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Can Alfalfa Nutrient Concentrate Serve as a Feed Ingredient for Feeding Juvenile Yellow Perch (Perca Flavescens)?

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CAN ALFALFA NUTRIENT CONCENTRATE SERVE AS A FEED INGREDIENT FOR FEEDING JUVENILE YELLOW PERCH *(PERCA FLAVESCENS*)?

by

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ABSTRACT

CAN ALFALFA NUTRIENT CONCENTRATE SERVE AS A FEED INGREDIENT FOR FEEDING JUVENILE YELLOW PERCH *(PERCA FLAVESCENS*)?

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William Sullivan

The University of Wisconsin-Milwaukee, 2023
Under the Supervision of Professor Dr. Dong-Fang Deng

This study evaluated the potential of alfalfa nutrient concentrate (ANC) used in feed for yellow perch. We assessed the quality of ANC based on pellet functionality, digestibility, and growth performance of perch fed with diets including various levels of ANC (0-20 g/100 g diet) to replace fishmeal protein in a control diet based on a 9-week feeding with three replications per diet. Pellet bulk density, durability, water stability, and oil retention capacity increased with fishmeal replacement. Growth rate, feed conversion ratio, satiation feed intake, and protein retention were similar for fish fed different diets ($P>0.05$). Fish fed ANC-20 had lower ash, phosphorus, calcium, and manganese than those fed ANC-0 ($P<0.05$). A lower phosphorus apparent digestibility coefficient was determined in ANC than in menhaden fishmeal, which partially explains the lower phosphorus content in fish fed ANC-20. This study suggests that ANC can be used as a partial protein source in perch feed, but more research is needed to address the concerns of low nutrient digestibility. Research on other species and longer-term feeding trials are warranted to evaluate the application of this ingredient in aquatic feed.

TABLE OF CONTENTS

Abstract……..……..ii

Lists of Figures…….…..……v

Lists of Tables…….……..vi

Lists of Abbreviations……...…..vii

Acknowledgments……...…viii

1. **Introduction**…….……1
2. **Materials and Methods**……6
   - **2.1.** Experimental diet formulation and processing..……6
   - **2.2.** Physical Quality……..……9
   - **2.3.** Growth Study…..…….……...…10
   - **2.4.** Ingredient Nutrient Digestibility…..……12
3. **Results**……14
   - **3.1.** Physical Quality…….…….…..14
   - **3.2.** Growth Study……..……..15
4. **Discussion**……..…….20
   - **4.1.** Impact of Fishmeal Replacement on the Physical Quality of Feed Pellets..……20
   - **4.2.** Effect of ANC on the Growth Performance of Fish.……22
   - **4.3.** Impact of ANC on the Nutrition Quality of Yellow Perch…….……..23
   - **4.4.** Ingredient Nutrient Digestibility..…….24
5. **Conclusion**……..……24
6. **Future Research**…….……...25
7. **References**……...…27

LIST OF FIGURES

Figure 1: Carcass phosphorus and calcium levels…….……19

Figure 2: Carcass iron and manganese levels……..……..19

Figure 3: Apparent digestibility coefficient (ADC, %)…….……20

LIST OF TABLES

Table 1: Test diet formulations…….…….7

Table 2: Physical properties of test diets…….....8

Table 3: Test diet amino acid profiles……8

Table 4: Test diet mineral analysis……..……15

Table 5: Growth performance of yellow perch fed test diet for 9 weeks……16

Table 6: Proximate composition of yellow perch fed test diet for 9 weeks…….……..….17

Table 7: Mineral content of yellow perch carcasses……..……..18

LIST OF ABBREVIATIONS

ANC	Alfalfa Nutrient Concentrate
CF	Condition Factor
CSI	Carcass index
FCR	Feed conversion ratio
FBW	Final body weight
HSI	Hepatosomatic index
IBW	Initial body weight
MS-222	Tricaine methane sulfonate
PER	Protein efficiency ratio
PR	Protein retention
RIR	Relative intestine ratio
SGR	Specific growth rate
VFI	Visceral fat index

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1. Introduction

Yellow perch (*Perca flavescens*) is a cool water species native to the Great Lakes region of North America and a staple entrée for the region's traditional Friday fish fries. In the past yellow perch were abundant within the Great Lakes supporting a large commercial fishery however due to several reasons over the past few decades the population has declined greatly closing commercial fishing of the species in most of the great lake region and leaving a shortage within the market for this high demand fish (Marsden & Robillard 2004) (Schaeffer et al 2011). The reduction in supply has led to inflated prices as high as $35 per pound for skin-on fillets (WalleyeDirect.com 4/03/2023). Due to this high price, there has been increased interest in the aquaculture of yellow perch, the species is lacking in species-specific formulated diets and is often fed diets formulated for salmonids (Brown et al 1996). The concern regarding salmonid diets is the high levels of fishmeal and fish oil inclusion within the diets.

Fishmeal traditionally was a main ingredient in the production of aquacultural feeds. As the name suggests, fishmeal is comprised of ground fish, sourced from the wild catch industry. Annual totals for the wild catch industry are approximately ninety million tons which have seen little change over the past few decades (European Commission 2021). Aquaculture production has exceeded the annual catch total for the wild catch industry since 2013, and only fifteen million of the wild catch was used for fishmeal production (FAO 2022). There is not enough fishmeal to feed all the fish produced leading us to need alternatives to this ingredient. This can be seen with the fish-in fish-out ratio (Kg fish produced per Kg fish consumed) which was as high as 1.4 in 2005 and has dropped to an average of 0.27 – 0.36 with the industries moving away from fishmeal (Kok et al 2020). Fishmeal which contains 60-72% crude protein is utilized as a protein source within aquacultural feeds with grow-out diets for carnivorous species like

yellow perch containing 32-45% crude protein (Miles & Chapmen 2021). Any alternative source of protein would need to have an adequate protein content to achieve these concentrations.

Many sources of alternative ingredients from terrestrial animals, insects, and algae, to different types of plants, have been investigated for aquatic feed. For an ingredient to be able to be used in aquaculture feed a few basic criteria need to be met. Foremost it must be available in large quantities and at a low cost to not raise the cost of production. To be used as a feed ingredient, five criteria including palatability, digestibility, characterization, functionality, and nutrient utilization by the cultivated species need to be investigated (Glencross et al 2007). Palatability relates to the attractiveness of the feed to the cultivated species and may include its texture, appearance, taste, size, or buoyancy. Digestibility is the ability of cultured species to break down nutrients for absorption. It is also necessary to characterize the chemical composition of the ingredient and consider any variability in its quality due to various factors. Functionality accounts for the impact of the ingredient on the physical quality of the feed pellet, such as its durability, density, water stability, and oil retention. Lastly, a feeding trial should be conducted to determine nutrient utilization by the cultured species (Glencross et al 2007).

Terrestrial animal protein sources used in aquacultured diets include poultry byproduct meal, feather meal, blood meal, meat, and bone meal. Poultry products are well-researched and utilized within commercial aquaculture diets due to their high nutrient quality, low cost, and local availability as poultry is raised in every country around the world. Acceptance of poultry byproduct meal (PBM) varied by species however most freshwater fish species evaluated were able to grow without inhibiting growth performance with 100% replacement of fishmeal with PBM and thirteen tested freshwater species were able to tolerate 50% replacement of fishmeal with PBM. One disadvantage of PBM similar to other alternative protein sources is the

incomplete amino acid profile mainly lysine and methionine but they provide good sources of ash (Galkanda-Arachchige et al 2020). Utilizing another byproduct of poultry production researchers found that enzymatically treated feather meal (EFM) replacement of fishmeal had no significant difference in the growth performance of juvenile turbot when 80g/kg of EFM was used to replace 160g/kg of fishmeal. Additionally, these researchers supplemented methionine and lysine into a diet with 240g/kg of EFM replacement of 480g/kg of fishmeal and found this increased growth performance and nutrient utilization compared to a similar diet without amino acid supplementation (Cao et al 2020). An ingredient that has a more balanced amino acid profile for aquaculture species is insect meal.

There are many species of insects, however, only a handful are currently being researched for use in aquaculture which includes black soldier flies, house flies, house crickets, meal worms, super worms, wax worms, and silkworms (Hameed et al 2022). Insects show great potential as many have complete amino acid profiles for use in aquaculture and high protein levels and chitin has been shown to lead to improvements in the immunology of the cultured species (Mousavi et al 2020). Insect meal does have some negatives associated with it unfortunately primarily there is a large amount of variability in the nutrition of the insect meal based on the diet, life stage, and processing method which would require standardization prior to introduction into commercial aquaculture to have consistent nutrient quality; additionally the infrastructure of production of insects is currently inadequate to achieve the volumes necessary for widespread aquaculture use (Nogales-Mérida 2019).

The two most cultivated commercial crops, corn, and soy are common ingredients in aquaculture diets including salmonids due to their consistent nutritional quality and large abundance (Sarker et al 2013). In the unprocessed form corn and soy like most plants have too

low of protein content to achieve the content needed for aquaculture, however, protein concentrates can be made with adequate protein content. By concentrating the protein, several parts are reduced including many antinutrient factors which would otherwise impair the growth performance of the cultured species (Krogdahl et al 2010). Creating protein concentrates has allowed for the use of ingredients that would otherwise impair the growth performance in their whole form. An experiment that replaced fishmeal with corn protein concentrate in the feed of rainbow trout found that there was no significant difference in growth performance with the replacement of 90g/kg of the 452g/kg fishmeal diet, although reduced growth performance was seen at 120g/kg replacement and yellow colored fillets were found in fish with increasing corn concentrate (Hosseini Shekarabi et al 2021). A previous study found that there was increased growth performance of yellow perch fed a diet containing 50% replacement of the 480g/kg fishmeal with modified soybean meal (enzymatically pretreated to remove antinutrient factors) compared to the control. However, reduced growth performance was observed at 100% replacement of fishmeal, demonstrating the replacement of fishmeal is dose-dependent (Kumar et al 2019), which is an important approach to determining the optimal level of an ingredient replacement.

Alfalfa (*Medicago sativa)* is a legume perennial plant species that is the fourth most-grown commercial crop in the United States and is used extensively within the production of ruminant livestock such as cattle (Fernandez et al 2019). This plant is grown for a variety of reasons, primarily it is used as a feed ingredient for ruminant livestock production as well as for its ecological benefits (Coburn et al 2021). Alfalfa plants form a symbiotic relationship with bacteria to fix nitrogen from the atmosphere which reduces the need for fertilization. They also produce the deepest roots of any commercial crop which benefits soil stabilization and nutrient

retention (Fernandez et al 2019). Furthermore, alfalfa produces more protein per acre than any other commercial crop (Coburn et al 2021). While unprocessed alfalfa leaf, due to its high levels of fiber, is generally not suitable as a feed ingredient for most carnivorous fish species, it can be processed into alfalfa nutrient concentrate (ANC) with low fiber and high protein concentration (Coburn et al 2021).

ANC is a new ingredient used in aquatic feed with limited research information on the market compared to its competitors such as corn and soy protein concentrates. Replacement of fishmeal (35-40%) by ANC has been found to increase growth performance in tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*), indicating its potential as an ingredient in aquatic feed (Olasunkanmi et al 2021). However, reduced growth performance was found when sharp snout sea bream (*Diplodus puntazzo*) was fed 100% replacement of fishmeal with ANC, suggesting that the fiber and other antinutrients might be the source of these negative impacts (Chatzifotis et al 2006). A recent study, the replacement of fishmeal with ANC in the feed of yellow perch resulted in a reduced specific growth rate in the fish fed the ANC diet (Coburn et al. 2021). However, for that study, fishmeal was completely replaced with ANC, which may have caused nutrient deficiency for the perch. This also suggests that a dose-response experiment should be conducted to evaluate the potential of fishmeal replacement in this species.

Therefore, in the current study, we investigated the potential of ANC as an ingredient in the feed of yellow perch based on a dose-response approach. We evaluated the impact of ANC on the physical quality of feed pellets, and the nutrient digestibility of fishmeal and ANC, and conducted a feeding trial to determine the optimal replacement level of fishmeal with ANC. The overall goal of this experiment was to conduct a comprehensive investigation of the ANC for use in aquatic feed.

2. Materials and Methods

2.1. Experimental Diet Formulation and Processing

Five test diets were formulated to include differing levels (0, 5, 10, 15, and 20%) of alfalfa nutrient concentrate (ANC) to replace fishmeal protein at an equivalent amount of digestible protein (Table 1). Test diets were manufactured at the Bozeman Fish Technology Center via heated extrusion (DNDL-44, Buhler AG, Uzwil, Switzerland). Mixed ingredient mash was exposed for 18-s to an average of 127 °C in the sixth extruder barrel section. The die plate was heated to an average temperature of 90 °C and pressure at the die head was maintained at 50-70 bar depending on the test diet formulation. Feed pellets of 2.5 mm diameter were produced and dried in a pulse-bed drier (Buhler AG, Uzwil, Switzerland) for 25 minutes at 102 °C, followed by cooling on a forced air cooling table until reaching a final moisture level of less than 7%. Pellets were then top coated with added oil using a vacuum coater (A.J. Mixing, Ontario, Canada). Test diets were delivered to the University of Wisconsin-Milwaukee and stored at -20 °C until use. The diet formulations and nutrient compositions are presented in Tables 1, 2 & 3.

Table 1: Test diet formulation for physical properties and the growth trial

Ingredients	ANC-0	ANC-5	ANC-10	ANC-15	ANC-20
	g/kg				
Menhaden fish meal (FM)	320.0	291.0	262.2	233.2	204.5
Corn Protein Concentrate	60	60	60	60	60
Soy protein concentrate	160	160	160	160	160
Poultry blood meal	80	80	80	80	80
Menhaden fish oil	90.0	92.4	94.6	96.9	99.1
Soybean oil	30.0	23.6	17.1	10.7	4.3
Wheat gluten meal	0	1.1	5.5	8.4	11.3
Alfalfa nutrient concentrate (ANC)	0	50	100	150	200
Wheat flour	163.4	143.3	120.5	99.3	77.9
Lecithin	10	10	10	10	10
Stay-C35	3	3	3	3	3
Vitamin premix[1]	10	10	10	10	10
Sodium chloride	2.8	2.8	2.8	2.8	2.8
Magnesium Oxide	0.6	0.6	0.6	0.6	0.6
Potassium chloride	5.6	5.6	5.6	5.6	5.6
Monocalcium Phosphate	20.0	22.0	23.5	25.0	26.6
Choline chloride	10	10	10	10	10
DL-Methionine	3.2	3.4	3.5	3.6	3.7
Lysine hydrochloride	13	13	12.9	12.8	12.7
Threonine	1.4	1.3	1.2	1.1	1.0
Taurine	5	5	5	5	5
Mineral mix[2]	1	1	1	1	1
Yttrium oxide	1	1	1	1	1
Grobiotic A	10	10	10	10	10
Proximate composition	*g/kg as fed*				
Moisture	42.6	41.6	44.2	43.6	44.4
Crude protein	508	514	535	520	536
Lipid	149	149	137	147	138
Ash	102	106	113	116	118
Fiber	16	46	41	41	41

[1] Vitamin mixture (ARS 702): Vitamin A Palmitate 965 IU, vitamin D3 660 IU, D-Alpha vitamin E 13.2 IU, vitamin K 0.470 mg, thiamine mononitrate 0.910 mg, riboflavin 0.960 mg, pyrodoxine HCl 1.370 mg, DL-pantothenate calcium, 10.110 mg, Cyanocobalamin 0.003 mg, Nicotinic Acid 2.180 mg, biotin D 0.033 mg, folic acid 0.250 mg. Wheat flour was added to bring it up to 1 g.

[2] Trace mineral mix (ARS 1440, g/kg mix): $CaCO_3$ 349.18 g, $CuSO_4 \cdot 5H_2O$ 59.00 g, $FeSO_4 \cdot 7H_2O$ 398.50 g, $MnSO_4 \cdot H_2O$ 52.60 g, KI 7.86 g, Na_2SeO_3 0.96 g, $ZnSO_4$.7H2O 131.90 g.

Table 2: Amino acid profile of test diets

Measurements	ANC-0	ANC-5	ANC-10	ANC-15	ANC-20
Indispensable amino acids	*g/kg diet as fed (g/kg amino acids)*				
Arginine	29.4	31.7	32.4	27.9	30.9
Histidine	18.0	16.8	17	16	17.9
Isoleucine	21.7	19.6	19.8	19.8	21.2
Leucine	51.7	50.6	52.6	48.6	46.4
Lysine	47.2	45.7	45.9	42.2	42.4
Methionine	10.3	10.6	10	9.8	10.1
Phenylalanine	28.3	26.6	28.6	27.7	30.6
Threonine	19.5	19.8	19.6	19.9	21.4
Tryptophan	8.2	8.6	8.6	8.4	8.6
Valine	28.8	28.4	29.7	27.6	30.3
Dispensable amino acids					
Alanine	35.0	31.2	32.2	33.5	35.1
Asparagine	52.4	49.5	50.6	50	51.6
Cystine	5.7	5.4	5.9	5.1	5.4
Glutamic acid	89.1	77.8	78.4	80.3	77.7
Glycine	35.2	26.9	27.3	28.1	29.1
Proline	31.9	28.6	29.4	29.3	31.2
Serine	25.8	23.2	24.5	24.1	25.8
Tyrosine	18.9	17.9	19.2	17.3	19.3

ANC: alfalfa nutrient concentrate; ANC-0, ANC-5, ANC-10, ANC-15, and ANC-20 are designated the test diets containing 0, 5, 10, 15, 20% ANC respectively to replace fishmeal within the diet.

Table 3: Mineral content of test diets

Analysis	ANC-0	ANC-5	ANC-10	ANC-15	ANC-20
Macro-minerals	*g/kg, as fed*				
Sulfur	7.6	7.5	7.7	7.7	7.5
Phosphorus	16.4	16.5	16.9	16.9	16.6
Potassium	10.6	10.8	11.3	11.3	10.9
Magnesium	1.9	2.0	2.1	2.2	2.2
Calcium	20.0	21.2	23.3	24.8	25.7
Sodium	4.3	4.2	4.1	3.9	3.6
Trace minerals	*mg/kg, as fed*				
Iron	756	813	911	969	1010
Manganese	44.0	49.9	56.4	62.6	66.8
Copper	38.3	39.5	40.9	43.4	42.4
Zinc	96.1	91.9	91.3	90.4	89.6

ANC: alfalfa nutrient concentrate; ANC-0, ANC-5, ANC-10, ANC-15, and ANC-20 are designated the test diets containing 0, 5, 10, 15, 20% ANC respectively to replace fishmeal within the diet.

2.2. Physical Quality

Water stability: Two grams of each diet was placed into a 40-mesh which was submerged into four liters of freshwater at 22°C for a duration of 15 and 30 minutes. Following this step, the pellets were rinsed in the freshwater of the same temperature and then filtered through a #3 Whiteman filter paper and dried at 105°C for 24 hours to obtain the dry matter weight. *The pellet water stability is defined as (%): 100* retention of dry matter (g) / dry weight of the pellet (g).*

Physical Durability: Pellets were placed into a commercial pellet tumbler (Continental-Agra Grain Equip., Inc. Newton, KS) for ten minutes at 50 RPM. For adequate testing of aquaculture feeds 5 – ¾ inch nuts are added into each compartment. After finishing tumbling the pellets were sieved to remove fine particles and the remaining pellets were weighed. *Pellet durability index (PDI) is defined as 100*weight of the pellet after tumbling (g)/weight of the pellet before tumbling (g).*

Oil Loss: Ten grams of each diet were placed into an aluminum pan lined with a pre-weighed oven-dried paper towel (Twenty minutes at 105°C) and then placed into an oven at 40°C. The samples were removed after twenty-four hours and allowed to cool to room temperature in a desiccator. Once cool the paper towel was removed and weighed once more. Total oil loss calculated by:100* (final paper towel weight (g) – initial paper towel weight (g).)/original feed fat content

Bulk Density: Was determined by filling a one-liter graduated cylinder with feed pellets. Once the container was filled to the appropriate volume the sample was weighed. Bulk Density is determined by dividing the total weight (g) by the total volume (L).

2.3. Growth Study

2.3.1. Fish Source and Maintenance

Juvenile yellow perch (71 days post-hatch, hatched in July 2020) were produced from the broodstock (origin from the Sassafras River stock) maintained at the School of Freshwater Sciences, University of Wisconsin-Milwaukee. The protocol for fish care and maintenance was similar to the protocol described by Jiang et al (2020). Fish were raised in a flowthrough system and fed on a commercial diet until used for the study.

Before the feeding trial, fish were conditioned in an indoor culture system for two weeks before the feeding trial. The culture system was run with flowthrough dechlorinated municipal tap water at a flow rate of 5 L/min per tank (250L water volume) held at a temperature of 23±1°C. During conditioning fifty-five fish were stocked into each of eighteen tanks. Fish were fed a commercial diet (Skretting, 52% protein &16% lipid) for one week, and then they were weaned to a mix of equal portions of all the experimental diets over the course of 7 days; the first 3 days (25% experimental / 75% commercial; 2 days 50% experimental / 50% commercial; and 2 days 75% experimental / 25% commercial.

At the end of the conditioning, fish were fasted for 24 hours and then randomized, weighed, and distributed amongst the same eighteen tanks used for conditioning with 35 fish per tank (initial body weight: 1.98±0.08g). Eighteen tanks were randomly assigned to each treatment (3 tanks per treatment). Water parameters were checked daily for dissolved oxygen (7.13±0.31 mg/L) and temperature (22.50±0.95°C), while ammonia (0.05±0.00 mg/L), pH (7.81±0.32), and total density of solids (168.14±6.99 mg/L) were checked weekly. Fish were fed four meals per day using automatic disc feeders. The feeding rate was based on the percentage of total biomass

(3.5 – 9.0% daily) of each tank. Fish were group-weighed once every three weeks in water treated with stress coat (API) and MS-222 (Syndel, 50mg/L) to reduce stress due to handling.

2.3.2. Sample Collection

At the beginning of the feeding trial, three groups of ten fasted (24-hour) fish were collected randomly and euthanized in buffered MS-222 (500mg/L). These fish were individually measured for body weight and full body length and then stored at -20°C until used for nutrient analysis.

At the end of the nine-week feeding study, all fish were fasted for 24 hours prior to being counted and weighed by the tanks to determine final survival and growth. MS-222 (100mg/L) was used to reduce handling stress during this process. To obtain individual body weight and length, five fasted fish (fasted for 40 hours) were randomly selected from each tank and euthanized in MS-222 (500mg/L). The fish were then stored at -20°C until used for nutrient analysis. Five additional fish were randomly euthanized in the same method and were dissected for carcasses, gonads, liver, gastrointestinal tract, and visceral fat. The carcass was stored at -20°C until used for nutrient analysis while the tissues were stored at -80°C for biomarker analysis.

2.3.3. Nutritional Analysis

Whole fish, carcasses, and feed samples were analyzed for the proximal composition (protein, lipid, dry matter, and moisture). Prior to analysis the samples were freeze-dried in a Labconco bulk tray freeze drier for 48 hours and homogenized in an IKA A11 analytic mill to produce a fine powder. Dry matter and moisture content was determined by initially freeze-drying the samples, and further drying the samples in an oven at 105°C for 24 hours. Ash content was determined by heating samples at 500C in a muffle furnace for 10 hrs. Total nitrogen was analyzed with an elemental analyzer. The crude protein was calculated by total nitrogen * 6.25.

The lipid content was determined using an ether lipid extractor (Foss Soxttec 8000 Extraction Unit). Fiber is calculated from the collected metrics subtracted from 100%.

2.3.4. Data Calculations

The following body indexes were utilized to gain insight into the growth performance of the fish during the study.

1. *Feed conversion ratio (FCR) = total dry feed weight (g) / total weight gain (g)*
2. *Specific growth rate (SGR (% day^{-1}) = 100 Ln (final body weight (g) / initial body weight (g)) / feeding period (day)*
3. *Condition factor (CF) = (total weight (g) * 100) / total length (cm)^3*
4. *Visceral Index (VI) = 100* visceral weight (g) / body weight (g)*
5. *Gonadal Somatic Index (GSI) = 100* gonadal weight (g) / body weight (g)*
6. *Hepatosomatic Index (HSI) = 100* liver weight (g) / body weight (g)*

The same statistical method was applied to experiments 1 & S. All data were presented in mean ± standard error, N=3. All data were evaluated with one-way ANOVA. Significance was detected using Tukey's post hoc test ($P \leq 0.05$). Polynomial regression was applied to determine the dose response of performance.

2.4. Ingredient Nutrient Digestibility

2.4.1. Diet Formulation

A complete reference diet was formulated to meet or exceed all known nutritional requirements of yellow perch which was blended with the test ingredients (fishmeal or alfalfa nutrient concentrate) in a 70:30 ratio (dry-weight basis) to form two test diets. Feed processing followed the same method described in section 2.1.

2.4.2. Fish Source and Maintenance

Juvenile yellow perch (664 days post-hatch, hatched July 2020) were produced from the broodstock maintained at the School of Freshwater Sciences, University of Wisconsin Milwaukee. The protocol for fish care and maintenance was similar to the protocol described by Jiang et al (2020). Fish used for the experiment were raised in the same facility within a flowthrough system and fed on a commercial diet until the study.

Fish were conditioned in an indoor culture system for one week. The culture system was run with flowthrough dechlorinated municipal tap water at a flow rate of 10 L/min per tank (360L water volume) held at a temperature of 22±1°C. During initial stocking, fish were gendered by external features to have 10 males and 10 female yellow perch in each tank (initial body weight: 211±7.5). At the end of conditioning, two tanks were randomly assigned to each treatment. Fish were hand-fed twice daily (10:00 & 17:00) at a rate of 1% biomass per day.

Water parameters were checked daily for dissolved oxygen (8.50±0.35 mg/L) and temperature (22.50±0.95°C), while ammonia (0.05±0.00 mg/L), pH (7.75±0.24), and total density of solids (170.61±5.84 mg/L) were checked weekly.

2.4.3. Sample Collection and Analysis

The fish were fed the experimental diet for 16 days before fecal samples were collected. All fish from each tank were caught and placed in buffered MS-222 (100ppm) to be anesthetized prior to fecal stripping. Once ready the fish was taken out of the water and lightly dried with a paper towel and then the fecal was collected by pressing on the lower abdomen. Fecal samples were collected in aluminum foil. Fecal were collected on 17- and 24-days post-feeding and they were pooled by tank number, then frozen in liquid nitrogen and stored in a -80ºC freezer until

used for analysis. Nutritional analysis on feed and fecal samples was conducted using the same methods described in the growth study. The following equation was used for the calculation of apparent digestibility.

$$\textit{Apparent digestibility coefficient (ADC) of a nutrient in an ingredient (\%)} = ADC_{test} + ((ADC_{test} - ADC_{ref}) * (Nutr_{ref} * 0.7) / (0.3 * Nutr_{ingredient}))$$

T-test was used to compare the difference in the nutrient ADC between fishmeal and ANC.

3. Results

3.1. Physical Quality

The results for physical quality are shown in (Table 4). All metrics of physical quality were impacted significantly by treatment. The durability index of ANC-0 and ANC-5 were similar ($P>0.05$) while being significantly lower than that of ANC-10, ANC-15, & AND-20 ($P<0.05$). Pellet density increased as ANC level raised increased, with ANC-0 showing a significantly lower density than ANC-5 and ANC-10, which were statistically similar. ANC-5 and ANC-10 both had significantly lower densities than ANC-15. The highest density was observed in ANC-20, which was significantly greater than ANC-15. Oil loss was found to be significantly different for ANC-0 and ANC-20. ANC-5, ANC-10, and ANC-15 were not significantly different from either ANC-0 or ANC-20. Water stability was measured at 15 and 30 minutes, and a similar trend was observed at both sampling time points. At the 15-minute sampling time point, ANC-0 and ANC-5 showed similar water stability, which was lower than the stability of ANC-10 and ANC-15. Both ANC-10 and ANC-15 were similar and had significantly lower water stability than ANC-20.

Table 4. Physical properties of test diets for yellow perch[1].

Measurements	ANC-0	ANC-5	ANC-10	ANC-15	ANC-20
[2]Durability index (%)	91.7±0.3[a]	90.8±0.3[a]	94.5±0.3[b]	94.4±0.2[b]	94.2±0.2[b]
[3]Density (g/L)	495.3±2.1[a]	535.9±3.1[b]	541.4±0.5[b]	580.4±2.0[c]	598.3±1.4[d]
[4]Oil loss (%)	9.98±3.19[b]	6.47±1.39[ab]	4.05±1.14[ab]	4.67±1.13[ab]	0.00±0.00[a]
[5]Water stability					
15 min	88.9±0.3[a]	88.7±0.2[a]	90.5±0.0[b]	90.6±0.3[bc]	91.4±0.1[c]
30 min	85.6±0.1[a]	85.3±0.1[a]	87.2±0.0[b]	87.4±0.8[b]	89.4±0.1[c]

ANC: alfalfa nutrient concentrate; ANC-0, ANC-5, ANC-10, ANC-15, and ANC-20 are designated the test diets containing 0, 5, 10, 13, or 20% ANC, respectively, to replace fishmeal of the test diets.

[1]Data were presented as mean ± SE, n=3. The different letters within the same row indicate significant difference tested by Tukey HSD test ($P<0.05$).

[2]Durability index (PDI): 100*weight of pellet after tumbling (g)/weight of pellet before tumbling (g).

[3]Bulk density (g/L): pellet weight (g) divided by a given volume of pellets (L).

[4]Oil leaking: 100* weight of fat absorption by the absorbent paper (g)/fat content of feed pellet (g).

[5]Water stability: 100*dry pellet retention weight (g) during a given time period original pellet weight (g).

3.2. Growth Study

3.2.1. Growth Performance

During the nine-week feeding trial, no mortalities were observed among the fish. There was no significant difference between treatments in terms of final body weight (FBW), specific growth rate (SGR), and feed conversion ratio (FCR) (Table 5). Morphological parameters including, condition factor (CF), carcass index (CSI), visceral fat index (VFI), and relative gut ratio (RIR), were similar for fish fed different test diets, However, there was a significant decrease in the hepatosomatic index (HSI) ($P<0.05$), with increasing level of ANC replacement of fishmeal (Table 5).

Table 5. Growth performance of yellow perch fed test diets for 9 weeks[1]

Analysis	Commercial	ANC-0	ANC-5	ANC-10	ANC-15	ANC-20
[2]FBW	17.35±1.02	17.49±0.73	17.59±1.01	18.30±0.91	16.66±0.74	16.73±1.04
[3]SGR	3.50±0.09	3.51±0.07	3.52±0.10	3.59±0.08	3.43±0.07	3.44±0.10
[4]FCR	1.10±0.07	1.05±0.05	1.08±0.05	1.05±0.03	1.09±0.07	1.08±0.04
[5]CF	1.07±0.03	1.10±0.02	1.10±0.02	1.11±0.03	1.10±0.03	1.15±0.03
[6]CSI	87.21±0.77	86.90±0.38	87.30±0.34	87.81±0.32	88.45±1.28	87.48±0.72
[7]HSI	1.53±0.13	1.86±0.12^{A}	1.63±0.10^{AB}	1.48±0.08^{AB}	1.38±0.09^{B}	1.34±0.12^{B}
[8]VFI	6.78±0.55	6.03±0.45	6.14±0.42	5.48±0.44	5.76±0.48	6.47±0.72
[9]RIR	0.51±0.02	0.45±0.02	0.51±0.02	0.47±0.05	0.49±0.05	0.54±0.03
[10]PR	34.4±2.7	31.3±2.5	29.5±0.9	29.8±0.9	30.3±2.7	29.0±0.3
[11]PER	3.18±0.22	3.20±0.18	3.18±0.19	3.18±0.09	3.08±0.32	3.13±0.15
[12]FI	0.82±0.14	0.77±0.02	0.72±0.11	0.80±0.08	0.97±0.18	0.95±0.13

[1]Data were presented as mean ± SE, n = 3. Means in the same row sharing different superscript letters are significantly different ($p < 0.05$), as determined by Tukey test. Initial body weight of fish, 1.98 ± 0.08 g, n=30. ANC: (alfalfa nutrient concentrate); ANC-0, ANC-5, ANC-10, ANC-15, and ANC-20 are the test diets containing 0, 5, 10, 15, or 20% ANC levels of replacement of fishmeal.

[2]FBW, final body weight (g)

[3]SGR (Specific growth rate) = 100* LN(Final body weight, g/initial body weight, g)/62 days.

[4]FCR (Feed conversion ratio) = (dry feed weight per tank, g) / (total weight gain per tank, g)

[5]CF (Condition factor, g/cm^3) = (body weight, g) / (body length, cm)^3 × 100.

[6]CSI (Carcass index, %) = (carcass weight, g) / (body weight, g) × 100.

[7]HSI (Hepatosomatic index, %) = (liver weight, g)/ (body weight, g).

[8]VFI (Visceral fat index, %) = (visceral fat weight, g)/ (body weight, g).

[9]RIR (Relative intestine ratio) = total length of intestine (cm)/full body length (cm).

[10]PR (Protein Retention, %) = 100 x (Final fish body protein, g – initial fish body protein, g)/ protein fed, g

[11]PER (Protein Efficiency Ratio) = (Fish weight gain, g) / (protein fed, g)

[12]FI (Food intake, %) = 100*(total feed fed in 30 minutes, g) / (total biomass)

The HSI of fish fed ANC-0 was significantly higher than those fed ANC-15 and ANC-20, which were similar to the fish fed ANC-5 and ANC-10 ($P<0.05$). Similarly, ANC-15 and ANC-20 were significantly different from ANC-0 but similar to ANC-5 and ANC-10. Protein retention (PR) and protein efficiency (PER) were also similar among the different dietary treatments. Moreover, except for the HSI, all parameters were comparable between the commercial diet treatment and the test diet treatments.

3.2.2. Fish nutrition composition and ingredient digestibility

Table 6 presented the proximate composition of whole fish and carcass. The results showed that different test diets did not have a significant impact on the level of moisture, crude protein, and lipid content ($P > 0.05$) in both whole fish and carcass. However, the ash content in the whole fish and carcass, in general, was found to decrease with an increased level of ANC in the test diets. The ash content of whole fish in ANC-20 was significantly lower than that in ANC-0 treatments. All treatments with ANC supplementation (5-20%) were not significant differences in the whole fish ash content. In carcass, the ash content was significantly lower in the fish fed ANC-10 or ANC-15 compared to fish fed the ANC-0. The moisture and protein content of commercial diet-fed fish was similar to those fed the test diets, while the lipid and ash content seemed higher for the commercial fish than most of the test diet-fed fish.

Table 6. Proximate composition of yellow perch fed test diets for 9 weeks

Analysis	Commercial	ANC-0	ANC-5	ANC-10	ANC-15	ANC-20
Whole fish	g/kg as wet					
Moisture	700±1.5	699±2.2	699±1.6	711±6.2	707±1.9	712±2.7
Crude Protein	155±2.1	163±1.5	159±3.5	163±0.7	166±0.3	164±4.3
Lipid	93±2.6	94±2.9	95±0.6	84±4.3	87±1.8	86±0.4
Ash	37±01.0	35±0.6^{A}	34±0.5^{AB}	34±0.6^{AB}	33±0.6^{AB}	32±0.4^{B}
Carcass	g/kg as wet					
Moisture	737±1.6	739±2.0	736±2.0	739±2.0	737±1.0	744±2.0
Crude Protein	189±1.7	200±2.1	199±2.1	194±1.6	198±3.6	195±2.1
Lipid	38±1.4	35±1.0	34±1.0	30±1.0	30±1.0	32±1.0
Ash	44±1.5	38±1.0^{A}	37±1.0^{AB}	35±1.0^{B}	35±1.0^{B}	36±0.0^{AB}

ANC (alfalfa nutrient concentrate). ANC-0, ANC-5, ANC-10, ANC-15, and ANC-20 are the test diets containing 0, 5, 10, 15, or 20% ANC levels of replace of fishmeal.
Data presented in mean ± standard error (n=3)

Table 7 presented the mineral composition of carcasses. No significant difference was found in all treatments for sulfur, potassium, magnesium, and sodium concentrations Phosphorus concentrations were negatively correlated with ANC concentration, which can be

seen in (Figure 1). The phosphorus content was similar between ANC-0 and ANC-5 treatments, which were significantly different from ANC-10, ANC-15, and ANC-20 treatments. The phosphorus level was also similar among ANC-5 ANC-10 and ANC-15 treatments, but the level from the ANC-20 treatment was significantly lower than that of the ANC-5 treatment.

Table 7. Minerals content of yellow perch carcasses.[1]

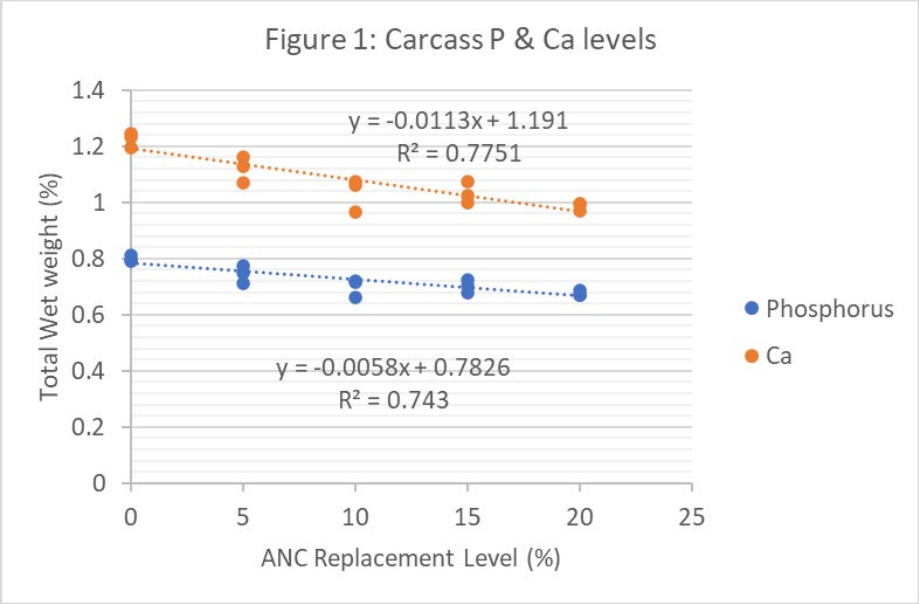
Analysis	Commercial	ANC-0	ANC-5	ANC-10	ANC-15	ANC-20
Macro mineral	g/kg as wet					
Sulfur	2.3±0.0	2.4±0.0	2.5±0.0	2.4±0.0	2.5±0.0	2.4±0.0
Phosphorus	8.1±0.0	8.0±0.0^{A}	7.4±0.0^{AB}	7.0±0.0^{BC}	7.0±0.0^{BC}	6.8±0.0^{C}
Potassium	3.6±0.0	3.6±0.0	3.6±0.0	3.6±0.0	3.7±0.0	3.6±0.0
Magnesium	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
Calcium	12.2±0.0	12.2±0.0^{A}	11.2±0.0^{AB}	10.3±0.0^{BC}	10.3±0.0^{BC}	9.9±0.0^{C}
Sodium	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Trace mineral	mg/kg, as wet					
Iron	5.8±0.3	6.2±0.3^{A}	4.8±0.2^{B}	4.9±0.4^{AB}	5.1±0.4^{AB}	4.2±0.1^{B}
Manganese	3.5±0.2	3.3±0.1^{A}	3.6±0.1^{AB}	3.7±0.2^{ABC}	4.0±0.2^{BC}	4.4±0.1^{C}
Copper	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
Zinc	18.5±0.7	17.8±1.2	16.9±0.2	16.1±0.2	16.8±0.5	17.0±0.6

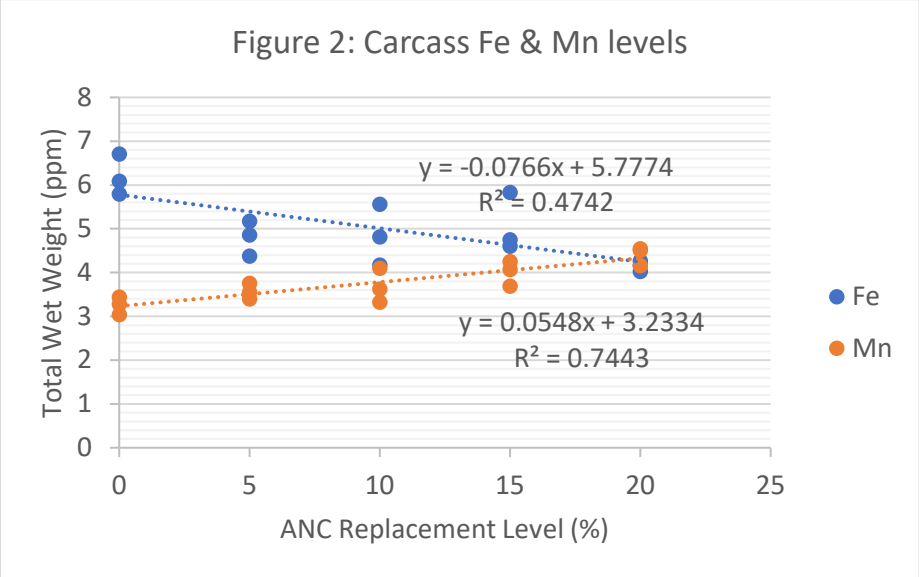
ANC (alfalfa nutrient concentrate). ANC-0, ANC-5, ANC-10, ANC-15, and ANC-20 are the test diets containing 0, 5, 10, 15, or 20% ANC levels of replace of fishmeal.

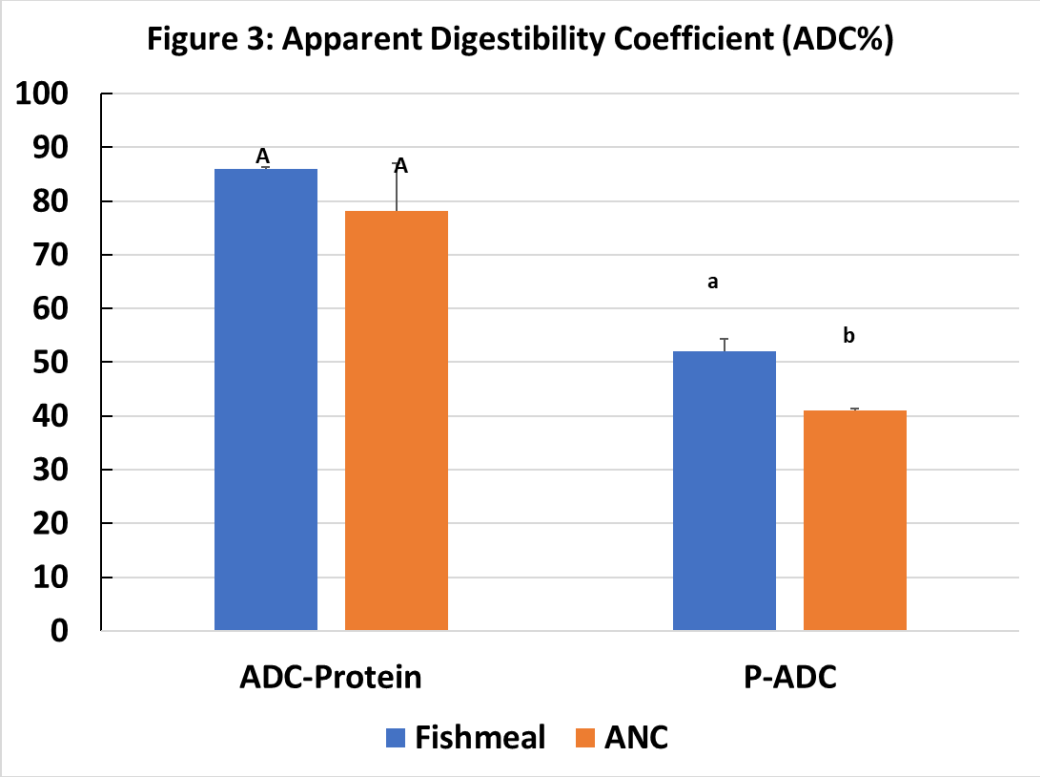
[1]Data presented in mean ± SE, n=3. Superscript lettering within same row dictates significant difference tested by Tukey HSD test ($P<0.05$).

The response of calcium concentrations to different test diets followed a similar pattern that was observed for phosphorus (Figure 1). The calcium concentration in the ANC-0 treatment was similar to that in the ANC-5 but significantly different from those in the ANC-10, ANC-15, and ANC-20 treatments. The calcium concentration in the ANC-5 treatment was similar to that in the ANC-10 and ANC-15 treatments but significantly different from that in the ANC-20 treatment. Similarly, iron concentrations were found to increase with the level of ANC in the test diets. On the other hand, the manganese level was positively correlated to the dietary ANC levels as shown in Figure 2. However, the copper and zinc levels were not affected by the fishmeal replacement with ANC. The mineral contents in fish fed the commercial diets were within the

ranges of those fed the test diets, with most of them being similar to the ANC-0 diet.







The apparent digestibility coefficient (ADC) for protein was not significantly different between fishmeal and ANC. However, the phosphorus ADC of ANC was significantly lower than that of fishmeal. (Figure 3).

4. Discussion

4.1. Impact of Fishmeal Replacement on the Physical Quality of Feed Pellets

The results of this study demonstrate that the physical quality of the feed pellets significantly improved as the level of ANC increased. The durability index, which indicates the ability of the pellet to remain intact under physical stress during transportation and handling, was found to increase as the ANC level increased. This increased durability is likely due to the higher fiber content in ANC compared to fishmeal (Buchanan et al., 2010). The increased density of the feed pellets may be attributed to higher-density ingredients, and this was observed with ANC as well, as the bulk density increased as the level of ANC increased, with ANC-20 having the highest

density. On the other hand, the significant increase in density may raise concerns about the difference in pellet buoyancy, which is important in relation to feeding behavior since different species prefer different sink rates. Therefore, when using ANC in aquaculture feed, considerations must be made to achieve the desired buoyancy to counteract the high density of the cultivated species. The capacity of an ingredient to bind oil is essential for aquaculture, as any loss of oil becomes effluent and results in wasted nutrients. In the current study, the decrease in oil loss as the level of ANC increased suggests that this ingredient benefits oil retention in the extruded feed pellets. Water stability is vital in testing an ingredient for the same reason as oil loss-dissolved feed equals wasted feed. The increased density of the ANC-based feed pellets resulted in increased water stability, with the highest values achieved from the highest fishmeal replacement level, which also coincides with the highest density. This finding is similar to a previous study by Cai et al (2022). The observed improvement in durability, water stability, and reduced oil loss of the feed pellets suggests that the application of ANC can help to reduce the nutrient loss that may occur during handling, feeding, and storage, thereby maximizing the nutrient value of the ANC-based feed.

4.2. Effect of ANC on the Growth Performance of Fish

The study findings suggest that the use of alfalfa nutrient concentrate (ANC) as a replacement for fishmeal up to 20% in the diet of yellow perch does not have any adverse effects on the growth of the fish. The study also found that the fish accepted the ANC-based diet well, and there were no negative effects on their satiation feed intake after 9 weeks of feeding. The weight gain of the fish was over 600% and the feed conversion ratio (FCR) was around 1.0, indicating that the ANC-based diet met the basic requirements for the growth performance of the fish. Moreover, the study found that the protein efficiency ratio and protein retention were not

negatively impacted using ANC, indicating that the protein in the test diets was well utilized to support the growth of the fish. The similar protein digestibility observed between fishmeal and ANC further supports the conclusion that ANC can be a viable alternative to fishmeal in the diet of yellow perch. These results suggest that the ANC-based diet can be a viable protein source alternative to fishmeal in the diet of yellow perch, which can help reduce the reliance on fishmeal and improve the sustainability of aquaculture feed production.

In a previous study, 18% ANC was used to completely replace fishmeal in the test diets for yellow perch and caused negative ingrowth (Coburn et al 2021). It is common to observe differences in the results of different studies, especially when the experimental conditions and methodologies are different. In the case of the study that found poor growth of yellow perch fed an ANC-based diet (Coburn et al 2021), it is possible that the feed used in that study did not provide enough nutrients required by the fish. Additionally, a complete replacement of fishmeal by 18% ANC in that formulation may have impacted on nutrient utilization and thus growth performance of the fish. Furthermore, the culture conditions between the two studies were different, such as fish size, feed management, and culture systems. These differences can significantly affect the growth and performance of the fish. For instance, the fish body weight in the current study was 2.5g, whereas the size of fish in the previous study is 20-24 g. Moreover, the feeding regime in the current study was four meals daily, while the feeding regime in the previous study was two meals to satiation. Finally, the culture system was different, with the current study utilizing a flow-through system and the previous study utilizing an aquaponic system. All these factors can influence the results of the studies and can account for the differences observed.

Yellow perch are prone to developing fatty liver and accumulating visceral fat, which is dependent on the quality of feed they receive, varying in levels and types of carbohydrates and lipids (Jiang et al., 2019a; Jiang et al., 2019b). In the current study, the values of the hepatosomatic index (HSI) decreased in response to increased levels of ANC in the test diets, with no negative impact on the growth of yellow perch. Additionally, the HSI values were within the ranges reported in previous studies (Jiang et al., 2019a), suggesting that perch might not experience clinical health concerns at this stage. It has been reported in other fish species that replacing fishmeal with plant protein sources can lead to fatty and enlarged livers. In a previous study, soybean meal was found to increase HSI, likely due to liver lipid deposition (Kumar et al., 2019). In our study, we did not measure the nutritional composition of the liver, which will be addressed in future studies to better understand the mechanisms by which ANC can maintain or decrease liver size. Similarly, the inclusion of ANC did not result in any extra accumulation of visceral fat, and fish fed the test diets had lower visceral fat levels than those fed the commercial diet. This could be another indicator of good fish health.

4.3. Impact of ANC on the Nutrition Quality of Yellow Perch

Although the protein and lipid levels were not affected by fishmeal replacement with ANC in the current study, there was a significant decrease in ash content. The exact reasons for this decrease are still unclear, but one possibility is the presence of phytic acid or phytates in ANC (Mikkelsen, 2004). Phytates are the primary storage form of phosphorus in plants and are indigestible by monogastric animals, which may explain the lower phosphorus digestibility in ANC compared to fishmeal and the resulting lower phosphorus levels in fish fed the ANC-based diets. Additionally, the existing fiber in ANC may interfere with divalent cations, such as calcium and iron, which are not available for absorption and are thus expelled as effluent (Katya

et al., 2017). The increased Mn levels may be due to the higher levels of test diets when ANC was used to replace fishmeal.

4.4. Ingredient Nutrient Digestibility

The similar apparent digestibility coefficient (ADC) of protein between ANC and fishmeal suggests that the protein in ANC is well digested by yellow perch, as supported by observations on protein efficiency ratio (PER), protein retention (PR), and protein content in fish fed the test diets, which were not affected by fishmeal replacement. However, the lower ADC of phosphorus and other minerals in ANC-based diets raises concerns about the potential for phosphorus deficiency in fish with long-term feeding, which could compromise fish health. This also raises concerns about nutrient discharge and potential environmental issues. Therefore, it is essential to address these drawbacks when using ANC in fish feed to ensure optimal fish health and mitigate environmental risks.

5. Conclusion

The findings of this study suggest that ANC has potential as a viable alternative to fishmeal in aquaculture feed. The physical quality of feed pellets was improved when fishmeal was replaced by ANC, which could result in reduced losses during handling, storage, and feeding. Additionally, an inclusion level of up to 20% did not negatively affect the growth performance or protein utilization of yellow perch, which is an important aquaculture species.

However, it is important to note that the mineral availability, particularly phosphorus, calcium, and iron, was reduced in the fish fed the ANC-based diets. The mechanism behind this reduction is not clear and warrants further investigation. Additionally, the study was conducted over a short duration, and a longer-term study is needed to assess the potential long-term health impacts of feeding yellow perch with ANC-based diets.

Despite these limitations, the findings of this study are promising, as they suggest that ANC could be a sustainable alternative to fishmeal in aquaculture feed formulations. This could benefit not only feed producers in the expanding aquaculture industry but also alfalfa producers, who may be able to tap into a new source of income.

6. Future Research

In order to address the issue of low phosphorus digestibility, further research is needed to determine the effectiveness of phytase supplementation in improving the digestibility of phosphorus in ANC-based diets. Phytase is an enzyme that breaks down phytic acid, the main phosphorus storage molecule within plants such as alfalfa. By breaking down the phytic acid, more phosphorus can become available for absorption by the fish. Therefore, a study could be conducted where the current diet formulation with and without phytase supplementation is tested in order to determine the effect on phosphorus digestibility.

Additionally, the current study was conducted for a relatively short period of time and was not able to determine the long-term effects of ANC on the health of the fish. It is important to conduct longer-term studies in order to determine if reduced ash content caused by ANC leads to a mineral deficiency in the fish. Furthermore, additional research can be conducted to determine the optimal inclusion level of ANC in fish feed for optimal growth performance and health.

Comparative studies with other plant protein sources, such as soy or corn protein isolates, would also be useful in determining the performance of ANC relative to other commonly used plant protein sources in aquaculture. This can help to provide a broader understanding of the potential applications of ANC within the industry.

Lastly, it would be beneficial to test the effects of ANC on other aquaculture species in order to determine its widespread uses within the industry. This would provide a better understanding of the potential benefits and drawbacks of using ANC in fish feed and its potential impact on varied species.

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