SWITCHING IT UP: MIXED FEEDING PLAN MAY LEAD TO IMPROVED UTILIZATION OF DIETARY PLANT PROTEIN

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Introduction

Increasingly popular as a fishmeal (FM) replacement in aquafeeds, soybean meal (SBM) is widely available, cost effective, and high in protein (Park et al. 2017, Lin and Luo 2011, Zhou et al. 2005). However, there are negative attributes to dietary SBM due to the presence of several anti-nutritional factors (Makkar 1993, Francis et al. 2001). These negative effects can present as reduced feed intake, reduced growth performance, and the development of SBM induced enteritis (SBMIE; Hua et al. 2019, Francis et al. 2001, Zhou et al. 2005, Krogdahl et al. 2010). Typical histological signs of SBMIE include shortening of mucosal folds, thickening of lamina propria, and infiltration of inflammatory cells (Gu et al. 2016, Fuentes-Quesada et al. 2018, Agboola et al. 2022a), all of which interfere with the ability of the intestine to efficiently digest and absorb nutrients.

Past studies have shown varying time frames for the onset of enteritis symptoms. One study of grass carp (*Ctenopharyngodon idellus*), a herbivorous species, found the symptomatic stage of SBMIE to occur between 7 to 10 days of feeding with a 70% SBM diet (Peng et al. 2020). In omnivorous species, the onset may occur earlier for lower levels of SBM inclusion. A study of the common carp (*Cyprinus carpio*) found the first visible morphological evidence of enteritis after 7 days of feeding with a 20% SBM diet (Urán et al. 2008). In the case of zebrafish (*Danio rerio*), signs of intestinal inflammation have arisen between days 4 (Hedrera et al. 2013) and 10 (Rehmann et al. 2022) when fed 50% SBM diets. Carnivorous species are less equipped to consume plant feedstuffs (Oliva-Teles et al. 2015) and signs of intestinal inflammation present at lower inclusion levels of SBM (Sales 2009). Morphological changes in the intestine were detected in Atlantic salmon (*Salmo salar*) after 5 days of feeding with a 20% SBM diet and 7 days of feeding with a 10% SBM diet (Urán et al. 2009).

While the onset of SBMIE varies between species and feeding habit, it typically takes a few days of consistent SBM diet feeding to occur. Thus, varying the feeding regimen and utilizing a pattern of alternating diets may allow for the intestinal tract to heal or cope with

the anti-nutritional factors present in SBM. Several studies have investigated the effects of feeding regimen on growth (Lawrence et al. 2012, Gonzales and Law 2013, Farias and Certal 2016), reproduction (Lawrence et al. 2012, Gonzales and Law 2013, Farias and Certal 2016), metabolism (Fang et al. 2013), and behavior (Dametto et al. 2018). However, not much is known on how varying feeding regimens affect SBM utilization and SBMIE. Thus, the objective of this study was to use the zebrafish, an omnivorous species with a middle-ground tolerance to SBM (Hedrera et al. 2013), as a model to investigate the effects of alternating feeding regimen with SBM diet on growth performance, feed utilization, and intestinal health.

Methods

Experimental Design and Feeding Regimen

Twelve 3.0 L tanks were assigned in triplicate (n = 3) to one of four treatment groups: fish that received the same FM-based diet every day (**FM-FM**), fish that received the same SBM-based diet every day (**SBM-SBM**), fish that received alternating FM- and SBM-based diets daily (**FM-SBM**), and fish that received alternating FM- and SBM-based diets weekly (**FM/SBM**). At 22 days-post-fertilization (dpf), zebrafish were randomly stocked into treatment tanks one fish at a time until all tanks contained 21 fish. Thirty initial fish samples were euthanized using an ice slurry of equal parts ice and water (Wallace et al. 2018), patted dry, and weighed to obtain an average initial weight. This average initial weight was used to calculate the starting biomass of each tank. The feeding trial was carried out from 23 dpf to 49 dpf, before zebrafish reached sexual maturation. This was done to prevent any biases in growth performance due to sexual dimorphism.

To avoid differences in experimental diet feed intake resulting from stress-related anorexia, the feeding trial began 24 hours after stocking at 23 dpf. Zebrafish were fed their respective diets to apparent satiation three times per day. The feeding periods were 09:30-10:30, 13:00-14:00, and 16:30-17:30. Due to the varying feeding regimens between tanks, care was taken to create a satiation feeding protocol that limited biases between treatments as well as negligible feed left uneaten. Within each feeding period, each tank was fed in three separate rounds. During each round, zebrafish were offered a small amount of food, and their feeding behavior was carefully observed. Tanks were provided additional small amounts of feed if "hunting" behavior was observed, and fish were monitored to ensure these offerings were consumed. This was repeated five minutes later and again ten minutes later. Overall, each tank had three carefully observed fifteen-minute feeding periods per day. For each tank, feed offerings were halted once signs of slowed feeding behavior in each tank were observed. More specifically, feeding ceased when only two fish remained feeding in the tank or when the feeding period ended.

Sampling

At 50 dpf, all fish were euthanized using an ice slurry. All fish were patted dry and individually weighed. Three fish per treatment tank were randomly selected for gene expression analysis; the digestive tracts of these fish were dissected, flash frozen in liquid nitrogen, and stored at

-80°C (Meyer et al. 2013). Five fish per treatment tank were randomly selected for histological analysis. Bodies were dissected at an angle below the anal fin and the anterior portion of the body was fixed in 10% neutral buffered formalin prior to histological processing (Sabaliauskas et al. 2006).

Statistical Analysis

Statistical analyses were performed using R software (R Foundation for Statistical Computing, Vienna, Austria, Version 4.2.1 "Funny-Looking Kid"). One-way ANOVA was used to assess differences in average individual weight, FCR, relative gene expression levels, and histological measurements among treatment groups. Normality was evaluated using the Shapiro Wilk's method. Levene's tests were used to test for homogeneity of variances. All data mentioned above were normal with equal variances. Pairwise Tukey tests were used in the case of significance. Differences among treatment groups were considered significant at p values < 0.05.

Percent survival was determined by dividing the final number of fish in each tank the initial number of fish in the tank and multiplying this value by 100. These values were analyzed using a Kruskal-Wallis one-way ANOVA due to non-normality. Feed conversion ratio (FCR) was also evaluated using a Kruskal-Wallis one-way ANOVA due to non-normality.

Results

Feeding regimen did not significantly affect survival ($2 = 1.6$, $p = 0.6594$).

There were no significant effects of dietary regimen on final individual weights ($F3,8 = 2.1363$, $p = 0.1751$). Numerically, the FM-SBM group had the highest weight per fish followed by FM/SBM, FM-FM, and SBM-SBM (Figure 1).

Feed conversion ratio (FCR) was not significantly different between treatment groups ($X2 = 6.5897$, $df = 3$, $p = 0.08619$; Figure 2).

Inflammation-related genes il-1b, tnfa, mmp9, and il-10 were examined. There was a significant effect of dietary treatment on the relative expression of tnfa ($F3,8 = 6.9036$, $p = 0.0131$). Pairwise Tukey tests revealed a significantly ($p = 0.0090$) higher expression of tnfa in the SBM-SBM group compared to the FM-FM group. There were no other significant effects of dietary treatment on the relative expression of inflammatory genes ($p > 0.05$; Figure 3).

Appetite-related genes ghrelin, leptin, and cck were also examined. There was a significant effect of treatment on the relative expression of leptin ($p = 0.0150$). Pairwise Tukey tests revealed significantly higher expression of leptin in the SBM-SBM group compared to the FM/SBM group ($p = 0.0119$). There were no significant effects on the relative expression of ghrelin or cck ($p > 0.05$; Figure 4).

Measurements of intestinal villi length, villi width, lamina propria, and muscularis ratio were taken from the proximal, middle, and distal portions of the intestine. There were no significant differences among treatment groups for any of the measurements ($p > 0.05$; Tables 1-3).

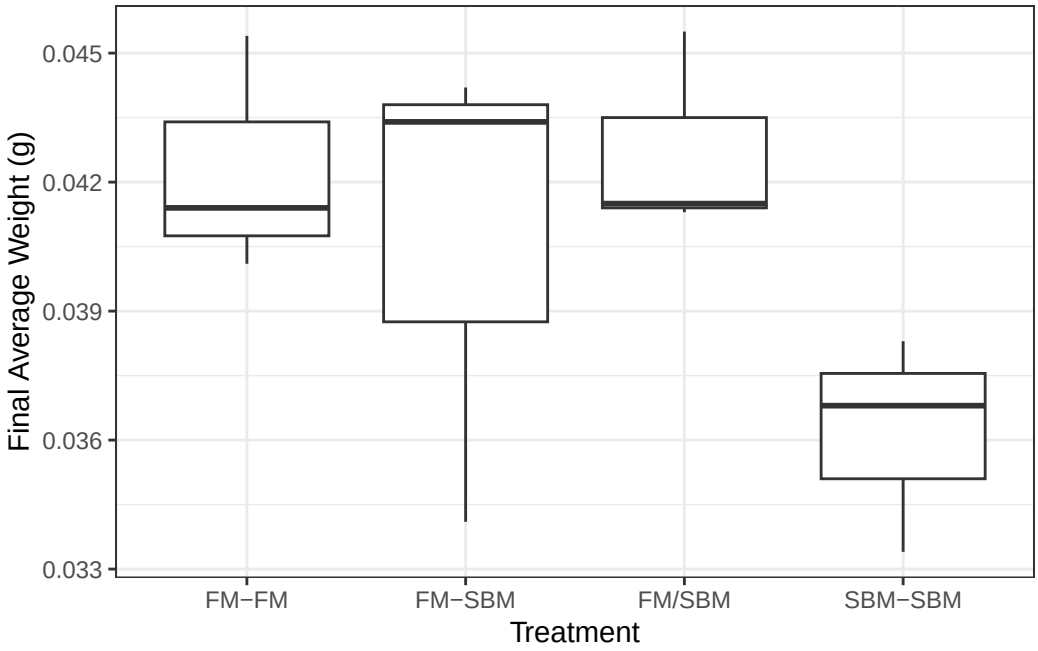

Figure 1: Average individual weight (g) of fish at experimental termination (50 days-post-fertilization).

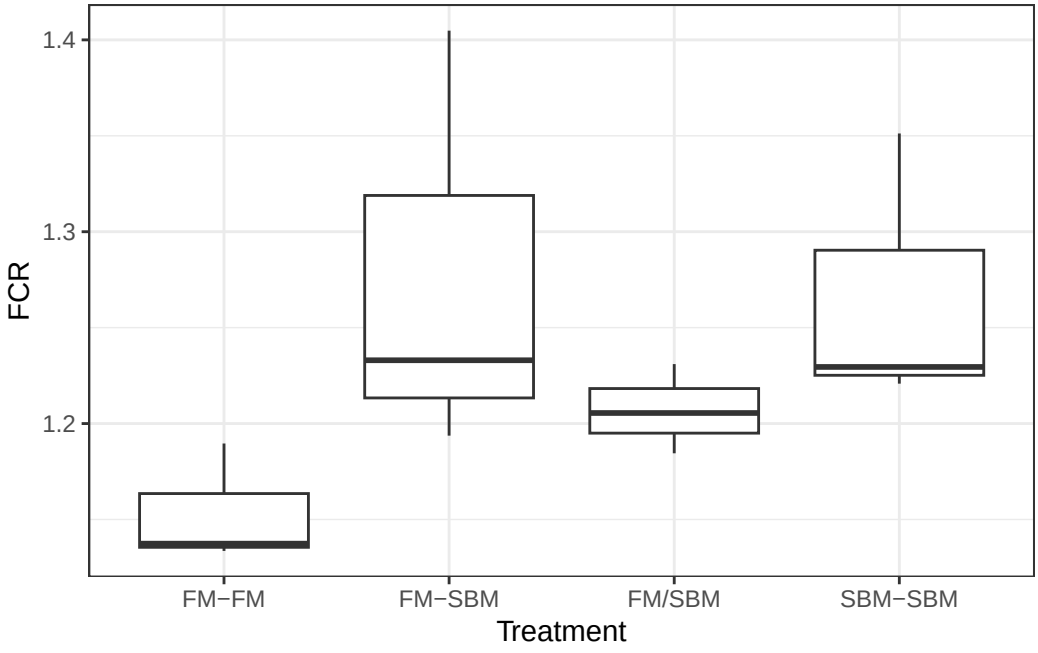

Figure 2: Average feed conversion ratio (FCR) after 26 days of feeding.

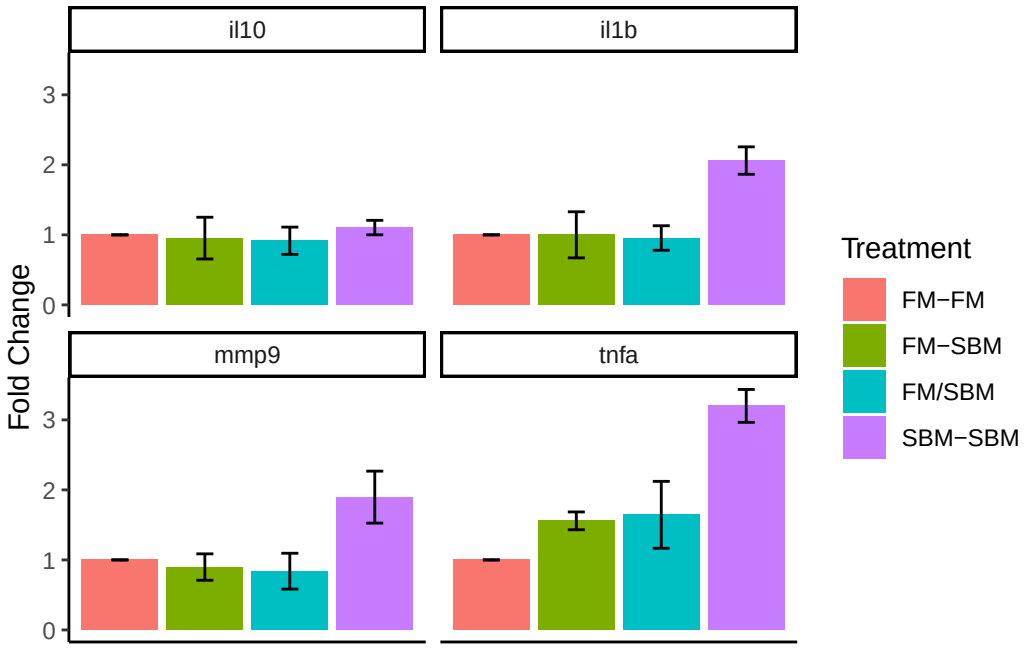

Figure 3: Expression of inflammation-related genes represented as average fold change in reference to the control group (FM-FM; n = 3). Error bars represent standard error of mean

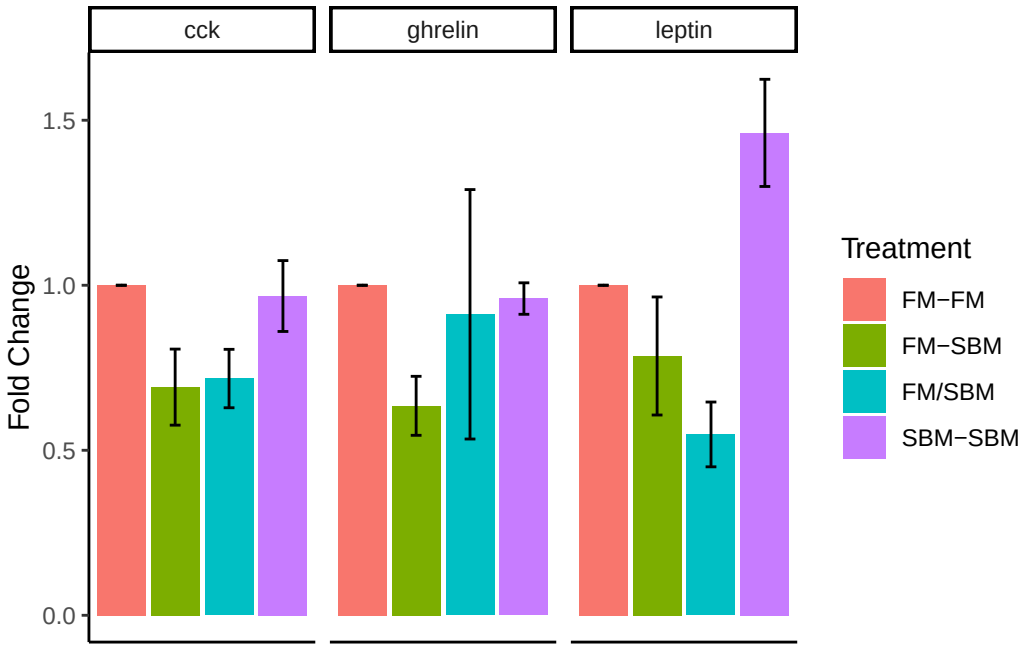

Figure 4: Expression of appetite-related genes represented as average fold change in reference to the control group (FM-FM; n = 3). Error bars represent standard error of mean.

Table 1: Histological measurements (µm) of the proximal intestine. Values are presented as average and standard deviation. There were no significant differences between treatment groups ($p > 0.05$).

	Proximal									
Treatment	Villi Length	sd	Villi Width	sd	Villi Length:Width	sd	Lamina Propria	sd	Muscularis	sd
FM-FM	151.27	2.69	76.90	1.71	2.02	0.03	12.82	3.41	9.19	2.84
FM-SBM	140.27	26.18	73.36	12.63	2.14	0.77	11.70	1.08	10.37	1.89
FM/SBM	141.25	35.18	70.78	8.66	2.07	0.53	13.33	0.56	10.58	1.46
SBM-SBM	123.87	13.91	67.92	7.01	1.88	0.32	14.74	3.07	9.14	0.21

Table 2: Histological measurements (µm) of the middle intestine. Values are presented as average and standard deviation. There were no significant differences between treatment groups ($p > 0.05$).

	Middle									
Treatment	Villi Length	sd	Villi Width	sd	Villi Length:Width	sd	Lamina Propria	sd	Muscularis	sd
FM-FM	118.95	5.18	63.52	12.82	1.97	0.42	10.08	1.32	7.17	1.89
FM-SBM	115.08	13.34	64.94	12.10	1.83	0.24	12.17	3.14	7.19	0.66
FM/SBM	108.38	12.92	64.56	7.47	1.76	0.33	15.25	2.28	7.77	1.11

SBM-SBM	107.42	3.78	68.81	12.98	1.64	0.33	16.62	8.54	6.64	1.11

Table 3: Histological measurements (µm) of the distal intestine. Values are presented as average and standard deviation. There were no significant differences between treatment groups ($p > 0.05$).

	Distal									
Treatment	Villi Length	sd	Villi Width	sd	Villi Length:Width	sd	Lamina Propria	sd	Muscularis	sd
FM-FM	137.92	29.15	67.49	19.65	2.16	0.21	13.63	4.50	6.67	3.00
FM-SBM	123.37	44.06	71.73	10.59	1.72	0.40	12.03	1.97	6.24	1.41
FM/SBM	122.52	37.39	78.80	10.36	1.62	0.32	15.06	2.77	7.15	1.09
SBM-SBM	106.55	8.57	75.00	7.82	1.46	0.25	13.55	1.44	10.04	2.49

Discussion

Growth Performance

Feed Utilization

Intestinal Health

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