakes, New Jersey). Blood samples were then centrifuged to separate the plasma. Head kidney samples were aseptically removed and stored on ice in 2 mL of Leibovitz-15 (L-15) medium containing 2% Fetal Calf Serum (FCS) (Sigma-Aldrich, St. Louis, Missouri), 100 i.u. penicillin-streptomycin per mL (Sigma-Aldrich) and 10 units of heparin per mL (Fisher Scientific, Waltham, Massachusetts), according to formulations described by Secombes (1990). Within 12 h of extraction, head kidney tissues were passed through 100 μm mesh in 2 mL of the modified L-15 medium. The samples were then centrifuged at 1000×g for 10 min and the pellet was re-suspended in fresh L-15 with 2% FCS. The cells were washed a second time and the cells resuspended in 0.1% FCS for increased adherence. The cells were then utilized in the respective phagocytosis and respiratory burst assays. Isolated plasma was analyzed for lysozyme activity.

Phagocytic evaluation was performed using methods described by Mustafa *et al*. (2008) and Mathews *et al*. (1990). Sample aliquots of 100 μL were placed on double-etched slides (Fisher Scientific) for cellular adherence and incubated at 18°C for 120 min. Previously prepared formalin-killed *Escherichia coli* was placed on the incubated slides and allowed to incubate for an additional 90 min at 18°C. Following incubation, the slides were washed with Phosphate Buffered Saline (PBS), fixed using methanol and stained using Wright-Giemsa (Sigma-Aldrich). The proportion of macrophages containing *E. coli* was determined using the 100x oil immersion objective lens on a light microscope.

The Respiratory Burst Assay (RBA) was performed using Nitroblue Tetrazolium (NBT) reduction via intracellular O_2 as described by Secombes (1990). Sample aliquots of 200 μL were placed in 48-well plates and allowed to adhere for 2 h in L-15 medium, supplemented with 0.01% FCS. The cells were then washed twice and subject to the RBA protocol. The RBA reagent solution was prepared by adding Phorbyl 12-myristate 13-acetate to L-15 medium at a concentration of 1 μg mL^{-1} followed by the inclusion of NBT at 1 mg mL^{-1}. The reagent solution was then mixed as described by Secombes (1990). The RBA reagent was added to the washed macrophages and allowed to react for 1 h. The wells were then emptied, the cells fixed with methanol and the wells were washed twice with a 70% methanol rinse. Each well was filled with 120 μL of 2 M KOH and 140 μL of DMSO and the

plates were agitated and read in a microplate reader (BioTek Corp., Winooski, Vermont) at 620 nm, using KOH/DMSO wells as blanks. OD_{620} readings were adjusted for the blanks and were representative of the respective RBA.

Lysozyme activity was determined using the turbidimetric assay, originally described by Parry *et al.* (1965) and modified by Lie *et al.* (1989). Briefly, a 0.2 mg mL^{-1} suspension of *M. lysodeikticus* (Worthington Biochemical Corp., Lakewood, New Jersey) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with previously isolated plasma concentrations of 10, 20 and 30 μL replicates. This particular range was chosen based on results by Lie *et al.* (1989) where 10 μL plasma sample additions were found to provide the optimal assay outputs. The plates were then kinetically analyzed in a microplate reader (BioTek Corp.) for 4 min at 530 nm with readings performed at 0.5 and 4.5 min. Units of lysozyme activity were defined and reported as an absorbance decreased of 0.001 per min.

All data were analyzed using the SPSS (9.0) statistical analysis program (SPSS, Chicago, Illinois). Rearing, fish health assessment and mortality data were analyzed using t-tests. Histological scores were analyzed using a Mann-Whitney test and cumulative mortality curves were analyzed using a two-sample Kolmogorov-Smirnov test. Immunological data was analyzed with two-way analysis of variance to determine the relative effects of rearing location and diet. All percentage data were arcsine transformed prior to analysis to stabilize the variances (Kuehl, 2000). Significance for all analyses was predetermined at $p<0.05$.

RESULTS

Mortality during tank room rearing was negligible with only two fish lost throughout the entire 30 days but increased during rearing in the raceway cages. Mortality started 18 days after moving the fish from the tank room to the raceways and continued for approximately the next 7 weeks (Fig. 1). Cumulative mortality curves were not significantly different between the fish fed either of the two dietsand there was also no significant difference in overall mortality. The fish groups receiving the fish meal-based diet had a mean±SE overall mortality of 4.46±1.32%, compared to 4.32±0.83% in the groups fed the FSBM diet. Mortality ranged from 2.16-8.11 and 2.16-6.21% in the cages fed the fish meal-based diet and the diet containing FSBM, respectively.

Erosion of the caudal peduncle and epidermal ulcerations were observed on some of the moribund and

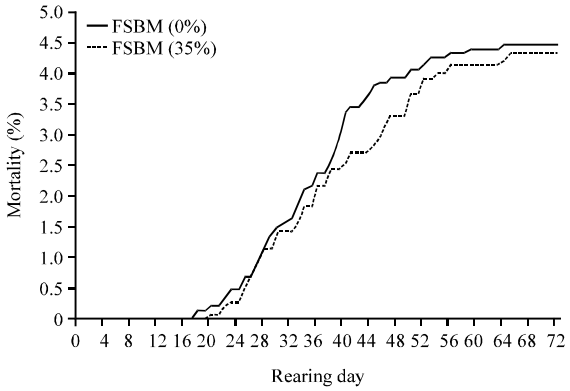

Fig. 1: Cumulative mortality during rearing in raceway cages of rainbow trout receiving a diet with either 0 or 35% Fermented Soybean Meal (FSBM) (n = 4)

dead fish. Increased pigmentation ascites, spiral swimming behavior and lethargy were also observed. However, none of the bacteriological culture or molecular methods confirmed the presence of *Flavobacterium psychrophilum*. Histopathology microscopic evaluations did not find any consistent pathological lesions in the fish examined with findings varying from myopathy to inflammation of several organs. One fish had lesions and filamentous Gram negative rod-shaped bacteria forming mats on the surface of ulcerated lesions, bacteria suggestive of *Flavobacterium psychrophilum* but not pathognomonic. Another fish had severe multifocal erosive ulcerative epidermitis with large numbers of hyphal organisms consistent with a secondary *Saprolegnia* sp., infection.

The fish fed the FSBM diet grew slower during both the tank room (Table 4) and raceway cage rearing phases of the experiment (Table 5). At the end of tank room rearing, mean tank weight and weight gain were significantly less and feed conversion ratio was significantly higher for the FSBM diet treatment. At the end of raceway cage rearing, the rainbow trout were significantly shorter and lighter in the cages receiving the diet containing FSBM and there was no significant difference in condition factor. However, condition factor had improved since transfer from circular tanks.

No significant differences were observed between treatment fish after tank room rearing for any of the qualitative assessments of fish health (Table 6). VSI and HSI were also not significantly different. No significant difference was observed in the histological scoring of lamina propria thickness, connective tissue width and the number of absorptive vacuoles (Table 7).

Immunological sampling 25 days after transfer to the raceway cages indicated no significant difference in

Table 4: Total tank rearing data (means±SE) including Feed Conversion Ratio (FCR) of rainbow trout fed diets containing either 0 or 35% Fermented Soybean Meal (FSBM). Means in a row with different letters are significantly different (N = 4, p<0.05)

FSBM (%)	Diet 1 (0)	Diet 2 (35)
Start weight (g)	1,723	1,723
End weight (g)	2,662±41^{z}	1,935±36^{y}
Gain (g)	939±41^{z}	212±36^{y}
Food fed (g)	1,814	1,814
FCR	1.94±0.09^{z}	9.38±1.66^{y}
Mortality (%)	0.0±0.0	0.12±0.07

Table 5: Mean (±SE) lengths (mm), weights (g) and condition factors (K)[a] at 30 and 104 days for rainbow trout fed diets containing 0 or 35% Fermented Soybean Meal (FSBM). Means with different letters across a row are significantly different (N = 4, p<0.05)

Dietary FSBM (%)	0	35
Length		
Day 30	77±3	76±1
Day 104	110±5^{z}	88±4^{y}
Weight		
Day 30	4.8±0.8	3.9±0.1
Day 104	13.4±2.0^{z}	7.2±1.0^{y}
K[a]		
Day 30	0.98±0.05	0.88±0.03
Day 104	0.96±0.03	0.95±0.03

[a]Condition factor (K) = 10^{5}×(Weight)/(Length^{3})

Table 6: Mean (±SE) liver weights (g), Hepatosomatic Index Values (HSI)[a], viscera weights (g), Viscerosomatic Index (VSI)[b] and fish health assessmentscat the end of tank room rearing (30 days) for rainbow trout fed diets containing 0 or 35% Fermented Soybean Meal (FSBM) (N = 4)

Dietary FSBM (%)	0	35
Viscera weight	0.43±0.17	0.39±0.05
VSI	10.45±1.23	9.49± 0.91
Liver weight	0.07±0.02	0.04±0.01
HSI	1.32±0.20	1.04±0.09
Fat	1.0±0.2	0.6±0.2
Fins	0.8±0.1	1.2±0.2
Gills	0.2±0.1	0.2±0.1
Opercles	0.1±0.1	0.4±0.2
Liver	0.0±0.0	0.0±0.0
Pseudo branchs	0.0±0.0	0.0±0.0
Eyes	0.0±0.0	0.0±0.0
Gut	0.0±0.0	0.0±0.0
Kidney	0.0±0.0	0.0±0.0
Spleen	0.0±0.0	0.0±0.0

[a]Hepatosomatic Index (HSI) = 100×(Liver weight/Body weight). [b]Viscerosomatic Index (VSI) = 100×(Viscera weight/Body weight). [c]Fish health assessments rating system described in Table 2

Table 7: Mean (±SE) distal intestine morphological scores from rainbow trout feddiets containing 0 or 35% Fermented Soybean Meal (FSBM) (N = 4)

Dietary FSBM (%)	0	35
Lamina propria of simple folds	1.83±0.17	2.08±0.16
Connective tissue at base of folds	2.00±0.13	2.25±0.16
Absorptive vacuoles	2.25±0.08	2.58±0.16

spleen somatic index, macrophage activity, respiratory burst or plasma lysozyme activity between trout fed either of the diets (Table 8). Macrophage activity, respiratory burst and plasma lysozyme activity were also not significantly different between the fish remaining in the tank room compared to the fish moved to the raceway cages. However, spleen somatic index was significantly elevated in the fish moved to the raceway cages with index levels approximately 150-400% greater than the fish remaining in the tank room.

Table 8: Mean (±SE) Spleen Somatic Index (SSI), macrophage activity (%), respiratory burst (%) and lysozyme activity (units/mL) from rainbow trout feddiets containing 0 or 35% Fermented Soybean Meal (FSBM) and sequentially reared in two different systems. Means with different letters across a row are significantly different (p<0.05)

	Tank room		Raceway cages	
Location	----------	----------	----------	----------
Dietary FSBM (%)	0	35	0	35
SSI	0.06±0.01^{z}	0.07±0.01^{z}	0.30±0.11^{y}	0.18±0.03^{y}
Macrophage activity (%)	37.8±4.0	33.8±2.6	32.4±2.6	27.8±2.0
Respiratory burst (%)	17.7±0.6	18.6±1.4	18.6±1.4	23.9±4.8
Lysozyme activity (units/mL)	692±279	202±82	212±82	293±58

DISCUSSION

The inability to isolate *Flavobacterium psychrophilum* and subsequently confirm that the observed mortality could be attributed to BCWD was unexpected and inconsistent with previous years when outbreaks occurred following transfer between rearing environments. The caudal peduncle erosion observed in some of the fish is a classic characteristic of BCWD (Davis and Calabrese, 1964). Other observations from the moribund or dead fish such as increased pigmentation, ascites, spiral swimming behavior and lethargy, although not unique to BCWD are also clinical signs of BCWD infection (Rucker *et al*., 1954; Kent *et al*., 1989; Santos *et al*., 1992; Bruno and Ellis, 1996; Madsen and Dalsgaard, 1999). In addition, the timing of the beginning of the mortality approximately, 3 weeks after moving the rainbow trout from the tanks to the raceway cages fits the classic pattern of BCWD disease outbreaks at McNenny and in other hatcheries. *F. psychrophilum* became established at McNenny Hatchery in 2006 and has infected every lot of small rainbow trout moved from the tank room to raceways at McNenny hatchery since that time. However, the pathological evidence produced in this study while not completely excluding the presence of *F. psychrophilum* does not support that BCWD was the major cause of the observed fish mortality. A portion of the observed mortality may have been due to *Aeromonas* sp., *Pseudomonas* sp. or other opportunistic, endemic pathogens at the hatchery. Because the spleen is responsible for antibody production and may enlarge during immunological responses (Hadidi *et al*., 2008), the

increased spleen sizes in the raceway cage fish in relativeto those fish remaining in the indoor tanks indicate probable exposure to *F. psychrophilum* or some other pathogen.

The slower growth observed in the trout receiving the FSBM diet was also very unexpected and surprising. Very similar diets have been used in prior studies with McConaughy strain rainbow trout at McNenny Hatchery and produced growth results equivalent to fish meal-based diets. FSBM has also been shown to be a good replacement for fish meal in rainbow trout diets through a wide range of dietary inclusion levels without any negative effects on growth or feed conversion (Yamamoto *et al*., 2010; Barnes *et al*., 2012, 2013). However, the rainbow trout used in this study were considerably smaller than that used in other studies (Yamamoto *et al*., 2010; Barnes *et al*., 2012, 2013) which may have influenced the results.

While, the protein and energy values of both diets used in this study were similar and acceptable for rainbow trout (NRC, 1993), palatability may have been an issue, particularly with the FSBM diet and particularly during tank room rearing. Because, this study was focused on mortality and not growth, feeding amounts were predicated on the highest consumption per tank and then kept the same between the treatments. The lack of growth and inordinately high feed conversion ratios observed with the FSBM diet are uncharacteristic of the McConaughy strain reared on either fish meal-based or FSBM-based diets (Barnes *et al*., 2012, 2013). In addition, the feed conversion ratios of the fish fed the fish meal-based diet were also higher than that reported for rainbow trout in studies feeding non-FSBM diets (Adelizi *et al*., 1998; Cheng *et al*., 2003a, b; Barrows *et al*., 2008), likely due to the residual feed waste. However, the VSI and HSI values of fish fed either of the two diets were similar to that reported previously for rainbow trout fed fish meal-based and FSBM-based diets (Barnes *et al*., 2012, 2013).

Although, the FSBM diet may have been suboptimal, it did not appear to have a negative effect on mortality rates. This finding is contrary to the suggestion by Post that malnutrition is a probable primary etiology of BCWD, although, we cannot say that BCWD was the primary reason for the trout mortality observed in this study. The results do suggest that dietary FSBM does not lead to an increased susceptibility to disease, unlike that reported for dietary soybean meal (Krogdahl *et al*., 2000). The lack of dietary FSBM-induced intestinal morphological changes or enteritis may also help explain the similar mortality levels observed between the diets. If FSBM had caused intestinal enteritis such as that observed with higher levels of dietary soybean meal (van den Ingh *et al*., 1991; Burrells *et al*., 1999; Nordrum *et al*., 2000; Buttle *et al*., 2001; Sealey *et al*., 2013), it may have likely facilitated the invasion of microbial pathogens such as *F. psychrophilum* that enter the fish via damaged tissue (Decostere *et al*., 2000; Krogdahl *et al*., 2000; Madetoja *et al*., 2002; Miwa and Nakayasu, 2005).

The FSBM diet had no apparent effect on rainbow trout immune function, at least in comparison to the fish meal-based control. Respiratory burst potential is an integral component of effective bactericidal ability (Chettri *et al*., 2010) and the non-compromised respiratory burst response in both diets indicates normal bacterial clearance ability and phagocytosis through the use of superoxide production. Increased macrophage activity is indicative of increased bacterial clearance competency within the teleost innate immune system and may increase adaptive immunity through antigen presentation (Novoa *et al*., 1996; Magnadottir, 2006). Lysozome activity analysis, although likely limited by the small number of fish sampled and the small amount of plasma collected, demonstrated a relatively similar bacteriolyticability between the diets (Caruso *et al*., 2002) and is within the range of activity reported previously (Verlhac *et al*., 1996). It is possible that other measures of immune function such as plasma IgM, β_2-microglobulin or gene expression may be required to detect any differences in immune response between fish-meal and plant-based diets as well as any possible immunostimulatory effect induced by a major feed ingredient (Magnadottir, 2010; Henriksen *et al*., 2015).

CONCLUSION

The results of this study indicate that a diet containing FSBM as the primary protein source will not lead to increased mortality during a disease event in rainbow trout, even if fish growth is negatively affected. Additional research is needed to determine what effects, if any, on mortality would occur if rainbow trout were fed FSBM diets that produced similar growth to fish meal-based diets. In addition, the response of rainbow trout fed FSBM to specific pathogen challenges should be evaluated in biosecure laboratory facilities to eliminate the uncertainties inherent in production hatcheries.

ACKNOWLEDGEMENTS

Researchers thank the South Dakota Soybean Research and Promotion Council, South Dakota Agricultural Experiment Station, Department of Natural

Resource Management at South Dakota State University and the South Dakota Department of Game, Fish and Parks for funding, facilities, equipment and supplies. Furthermore, the assistance of R. Ray, A. Davis, E. Krebs, P. Nero, M. Wipf and S. Zimmerman is greatly appreciated. Nutraferma Inc. of North Sioux City, South Dakota provided the fermented soybean meal for this trial.

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