Black mulberry fruit as a nutraceutical source for juvenile Dusky kob: Physiological and tissue nutrient responses

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Master of Science degree in Agriculture (Animal Science)

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DECLARATION

I, Thabang Mashiloane, affirm that:

- this is my original research work.
- no part of this thesis has been submitted for any degree or examination anywhere else other than at the University of Mpumalanga.
- the results presented in this paper have been generated and analyzed by me.
- information and material from other sources have been fully acknowledged.

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GENERAL ABSTRACT

There is a need for the aquaculture industry to engineer socially, economically, and environmentally sustainable aquafeeds. This includes minimizing the use of chemotherapeutics and other synthetic feed additives to improve the economic viability of aquaculture, mitigate microbial resistance induced by antibiotic growth promoters, reduce the accumulation of antibiotic residues in fish products, and protect the environment. Accordingly, this dissertation evaluated the potential utility of black mulberry fruit powder (BMFP) as a sustainable nutraceutical source in juvenile Dusky kob fish (*Argyrosomus japonicus*). Black mulberry fruit (*Morus nigra*) powder has unique beneficial bioactive compounds with putative antioxidant, immunostimulant, antimicrobial, anti-inflammatory, appetite stimulation, and growth-promoting properties. These bioactive compounds in the powder may also enhance fish health and the shelf life of fish fillets and the nutritional value of fish products for human consumption. However, despite the potential utility of this natural nutraceutical source, it is yet to be evaluated in the Dusky kob. The study is divided into two experimental chapters (3 and 4). Chapter 3 evaluates the utility of BMFP-containing diets and the effect of BMFP-containing diets on feed utilization, growth performance, and haemo-biochemical parameters in juvenile Dusky kob. Chapter 4 gives a narrative of the evaluation of the dietary effects of BMFP-containing diets on the proximate composition, amino acids, minerals, and fatty acids profiles of Dusky kob fish fillets. The fish feeding trial was carried out in a recirculating aquaculture system (RAS) using four isonitrogenous and isoenergetic experimental diets formulated by including BMFP in a Dusky kob commercial diet at 0 (BMFP0), 5 (BMFP5), 10 (BMFP10), and 12% (BMFP12) w/w. In chapter 3, dietary treatment groups were chemically characterized for proximate, amino acid, phytochemical, mineral, and fatty acid content. At the end of the feeding trial, blood samples were collected from five randomly selected kob fish under anaesthesia sampled from each experimental tank for haemo-biochemical analysis. In chapter 4, twenty fish were randomly selected from each tank to characterize the proximate composition, amino acid profile, mineral composition, and fatty acid of kob fillets. Diet × week interaction did not show any ($p > 0.05$) influence on feed intake, feed conversion ratio (FCR) from weeks 1 – 3 and specific growth rate (SGR), but an increase ($p < 0.05$) of weight gain in weeks 2 and 4, and FCR in week 4 was observed. Weight gain in week 2 showed a positive linear trend in response to incremental levels of BMFP (Figure 3.1). Experimental diets influenced ($p < 0.05$) lymphocytes, monocytes, and eosinophils counts but not ($p > 0.05$) haematocrit, thrombocytes, neutrophils, and basophils (Figure 3.2). A positive quadratic trend for lymphocytes and a negative quadratic trend for monocytes was observed, whereas a positive linear trend for eosinophil counts was observed in response to incremental levels of BMFP. A negative quadratic response was observed for blood urea in kob fish reared on BMFP-containing diets. The results suggest that while dietary inclusion of BMFP did not enhance feed utilization and growth performance as expected, it had beneficial effects on some haemo-biochemical parameters (lymphocytes, monocytes, and blood urea) in juvenile Dusky kob. At the end of the four-week feeding trial, quadratic trends were observed for lysine and phenylalanine content. Lower dietary levels of BMFP significantly enhanced lysine (Figure 4.1) and phenylalanine (Figure 4.2) deposition in kob fish fillets. The results also revealed a significant response in the deposition of Mg, Mn, and Zn in fish filets, in which BMFP10 promoted the highest concentration of these minerals. The experimental diets did not affect the fatty acid content of the fish fillets except for stearic acid. It was calculated that the levels of BMFP DM inclusion that optimize lymphocytes, monocytes, and blood urea in juvenile Dusky kob were 6.1, 8.4, and 7.5%, respectively. From the quadratic equation, it was also calculated that 5.6 and 3.9% were the optimal inclusion levels of BMFP that maximized lysine and phenylalanine content in Dusky kob fish fillets, respectively.

Keywords: Black mulberry fruit powder; Growth performance; Haemobiochemical parameters; Amino acid profile; Mineral content; Fatty acid content

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All glory to my God, who has made the materialization of this dissertation and everything else possible. Thank you, Abba-Father.

DEDICATION

I want to dedicate this dissertation to my beloved brother Lefa Mashiloane who passed on during my time of data collection.

May his golden soul rest in peace.

LIST OF ABBREVIATIONS

ALKP	Alkaline Phosphatase
ALT	Alkaline Aminotransferase
AOAC	Association of Official Analytical Chemists
AST	Aspartate Aminotransferase
BMFP	Black Mulberry Fruit Powder
CF	Crude Fibre
CP	Crude Protein
DFFE	Department Forestry, Fisheries and Environment
DM	Dry Weight
EAA	Essential Amino Acid
EE	Ether Extract
EDTA	Ethylenediaminetetraacetic Acid
FA	Fatty Acid
FAO	Food Agriculture Organisation
FCR	Feed Conversion Ratio
FM	Fish Meal
MUFA	Mono-unsaturated Fatty Acid
NEAA	Non-essential Amino acid
NFE	Nitrogen-Free Extract
PPT	Parts Per Ton
PUFA	Poly-unsaturated Fatty Acid
RAS	Recirculating Aquaculture System
RBC	Red Blood Cell
RPM	Revolutions Per Minute
SAS	Statistical Analysis System
SASI	Southern African Sustainable Seafood Initiative
SFA	Saturated Fatty Acid
SGR	Specific Growth Rate
WBC	White Blood Cell

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1 CHAPTER ONE: INTRODUCTION

1.1 Background

The past few decades have seen a significant increase in the human population resulting in the expansion of the middle class (Martin & Zufia, 2016). This has increased the global average per capita fish consumption, reaching 19.7 kg in 2013 (Nadarajah & Flaaten, 2017) and is projected to surpass 21.5 kg per capita by 2030 (Ahmad, Abdullah, Hasan, Othman, & Ismail, 2021). Since most of the fish consumed by humans comes from captured wild caught fish, this has resulted in the over-exploitation of wild fish stocks. In addition to overfishing, fish stocks have been depleted due to toxic contaminants, habitat depletion, and climate change (Caipang, Mahuhay-Omar, & Gonzales-Plasus, 2019). In 2004, efforts were made to safeguard wild fish stocks by establishing the Southern African Sustainable Seafood Initiative (SASI) to alter consumer consumption towards more sustainable seafood diets (Cawthorn, Steinman, & Witthuhn, 2011).

The over-exploitation of wild fish capture has increased opportunities in aquaculture production (Hara, Njokweni & Semoli, 2017), with about 600 aquatic species being farmed worldwide (Caipang *et al.*, 2019) to plug the demand-supply gap of fish products. Accordingly, aquaculture production is currently the fastest-growing industry worldwide (Fry, Mailloux, Love, Milli & Cao, 2018; Lucas, Southgate & Tucker, 2019; Giri, Sukumaran & Park, 2019), posting growth rates of about 5.8% yearly between 2000 and 2016 (Hua, Cobcroft, Cole, Condon, Jerry, Mangott, Praeger, Vueko, Zeng, Zenger & Strugnell, 2019). This shows that the aquaculture industry has the potential to bridge the gap between the demand and supply of fish products for human consumption. Indeed, aquaculture production is projected to overtake wild fish capture as a significant source of fish for human use (Amosu *et al.*, 2013), providing about 16 to 47 million additional tons of fish for the global market by 2030 (Belton & Thilsted, 2014). However, South Africa's aquaculture industry is still in the developmental stage producing about 5000 tons of products annually valued at R200-300 million, compared to 600 000 tonnes valued at R2 billion from wildlife capture fisheries (Hara *et al.*, 2017). In South Africa, Dusky kob (*Argyrosomus japonicus*) farming has been earmarked for expansion to meet the increasing demand for fish products. The Dusky kob is known to have good growth performance, reasonable survival rates in different water salinities, tolerance to high stocking densities, formulated feed, low oxygenated water levels, and different water temperatures in aquaculture systems (Madibana *et al*., 2020). Moreover, there is still a need to develop new commercial fish diets to support the rapid expansion of aquaculture to meet the demand for low-cost, safe, and high-quality fish and other aquaculture products (Craig, Helfrich, Kuhn & Schwarz, 2017).

1.2 Problem statement

Efforts to expand Dusky kob aquaculture in South Africa face several challenges that must be overcome to ensure an economically, environmentally, and socially sustainable enterprise. Dusky kob aquaculture is vulnerable to disease infestation due to several stressors associated with the prevailing conditions in the intensive production system (Reverter, Bontemps, Lechinni, Banaigs & Sasal, 2014). The use of prophylactic antibiotics to forestall the proliferation of pathogenic microbes in aquaculture systems has been the primary mitigation strategy. However, this practice has been demonstrated to be detrimental to enterprise profitability, human health, and good environmental stewardship (Pepi & Focardi, 2021; Ferri, Lauteri & Vergara, 2022; Okeke, Chukwudozie, Nyaruaba, Ita, Oladipo, Eieromedoghene, Atakpa, Agu & Okoye, 2022). In addition, using chemotherapeutics and other feed additives has multiple negative impacts, such as the emergence of drug-resistant bacterial strains that affect humans and the residual accumulation of chemical agents in fish tissues destined for human consumption.

The viability of Dusky kob aquaculture production is also threatened by high feed costs caused by overreliance on expensive fishmeal and fish oil products, whose supply is increasingly becoming erratic (Tacon & Metian, 2008; Mdhluvu, Mlambo, Madibana & Mwanza, 2021; Sáez-Royuela, García, Carral & Celada, 2022). The cost of Dusky kob aquaculture as an enterprise is high due to the use of expensive feed additives among them antibiotic growth promoters, synthetic antioxidants, exogenous enzymes, and other inputs designed to boost feed utilization efficiency (Cabello, 2006). Yet another challenge facing aquaculture is poor fish product stability at harvest, leading to shorter shelf life, especially in fish reared on diets rich in polyunsaturated fatty acids (Sampels, 2013). After harvest, most fish products undergo rapid lipid oxidation, leading to a rancid taste, off-flavours and the build-up of toxic substances, which can have detrimental effects on human health. Therefore, a promising solution to these challenges is using plant-based bioactive compounds as feed additives in Dusky kob diets. Indeed, Reverter *et al*. (2014) report on the potential use of plant products to stimulate fish appetite and boost fish growth performance. Black mulberry (*Morus nigra*) is ubiquitous in many parts of South Africa and its fruits are a potential source of bioactive compounds that can be used to fortify fish diets used in aquaculture including for Dusky kob.

1.3 Justification

The formulation of new, advanced, and sustainable diets can help support the fish aquaculture industry as it expands to meet the fast-growing demand for fish (Craig *et al*. 2017). This approach has placed increased attention on using locally available plant-based products as a source of both nutrient and bioactive compounds to promote organic fish aquaculture (Caipang *et al.* 2019). Plant products are rich in compounds with various health beneficial biological activities, including among many, immune-system stimulation, appetite stimulation, anti-pathogenicity and growth promotion that can be harnessed in fish aquaculture production (Reverter *et al.* 2014). The plant-derived compounds with health beneficial biological activities include alkaloids, tannins, glycosides, terpenoids, steroids, flavonoids, saponins, essential oils, phenolics, and anthocyanins (Citarasu, 2010). Reverter *et al.* (2014) report that a study focused on haematological parameters on fish health found that lymphocyte, haematocrit, monocyte, haemoglobin, and erythrocyte levels increased after treatment with plant-based feed extracts as compared to the control fishmeal diet.

Black mulberry fruits (*Morus nigra*) have long been associated with human health benefits. Indeed, Farahani, Salehi-Arjmand, Khadivi & Akramian (2019) reported that mulberries could offer protection against kidney and liver failure, strengthening the joints, improving eyesight, and slowing aging process. The phytochemical components of mulberries include alkaloids, phenols, anthocyanins, polysaccharides, and flavonoids (Sânchez-Salcedo, Mena, Gracîa-Viguera, Hernandez & Martinez, 2015; Khalifa, Zhu, Li & Li, 2018; Wen, Hu, Linhardt, Liao, Wu & Zou, 2019). In addition, black mulberries exhibit high levels of antioxidant activities, which can be helpful to neutralise free radicals in fish tissue before and after harvesting (Chen, Chen, Yang, Chen & Gao, 2016). Using black mulberry fruits in Dusky kob diets could boost feed utilization efficiency, product quality, and product stability while promoting environmental and human health. However, there have been no scientific investigations into using black mulberry fruits as a nutraceutical source to boost fish growth and enhance the quantity and quality of fish products for human consumption. Therefore, this study was designed to evaluate the effectiveness of using black mulberry fruit powder as a nutraceutical for juvenile Dusky kob reared in a recirculating aquaculture system.

1.4 Broad aim

To evaluate the utility of mulberry fruit powder as a nutraceutical source for juvenile Dusky kob.

1.5 Specific objectives

The studies specific objectives were to:

- determine feed utilization, growth performance, and haemo-biochemical parameters in juvenile Dusky kob-fed diets containing incremental levels of black mulberry fruit powder.
- determine tissue nutrient composition in juvenile Dusky kob-fed diets containing incremental levels of black mulberry fruit powder.
- identify inclusion levels of black mulberry fruit powder that optimize feed utilization, haemo-biochemical parameters, growth performance, and tissue nutrient composition in juvenile Dusky kob diets.

1.6 Hypothesis

Dietary inclusion of black mulberry fruit powder in juvenile Dusky kob will significantly improve growth performance, feed utilization, haemo-biochemical parameters, and tissue nutrient composition.

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2 CHAPTER TWO: LITERATURE REVIEW

2.1 Global aquaculture production

Aquaculture is farming aquatic organisms such as molluscs, oysters, fish, crustaceans, lobsters, and aquatic plants, for example, seaweeds and algae (Lucas, Southgate & Tucker, 2019; Ahmad, Abdullah, Hasan, Othman, & Ismail, 2021). It can be subdivided into “fed” and “un-fed” aquaculture. Unfed aquaculture relies on naturally available feeds such as filter feeders, bivalves and grass carp whereby feed is not introduced by humans but originates naturally from the production ecosystem. Fed aquaculture involves feeding fish commercially prepared feeds. Traditionally, fed fish are cultured on a diet formulated from fishmeal and fish oil, in which the by-products are processed from pelagic fish sourced from wild-life capture (Hua, Cobcroft, Cole, Condon, Jerry, Mangott, Praeger, Vueko, Zeng, Zenger & Strugnell, 2019).

Today, the aquaculture industry is the fastest-growing animal sector worldwide (Ahmad *et al.,* 2021). Africa has the fastest growth rates in the aquaculture industry in terms of market value and quantity, though still showing a base low of 9.28% in value and 7.35% in growth per annum, compared to the Asian region (Nadarajah & Flaaten, 2017). This suggests that there are challenges still faced by the aquaculture industry that need to be overcome to ensure a sustainable enterprise. An estimated 54.8 million people are actively employed in the wild fish capture and aquaculture production sectors worldwide (Madibana, Mlambo, Lewis & Uys, 2020). A total of 178 million tonnes of marine species (molluscs, freshwater fish, marine fish, crustaceans) were farmed and harvested through aquaculture and wild capture worldwide, whereby capture fisheries contributed 90 million tonnes and aquaculture 88 million tonnes (Food and Agriculture Organisation, 2008; FAO, 2022). Fish are an essential source of fatty acids, high-quality protein and other vital micronutrients necessary for human health. In addition, fish is considered as one of the most cheapest and commonly found form of animal food source in food deficit and low-income countries, thus contributing to the diversity of diets dominated by starchy staples (Belton & Thilsted, 2014; Akpalu & Okyere, 2022). Moreover, most coastal areas in Asia, Europe, and America have been active in aquaculture development for the past decades, although Africa has made little progress in aquaculture production (Amosu, Robertson-Andersson, Maneveldt, Aderson & Bolton, 2013).

Aquaculture has gained interest over the past decades and is estimated to overtake wild fish capture (Amosu *et al.*, 2013). The rising per capita consumption and increasing human populations are fuelling the demand for fish and other fish products worldwide (Brugere & Ridler, 2004). Globally, in 2014, the total supply of fish production for human consumption was reported at approximately 73.8 million tons, and in 2018, aquaculture was responsible for about 82 million tons, which is expected to increase further to about 109 million tons by 2030 (Ahmad *et al.*, 2021). Over the past years, China has been considered the single largest fish producer (Brugere & Ridler, 2004; Olsen & Hasan, 2012), contributing 34.4 million tons or 65.5% of the global aquaculture production (FAO, 2008). Notably, between 1980 and 2012, global fish supply from aquaculture production increased at an average of 8.6% per annum, while growth in wildlife fish capture remained stagnant (Nadarajah & Flaaten, 2017). In 2014, the global aquaculture industry outperformed wildlife capture in the supply of seafood destined for human consumption. According to Nadarajah & Flaaten (2017), this trend is projected to increase further in regions like Africa, America, Asia, and Europe between 2010 and 2030. Therefore, it is predicted that the rapid growth of aquaculture production will need to provide about 16 – 47 million additional tonnes of fish by the year 2030 (Belton & Thilsted, 2014).

2.2 South African aquaculture production

South Africa is regarded as one of Africa's epicenters of fish trade and production. Cawthorn, Steinman & Witthuhn (2011) found that South Africa has four major fish production sites: Cape Town, Eastern Cape, KwaZulu-Natal, and the Northern Cape. Figure 2.1 shows the total quantity of marine fish captured between 2003 and 2008 in South Africa, Namibia, and Angola. Indeed, South Africa averaged 689 681 tons (live weight) annually (see Figure 2.1), which is considered as the highest in the Southern African region, as found by Cawthorn *et al.* (2011). Figure 2.2 shows Africa's top ten aquaculture producers in 2018. Egypt, Nigeria and Uganda are ranked as the top 3 producers, posting 1 561 457, 291 233, and 103 737 metric tons of aquaculture production, while South Africa lags behind as it is ranked 10th with a total production output of 6181 metric tons, as found by Adeleke, Robertson-Andersson, Moodley & Taylor (2020). This suggests that South African aquaculture still lags in production compared to other African countries. Additionally, the South African aquaculture industry is divided into two farming practices; freshwater and marine farming (DAFF, 2011; DAFF, 2013). Moreover, the country's aquaculture industry produces both plant (seaweed) and aquatic animal products (marine, fresh, and mariculture fish) (Ahmad *et al.,* 2021).

The growing demand for healthy and nutritious animal protein foods has increased the demand for fish and other fish products (Hara, Njokweni & Semoli, 2017). Since most of the fish species consumed by humans come from wildlife capture, the sustainability of South African wildlife capture is threatened by the exhaustion of most wild fish stock populations. Amosu *et al.* (2013) suggest that the exhaustion of wild fish stocks can be accounted for by several factors, namely, high levels of fishing, the growing human population, toxic contaminants habitat depletion, and climate change (Caipang, Mahuhay-Omar & Gonzales-Plasus, 2019). Thus, to safeguard the local fish stocks, in 2004, the Southern African

Sustainable Seafood Initiative (SASI) was established to shift consumer behaviour towards consuming more sustainable seafood products (Cawthorn *et al.*, 2011). Yet another challenge is the continued use of antibiotics and chemotherapeutics that have primarily contributed to increased environmental hazards, suppression of fish immune systems, and the proliferation of antibiotic/drug-resistant pathogens (Giri, Sukumaran & Park, 2019). Moreover, other concerns in the aquaculture industry are related to using hormones, vitamins, and antibiotics used as feed additives to stimulate and to promote fish growth (Ahmad *et al.*, 2021). Residues from these compounds are deposited in fish meat and waste thus potentially causing public health and environmental challenges.

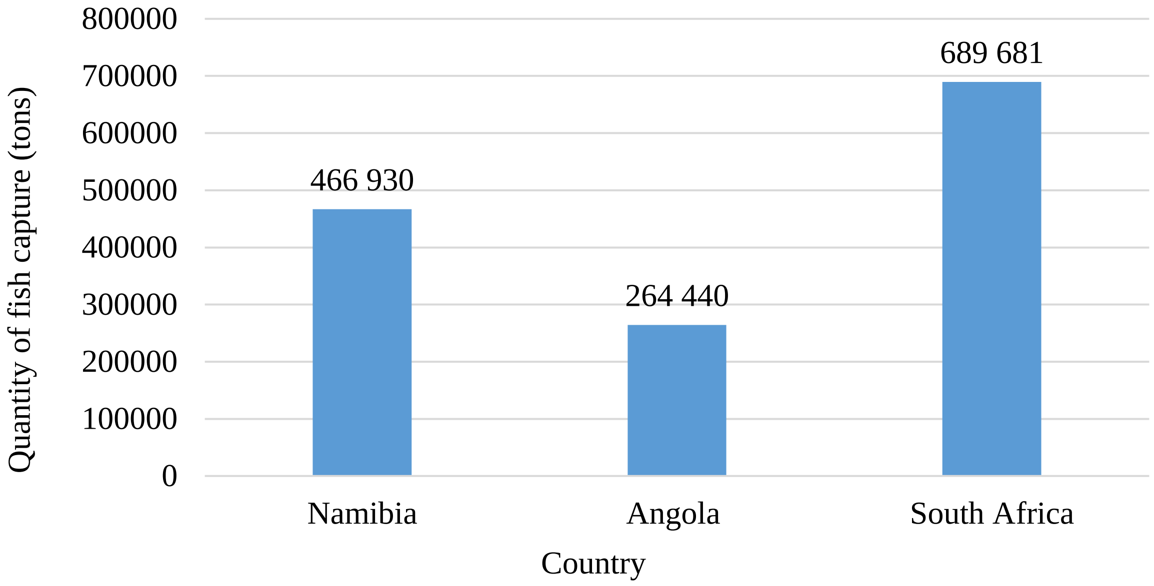

Figure 2.1: Total marine fish capture in some Southern Africa Development Community countries between 2003 and 2008 (Cawthorn *et al.*, 2011).

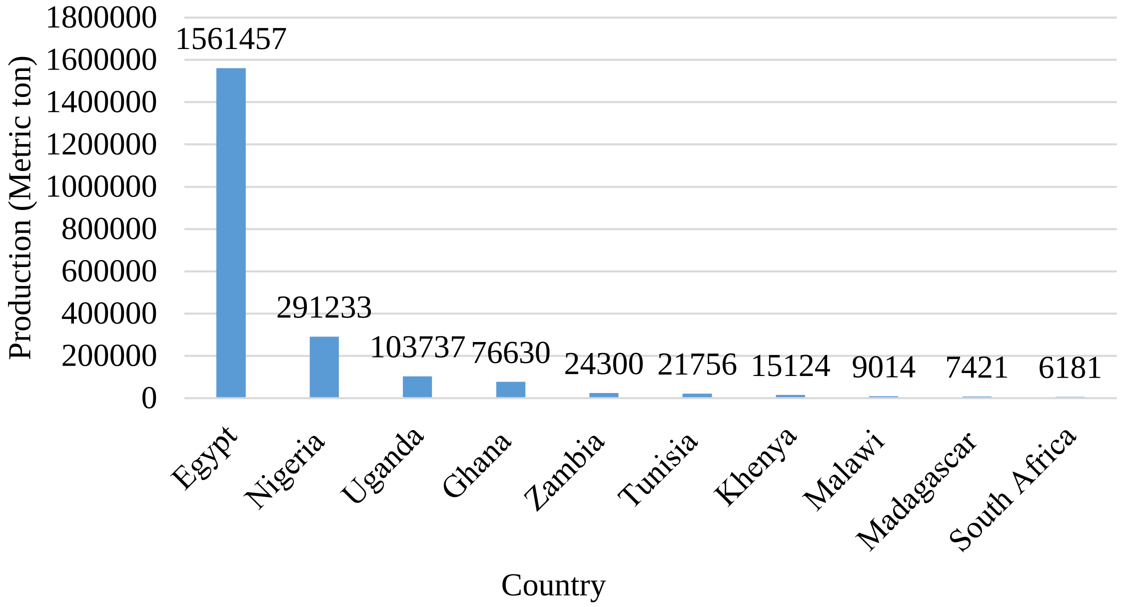

Figure 2.2: Top 10 African aquaculture producers in 2018 (Adeleke *et al.*, 2020)

2.3 The nature, biology, and distribution of Dusky kob

2.3.1 Nature and biology of Dusky kob

The Dusky kob (*Argyrosomus japonicas*) is a carnivorous finfish that belongs to the *Sciaenidae* family exhibiting several essential traits for a successful aquaculture species (Griffiths, 1996; Booth, Allan, and Smullen, 2013; Madibana *et al*., 2020). Dusky kob fish can grow up to 75 kg live weight (Cowley, Kerwath, Childs, Thorstad, Okland & Naesje, 2008) and reach a maximum length of 175 cm and an age of 42 years in the wild (Silberschneider & Gray, 2007). The *Sciaenidae* family has an affluent population of 270 extant species and 70 genera, thus receiving worldwide interest throughout the aquaculture industry (Nelson, 1994). Nevertheless, in South Africa, it is considered a recruitment overfish finfish (Silberschneider & Gray, 2007).

In South Africa, Dusky kob fish are known as the Kabeljou; in Australia, they are known as the mulloway (Fielder & Heasman, 2011; Cawthorn *et al*., 2011). Previously, Dusky kob fish were misclassified, mainly because of their anatomical features, which closely resembled the silver kob (*Argyrosomus inodorus*) (Branch, Griffiths, Branch & Beckley, 2007). Silberschneider & Gray (2007) found that in the past, the *Argyrosomus japonicus* was known by at least 15 other terms, whereby it was misclassified as *Argyrosomus hololepidotus* in some areas. Plate 2.1 shows an image of the Dusky kob (*Argyrosomus japonicus*) taken at the Marine Research Aquarium, Sea Point, Cape Town.

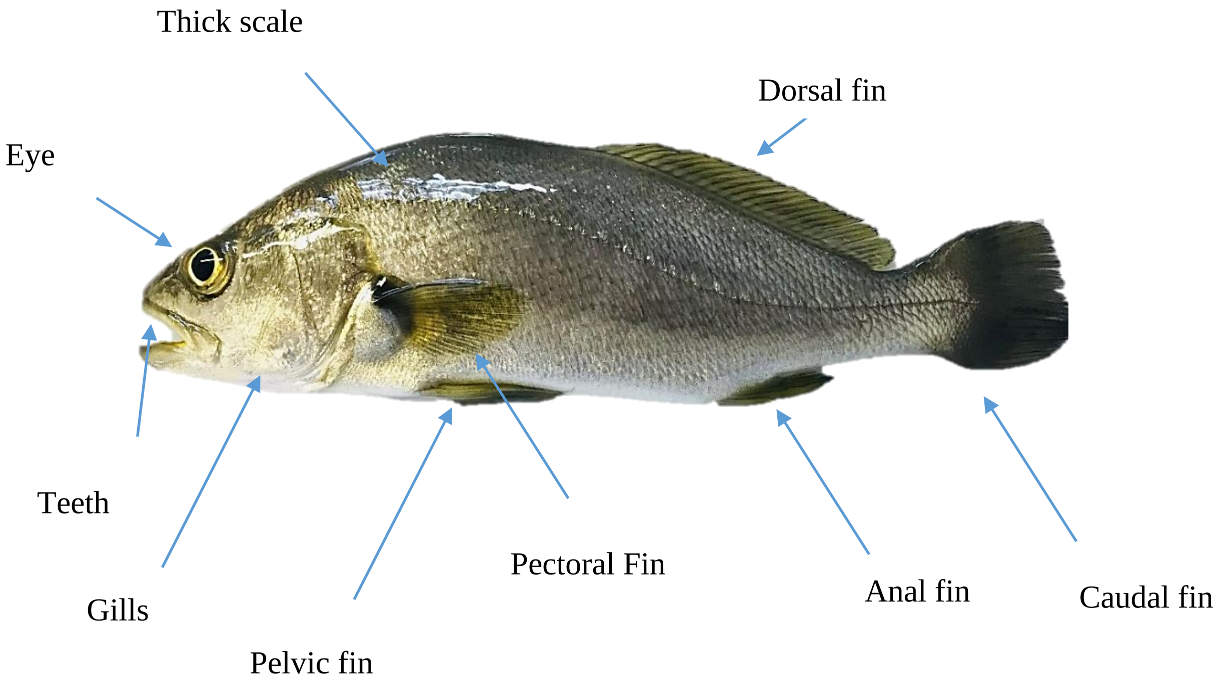

Plate 2.1: Image showing juvenile Dusky kob (*Argyrosomus japonicus*)

The geographical region largely influences the sexual maturity and time of spawning of the Dusky kob. In South African waters, mature Dusky kob fish register an average length of 92 cm at more than three years and 107 cm at more than seven years for males and females, respectively (Fielder & Heasman, 2011). Spawning is common in nearshore coastal regions, but there is some evidence that this also occurs in the lower reaches of estuary waters (Silberschneider & Gray, 2007). In addition, Benatzeder *et al.* (2010) found that adults naturally spawn near-shore marine environments, beyond the surf zone but at depths less than 100 m, while juvenile Dusky kob recruits naturally into estuaries and move to the upper reaches, whereby levels of water salinity ranges between 0 – 5 ppt. In South Africa, spawning occurs in the Northern KwaZulu-Natal region (30°N - 31°S) from August to November (winter to spring) and in the Southern and Southeast Cape regions (33°N - 35°S) from October to January (summer), as found by Fielder & Heasman (2011). The juvenile stage of the Dusky kob may vary between different regions, with differences caused by freshwater flows, salinity, depth of water and turbidity. Juveniles have fast growth performances, reaching about 35 cm full length in the first year and between 87 – 90 cm full length within five years in both South African and Australian waters (Griffiths,1996).

2.3.2 Distribution of Dusky kob

The biology of the Dusky kob is well-documented in South Africa and Australia, however, information on their distributional range in other parts of the world is limited (Silberschneider & Gray, 2007). They are spatially distributed in both the Southern and Northern hemispheres. It is a demersal fish whose habitats include estuarine waters, freshwaters, and coastal marine waters (Fielder & Heasman, 2011). The Dusky kob enjoys estuaries, surf zones, and further offshore water depths but not more than 100 m (Bernatzeder *et al.*, 2010). Figure 2.3 shows the global distributional range of the Dusky kob. In the Southern region, Dusky kob are usually distributed along the coastal seaboard of Southern Africa from Cape of Good Hope (Cape Point) to Mozambique, Australia (along the entire Southern seaboard), Chinese sea coast to the Southern Sea waters of Japan and Korea, as found by Silberschneider & Gray (2007). However, the kob appears more abundant between Cape Angulhas and KwaZulu Natal (Bernatzeder *et al.* 2010; Griffiths, 1996).

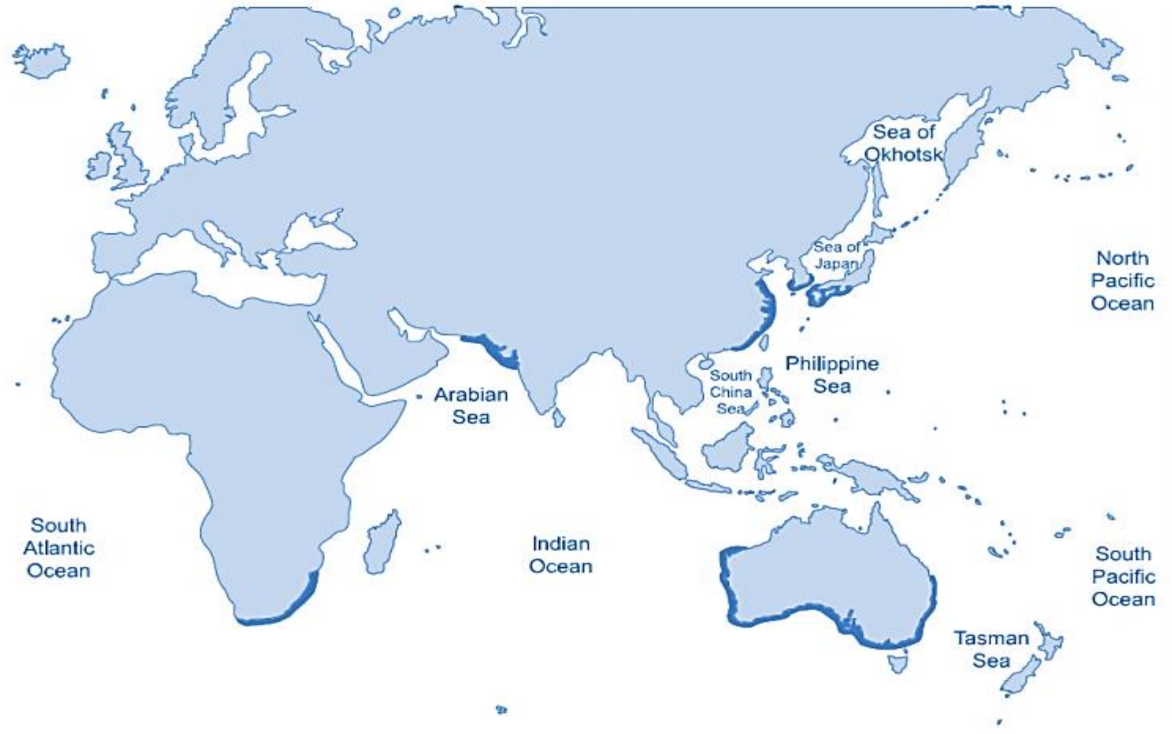

Figure 2.3: Global distribution of Dusky kob (*Argyrosomus japonicus*) fish (Silberschneider & Gray, 2007).

Over the past years, several finfish species have been under study to assess their suitability for aquaculture production and market access. Until today, the only marine finfish farmed commercially in South Africa is the Dusky kob (DAFF, 2013). An investigation by Cawthorn *et al.* (2011) on the commercial availability of Dusky kob fish to South African consumers from a sample population of 215 restaurants and 200 retail outlets (fish markets and supermarkets) suggests that future investment in the Dusky kob is promising. In Figure 2.4, the kob ranked 7th regarding fish availability in restaurants from four South African provinces. However, regarding its frequency in retail outlets in South Africa, the Dusky kob ranked 12th, as found by Cawthorn *et al.* (2011).

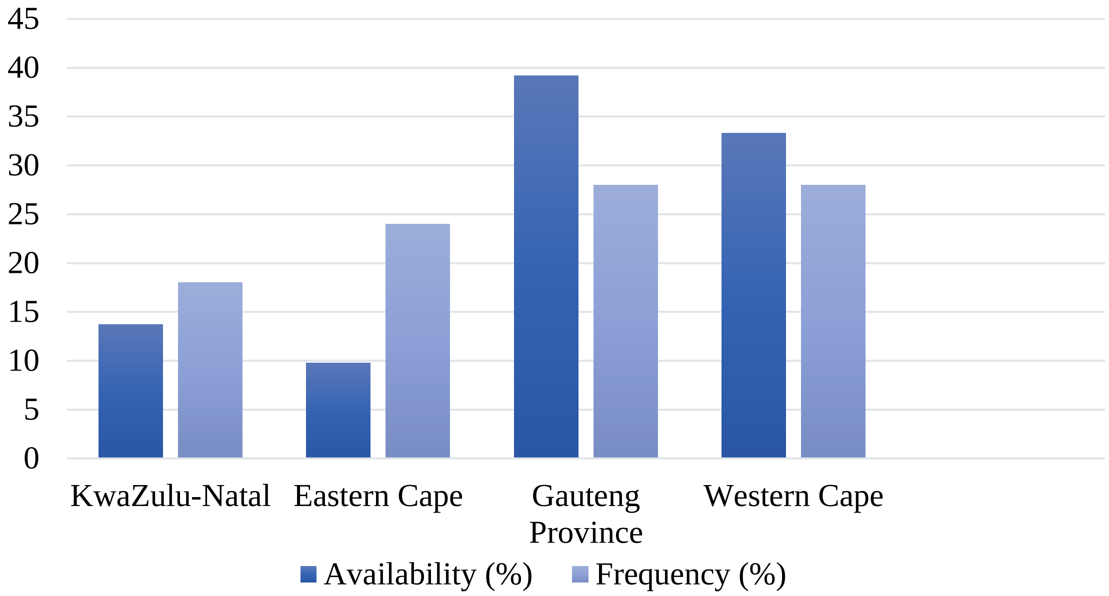

Figure 2.4: Fish availability in restaurants and frequency in retail outlets in Eastern Cape, KwaZulu-Natal, Western Cape, and Gauteng provinces of South Africa (Cawthorn *et al.*, 2011).

2.4 Suitability of Dusky kob for aquaculture production in South Africa

As shown in Figure 2.4, the Dusky kob is more prevalent in the Gauteng Province (39.2%) and less available in Eastern Cape (9.8%) restaurants (Cawthorn *et al.*, 2011). However, it is equally frequent in both Gauteng Province and Western Cape and less frequent in KwaZulu-Natal. Table 2.1 shows a list of traits that make the Dusky kob a favourite finfish for aquaculture species in South Africa.

Table 2.1: Attributes of the Dusky kob that make it suitable for aquaculture species

Trait	**References**
Good growth performance	Madibana *et al.,* 2020; Silberschneider & Gray, 2007; Booth *et al.,* 2013; Adesola, Jones & Shipton, 2017.
Good feed conversion ratios	Madibana *et al*., 2020.
Tolerate different levels of salinity	Booth *et al.,* 2013; Adelosa *et al.,* 2017.
Tolerate low oxygenated water	Fitzgibbon, Strawbridge & Seymour, 2007; Adelosa *et al.,* 2017.
Accepts formulated feed	Adelosa *et al.,* 2017.
Tolerate high stocking densities	Silberschneider & Gray, 2007; Booth *et al.,* 2013; Adelosa *et al.,* 2017.
Tolerance to disease	Silberschneider & Gray, 2007; Booth *et al.,* 2013.
Tolerance to a wide range of temperatures	Silberschneider & Gray, 2007; Adelosa *et al.*, 2017.
High market value	DAFF, 2013.
Tolerates different pH levels	Fielder & Heasman, 2011.

Water parameters are essential for the optimal growth and development of Dusky kob. The aquatic system tends to be polluted easily by unfed materials. The excess feed can decompose gradually in the water medium, settling at the bottom as sediment and consuming oxygen. This negatively impacts water quality. Table 2.2 shows the recommended water quality parameters for Dusky kob culture.

Table 2.2: Optimal range and tolerance levels of water quality parameters for Dusky kob

Parameter	Optimal range	Tolerance	References
Water temperature	24 – 26	15 – 30°C	Fielder & Heasman, 2011; Collett, Vine, Kaiser, & Baxter, 2008; Bernatzeder & Britz, 2007.
pH	7.8 – 8.2	7.8 – 8.2	Fielder & Heasman, 2011.
Salinity	14 – 35 ppt	5 – 35 ppt	Fielder & Heasman, 2011; Silberschneider and Gray, 2007.
Dissolved oxygen	5.5 – 6 mg/L	> 6 mg/L	Madibana *et al*., 2020.

2.5 Dusky kob: digestive system development and nutrient requirements

Dusky kob have large mouths equipped with gill rakers, sharp canine inform teeth, short intestines and a large distensible stomach specialised for ripping and digesting pelagic flesh (Silberschneider & Gray, 2007). According to Musson & Kaiser (2014), the anatomical and phycological development of the digestive system is sub-divided into three distinct phases:

- the first phase is the yolk sac phase, also known as the eleuthero-embryonic stage
- towards the final phase, exogenous feeding begins wherein the fish relies entirely on exogenous feeding with a fully functional mouth and anus and the buccopharyngeal cavity is well distinguished with goblet cells and rudimentary taste buds.
- the final phase is where all the accessory organs increase in size, and there is only exogenous feeding. The digestive tract is fully developed and functional.

Understanding the dietary requirements of any animal can help formulate cost-effective diets. Protein is the single most expensive nutrient essential for growth (Adelosa *et al.*, 2017). The Dusky kob is considered a benthic carnivore but has been reported to also feed throughout the water column. Crustaceans (shrimp and prawns) are an essential diet of Dusky kob (Silberschneider & Gray, 2007). According to Adelosa *et al.* (2011), juvenile Dusky kob requires 31.7 g/kg lysine DM. Table 2.3 shows a list of protein and lipid nutrients for different marine finfish species. These recommendations can be used to formulate a diet for the Dusky kob.

Table 2.3: Lipid and protein (%) requirements for different carnivorous marine finfish species

Fish species	**Lipids**	**Protein**	**References**
Haddock (*Melannogrammus aeglefinus)*	12	45	Kim & Lall, 2001.
Red drum (*Sciaenops ocellatus)*	8 -13	44 – 45	Thoman, *et al.*, 1999; Turano *et al.*, 2002; Webb & Gatlin, 2003.
Asian sea bass (*Lates calcarifer)*	12	42.5	Catacutan & Coloso, 1995.
Striped bass (*Morone saxatilis)*	16.5	55	Milliken, 1983.
Grouper (*Epinephelus malabaricus)*	8	47.8	Chen & Tsai, 1994.

2.6 Fishmeal as an aquafeed

Fishmeal is a much sought-after feed ingredient owing to its high-quality protein content and high biological value of essential amino acids. Fishmeal is produced from small pelagic oceanic fish, which includes anchovies, sardines, herrings, and menhaden, which are pulverized before the removal of oil and water (Bandara, 2018). Residues of oil and water extraction are then cooked and converted into a palatable meal. According to Olsen & Hasan (2012), it is also considered an essential source of vitamins (e.g., niacin, riboflavin, and vitamins A and D), minerals (e.g., iodine, zinc, calcium, selenium, iron, and phosphorus), as well as other nutrients such as essential amino acids. Moreover, fishmeal has also been reported to contain several unique nutrients, such as taurine and other feed constituents, that still need to be classified and well-understood (Bandara, 2018). Therefore, attempting to replace fishmeal in animal diets completely may not be a sustainable strategy (Luthada-Raswiswi, Mukaratirwa & O'Brien, 2021), especially for carnivorous fish that rely solely on the supply of fishmeal.

Hua *et al.* (2019) classify fishmeal and fish oil products as unsustainable aquafeeds because, by 2025, about 37.4 million tons of fish will be needed to meet the growing demands of fish and other aquatic products. Testing different strategies that might improve feed utilization efficiency in fish to reduce the amounts of fishmeal required to attain production objectives is the desired mitigation strategy (Booth *et al.,* 2013). However, recent attempts to reduce feeding costs have resulted in the utility of alternative protein sources, such as poultry by-products, to replace fishmeal in fish diets. This approach has several drawbacks, with some studies suggesting that the risk of microbial infections in fish is high when offered meat-based feed ingredients, which is a significant concern for growers and consumers (Hua *et al.,* 2019).

Progress in the fish industry has been countered by high feeding costs that include the purchase of fishmeal, antibiotic growth promoters, exogenous enzymes, and synthetic antioxidants (Caipang *et al.*, 2019). There is mounting evidence suggesting that the sustainability of fishmeal is under threat in aquaculture production. The supply and cost of feed ingredients, such as fishmeal, produced from captured wild fish is a major threat to sustainability (Bandara, 2018). Traditionally, due to the high-quality nutrients and low price, fishmeal was the primary feed ingredient of choice in aquaculture production (Olsen & Hasan, 2012). About 70% of the global fish industry is dependent on commercially formulated feed ingredients (Fry, Mailloux, Love, Milli & Cao, 2018). The limited supply of fishmeal and the continued increase in its price has negatively impacted the production of carnivorous fish species that solely rely on fishmeal and fish oil to meet their basic nutritional requirements (Booth *et al.*, 2013).

Aquaculture production has also been described as a vulnerable industry with high incidences of infestation with microbes due to several stressors associated with the intensive production system (Reverter, Bontemps, Lechinni, Banaigs & Sasal, 2014). It has also been noted that high-stocking densities within intensive production systems often lead to the accumulation of toxic residual chemical agents in fish tissue, which are taken in from the surrounding aquatic ecosystem. Caipang *et al.* (2019) found that higher risks of disease spread were prevalent when meat wastes were used as feed ingredients in aquafeeds. The most common fish toxic contaminants are methylmercury and organ-halogenated toxins (Cawthorn *et al.*, 2011). In the aquaculture industry, fish feed is the single most expensive variable cost. In an attempt to mitigate the high cost of fish feeds, alternative feed ingredients, have and continue to be evaluated in fish diets. However, there are several drawbacks to the utility of food waste materials in aquafeeds. Food wastes have a high moisture content, are perishable, and may also carry pathogens. However, this has been addressed through sterilization using chemical feed additives, for example, enzymes or improving the technology used to collect food waste through solid-state-fermentation (Hua *et al.*, 2019). Several studies suggest that the risk of microbial infections in fish is high when offered meat-based feed ingredients, which is a significant concern for growers and consumers (Dórea, 2006; Caipang *et al.*, 2019).

However, the ever-increasing cost of fishmeal has led to the use of alternative feed ingredients such as plant-based protein, animal protein, and single-cell protein for partial or complete replacement in fishmeal-based diets (Luthada-Raswiswi *et al.*, 2021).

2.7 Plant-based feed additives in fish diets

Today, there is increasing interest in the potential use of plant-based feed additives as nutraceutical sources in animal diets (Caipang *et al.* 2019). Plant additives constitute several unique bioactive compounds with putative biological activities such as growth promotion, immunostimulation, anti-pathogenicity, enhancement of tonicity, appetite stimulation, stress mitigation, and maturation of culture species in shrimp and fish (Reverter *et al.*, 2014). Faehnrich, Franz, Nemaz & Kaul (2021) reported that the nutraceutical properties of plants are due to several bioactive compounds such as tannins, essential oils, alkaloids, saponins, anthocyanins, phenolics, glycosides, phenolics, terpenoids, or steroids. Recent research focused on using plant-based bioactive compounds prove that they can stimulate appetites and boost fish growth performance which translates to increased meat yield (Reverter *et al.*, 2014).

Plant-based extracts can help reduce feeding and therapeutic costs while promoting environmental stewardship because they are more biodegradable compared to synthetic prophylactics (Reverter *et al.*, 2014) and other feed additives. Indeed, there has been several studies cited on the successful use of plant-based feed additives in different aquaculture species (see Table 2.4). The chemical analysis of black and red mulberry fruits shows that they are rich source of bioactive compounds such as phenolic acids, flavanols, and anthocyanins, as shown in Table 6 (Sânchez-Salcedo, Mena, Gracîa-Viguera, Hernandez & Martinez, 2015; Chen, Chen, Yang, Chen, Gao & Lu, 2016.). Mulberry fruit powder as a nutrient might have a potentially positive effect on meat tissue (Xu, Zhu, Liu & Cheng, 2018). The possible use of the mulberry fruit for feed optimisation has generated much interest in research due to their nutritional content, with both immature and mature fruits have been explored for medicinal use, such as antibiotics, and as immunostimulants. Therefore, the formulation of new advanced aquafeeds can help support the fast-growing fish industry (Luthada-Raswiswi *et al*., 2021) as it expands to meet the growing demand for low-cost, safe, and high-quality fish and other seafood products (Craig, Helfrich, Kuhn & Schwarz, 2017).

Table 2.4: The utility of plant-based feed additives in different aquaculture species

Plant	**Fish**	**Response**	**References**
Bushy Matgrass *Lippia alba* (Mill)	Juvenile silver catfish (*Schilbe mystus*)	It decreased lipid peroxidation; increased the lactate reserves and glycogen, and antioxidative power.	Saccol, Uczay, Pês, Finamor, Ourique, Riffel, Schmidt, Caron, Heinzmann, Llesuy, Lazzari, Baldisserotto & Pavanato, 2013.
Peppermint (*Mentha piperita*)	Fry Caspian white fish (*Rutilus frisii kutum*)	Promotes growth, increased haematological and humoral immune parameters.	Adel, Amiri, Zorriehzahra, Nematolahi & Esteban, 2015.
Seaweed (*Ulva* sp.)	Dusky kob, (*Argyrosomus japonicus*: Sciaenidae)	Blood cholesterol and alkaline phosphatase were decreased.	Madibana, Mlambo, Lewis & Fouchê, 2017.
African wormwood (*Artemisia afra*)	Sub-adults of (*Oreochromis mossambicus)*	Increased count of white blood cells; enhanced immunity; increased feed intake and weight gain	Mbokane & Moyo, 2018.
Black mulberry *(Morus nigra)* powder	Rainbow trout (*Oncorhynchus mykiss*)	Increased muscle pigmentation, growth performance, and health status. Increased blood carotenoid concentration, decreased blood glucose, alanine aminotransferase, and aspartate aminotransferase.	Shekarabi, Omidi, Dawood, Avazeh & Heidari, 2019.

2.8 Challenges of plant-based feed additives

Amid the potential use of natural plant feed additives in fish nutrition, plant-based feed alternatives have been reported to contain antinutritional compounds with adverse effects on fish. Akande *et al.* (2010) found that the most common anti-nutrients in plant protein sources include toxic tannins, saponins, amino acids, gossypol, phytic acid, oxalates, glycosides, cyanogenic glycosides, amylase inhibitors, lectins (phytohemagglutinins), protease inhibitors, and chlorogenic acid. Anti-nutrients are substances that by themselves or through their by-products, impede feed utilization health, and performance of animals. This is especially true for carnivorous animals like the Dusky kob, which are not adapted to digest and utilize plant material effectively. Bandara (2018) reported several major anti-nutrients in plants which have a potentially negative effect on fish. Table 2.5 lists the most common anti-nutrients and describes their mode of action and reported effects on fish performance and health.

Table 2.5: Selected plant anti-nutrients, their mode of action, and influence on fish health and performance

Anti-nutritional factor	**Mode of action in fish**	**Effect on fish**	**References**
Tannins	Bind with minerals, proteins, and other essential feed constituents.	Diminish the absorption of vitamin B-12; interferes with the digestive process.	Freeland, Calcott, & Anderson, 1985.
Phytates	Chelate the divalent and trivalent cations (e.g., Zn^{2+} and Mg^{2+}, etc.), thus forming a phytate-mineral complex.	Affects the bioavailability of phosphorus, zinc, and some other divalent cations. Thus, disrupting growth performance.	Hajra, Mazumder, Verma, Ganguly, Mohanty & Sharma, 2013; Richardson, Higgs, Beames & Mcbride, 1985.
Gossypol	Forms gossypol-protein complex; it reacts with Fe^{2+} to form an inactive ferrous gossypolate complex.	Limits the bioavailability of lysine; results in amino acid deficiencies such as methionine. Reduction of succinic dehydrogenase and cytochrome-c activity.	Smith, 1970.
Protease inhibitors	It acts by binding with digestive enzymes like trypsin, thus rendering them partially or fully inactive.	Diminishes digestion of proteins.	Hajra *et al.*, 2013.
Oxalates	Binds with calcium to form an oxalate complex.	It affects the bioavailability of minerals in fish (e.g., calcium)	Hajra *et al.*, 2013.
Mimosine	Interferes with thyroxine synthesis	It affects the production of thyroxine, thus affecting fish growth.	
Phytoestrogens	Have estrogenic properties which can attach to oestrogen receptors or transform into compounds with an estrogenic effect.	Oestrogen exhibits a wide range of effects in fish on several physiological processes, such as increased plasma vitellogenin level or induced vitellogenesis.	Hajra *et al.*, 2013.
Saponins	Increased permeability of intestinal mucosal cells (enterocytes) increased infiltration of different cells and inhibit nutrient transport; Contain hydrophobic and hydrophilic segments that form micelles in an aqueous environment.	Damage the respiratory epithelium of the gills; Reduces fish growth performance.	Freeland *et al.*, 1985

2.9 Mulberry

2.9.1 Distribution and common mulberry tree species

The mulberry tree (*Morus nigra*) is a hardy, flowering, and perennial woody plant that belongs to the Moraceae family (Farahani, Salehi-Arjmand, Khadivi & Akramian, 2019; Ghosh, Gangopadhyay & Chowdhury, 2017). There are 24 identified *Morus* species and one subspecies, with a diverse gene pool of about 100 known cultivars (Kamiloglu, Serali, Unal & Capanoglu, 2013), spatially distributed in the temperate and tropical Northern Hemisphere and tropical Southern Hemisphere (Okatan, Polat & Aşkin, 2016). In addition, more than fifteen species of the *Morus* are found in temperate, tropical, and subtropical regions of Africa, Asia, and North America (Ghosh *et al.,* 2017). The most common mulberry species are *nigra, alba, rubera, cathayana, australis, mesosygia, atiopurpurea,* and *natabilis.* However, the three major species of the *Morus* genus are the black (*Morus nigra*), white (*Morus alba*), and red (*Morus rubra*) mulberry (Sânchez-Salcedo *et al.,* 2015). Globally, about ninety percent of the total raw silk production comes from mulberry trees, making the mulberry economically attractive in most rural communities. Moreover, mulberry is also used in producing several goods fit for human consumption, with its bark and wood found useful for manufacturing certain compounds and medicines (Ghosh *et al.* 2017).

2.9.2 A comparison of different mulberry fruits

Black mulberry fruits have a purple-to-black colour and are juicy with an acidic taste when fully ripe (Farahani *et al.,* 2019). They have an oval shape and measure between 1 to 4 mm in length when fully ripe (Chen *et al.,* 2016). The fruits are widely known for their tasty flavour, rich nutritive properties, and therapeutic value as traditional medicine (Darias-Martín, Lobo-Rodrigo, Hernández-Cordero, Díaz-Díaz & Díaz-Romero, 2003). Mulberry fruits consist of almost all the important minerals, essential amino acids, organic acids, fatty acids, and vitamins. Moreover, black mulberry fruits taste fruity, sweet, sour, musky, and woody (Iqbal, Khan, Jilani & Khan, 2010; Rohela, Shukla, Kumar & Chowdhury 2020) and contain phenolic acids, anthocyanins, and bioflavonoids, which have antioxidant properties (Okatan *et al.*, 2016). Other organic bioactive compounds found in these fruits are known to have anti-diabetic, anti-inflammatory, and anti-hyperlipidaemic properties (Sanchez-Salcedo *et al.,* 2015). In a study by Gundogdu, Muradoglu, Sensoy & Yilmaz (2011), chlorogenic acid and antioxidant compounds were found to be higher in black mulberry fruits compared to red and white mulberry fruits (Table 2.6). Phenolic compounds are defined by their small molecular structure having at least one phenol unit (Naiel, Alagawany, Patra, El-Kholy, Amer & Abd El-Hack, 2021). Anthocyanins are flavonoids that give off the colour of flowers, vegetables, and fruits, including red, orange, and blue. They are also considered the largest and most essential water-soluble phenolic pigments that give-off protons to highly reactive free radicals, thus rendering them very useful in the fight against several diseases that threaten animals (Shekarabi *et al.,* 2020). They scavenge for free radicals by directly inhibiting the oxidation of light lipoproteins (Rohela *et al.*, 2020), thus having the potential to increase food stability for the longer shelf-life of fish. Moreover, anthocyanins are classified as organic bio-colourants that naturally replace synthetic ones (Kamiloglu *et al.*, 2013).

Table 2.6: Physiochemical properties of red, white, and black mulberry fruits

Physiochemical property	**Black mulberry**	**White mulberry**	**Red mulberry**	**References**
pH	3.52	5.60	4.04	
Total acidity (%)	1.40	0.25	1.37	
Fat (%)	0.95	1.10	0.85	
Fe mg/100g	4.2	4.2	4.5	
Ascorbic acid mg/100 g	21.8	22.4	19.4	
Moisture (%)	72.6	71.5	74.6	
Mg (mg/100g)	106	106	115	
Total dry weight	27.0	29.5	24.4	Ercisli and Orhan (2006)
Calcium mg/100g	132	152	132	
K mg/100g	922	1668	834	
Total phenolics (mg QE/100 g	1422	181	1035	
Total flavonoids (mg QE/100 g)	276	29	219	
Antioxidant capacity (mol TE g/fw)	13.999	4.494	5.497	
Hunter b*	1.72	16.2	2.02	
Hunter L*	14.3	78.4	27.3	
Hunter a*	7.02	-13.6	8.55	
Ash g/100 g	0.50	0.57	2.45	Imran, Khan, Shah, Khan & Khan (2010)
Fiber g/100 g	11.75	1.47	-	
Glucose g/100 g/fw	7.748	6.864	6.068	
FRAP µmol TE/1 g fw	12.9	4.494	6.4	Gundogdu *et al.* (2011)
Fructose g/100 g/fw	5.634	6.269	5.407	
Citric acid g/100 g/fw	1.084	0.383	0.762	
Succinic acid g/100 g/fw	0.342	0.168	0.132	
Malic acid g/100 g/fw	1.323	3.095	4.467	
Total organic acid g/100 g/fw	2.951	3.983	5.812	
Total soluble solids %	11.60	7.27	19.20	Aljane & Sdiri (2016)
Total anthocyanins content C3G mg/g	719	911.8	109	Natić, Dabić, Papetti, Akšić, Ognjanov, Ljubojević & Tešić (2015)

2.9.3 Mulberry as a food/feed source

Black mulberry fruits are reported to be safe for human consumption in both fresh and dry states (Iqbal *et al.,* 2010). The fruits are commonly processed into several marketable products such as syrup, alcoholic beverages, ice cream, pulp, concentrates and juice, molasses, jam, and vinegar purposed for human consumption (Gundogdu *et al.,* 2011; Kamiloglu *et al.,* 2013; Okatan *et al.,* 2016; Farahani *et al.,* 2019). Traditionally, the mulberry tree has been cultivated for its leaves, which are used as diets for silkworms (*Bombyx mori* L.) for silk yarn production (Ghosh *et al.,* 2017). On the other hand, the mulberry tree itself has been used as an ornamental crop (Sânchez-Salcedo *et al.,* 2015). Traditionally, mulberry leaves have been long used as significant sources of protein in animal production (viz. serein and fibroin) and vitamins for silkworms. The silk harvested from the farmed silkworms is used to manufacture luxurious silk garments. On the other hand, scarcity of grazing pastures is a common problem in most developing countries associated with rapid deforestation, increased human population, and industrialization. Mulberry leaves and shoots are also used as dietary sources for young and late-aged silkworm instars. The mulberry leftovers such as twigs, leaf stalks, and shoots are deemed as waste materials during processing but can be potentially used as a nutritive feed source for domesticated herbivores (Ghosh *et al.,* 2017). Evidence suggests that plant-based products are a proven rich source of special bioactive compounds that possess health beneficial biological activities, among many, appetite and immune simulation, anti-pathogenicity, and growth promotion which can be harnessed in fish aquaculture (Reverter *et al.,* 2014). These potential benefits are due to the presence, in plant extracts, of phytochemicals alkaloids, tannins, glycosides, terpenoids, steroids, flavonoids, saponins, essential oils, phenolics, anthocyanins (Citarasu, 2010).

2.9.4 Mulberry as a pharmaceutical

Black mulberries are reported to have medicinal value, with most work focusing on their positive impact on human health. Indeed, several studies have shown that black mulberry fruits contain phytochemicals that exhibit antioxidant, anti-inflammatory, antiviral, hypolipidemic, neuroprotective, anti-hyperglycaemic, anti-HIV, cytotoxic, and anti-hypotensive properties (Ghosh, *et al.,* 2017). Furthermore, mulberry fruits have been shown to possess several significant health benefits: hepato- and reno-protective, eyesight improvement, joint-strengthening and anti-aging in humans (Lee *et al.,* 2013). Okatan *et al*. (2016) also found that black mulberry fruits can be used to treat fever, lower blood pressure, and facilitate urine discharge in humans. Further investigations, also in humans, by Chen *et al.* (2016) revealed that mulberries can also potentially treat weaknesses, fatigue, anaemia, and premature greying of hair.

2.10 Haematological and clinical biochemistry of Dusky kob

Blood parameters provide information on the status of health, levels of toxicity, and physiological derangements of cultured fish (Fazio, 2019). Haematological parameters can be used as a biological index to examine any pathological and physiological alterations in fish (Madibana & Mlambo, 2019). Blood parameters are crucial for the diagnosis and prognosis of diseases (Oliveira-Júnior, Tavares-Dias & Marcon, 2009). Studies that focus on haematological parameters provide information on fish health by observing blood cell counts and serum biochemistry (Reverter *et al.,* 2014; Sayed, Mahmound & Muhammad, 2020). In order to generate reliable and accurate results, drawn blood samples must be placed in ethylenediaminetetraacetic acid (EDTA) or lithium heparin coated blood collection tubes although lithium heparin is preferable as it has been reported to have the least possible risks of causing artefacts in the blood sample (Fazio, 2019). Coating with sodium heparin has been found to change the plasmic ionic composition, while EDTA changes red blood cells morphology but is widely used in fish (Fazio, 2019). The accuracy of the cell counts in the diagnostic haematology is one of the most challenging aspects (Hoffman-Lehmann, Holznagel, Ossent & Lutz, 1997), however, similar techniques used for mammals can also be applied in for fish with slight modifications (Hoffman *et al.,* 1997). Moreover, manual assessment of blood parameters is favoured by most researchers since it eliminates the confusion between thrombocytes, nucleated erythrocytes, and (total leukocytes) (Oluyemi, Adeparusi & Olanrewaju, 2008; Lorenz, Barone, Franca, Sabioni, Koch & Cyrino, 2018).

2.11 Aquaculture production systems used for rearing Dusky kob

2.11.1 Recirculating Aquaculture System

The recirculating aquaculture system (RAS) is a ubiquitous system used worldwide for the commercial production of aquatic organisms. The RAS was developed as a production technology for fish farmed intensively, where water supply or access is largely limited (Martins, Eding, Verdegem, Heinsbroek, Schneider & Blancheton, 2010). The system can be used to culture both saltwater and freshwater aquaculture species. For maximum profits, mostly high-value fish are farmed using the RAS system. The Dusky kob has several attributes that make it suitable aquaculture species in a RAS system (Table 2.1). The RAS system ensures environmental sustainability, given that about 90 – 99% of the water can be reused. In addition, the system operator has control over water quality parameters such as temperature, salinity, water pH, and dissolved oxygen. The disadvantages are that the system requires a high initial capital investment, as well as constant and careful monitoring (Martins *et al.,* 2010; Badiola, Mendiola, & Bostock, 2012; Almeida, Magalhaes, Sousa, Borges, Silva & Blanquet, 2021).

2.11.2 Cage system

According to Masser (1988), cage aquaculture uses existing water bodies, whereby fish are kept in a cage or basket which allows free flow of water to pass through between the fish and the system. In South Africa, cage aquaculture is a relatively new concept for farming aquatic organisms. The commercial offshore cage systems from AKVA Group, Fusion Marine LTD and a few different designs procured from China were the most used cage systems in South Africa (Viljoen, 2019). The technology needed to run a successful cage system in more challenging environments is difficult compared to RAS system. There are only two operational cage systems in South Africa focused on commercial Dusky kob production using offshore cages. The two operations were considered as essential pilot studies coordinated by Stellenbosch University. (Viljoen, 2019). In considerations of the cage system there are several pros and cons farmers need to contend with (Masser,1988). The pros are that (1) the system can be established on many different water bodies (dams, lakes, sea, river, ponds, pits, strip, and reservoirs), (2) requires low initial capital investment compared to the RAS, (3) simplified harvesting, as fish are trapped in one area, and (4) simplified observation and sampling of fish unlike the pond system water may often be murky causing difficulty in spotting fish in the water. The cons are as follows (1) feed must be kept fresh and adequate, (2) fish may suffer from low oxygen syndrome which may require the farmer to implement mechanical aeration, (3) pond fish make use of readily available naturally food, while with the cage system fish have limited access to naturally occurring food; thus, cannot forage for themselves but need to be supplied with feed, (4) feed refusals and metabolic waste may potentially lead to eutrophication (5) the system is highly vulnerable to poaching as fish are confined in one area (Halide, Stigebrandt, Rehbein & McKinnon, 2009).

2.11.3 Pond system

Ponds are one of the several production systems used to farm aquatic organisms such as aquatic plants, fish, and other aquatic animals worldwide. Traditionally, extensive production systems are often used for the farming of more the one aquaculture species. Ponds are often enriched with a balanced application of fertilisers to improve the natural productivity of the water. The purpose of nutrient enrichment is to support the proliferation of algae (Viljoen, 2019). In addition, fertilization is usually achieved by administering animal manure (i.e., cow dung) into to aquatic ecosystem. Some farms also use pigsties or chicken cops suspended above the water. In South Africa, Zini Fish Farms (Pt) Ltd, based in KwaZulu Natal, makes use of earthen pond system to farm Dusky kob commercially (Viljoen, 2019).

2.12 Fillet quality for human consumption

The fish fillet quality is the most important aspect of human consumption. Human growth, key organ maturation and health is directly influenced by the type and quality of food ~~humans~~ consumed (Chen *et al.,* 2022). Food quality involves a complex set of attributes influenced by numerous endogenous and exogenous factors (Marina *et al.,* 2017). The nutrients contained in fish diets are usually transferred to the fish fillet. Therefore, the quality of raw feed ingredients used in fish feed formulations are strictly associated with the quality of the final product (Schafberg, Loest, Müller-Belecke & Rohna, 2020). Nevertheless, the nutritional status and organoleptic properties in combination with freshness give fish quality perception (Marina *et al.,* 2017).

Consumers tend to value more of wild fish compared to cultured fish as he former are considered to be more naturally grown. However, fillets from both wild fish and farmed fish contain a diverse range of nutrients that make them an important sources of nutrients in human foods worldwide (Chen *et al.,* 2022).

Human consumption of fish has been associated with several metabolic and physiological health benefits (Marina *et al.,* 2017). Consumption of fish-based diets has been proven to contain several health benefits such as cardio-, hepato- and neuro-protection, promotion of wound healing, , anti-oxidation, and anti-inflammation properties (Chen *et al.,* 2022). These health benefits are ascribed to presence of a wide range of essential nutrients (omega-3 PUFA and vitamins D and B_2) with functional properties. Omega-3 fatty acids exhibit antioxidant and anti-inflammatory properties (Chen *et al.*, 2022). Fish peptides and hydrolysates have been commonly investigated for antioxidant activities (Chen *et al.,* 2022). A lipidomic investigation on *Sparus aurata* and *Dicentrarchus labrax* as carried out by Costa, Albergamo, Piparo, Zaccone, Capillo, Manganaro, Dugo & Mondello (2016) revealed that wild fish contain higher quantities of omega-3 fatty acids compared to cultured fish, however, higher concentrations of omega-6 and oleic acids are found in cultured fish due to feed supplementation. Nettleton & Exler (1992) found that when wild fish and cultured Coho salmon, red swamp crayfish, channel catfish, rainbow trout, white river crayfish and eastern oysters were analysed (raw and cooked) for proximate composition and vitamin content, farmed catfish showed 5 – 2.5 times fatter than their wild fish counterparts. When compared to that from wild fish, the lipid content of fillets derived from farmed fish is more constant and less affected by seasonal variations and closely mirror the dietary fatty acid profile (Cahu *et al.,* 2004). Generally, farmed fish have a higher total lipid content than wild fish, and contain higher amounts of docosahexaenoic acid and eicosapentanoic acid per 100g of fillet (Cahu *et al.,* 2004). However, it is essential to note that sensory analyses performed by trained consumer panellists (flavour, aroma, aftertaste and odour) have not reported any differences between wild and farmed fish (Cahu *et al*., 2004).

Fillet colour is another meat quality parameter that is determined by lightness (L*), yellowness (b*), and redness (a*), according to Kotzamanis, Kumar, Tsironi, Grigorakis, Ilia, Vatsos (2020) and Skalechi, Florek, Pyc, Kaliniak & Staszowska (2016). The diets affect the sensory properties (colour, taste, texture) and freshness of fish fillets as found by Monge-Ortiz *et al.* (2020). In a study conducted by Shekarabi *et al.* (2019), incorporation of black mulberry juice powder (BMP) into rainbow trout fish diets as an organic pigment source resulting in fish fillets with higher redness (a*) and yellowness (b*) values but reduced lightness (L*). Thus, there is some research evidence suggesting that black mulberry has the potential to affect fillet quality and consumer acceptability positively.

2.13 Summary

The sustainability of Dusky kob aquaculture is under threat due to a variety of stressors induced by intensive rearing conditions, high feeding costs, and the use of chemotherapeutic agents loathed by consumers. To ensure an economically, environmentally, and socially sustainable Dusky kob farming enterprise in South Africa, it is important to search and develop plant-based feed additives that can be harnessed to increase production efficiency while enhancing fish products shelf stability and safeguarding consumer health. Evidence suggests that growth of the fish industry has been hindered by high feed costs due to over-reliance on fishmeal as a feed ingredient and fortification of feeds with antibiotic growth promoters, exogenous enzyme supplements and synthetic antioxidants (Caipang *et al.*, 2019). Utilising locally available plant products such as black mulberry fruits powder that is rich in phytochemicals with health beneficial biological activities (Citarasu, 2010), can reduce the cost of feeding Dusky kob while promoting good environmental and human health. However, to my knowledge there has been no scientific investigations into the use of the black mulberry fruits as a nutraceutical source in Dusky kob to boost growth performance and improve fillet quality destined for human consumption. Therefore, this study is designed to plug this gap and provide information on the utility of black mulberry fruit powder as a nutraceutical in juvenile Dusky kob fish.

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3 CHAPTER THREE: FEED UTILIZATION, GROWTH PERFORMANCE, AND HAEMO-BIOCHEMICAL PARAMETERS OF JUVENILE DUSKY KOB OFFERED DIETS CONTAINING BLACK MULBERRY FRUIT POWDER

Abstract

Black mulberry (*Morus nigra*) fruit powder (BMFP) has bioactive compounds that can potentially enhance feed utilization, growth performance and health of fish. However, the utility of this nutraceutical source has yet to be evaluated in Dusky kob aquaculture. Therefore, the current study investigated the effect of incremental levels of dietary BMFP on feed utilization, growth performance and haemo-biochemical parameters in juvenile Dusky kob (*Argyrosomus japonicus)* in a recirculating aquaculture system (RAS) at the Marine Research Aquarium, Sea Point, Cape Town. One thousand and five hundred fish (initial body weight: 80 ± 4.48 g, initial fish length: 17 ± 0.30 cm) were procured from Kingfish Enterprise, a private hatchery from (East London, South Africa). The experimental system included 20 polyethylene tanks (experimental units), with each carrying 65 fish. Four isonitrogenous and isoenergetic experimental diets formulated by including BMFP in a Dusky kob commercial diet at 0 (BMFP0), 5 (BMFP5), 10 (BMFP10), and 12% (BMFP12) w/w. The four diets were randomly allocated to the 20 experimental tanks and offered to fish (with two instalments) at 2.8% live weight/day over a four week feeding pereiod. Twenty fish were randomly selected from each tank for weekly fish weight and length measurements to track growth performance. At the end of the four-week feeding trial, blood samples were collected under anaesthesia from five juvenile Dusky kob fish, randomly selected from each experimental tank for haemo-biochemical analysis. Diet × week interaction did not ($p > 0.05$) show any effect on feed intake, feed conversion ratio (FCR) from weeks 1 – 3, and specific growth rate (SGR), but significantly ($p < 0.05$) influenced weight gain for weeks 2 and 4, and FCR in week 4. Weight gain in week 2 showed a positive linear trend [$y = 7.90(\pm 1.992) + 0.24(\pm 0.843)x$; $R^2 = 0.3062$; $p = 0.0136$] in response to incremental levels of BMFP. From the observed results experimental diets influenced ($p < 0.05$) lymphocyte, monocyte, and eosinophil counts, but did not ($p > 0.05$) influence haematocrit, thrombocytes, neutrophils, and basophils. Quadratic trends were observed for lymphocytes [$y = 83.70(\pm 2.984) + 3.21(\pm 1.263)x - 0.26(\pm 0.102)x^2$; $R^2 = 0.2784$; $p = 0.0201$], and monocytes [$y = 11.41(\pm 1.459) - 1.92(\pm 0.617)x + 0.11(\pm 0.050)x^2$; $R^2 = 0.1520$; $p = 0.0352$], whereas a positive linear trend for eosinophil counts [$y = 1.60(\pm 0.701) - 0.23(\pm 0.296)x$; $R^2 = 0.3258$; $p = 0.0068$] were observed in response to incremental levels of BMFP. In conclusion, the results show that Dusk kob fish fed BMFP-based diets neither had enhanced feed utilization and growth performance nor compromised growth performance and feed utilization efficiency. The results reveal that weight gain and FCR of the fish was not significantly enhanced in response to BMFP-containing diets. Moreover, BMFP-containing diets increased lymphocytes, eosinophils, and blood urea, but reduced monocyte counts. From the quadratic responses observed it was determined that optimal dietary BMFP levels for lymphocytes, monocytes and blood urea were 6.1, 8.4, and 7.5%, respectively.

Keywords: Black mulberry fruit powder; Dusky kob; Feed utilization; Growth performance; Blood parameters

3.1 Introduction

Globally, commercial aquaculture is the fastest-growing animal food production enterprise because fish are an essential source of amino acids, vitamins, minerals, omega-3 fatty acids and highly digestible energy (Doĝan & Ertan, 2017) for humans. Aquaculture and wild fisheries cater for about 17% of the global animal protein needs, while in some developing countries, the contribution is more than 50% (Fry, Mailloux, Love, Milli & Cao, 2018). Due to a fast-growing human population, it is estimated that an additional 23 million tons of aquatic food products need to be produced to satisfy the current level of per capita consumption (Giri, Sukumaran & Park, 2019). To meet this rapidly increasing demand for fish and other fish products, aquaculture continues to expand to complement wild fisheries in bridging the gap between supply and demand (Lugert, Thaller, Tetens, Schulz, Krieter, 2014). The expansion of the aquaculture industry, while enhancing food and nutrition security has the potential to alleviate poverty and contribute to national economic growth. However, South African Dusky kob aquaculture is still lagging as it faces several challenges that need to be overcome to ensure that it becomes socially, economically, and environmentally sustainable (Madibana, Mlambo, Lewis & Uys, 2020). Dusky kob aquaculture depends mostly on commercially formulated feeds, which constitute over 50% of total production costs and thus negatively affect economic sustainability (Djissou, Adjahouinou & Koshio, 2016; Fry *et al.,* 2018). In addition, feed unavailability, rising feed costs, and deteriorating quality of feed ingredients are significant challenges facing most modern aquaculture production (Bhaskar & Pynne, 2015). Fishmeal and fish oil are essential primary sources of proteins and lipids in aquafeeds, respectively. However, the increased demand and erratic supply have resulted in a limited supply and high market prices of fishmeal and fish oil.

Intensive fish production systems employed for aquaculture expose fish to several stressors resulting in widespread disease outbreaks. As a result, antibiotics and chemotherapeutics have been widely used in commercial aquaculture to forestall the proliferation of fish pathogens (Citarasu, 2009) and to cut production costs by improving feed utilization efficiency. Unfortunately, the prolonged use of synthetic prophylactics to combat fish pathogens is also responsible for the emergence of microbial drug resistance and the presence of chemical residues in fish products destined for human consumption (Tang, Cai, Liu, Wang, Lu, Wu & Jian, 2014). The emergence of drug-resistant pathogens undermines the efficacy of synthetic antibiotics and chemotherapeutics (Cabello, 2006), reduces profit margins due to high fish mortalities (Giri *et al.,* 2019) while compromising human health. Consequently, the search for natural alternatives to synthetic antibiotics in aquaculture has gained momentum. The focus is on natural and readily available feed ingredients (mostly of plant origin) that can positively influence fish growth performance, feed utilization and health status (Reverter, Bontemps, Lechinni, Banaigs & Sasal, 2014; Thorarensen, Kubiriza & Imsland, 2015; Abdel-Latif, Abdel-Tawwabb, Khafaga & Dawood, 2020; Asgari-Kafrani, Fazilati & Nazem, 2020). Locally available plant products can be sources of nutrient and pharmaceutical compounds beneficial to fish (Caipang *et al.,* 2019).

One such plant-based potential feed additive is the black mulberry fruit (*Morus nigra*), whose consumption has been associated with human health benefits such as protection against kidney and liver damage, improved eyesight, strengthening of joints, and anti-aging effects as found by Farahani *et al.* (2019). Mulberry fruits contain alkaloids, phenols, anthocyanins, polysaccharides, and flavonoids (Sânchez-Salcedo, Mena, Gracîa-Viguera, Hernandez & Martinez, 2015; Khalifa, Zhu, Li & Li, 2018; Wen, Hu, Linhardt, Liao, Wu & Zou, 2019) phytochemicals with appetite- and immune-stimulation, anti-pathogenic, and growth promoting activities (Reverter *et al.*, 2014) which can be exploited in aquaculture production. The use of locally available black mulberry fruits has the potential to boost feed utilization efficiency, enhance product quality and stability, and reduce the cost of feeding Dusky kob while promoting environmental and human health. While there have been a few studies reporting on the nutraceutical benefits of black mulberry products in fish diets (Yilmaz, Ergon, Yigit, Yilmaz & Ahmadifar, 2020; Shekarabi, Omidi, Dawood, Avazeh, Heidari, 2019), none of them have investigated the utility of dehydrated black mulberry fruits as a nutraceutical source to boost growth performance and health of Dusky kob fish. Therefore, the current study sough to determine the effect of fortifying commercial Dusk kob fish diet with incremental levels of BMFP on feed utilization, growth performance, and haemo-biochemical parameters in juvenile Dusky kob. In addition, the study sought to determine an optimal dietary inclusion level of BMFP based on the response parameters. The study tested the hypothesis that dietary inclusion of BMFP would significantly improve growth performance, feed utilization, and haemo-biochemical parameters in juvenile Dusky kob.

3.2 Materials and methods

3.2.1 Ethical statement

The study was conducted in compliance with the South African Animals Protection Act of 1962 (Act 71 of 1962). Ethical clearance was provided by the Aquaculture Animal Ethics Committee (AAEC) at the Fisheries Branch of the Department of Forestry, Fisheries and Environment (DFFE) with approval number: **20201111_dk_01_Mlambo,** and by the Animal Research Ethics Committee of the University of Mpumalanga with approval number: **FANS20**.

3.2.2 Experiment system

The study was carried out at the Marine Research Aquarium (33.9169°S, 18.3875°E), Department of Forestry, Fisheries and the Environment (DFFE) at Sea Point, Cape Town, South Africa. The recirculating aquaculture system (RAS) previously described by Madibana

& Mlambo (2019) was used. The experimental system and filtration components are shown in Plate 3.1. Madibana *et al.* (2020) reported that the RAS uses seawater (24 – 26° C) carrying dissolved oxygen at 55-60 mg/L. The filtration system includes a biological media, protein skimmer, and sand filter. The system also uses ultraviolet lights (55 w) mounted on the water route between the experimental tanks and the filtration system is installed to reduce bacterial loads within the system (Madibana, Mlambo, Lewis & Fouchê, 2017). The system is supplied with pressurised water through 25 mm valve-controlled inlets as a horizontal spray bar or vertical downpipe with a terminal elbow to stir the water at the bottom tank for self-cleaning. The system also has a central 75 mm outlet with up-flow and vertical standpipe as a water level controller in tanks. The second connection is a standpipe connected to up-flow and pipe with a down-flow line linked to the return line from the system tanks to the filtration system. The bottom part of the up-flow standpipe is connected to an exit valve (Y-piece reduction to 32 mm valve). The function of this valve is to remove organic sediment that is too heavy to be removed via the up-flow vertical standpipe. Moreover, the water flow within the tanks was adjusted at an average of 2000 L per hour and 1200 L per hour of top-up water in the sump.

Plate 3.1: Experimental system showing the experimental tanks and the filtration components: (A) Foam fractionator; (B) Degassing chambers; (C) Sand filter; (D) Media for chemical filtration (E) A view of the 20 experimental tanks; (F) Biological filtration media

3.2.3 Black mulberries

Fresh mature black mulberry fruits were harvested from trees growing in Senwabarwana town (23.271°′S 29.123°′E) town, Limpopo, South Africa. The area has a hot semi-arid (Steppe) climate and loam soil with an average annual rainfall of ± 250 ml and average temperatures of 17°C (min) and 36°C (max). The ripe fruits, presenting with a deep purple-black colour, were hand-harvested. Clean plastic sheets were placed directly underneath the canopy of mulberry trees, followed by vigorous shaking of the trees to dislodge fruits onto the plastic sheeting. Immediately thereafter the ripe berries were manually selected and placed in cooler boxes containing ice before being transported to the laboratory where they were oven-dried at 60°C until constant weight. Thereafter, the dehydrated fruits were milled using a milling machine (Artlon Gold Mixer, Daesung Artlon Co., Poju, Korea) into a powder [black mulberry fruit powder (BMFP)] and stored at - 20°C.

3.2.4 Experimental fish

One thousand five hundred Dusky kob fingerlings (initial live weight: 80 ± 4.48 g; initial fish length: 17 ± 0.30 cm) were procured from Kingfish Enterprise (East London, Eastern Cape). The Dusky kob fish fingerlings were transported to the Marine Research Aquarium experiment site under anaesthesia (40 mg of oil per litre of water) as described by Madibana and Mlambo (2019). Upon arrival, the fish were weighed and then randomly and evenly transferred into the 20 RAS tanks (34 ppt salinity, 25°C water temperature and 60 mg/l dissolved oxygen) as described by Madibana *et al.* (2017). The fish were adapted to experimental conditions for 14 days before the commencement of the feeding trial. During this adaptation period, a commercial Dusky kob diet (SA feeds (Pty) Ltd) was offered to the fingerlings.

3.2.5 Experimental diets

Four iso-nitrogenous (~ 465.12 g/kg crude protein) and iso-energetic (~ 15.2 MJ/Kg ME) diets were formulated at SA feeds (Pty) Ltd (Hermanus, South Africa) by adding black mulberry fruit powder (BMFP) to a commercial Dusky kob diet at 0 (BMFP0), 50 (BMFP5), 100 (BMFP10), and 120 g/kg (BMFP12). After formulation, the mash diets were transformed into flakes at the Marine Research Aquarium, Sea Point, Cape Town, South Africa. To this end, diets were separately weighed into an empty bucket and mixed with water to form a paste. The paste was kneaded to produce a dough that was then thinly spread onto black flat boards. The thin layers were then sun-dried (~12 hours) to constant weight. Using a kitchen specular, the dried thin layers were scrapped off from the blackboards into flakes that were used to feed fish. The four diets were randomly allocated to 20 tanks, with each replicate tank carrying 65 juvenile Dusky kob fish. The diets and black mulberry fruit powder were analysed for dry matter, ash, moisture, crude protein, crude fat, gross energy, amino acid profile, mineral composition, and total phenolic content.

3.2.6 Chemical analyses

Black mulberry fruit powder and formulated diets were sampled for chemical characterisation. Dietary ingredients (gross composition) and proximate composition of formulated diets are shown in Table 3.1. Dry matter (DM), ash, crude protein (CP), crude fat, and gross energy were assayed according to the Association of Official Analytical Chemists (AOAC, 2010) methods. Dry matter content assay was determined by oven-drying at 105°C until all free water evaporated as indicated by the attainment of constant weight (AOAC method number 930.15). Organic matter (OM) was determined as the loss in weight upon incineration in a muffle furnace at (550°C) for 12 hours (AOAC method number 942.05). Crude protein was determined following the micro-Kjeldahl method using the Kjeltec system through digestion, distillation, and titration steps (AOAC method number 2001.11). The crude fat content was evaluated using the ANKOMXT15 fat extractor by refluxing samples with petroleum ether, an organic solvent. The residues after extraction were weighed and crude fat was determined by difference from the initial sample weight (AOAC method number 2003.05). Gross energy assay was performed by weighing samples and incinerating them in an oxygen bomb calorimeter with benzoic acid as a standard (AOAC method number 971.33).

Bioactive compounds of formulated diets and absolute BMFP are presented in Tables 3.2. Duplicate procedure was followed using a ratio of 1:5 (20g: 100 ml). Samples were milled using a mill machine using a sieve of 0.5 mm. After that, 20 g of each sample was mixed with 100 ml of distilled water. The mixture was poured into a glass beaker and covered with aluminum foil because the samples were light sensitive. Thereafter, using the magnetic stir the samples were stirred for 2 hours with water. Each mixture was poured into four centrifugal conical test tubes and rotated at 5000 rpm.

Table 3.1: Gross ingredient and proximate composition of experimental diets containing black mulberry fruit powder (BMFP)

	Diets[1]			
	BMFP0	**BMFP5**	**BMFP10**	**BMFP12**
Ingredients (g/kg)				
Black mulberry fruit powder	0.00	50.00	100.00	120.00
Fishmeal 65	300.00	285.00	270.00	264.0
Maize	187.1	177.7	168.4	164.7
Soya oilcake	100.00	95.00	90.00	88.00
Full fat soya 58	85.00	80.80	76.50	74.80
Blood meal 90	60.00	60.00	57.00	52.80
Maize gluten	109.10	103.60	98.20	96.00
Pork meal 28	81.70	77.60	73.50	71.90
Fish and poultry oil	50.40	51.00	48.30	47.20
Vit/ Min Premix	23.50	23.50	23.50	23.50
The proximate composition				
Dry matter (g/kg)	877.60	833.30	850.00	877.60
Ash (g/kg)	86.30	105.60	247.10	107.40
Moisture (g/kg)	122.40	166.70	145.00	119.40
Crude Fat (g/kg)	121.52	75.00	135.20	107.40
Crude Protein (g/kg)	465.80	415.70	421.60	405.90
Gross energy (Kcal/kg)	4276.26	4302.83	4245.78	4138.14

[1] Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg.

Table 3.2: Bioactive compounds (mg/g DM) in black mulberry fruit powder (BMFP) and BMFP-containing diets

	Diets[1]				
Compounds	**BMFP0**	**BMFP5**	**BMFP10**	**BMFP12**	**BMFP**
Acids					
Acetic acid	8.63	8.17	18.81	13.47	19.67
Butanoic acid	7.11	2.66	4.16	1.84	0.15
Hexanoic acid	0.00	0.60	0.93	0.46	0.70
Nonanoic acid	0.03	0.13	0.10	0.07	0.19
Pentadecanoic acid	0.37	0.14	0.38	0.21	0.16
Tetradecanoic acid	6.28	1.40	4.22	2.28	0.54
Hexadecanoic acid	2.35	16.35	35.91	21.47	16.02
Pentanoic acid	0.13	0.36	0.58	0.30	0.25
4-methyl-valeric acid	0.09	0.69	1.06	0.50	0.09
Larixic acid	0.13	0.28	0.45	0.25	0.42
Ketones					
Methyl pyrrol-2-yl ketone	0.0	0.13	0.29	0.16	0.86
Alletone	0.0	0.49	1.45	0.19	1.74
1-hydroxy-2-propanone	0.0	0.35	0.85	1.02	1.86
Alcohols					
5-methyl-2-Furanmethanol	0.0	65.37	0.36	0.22	0.50
2-Furanmethanol	0.0	0.24	1.47	0.92	5.85
Other compounds					
5-methyl-furfural	0.0	0.00	0.16	0.14	1.58
2-Cyclopentene-1,4-dione	0.0	0.08	0.41	0.26	1.03
Butyrolactone	0.0	0.00	0.37	0.14	0.77
Pyranone	0.45	7.89	19.65	11.51	48.99
Hydroxymaltol	0.0	0.64	1.02	0.67	2.38

[1] Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg.

3.2.7 Amino acid profile of diets

Analysis of amino acid profile in treatment groups was performed at the University of Limpopo, using HPLC as described by Dimova (2003) and Gheshlaghi *et al.* (2008). Serine, arginine, histidine, glutamic acid, alanine, leucine, glycine, tyrosine, threonine, phenylalanine, aspartic acid, valine, proline, methionine, tryptophan, isoleucine, and lysine were assayed and expressed in g/100 g. Table 3.3 shows the amino acid profile of experimental diets.

Table 3.3: Amino acid composition (g/100 g DM) of formulated diets and black mulberry fruit powder (BMFP)

	Diets[1]				
Amino acid	**BMFP0**	**BMFP5**	**BMFP10**	**BMFP12**	**BMFP**
Lysine	3.33	3.75	3.06	3.17	2.49
Methionine	1.04	1.13	1.08	1.00	0.96
Valine	2.71	2.84	2.35	2.42	2.11
Histidine	0.89	2.51	2.83	0.58	1.96
Leucine	4.37	4.60	3.82	3.97	3.25
Isoleucine	2.32	2.50	2.07	2.13	1.99
Arginine	3.36	3.67	3.00	3.10	2.33
Phenylalanine	2.34	2.54	2.16	2.19	2.09
Threonine	2.05	2.26	1.83	1.88	1.23
Tyrosine	1.60	1.74	1.60	1.79	1.42
Proline	3.01	3.28	2.66	2.76	2.41
HO-Proline	0.59	0.56	0.44	0.39	0.25
Serine	2.31	2.87	2.57	2.27	2.02
Aspartic acid	4.33	4.80	3.91	4.02	3.33
Alanine	3.13	3.44	2.73	2.77	2.23
Glycine	3.62	3.91	3.16	3.14	3.12
Glutamic acid	7.36	8.01	6.47	6.82	6.55

[1] Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg.

3.2.8 Mineral composition of diets

One gram of each treatment sample was digested in concentrated nitric acid using a microwave oven MarsXpress (CEM Corporation, Matthews). The digested samples were diluted in 25 mL of distilled water inside polypropylene tubes. The concentration of macro-elements (sodium, calcium, sulphur, phosphorus, potassium, and magnesium) and micro-elements (zinc, fluorine, cobalt, selenium, iodine, iron, manganese, and copper) were determined by flame atomic absorption spectrometry (FAAS; air-acetylene flame). The mineral composition of formulated diets and absolute black mulberry fruit powder is presented in Table 3.4.

Table 3.4: Mineral composition (mg/L dry weight) of formulated diets and black mulberry fruit powder (BMFP)

	Diets[1]				
Minerals[2]	**BMFP0**	**BMFP5**	**BMFP10**	**BMFP12**	**BMFP**
Ca	83.70	53.40	42.50	43.90	12.00
Cu	0.95	1.07	0.77	0.78	0.51
Fe	2.34	2.41	1.69	1.75	0.83
K	33.00	23.50	32.40	31.90	53.8
Mg	6.46	6.56	5.49	5.86	4.74
Mn	1.17	1.14	0.84	0.87	0.43
Na	12.80	17.00	8.73	8.52	1.46
P	34.30	29.90	20.90	21.70	2.63
Zn	1.44	1.19	0.94	1.00	0.36

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg. [2]Minerals: Ca = calcium; Cu = copper; Fe = iron; K = potassium; Mg = magnesium; Mn = manganese; Na = sodium; P = phosphorus; Zn = zinc.

3.2.9 Feed intake and growth performance

Dietary treatments were offered in two daily instalments (8 am and 3 pm) at 2.8% of fish live weight. Each diet was replicated five times, with each tank carrying 65 fish, being considered as the experimental unit. Twenty fish from each RAS tank (experimental unit) were randomly selected to determine initial body weight and length measurement at the onset of the experimental feeding trial. During the four-week feeding trial, twenty fish were randomly sampled from each tank weekly to determine the average fish weight gain (AWG) and length. Feed intake was calculated as the total amount of feed offered per fish per day because when fed at 2.8% feeding intensity, juvenile Dusky kob did not leave any feed refusals. Weight gain was calculated by subtracting the average live weight at the last measurement from the average live weight at the current measurement size.

The feed conversion ratio (FCR) was calculated using the following formula:

$$= \frac{total\ amount\ of\ feed\ consumed\ (g)}{total\ amount\ of\ fish\ weight\ gain\ (g)}$$

Specific growth rate (g/day) of Dusky kob was determined using the following formula:

$$= \frac{ln(Final\ weight) - ln(initial\ weight)}{Number\ of\ days}$$

3.2.10 Haematology and serum biochemistry

At the end of the four-week feeding trial, blood samples were collected under anaesthesia from five juvenile Dusky kob fish, randomly selected from each experimental tank. About 400 mg of clover oil/L of water was used to euthanize fish prior to blood sampling according to the guidelines described by the American Veterinary Medical Association (AVMA, 2020). The clove oil was mixed with warm water over a 5-minute period. Fish were then moved from experimental tanks into smaller buckets containing euthanizing agent (clover oil) and remained in the solution for at least 10 minutes. This was followed by ablation of the caudal fin to bleed the fish into two types of collecting tubes: one that contained the anticoagulant, ethylenediaminetetraacetic acid (EDTA) at 5 mg/ml of blood (blood meant for haematological analysis) and the other bottle with no coagulant (blood meant for serum biochemical analyses). Blood count was manually done using a light microscope (Olympus, Tokyo, Japan) under oil immersion at 100 × magnification. The analyses performed were for haematocrit, lymphocytes, thrombocytes, monocytes, neutrophils, basophils, and eosinophils. Serum biochemical parameters were analysed using blood samples from tubes that had no anticoagulant. Blood serum was pippeted into a clear sterilised bottle for further analysis. Serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) activities and creatinine, albumin, globulin, total protein (TP), cholesterol, triglycerides, and urea concentration were determined using standard methods as described by Reitman & Frankel (1957).

3.3 Statistical analyses

Growth performance and haemo-biochemical data were subjected to normality and homogeneity tests using the NORMAL option in the Procedure Univariate statement and Levene’s test, respectively. Data collected weekly from experimental units were analysed using repeated measures analysis to test for interactions between experimental diets and time (age of fish). Response surface regression (RSREG) analysis was done to mathematically define response curves of measured parameters as dietary BMFP levels increased. To estimate the optimum inclusion level of BMFP in Dusky kob diets the following quadratic model was employed: $y = a + bx + cx^2$, where y = response variable; a and b are the coefficients of the quadratic equation; c is intercept; x is mulberry fruit powder level (%) and $-b/2c$ is the x value that maximises/minimises response parameters.

The repeated measures analysis option of the general linear models (GLM) procedures of the Statistical Analysis System (SAS, 2010) was used for the weekly measurements of feed utilization and growth performance data. The following statistical linear model was employed:

$$Y_{ijk} = \mu + D_i + T_j + (D \times T)_{ij} + E_{ijk},$$

Where, Y_{ijk} = response parameter, μ = population mean, D_i = experimental diet effect, T_j = time effect (week), $(D \times T)_{ij}$ = Diet × time interaction effect, and E_{ijk} = random error.

The effect of experimental diets on overall feed utilization, overall growth performance, blood parameters and tissue nutrient composition was evaluated by the GLM procedures of SAS (2010) using the model:

$$Y = \mu + D_i + e_{ij},$$

Where, μ = the overall mean; D_i = the effect of experimental diet; and e_{ij}: the residual error. When experimental diets were detected to have a significant effect, means were separated using probability of difference (PDIFF) option in the Lsmeans statement. Statistical significance was declared at $P < 0.05$.

3.4 Results

3.4.1 Feed utilization and growth performance

The repeated measures analysis did not show a significant ($p > 0.05$) for feed intake (g/fish/day) and specific growth rate (SGR) were not significantly ($p > 0.05$) influenced by diets as shown in Table 3.5 While on the other hand, a significant interaction ($p < 0.05$) between diet × age of fish for weight gain (Table 3.6) and feed conversion ratio (FCR) (Table

3.7) was observed. The GLM procedure revealed that BMFP-containing diets (Table 3.6) significantly ($p < 0.05$) influenced weight gain in weeks 2 and 4 but not ($p > 0.05$) in weeks 1 and 3. In week 2, the effect of BMFP12 and control diet (BMFP0) on weight gain were significantly different. The results reveal that BMFP12 (16.4 g/fish/day) promoted the highest weight gain, while BMFP0 (7.5 g/fish/day) promoted the lowest weight gain as shown in Table 3.6. Dietary effects of BMFP5, BMFP10, and BMFP12 on weight gain were similar ($p > 0.05$), with no significant differences between their means. The means of weight gain for fish reared on control diet were similar ($p > 0.05$) to BMFP5 and BMFP10. The RSREG model revealed a positive linear trend [$y = 7.902(\pm 1.992) + 0.243(\pm 0.843)x$; $R^2 = 0.3062$; $p = 0.0136$] for weight gain (week 2) in response to incremental levels of dietary BMFP as depicted in Figure 3.1.

In week 4, weight gain for fish reared on BMFP5 (16.9 g/fish/day) and BMFP10 (7.3 g/fish/day) differed ($p < 0.05$). From the results it was revealed that BMFP5 promoted the highest weight gain compared to BMFP10. However, the effect on weight gain for fish reared on BMFP5 and BMFP10 were both similar to control diet and BMFP12. The RSREG did not reveal any significant ($p > 0.05$) pattern in week 4 for weight gain. Regarding feed conversion ratio (FCR), BMFP-containing diets only had a significant ($p < 0.05$) effect in week 4 (Table 3.6). The effect on FCR4 for BMFP10 differed ($p < 0.05$) compared to BMFP0 and BMFP5. BMFP10 (3.23) promoted the highest FCR, while BMFP0 (1.77) and BMFP5 (1.40) promoted the lowest FCR. It was also observed that the effect of BMFP12 on FCR was similar to BMFP0, BMFP5, and BMFP10. Table 3.7 shows that the RSREG model did not show any significant trend of FCR in response to BMFP-containing diets.

Table 3.5: Overall feed intake (g/fish/day) and specific growth rate (SGR) of juvenile Dusky kob fish in response to incremental levels of black mulberry fruit powder

	Diets[1]					Significance[3]		
Parameter	**BMFP0**	**BMFP5**	**BMFP10**	**BMFP12**	**SEM[2]**	**GLM[4]**	**Linear**	**Quadratic**
Feed intake	72.0	74.5	72.3	72.20	2.1	0.810	NS	NS
SGR[5] (%/day)	1.14	1.34	1.30	1.43	0.13	0.466	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean; [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [4]GLM: General Linear Model; [5]SGR; Specific growth rate.

Table 3.6: Weight gain (g/fish/day) of juvenile Dusky kob fish in response to incremental levels of dietary black mulberry fruit powder

	Diets[1]					Significance[3]		
Week	**BMFP0**	**BMFP5**	**BMFP10**	**BMFP12**	**SEM[2]**	**GLM[4]**	**Linear**	**Quadratic**
1	7.2	7.5	11.3	8.6	2.057	0.504	NS	NS
2	7.5^{a}	11.2^{ab}	10.9^{ab}	16.4^{b}	1.924	0.035	*	NS
3	7.8	9.0	9.50	8.4	2.041	0.946	NS	NS
4	11.6^{ab}	16.9^{b}	7.3^{a}	11.8^{ab}	1.687	0.010	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet; [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; * = $p < 0.05$; [4]GLM; General Linear Model; ^{a,b,c}Within rows means with similar subscripts do not differ ($p > 0{,}05$).

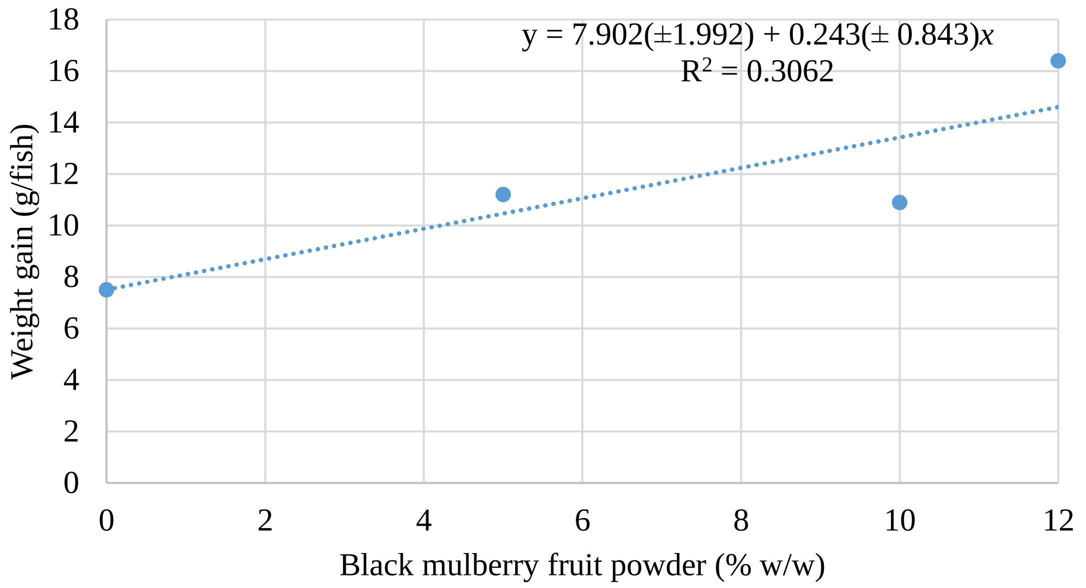

Figure 3.1: Weight gain in juvenile Dusky kob in response to incremental levels of black mulberry fruit powder.

Table 3.7: Weekly feed conversion ratio in juvenile Dusky kob in response to incremental levels of black mulberry fruit powder

	Diets[1]					**Significance[3]**		
Week	**BMFP0**	**BMFP5**	**BMFP10**	**BMFP12**	**SEM[2]**	**GLM[4]**	**Linear**	**Quadratic**
1	1.86	3.66	1.60	2.07	0.728	0.223	NS	NS
2	2.63	2.13	1.82	1.07	0.536	0.259	NS	NS
3	3.11	3.01	2.31	2.33	0.554	0.627	NS	NS
4	1.77^{a}	1.40^{a}	3.23^{b}	1.97^{ab}	0.353	0.012	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet; [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [4]GLM: General Linear Model; [a,b,c]Within rows means with similar subscripts do not differ ($p > 0.05$).

3.4.2 Hematological parameters

The GLM model showed that BMFP-containing diets significantly ($p < 0.05$) influenced lymphocytes, monocytes, and eosinophils, but not ($p > 0.05$) haematocrit, thrombocytes, neutrophils, and basophils in juvenile Dusky kob fish (Table 3.8). From the results, blood lymphocytes in BMFP10 and BMFP12 fish differed ($p < 0.05$). It was determined that BMFP10 promoted higher lymphocyte levels (93.8%) compared to BMFP12 (82.0%). However, fish fed BMFP12 and BMFP10 diets had similar ($p > 0.05$) lymphocyte counts as those fed BMFP0 and BMFP5 diets. Furthermore, blood monocytes in fish reared on BMFP5, BMFP10, and BMF12 diets were similar, while BMFP12 and BMFP10 fish also had a similar monocyte counts. Compared to BMFP0 with 11.2% monocytes, fish fed BMFP5 (5.4%) and BMFP10 (2.4%) had lower monocyte counts. The diet BMFP12 had a different effect on eosinophils compared to BMFP0 and BMFP5, but was similar to BMFP12. Treatment regimen BMFP12 (4.4%) promoted higher levels of eosinophils compared to BMFP0 (1.4%) and BMFP5 (1.6%), which had the lowest eosinophil counts. A positive linear trend [$y = 1.60(\pm 0.701) - 0.23(\pm 0.296)x$ $R^2 = 0.3258$; $p = 0.0068$] was observed for eosinophil counts, as dietary BMFP levels increased as depicted in Figure 3.2. Quadratic trends were observed for lymphocytes [$y = 83.70(\pm 2.984) + 3.21(\pm 1.263)x - 0.26(\pm 0.102)x^2$; $R^2 = 0.2784$; $p = 0.0201$] and monocytes [$y = 11.41(\pm 1.459) - 1.92(\pm 0.617)x + 0.11(\pm 0.050)x^2$; $R^2 = 0.1520$; $p = 0.0352$] as dietary BMFP levels increased as depicted in Figure 3.2. Based on the quadratic equation for lymphocytes and monocytes, it was calculated that 6.1 and 8.4% of BMFP were the optimal inclusion levels in Dusky kob diets, respectively.

Table 3.8: Haematology of juvenile Dusky kob in response to incremental levels of dietary black mulberry fruit powder

Parameter	**Diets**[1]					**Significance**[3]		
	BMFP0	**BMFP5**	**BMFP10**	**BMFP12**	**SEM**[2]	**GLM**[4]	**Linear**	**Quadratic**
Haematocrit (%)	42.7	40.8	40.0	43.1	1.497	0.4126	NS	NS
Thrombocytes (/hpf)	3.9	4.4	3.2	5.4	0.972	0.4845	NS	NS
Lymphocytes (%)	84.4^{ab}	90.8^{ab}	93.8^{b}	82.0^{a}	2.781	0.029	NS	*
Monocytes (%)	11.2^{b}	5.4^{a}	2.4^{a}	5.6^{ab}	1.460	0.005	*	*
Basophils (%)	1.8	1.6	0.6	1.2	0.570	0.481	NS	NS
Neutrophils (%)	1.0	0.8	0.0	0.4	0.316	0.160	NS	NS
Eosinophils (%)	1.6^{a}	1.4^{a}	3.2^{ab}	4.4^{b}	0.728	0.031	*	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet; [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [4]GLM: General Linear Model; [a,b,c]Within rows means with similar subscripts do not differ ($p >$ 0,05).

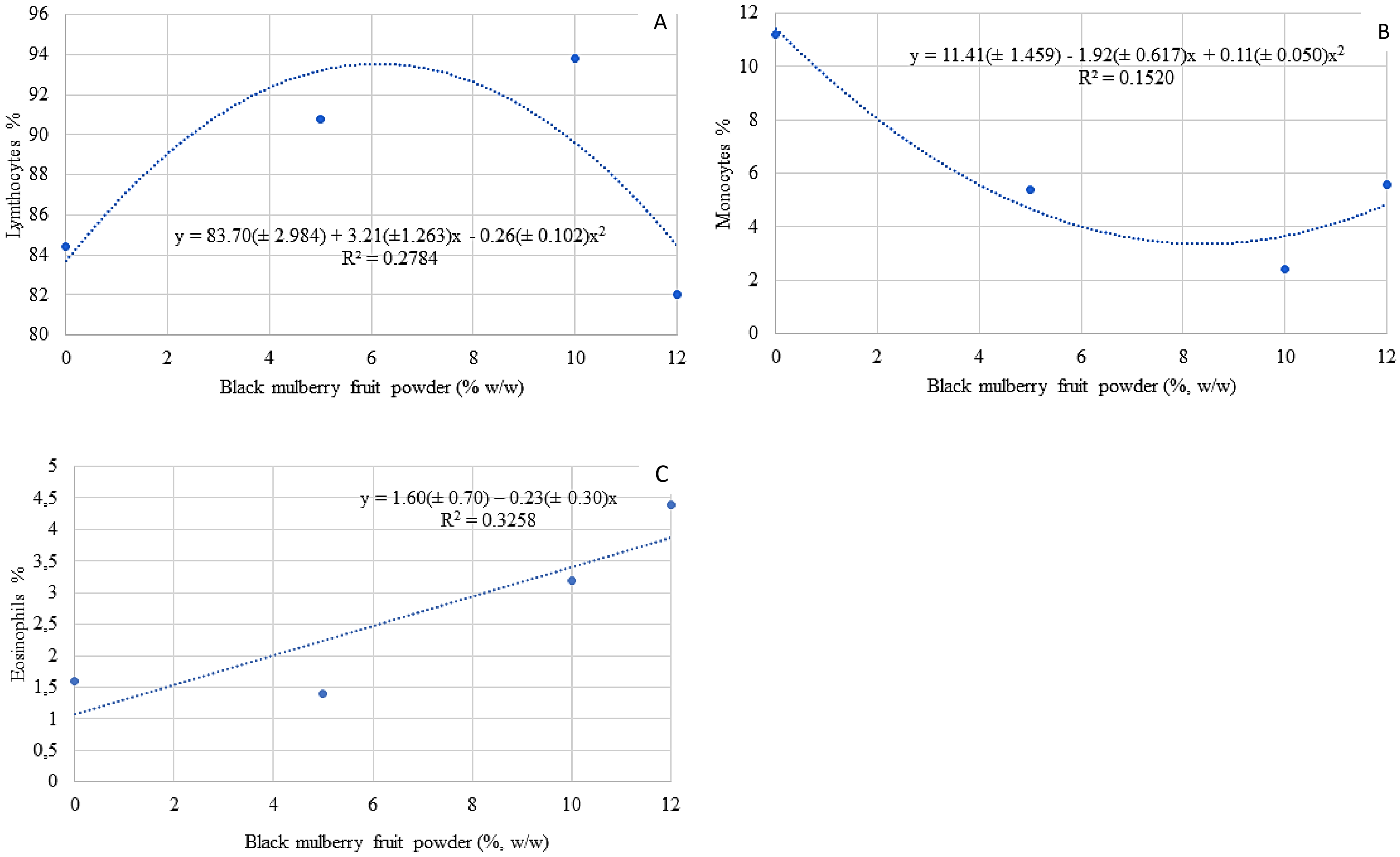

Figure 3.2: Changes in lymphocytes (A), monocytes (B), and eosinophils (C) in juvenile Dusky kob in response to incremental levels of black mulberry fruit powder

3.4.3 Serum biochemical parameters

The GLM procedure revealed that diets significantly influenced ($p < 0.05$) serum albumin, blood urea, globulin, triglycerides and cholesterol concentration as well as serum alkaline phosphatase, but not ($p > 0.05$) total protein (TP), triglycerides, alanine aminotransferase, aspartate aminotransferase activities. The inclusion of BMFP into Dusky kob diets significantly ($p < 0.05$) increased cholesterol concentration and alkaline phosphatase activity but reduced urea concentrations in the fish's serum. Table 3.9 shows that the blood urea levels in fish reared on BMFP0 significantly differed from those reared on BMFP-containing diets. The control diet (1.7 mmol/L) promoted higher ($p < 0.05$) blood urea concentration compared to BMFP5 (1.0 mmol/L), BMFP10 (1.0 mmol/L), and BMFP12 (1.2 mmol/L). Globulin levels of fish reared on BMFP10 differed compared to BMFP0 and BMFP5 fish, while BMFP12 had similar effect on globulins as BMFP0, BMFP5, and BMFP12. Table 3.9 reveal that BMFP10 (27.4 g/L) promoted the lowest levels of globulins compared to BMFP5 (30.2 g/L) and BMFP0 (30.6 g/L).

Regarding effects on serum cholesterol concentration, Table 3.9 shows that BMFP12 had a different effect compared to the control diet, however, the effect BMFP12 was similar to BMFP5 and BMFP10. While the control diet had a similar effect on serum cholesterol concentration of Dusk kob as BMPF10, findings showed that BMFP12 (2.6 mmol/L) and BMFP5 (2.5 mmol/L) promoted the highest ($p < 0.05$) serum cholesterol concentrations. Alkaline phosphatase (ALKP) in fish reared on BMFP0, BMFP5, BMFP12, and BMFP10 had similar effects. The fish reared on BMFP12 (54.0 U/L) had the highest serum ALKP activities and counterparts reared on the control diet had the lowest (41.0 U/L) ALKP activity. The RSREG model showed a negative linear trend was observed for blood urea [$y = 1.71(\pm 0.105) – 0.22(\pm 0.040)x + 0.01(\pm 0.004)x^2$; $R^2 = 0.3360$; $p = 0.001$] globulin [$y = 30.74(\pm 0.754) - 0.16(\pm 0.319)x$; $R^2 = 0.3882$; $p = 0.016$], and positive linear trends cholesterol [y =

2.14(± 0.092) + 0.09(± 0.039)x; $R^2 = 0.1904$; $p = 0.011$] and ALKP [$y = 40.72(\pm 3.126) + 3.26(\pm 1.320)x$; $R^2 = 0.1942$; $p = 0.062$] in response to incremental levels of BMFP as shown in Figure 3.3. Based on the quadratic equation for blood urea, it was calculated that 7.5% BMFP was the optimal level that resulted in the lowest serum blood urea concentration.

Table 3.9: Serum biochemistry of juvenile Dusky kob in response to incremental levels of dietary black mulberry fruit powder

Parameter[3]	**Diets**[1]					**Significance**[4]		
	BMFP0	**BMFP5**	**BMFP10**	**BMFP12**	**SEM**[2]	**GLM**[5]	**Linear**	**Quadratic**
Urea (mmol/L)	1.7^b	1.0^a	1.0^a	1.2^a	0.107	0.001	*	*
TP (g/L)	43.2	42.8	39.8	41.2	1.032	0.118	NS	NS
Albumin (g/L)	13.0	12.6	12.0	12.8	0.274	0.098	NS	NS
Globulin (g/L)	30.6^b	30.2^b	27.4^a	28.0^{ab}	0.735	0.016	*	NS
TRIG (mmol/L)	2.0	2.3	2.7	4.2	0.557	0.054	NS	NS
CHOL (mmol/L)	2.1^a	2.5^b	2.4^{ab}	2.6^b	0.088	0.011	*	NS
ALKP (U/L)	41.0^a	50.8^{ab}	54.0^b	48.6^{ab}	3.202	0.062	*	NS
ALT (U/L)	16.6	22.6	22.0	15.8	0.274	0.439	NS	NS
AST (U/L)	73.8	100.4	102.0	64.6	16.490	0.306	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet; [3]Parameter: TP = total protein; TRIG = triglycerides; CHOL = cholesterol; ALKP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; [4]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [5]GLM: General Linear Model; [a,b,c]Within rows means with similar subscripts do not differ ($p > 0.05$).

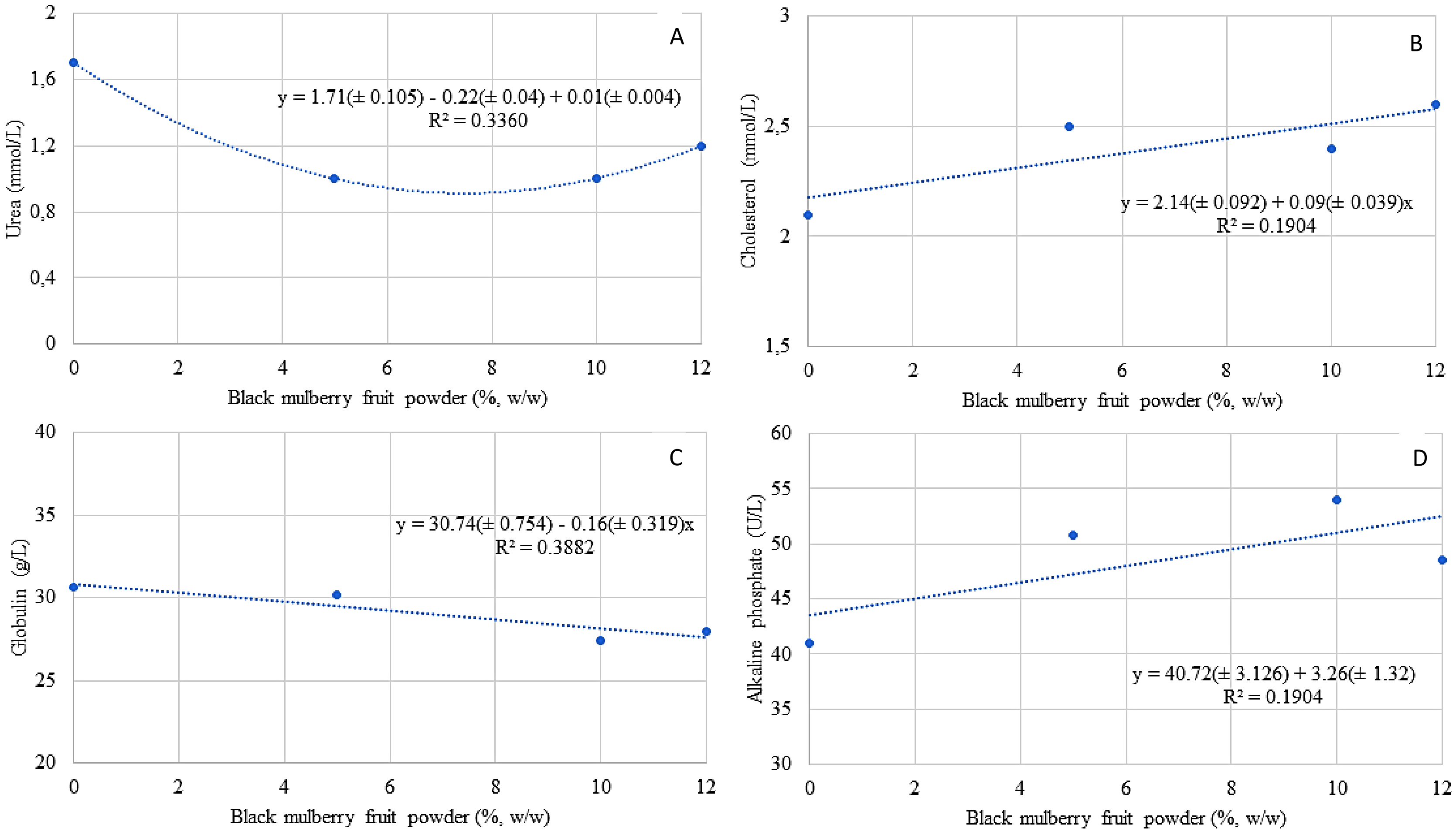

Figure 3.3: A – Blood urea, B – cholesterol, C – globulins, and D – alkaline phosphatase levels in juvenile Dusky kob blood serum in response to incremental levels of black mulberry fruit powder

3.5 Discussion

3.5.1 Feed utilization and growth performance

This study investigates possible effects of dietary supplementation with black mulberry fruit powder (BMFP) on feed utilization, growth performance, and blood parameters of juvenile Dusky kob. The potential utility of BMFP as an organic feed additive is based on the presence of beneficial bioactive compounds (Table 3.2), which could improve the health status and growth performance of Dusky kob fish. Black mulberry fruits and leaves have been reported to exhibit favourable biological processes such as appetite and growth stimulation, maturation of culture species and anti-pathogenicity in several animal models (Sharif & Pirsa, 2021; Jan, Parveen, Zahiruddin, Khan, Mohapatra & Ahmad, 2021; Li, Lu, Wu, Xiong, Luo, Ma & Liu, 2020; Yilmaz *et al.*, 2020; Shekarabi *et al.*, 2019; Khalid, Fawad & Ahmed, 2011). These biological benefits have been attributed to the presence in the BMFP of an array of unique bioactive compounds such as glycosides, terpenoids, saponins, alkaloids, flavonoids, phenolics, steroids, tannins, and essential oils (Reverter *et al.*, 2014). While BMFP is widely used for medicinal purposes (Lim & Choi, 2019), there has been no scientific reports on its use as a nutraceutical source in juvenile Dusky kob. Therefore, the current study evaluated the utility of this plant-based feed additive as a key component of an organic production strategy for Dusky kob.

Weight gain, FCR, and SGR are accurate growth performance parameters used to judge the potential use of natural feed additives in fish aquaculture. In aquaculture facilities, the growth performance of an organism is the most prominent factor that influences economic viability. In the current study, the expectation was that BMFP inclusion in juvenile Dusky kob diets would enhance feed utilization efficiency and growth performance by enhancing nutrient utilization in the digestive tract of fish through the action of various beneficial bioactive compounds (Shekarabi *et al.,* 2019). Indeed, the results from this study suggest that BMFP-containing diets enhanced weight gain (weeks 2 and 4) of juvenile Dusky kob fish, which is consistent with the results obtained by Shekarabi *et al.* (2019) and Yilmaz *et al.* (2020). However, SGR was not improved in the current study, possibly because feed intake was not increased and utilization was not enhanced .

There was no notable improvement in overall feed intake, FCR (1 – 3) and SGR, however a significant ($p < 0.05$) improvement in weight gain (weeks 2 and 4) and FCR in week 4 was observed in response to BMFP-containing diets. Indeed, a positive linear increase in weight gain in weeks 2 (Figure 3.1) was observed as the level of dietary BMFP increased, suggesting a positive influence of the nutraceutical source on growth of juvenile Dusky kob. However, this positive effect was not maintained in weeks 1 and 3, where feed utilization and growth performance were similar between control fish and those reared on BMFP-containing diets. Nevertheless, the evidence suggests an improvement on growth performance was expected. It is also evident that incremental levels of BMFP did not compromise the growth performance of Dusky kob. This is an important finding given that BMFP bioactive compounds may still positively influence fish product quality and stability.

Results for weight gain in week 2 (Table 3.6) from this study, are in agreement with the evidence provided by Yilmaz *et al.* (2020) that shows that dietary supplementation with black mulberry juice syrup positively promoted the growth performance of tilapia fish. This physiological benefit owes to the presence of flavanols, anthocyanins, and also cinnamic and benzoic acid derivates in black mulberry fruits. These compounds have been reported in enhancing the regulation of intestinal flora by activating digestive enzymes, or increasing nutrient intake (Yilmaz *et al.,* 2020). Similarly, Shekarabi *et al.* (2019) found that growth performance of rainbow trout was positively affected when offered diets enriched with black mulberry juice. In that study, weight gain and SGR were significantly increased while FCR was reduced, similarly weight gain was enhanced by BMFP-containing diets, but SGR was not influenced ($p < 0.05$). The FCR was significantly ($p < 0.05$) influenced, but not improved as the control and BMFP-containing diets were similar as shown in Table 3.7. The discordance between these previous studies and current findings in terms of SGR and feed intake can be attributed to the different levels of black mulberry inclusion used, as well as the difference in fish species studied. Omnivorous fish like tilapia, may be better able to digest and utilize plant-based feed additives compared to the carnivorous Dusky kob used in this study. In the current study, analysis of FCR results suggest that the efficacy of Dusky kob fish when converting nutrients from diets containing BMFP into body mass was similar between weeks 1 – 3 but changed with age of the fish in weeks 4 as BMFP5 and BMFP10 were statistically different. In general, feed intake increased every week over the course of the feeding trial for all dietary groups as expected. This occurred because fish liveweight increased over time, which also increased their feed intake due to higher nutritional requirements and an increase in energy demand with growth.

In aquaculture production, the main aim is to obtain high-quality muscle gains (Andersen, Waagbø & Espe, 2016). Fish growth is strongly associated with muscle gains, which is one of the most significant tissues for the deposition of nutrients in fish (Jiang, Li, Wang, Yang, Fu, Zheng & Li, 2016). In the current study, the supplementation with BMFP at incremental levels positively influenced ($p < 0.05$) weight gain and FCR in juvenile Dusky kob fish. While fish diets are increasingly dominated by plant-based protein diets in partial/full replacement of animal-based protein diets, this has been shown to reduce feed intake, protein utilization, and growth performance but increasing the requirement of some amino acids (Andersen *et al.*, 2016) which are limiting in plant-based dietary protein sources.

3.5.2 Blood parameters

Haemo-biochemical parameters are effective biological indices that provide information on potential health status, stress, disease infection, oxygen transport capacity, nutritional status, and intoxication levels (Shekarabi *et al.,* 2019; Yilmaz *et al.,* 2020; Ahmed & Ali, 2013) especially when novel dietary ingredients are offered to fish. Consequently, there has been growing interest in the study of haemo-biochemical parameters in fish (Zhao, Liu & Niu, 2021; Handayani *et al.,* 2020; Fazio, 2019; Adel, Amiri, Zorriehzahra, Nematolahi & Esteban, 2015), especially in nutrition trials. While it is widely confirmed that blood accounts between 1.3 – 1.7% of total live fish weight (Witeska, Kondera, Ługowska & Bojarski, 2022), it is for this reason that the effect of dietary BMFP on haemo-biochemical parameters in juvenile Dusky kob was evaluated. Some studies have established normal blood parameter ranges for several carnivorous fish such as red sea goatfish (*Parupeneus forsskali*), Rüppell's wrasse (*Thalassoma klunzingeri*), spotted rose snapper (*Lutjanus guttatus*), rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis niloticus*) (Zhao *et al.,* 2021; Sayed, Mahmoud & Muhammad, 2020; Hernandez, Olmeda-Guerrero, Chavez-Sanchez, Ibarra-Castro, Gaxiola-Cortez & Martínez-Cardenas, 2020; Handayani, Soegianto & Lignot, 2020), however, there is still sparse information on normal ranges of blood parameters for juvenile Dusky kob fish

3.5.2.1 Haematological parameters

In the current study, all haematological parameters (Tables 3.8 and 3.9) fell within the normal ranges of juvenile Dusky kob as reported in a few studies (Madibana *et al.,* 2017; Madibana and Mlambo, 2019; Madibana *et al.,* 2020; Mdhluvu *et al.,* 2021). Blood leukocyte (white blood cell) count is the most used parameter to investigate effects of an intervention on fish immune system. In vertebrates, white blood cells (WBC; leukocytes) are elementary indicators of pathological defense of the immune system (Ahmed & Ali, 2013). This means that the number and quality of WBC's can help determine whether immunostimulation has occurred in fish in response to diseases (Ahmed & Ali, 2013).

In the current study, findings showed that experimental diets influenced monocyte, lymphocyte, and eosinophil counts but not ($p > 0.05$) haematocrit, thrombocyte, neutrophils, and basophil counts in juvenile Dusky kob fish (Figure 3.2). The results obtained in the current study are not consistent with those obtained by Yilmaz *et al.* (2020) who reported that hematological parameters in Nile Tilapia did not differ ($p > 0.05$) when offered black mulberry syrup. Haematocrit (cluster cell volume) is a measure of red blood cell (RBC; erythrocyte) counts as a percentage in total blood fluid. In the current study, haematocrit count ranged between 40.0 and 43.1% but did not respond ($p > 0.05$) to incremental levels of BMFP. Erythrocytes are the most abundant form of fish blood cells, constituting between 98 – 99% of all blood cell types are made of a non-nucleated biconcave disk that consist of haemoglobin (Witeska *et al.*, 2022), the biomolecule responsible for transporting oxygen from the lungs to the rest of the body while carrying carbon dioxide from the rest of the body to the lungs for expulsion. High RBC counts are associated with rapid movements, high activity with streamlined bodies, and predaceous nature (Sayed *et al.*, 2020). Therefore, erythrocytes are potential indicators of the oxygen transport capacity of the fish. Like in the present study, feeding black mulberry syrup to trout fish, had no effect on haematocrit levels (Yilmaz *et al.*, 2020).

Fish leukocytes such as lymphocytes, monocytes and eosinophils are white blood cells responsible for combating pathogens and enhancing ~~fish~~ health (Ali, Hohn, Allen, Ford, Dail, Pruett & Petrie-Hanson, 2014). These cells are found in specific concentrations and locations in body tissues. Nahak & Sahu (2014) found that lymphocyte, monocyte, and eosinophil counts may be altered in nutritional trials through the activity of special plant-extracts such as terpenoids and flavonoids compounds that are also found in BMFP. It was found that lymphocytes, monocytes, and eosinophils were significantly ($p < 0.05$) influenced by *Ocimum basilicum L.*- containing diets (plant-based feed ingredient) (Nahak & Sahu, 2014). In the current study, similar results were obtained, as lymphocytes and eosinophils were significantly ($p < 0.05$) increased while monocytes were decreased when fish were BMFP (plant-based ingredient). Lymphocyte count showed a quadratic response ($p < 0.05$) with dietary incremental levels of BMFP (Figure 3.2). Higher lymphocyte counts are associated with healthy fish (Ahmed & Ali, 2013), while unhealthy fish tend to have lower lymphocyte and higher monocytes and neutrophils. In the current study, the evidence revealed that lymphocytes increased while monocytes tended to decline with incremental levels of BMFP, suggesting a positive influence of Dusky kob health status.

During inflammatory condition and degradation of resident macrophages, monocytes have been reported in contributing to tissue-resident of macrophage populations (Witeska *et al.,* 2022; Ahmed & Ali, 2013). In the current study, lower monocyte counts were observed in fish reared on BMFP-containing diets, while higher counts were observed in fish reared on the control diet (BMFP0). The higher monocyte counts in control fish may suggest that fish not supplemented with BMFP were producing more monocytes to fight against harmful pathogens that threatened their health. On the other hand, lower counts of monocytes in fish on BMFP-containing diets could be because black mulberry possess anti-pathogenic properties (Yilmaz *et al.,* 2020) that protected fish from prolonged exposure to pathogens. Therefore, fish offered BMFP-containing diets did not need to invest in robust immune responses to deal with pathogens and thus they produced less monocytes. However, it was observed that at the highest inclusion levels of BMPF, monocytes increased again, but it is not clear why this happened. Nevertheless, research evidence by Ahmed & Ali (2013) found that a significant increase of monocytes occurred in infested fish.

In addition, Figure 3.2 showed a linear increase in eosinophil counts with incremental levels of dietary BMFP. Eosinophils are essential for fish's defense against harmful parasitic infestations (Witeska *et al.*, 2022). The presence of harmful pathogens may result in higher levels of eosinophils in fish. On the other hand, eosinophil counts could have been high due to BMFP inclusion in fish diet without the threat of harmful parasites. Based on the evidence for lymphocyte and monocyte counts in fish reared on BMFP-containing diets, it can be concluded that eosinophils were high in BMFP reared fish because of special bioactive promoters found in mulberry instead of fish pathogen infestation. However, it is not clear whether there is a correlation between eosinophils counts in fish and parasitic infestation, since a microbial investigation was not performed. Moreover, since the lymphocytes were high and monocyte counts were low, this suggests that all experimental fish were in good health.

3.5.2.2 Serum biochemical parameters

Serum biochemistry varies between fish species and can be potentially influenced by biotic and abiotic factors such as age, diet, sex, temperature, and seasonal patterns (Sayed *et al.,* 2020). In the current study, inclusion of BMFP in juvenile Dusky kob diets influenced ($p < 0.05$) blood urea, cholesterol, globulins, and alkaline phosphatase, but not ($p > 0.05$) total serum protein, triglycerides, albumin, alanine aminotransferase, and aspartate aminotransferase (Table 3.8). The control diet promoted higher levels of blood urea compared to BMFP5, BMFP10, and BMFP12. Globulin count in kob fish reared on BMFP10 promoted the lowest levels compared to control, BMFP5, and BMFP12, although BMFP10 was similar to BMFP12. The results suggest that BMFP12 promoted the highest levels of blood triglycerides compared to BMFP0, but was similar to BMFP5, and BMFP10.

Alkaline phosphatase levels in fish reared on BMFP0 and BMFP10 were statistically different ($p < 0.5$), however, both were similar ($p > 0.05$) to BMFP5 and BMFP12.

Blood parameters such as alanine aminotransferase and aspartate aminotransferase are used to accurately assess liver damage, while blood urea and creatinine (kidney bio-markers) are used as indicators for renal failure in fish. According to Ajeniyi & Solomon (2014), nitrogen and creatine levels are parameters usually linked with kidney function. Blood urea is a non-protein nitrogenous by-product of enzymatic activity in the liver, which is derived from the breakdown of tissue and dietary protein turnover into amino acids (Ajeniyi & Solomon, 2014). Thereafter, blood urea will then travel via the bloodstream until it reaches the kidneys, the site of urea filtration and excretion outside the organism's body. In the current study, blood urea was reduced ($p < 0.05$) in fish fed BMFP-containing diets. Consequently, fish fed diets containing BMFP recorded lower levels of blood urea, while control fish recorded higher levels as seen in Table 3.9. Higher concentrations of blood urea in control fish suggest higher levels of protein catabolism in fish, in which excess urea is being removed from the body via urine (Wang, Ron & Jiang, 2014). On the other hand, low blood urea levels are also associated with environmental benefits as it leads to lower levels of undesirable ammonia in experimental tanks when excreted via urine into the water. The low blood urea levels in fish fed BMFP-containing diets may be due to growth-promoting bioactive compounds in BMFP that increase the efficacy of protein catabolism by enhancing nitrogen protein deposition into fish tissue. Nevertheless, to avoid blood urea intoxication, from the quadratic response observed for blood urea (Figure 3.3), it was calculated that 7.5% BMFP inclusion rate minimized blood urea in fish. This suggests a positive influence of dietary BMFP on the protein tissue metabolism shown by lower levels of globulins that were recorded in fish reared on 5.0 – 10.0% BMFP. The evidence suggests that BMFP-containing diets can supress serum urea levels in fish. On the other hand, total serum protein (nitrogen-based compound) did not vary across all dietary treatment groups, possibly because formulated diets were isonitrogenous in nature as shown in Table 3.1. Serum total protein is a biological marker of liver synthetic activity.

In the current study, globulin levels were significantly reduced in response to inclusion levels of BMFP in fish diets (Figure 3.4). Nahak & Sahu (2014) found that globulin levels are potentially reduced by the activity of steroids, terpenoids, cardiac glycerides, tannins, flavonoids, and saponins, all compounds that are found in plant-based feed ingredients (see Table 3.2). Globulins, synthesised by the liver are required for blood clotting and fighting infections hence the BMFP-induced reduction in globulin levels is concerning and also contradicts the recorded beneficial *in vivo* properties of BMFP (Yilmaz *et al.,* 2020). While the results reveal that globulin levels were reduced by the inclusion of BMFP in fish diets, they still fell within the normal ranges that were reported with diets containing black mulberry (Shekarabi *et al.,* 2019; Yilmaz *et al.,* 2020).

Cholesterol linearly ($p < 0.05$) increased with incremental levels of dietary BMFP (Figure 3.3), except ($p > 0.05$) for triglycerides. Cholesterol is important for cell membrane integrity, synthesis of hormones and vitamin D, as a promoter of antioxidant function, as a precursor of bile salts, and for optimal function of neurotransmitter receptors (Zampelas & Magriplis, 2019). Low serum cholesterol concentrations have been shown to mediate aggressive behaviour in fish (Aguiar & Giaquinto, 2018). In the current study, the observed increase in serum cholesterol concentration with increasing levels of BMFP may suggest an improvement of health status of fish. Increases in cholesterol and triglycerides do not necessarily indicate poor health in Dusky kob fish. Medium-chain triglycerides can preserve high temperature impairment in physical performance and muscle function (PLoS One, 2018). However, literature on the influence of BMFP-containing diets on triglycerides and cholesterol levels is currently limited.

Alanine aminotransferase and AST are liver enzymatic are enzymes resident in hepatocytes which are used as important indicators of health, liver protein enrichment, and toxin contamination of fish. Any increase in AP and AST above normal levels could be attributed liver parenchyma cell damage (Ahsan *et al.*, 2008), because these enzymes are released into the bloodstream because they leak from cells when damaged (Madibana *et al.*, 2020). Within range liver enzyme activities of AST and ALT suggest an improvement of health status and well-being fish (Shekarabi *et al.*, 2019). In the current study, there is evidence that dietary treatments did not influence serum AST and ALT activities suggesting that BMFP does not contain any toxic compounds that can cause liver parenchyma cell damage which would compromise fish health. This finding agrees with the results obtained by Yilmaz *et al.* (2020) who reported that AST and ALT not affected by black mulberry juice supplementation. However, these results contradict Shekarabi *et al.* (2019) who observed that AST and ALT were reduced ($p < 0.05$) when black mulberry juice powder was included in rainbow trout fish diets. The discordance maybe be due to the difference in fish species which (different species) may respond differently to the same plant-based feed ingredient. By lowering serum AST and ALT activities, black mulberry juice had a positive health effect on rainbow trout (Shekarabi *et al.*, 2019). Furthermore, it could be that the juice has a higher concentration of health beneficial phytochemicals than the mulberry fruit powder used in the current study. Moreover, according to the evidence from the current study, the results suggest that BMFP-containing diets improved the health of Dusky kob fish and did not compromise the health of the fish.

3.6 Conclusions

Black mulberry fruit powder has been reported to possess phytochemicals with health beneficial biological activities that could be tapped into to boost Dusky kob fish growth performance and feed utilization efficiency and promote better health. Findings from the current study suggest that fortifying a commercial Dusky kob fish diet with BMFP enhanced feed utilization and growth performance as expected and had beneficial effects on some haemo-biochemical parameters (lymphocytes, monocytes, and blood urea) in juvenile Dusky kob. In conclusion the optimum BMFP dietary inclusion levels for lymphocytes, monocytes, and blood urea were found to be 6.1, 8.4, and 7.5%, respectively.

3.7 References

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4 CHAPTER FOUR: NUTRIENT COMPOSITION OF FISH FILLETS FROM JUVENILE DUSKY KOB OFFERED BLACK MULBERRY FRUIT POWDER-CONTAINING DIETS

Abstract

The diet of cultured fish has a direct effect on the quality of fish products for human consumption as evidenced by differences between wild and farmed fish in terms of nutrient content, especially n-3 fatty acids. Therefore, the current study evaluated the effect of supplementing black mulberry fruit powder (BMFP) in juvenile Dusky kob diets on proximate, amino acid, mineral, and fatty acid content of Dusky kob fillets. The study was carried out as previously described in chapter 3. Four isonitrogenous and isoenergetic diets were formulated by including BMFP in a Dusky kob commercial diet at 0 (BMFP0), 5 (BMFP5), 10 (BMFP10), and 12 % (BMFP12) w/w. The four diets were each randomly allocated five experimental tanks and offered to fish (twice a day) at 2.8% live-weight/day over a four-week feeding period. Dietary treatment groups were chemically characterized for proximate composition, amino acid profile, and mineral composition, and fatty acid content. At the end of the four weeks feeding trial, twenty fish were randomly selected from each tank to characterize the proximate composition, amino acid profile, mineral composition, and fatty acid profile of fillets. Quadratic trends were observed for lysine [$y = 2.37(\pm 0,139) + 0.19(\pm 0.059)x - 0.02(\pm 0.005)x^2$; $R^2 = 0.0571$; $p = 0.0060$], and phenylalanine [$y = 1.34(\pm 0.100) + 0.07(\pm 0.042)x - 0.01(\pm 0.003)x^2$; $R^2 = 0.2366$; $p = 0.0367$] content. BMFP-containing diets significantly enhanced lysine and phenylalanine deposition in Dusky kob fish muscle. The quadratic equation calculated reveal that 5.6 and 3.9% of BMFP inclusion maximized lysine and phenylalanine content in kob tissue, respectively. A decline in these essential amino acids in fish muscle was observed when incremental levels of BMFP exceeded 6%. Additionally, the results also revealed a significant response in the deposition of Mg, Mn, and Zn in fish fillets, with fillets from fish reared on BMFP10 diet containing the highest amounts of the three minerals. At BMFP dietary inclusion levels above 10%, a decline in the fillets' Mg, Mn, and Zn content was observed suggesting that below his dietary inclusion level the BMFP he fillets' mineral content. Experimental diets did not affect fatty acid content of the fish fillets except for stearic acid. The means of BMFP5 and BMFP10 differed (p < 0.05), whereby. In conclusion, from the quadratic responses it was calculated that 5.6% and 3.9% were the optimum dietary inclusion levels of BMFP that maximized lysine and phenylalanine in juvenile Dusky kob fillets, respectively. However, fortification of a commercial Dusky kob fish diet with BMFP above 6% decreased he lysine and phenylalanine content in the fish's fillets. Overall, it is concluded that fortifying a commercial Dusky kob fish diet with BMFP did not compromise the proximate, amino acid, mineral, and fatty acid contents of the fish's fillets, but BMPF enhanced the lysine, phenylalanine, Mg, Mn, and Zn content of the fish's fillets.

Keywords: Black mulberry fruit powder; Amino acids; Minerals; Fatty acids; Dusky kob

4.1 Introduction

Fish is a unique source of nutrients compared to terrestrial farm animals (Korkmaz, Şen Agilkaya, Karaytug & Ozcan, 2022); they offer high-quality protein, minerals (selenium, phosphorus, iron) and essential fatty acids. It is a necessary dietary component for a nutritionally balanced diet for humans (Korkmaz *et al.,* 2022). The sustainable development of the aquaculture fish industry is a compelling strategy towards improved food security and nutrition while ensuring lower environmental impact (Baldissera, Souza, Zeppenfeld, Velho, Klein, Abbad, Ourique, Wagner, Da Silva & Baldisserotto, 2020). The successful production of farmed fish products depends on the availability of a nutritious, palatable, nutritionally balanced and highly digestible fish diets (Zafar & Khan, 2021). Nevertheless, aquaculture production still needs to be expanded to bridge the gap between the supply and demand for high-quality fish products. Aquaculture production is increasingly associated with using semi-intensive and intensive production systems that result in stressful conditions and subsequently lead to disease outbreaks (Baldissera *et al.*, 2020). In response, farmers administer prophylactic antibiotics and chemotherapeutics as a pre-emptive strike to forestall the proliferation of harmful fish pathogens. However, prolonged use of synthetic chemicals results in the emergence of drug-resistant bacterial strains that cause a public health challenge, for example, through antibiotic resistance. In addition, residues from metabolized prophylactics and therapeutic are deposited in fish muscle destined for human consumption and elicit negative health outcomes in consumers. Several countries have banned and others are banning the use of these synthetic pharmacological agents in the food/fish production chain (Purbosari, Warsiki, Syamsu & Santoso, 2019). Cabello (2006) argues that routine use of synthetic pharmacological agents as growth promoters and antioxidants in aquaculture, livestock and poultry diets is detrimental to enterprise profitability, human health and good environmental stewardship. The fish aquaculture industry in addition to facing the challenge of high feed costs has also to contend with the challenge of lipid peroxidation of fish fillets which results in reduced product shelf life especially when diets rich in long-chain polyunsaturated fatty acid (LC-PUFA) are used (Sampels, 2013). Lipid peroxidation of muscle causes the development of rancid taste, foul odours, off-flavours and loss of fat-soluble vitamins, essential amino acids, and other essential molecules (Shahidi & Zhong, 2015).

Another problem is that most fish products destined for human consumption come from fish reared on cheap animal-based alternative feed ingredients, which are used with little concern for human health. Of the several challenges faced by the aquaculture industry, the economic and ecological unsustainability of fishmeal as an animal-based fish feed is probably the most pertinent (Nyuliwe, Mlambo, Madibana, Mwanza & Wokadala, 2022). The increased demand, high market prices, depletion of wild fish, and erratic supply of fishmeal are compelling reasons why farmers are gradually shifting towards the utility of plant-based feed ingredients that are low-cost, safe, and nutraceutical and nutrient-rich as a strategic solution to these challenges, especially for carnivorous fish.

One such is locally available plant-based feed additive is black mulberry fruit powder (*Morus nigra*) that can be used to reduce the cost of feeding juvenile Dusky kob but could also improve fish fillet quality and stability. Plant-based feed additives are increasingly used to replace synthetic, expensive, and potentially toxic synthetic chemotherapeutic drugs (Baldissera *et al.*, 2020) in fish diets. According to Nyuliwe *et al.* (2022), the utility of plant-based feed ingredients in fish diets has been explored worldwide. However, sub-Saharan countries, such as South Africa, have yet to fully realize the potential benefits of plant products such as BMFP which contains an array of unique bioactive compounds, among many, essential oils, flavonoids, anthocyanins, phenols, polysaccharides, and carotenoids (Ercisli & Orhan, 2006; Sânchez-Salcedo, Mena, Gracîa-Viguera, Hernandez & Martinez, 2015; Khalifa, Zhu, Li & Li, 2018; Wen, Hu, Linhardt, Liao, Wu & Zou, 2019) Despite these potential benefits, there is no study that has interrogated the potential of BMFP to be used as a nutraceutical-rich dietary supplement that could improve the nutritional quality and shelf-life of Dusky kob fish fillet. Among many factors, diet is one of the major determinants of the quality of fish fillet (Mdhluvu *et al.,* 2021). Therefore, it is possible that dietary fortification with BMFP could alter fish fillet proximate, amino acid, mineral, and fatty acid content. Therefore, the objective of the current study was to determine tissue nutrient composition in response to incremental levels of dietary BMFP in juvenile Dusky kob fish. In addition, the study sought to determine the optimal inclusion level of BMFP in Dusky kob diets based on measured response parameters in juvenile Dusky kob fish fillets. Therefore, the study tested the hypothesis that dietary inclusion of BMFP in juvenile Dusky kob diets would significantly improve fish fillet quality.

4.2 Materials and methods

4.2.1 Experimental site and fish diets

Experimental site, experimental design, experimental fish, and fish feeding strategy, as well as the assay of amino acids, proximate composition, mineral content in diets are presented in chapter 3.

4.2.2 Fish fillet preparation

After 4-weeks of feeding, at the end of the feeding trial, twenty kob fish were randomly sampled from each tank to provide fillets for the analysis of proximate components, amino acids, minerals, and fillet fatty acid profile. Fish were euthanised using 0.25 g of clove oil/L. Thereafter, fish fillets were vacuum-packed and sealed in plastic bags and frozen in a minus twenty-degrees freezer pending chemical characterisation.

4.2.3 *Proximate composition*

Fish fillets were analysed for dry matter, crude protein, crude fat, and ash according to standard AOAC (2005) methods. Dry matter (method no. 930.15) was determined by oven-drying the samples at 105 ◦C for 12 h and ash content (method no. 942.05) was determined after incineration at 550 ◦C for 12 h. Nitrogen (N) content was determined using the Kjeldahl method (method no. 976.05) and crude protein (CP) content was calculated as N × 6.25. Gross energy was determined using an adiabatic oxygen bomb calorimeter (Parr Instruments, Moline, IL, USA) calibrated with benzoic acid. Amino acid analysis was done after acid hydrolysis, pre-column derivatisation, separation by HPLC, and detection using a fluorescence detector based on a method originally described by Einarsson, Josefsson & Lagerkvist (1983). Concentration of amino acids was expressed in g/100 g of sample. Lipids were extracted from the samples based on a method by Folch, Lees, Sloane Stanley (1957), and then methylated to fatty acid methyl esters (FAMEs) using BF3 in methanol. FAMEs were quantitatively measured by capillary gas chromatography against C11:0. Pyrogallic acid was added to minimize oxidative degradation of fatty acids during analysis. Total fat was calculated as the sum of individual fatty acids expressed as triglyceride equivalents.

4.2.4 *Amino acid profile*

Twenty Dusky kob fillet samples were provided to analyse the amino acid content. The amino acid assay was performed at the University of Limpopo using high-performance liquid chromatography (HPLC). Non-essential and essential amino acids were assayed and expressed in g/100g (Martínez, Losada, Franco & Carballo, 2011). The HPLC method, as described by Martínez *et al.* (2011), was used to quantify the total amino acids of fillets. Chapter 3 (Table 3.3) shows he amino acid content of the diets.

4.2.5 *Mineral composition*

One gram of kob fillet samples was digested in a concentrated nitric acid solution using a microwave oven MarsEcpress (CEM corporation, Mathews). Thereafter, 25 ml of distilled water was used to dilute the digested samples inside polypropylene tubes. The concentration of macro-elements calcium, phosphorus, sodium, magnesium, sulphur and potassium and microelements iron, zinc, manganese, selenium, iodine, fluorine and copper was determined using the flame atomic absorption spectrometry (FAAS; air-acetylene flame) as described by Mdhluvu *et al*. (2021). The diet mineral composition has already been presented in Table 3.4.

4.2.6 *Fatty acids*

The fatty acid (FA) profile was determined using a modified method of the AOAC (2005). The homogenised samples (*ca* 1 g) were weighed into digestion tubes and processed for gas chromatographic analysis according to the standard procedure described by Smichi, Kharrat, Achouri & Gargouri (2016). The results were quantified by comparing the FA peaks of kob fillets in comparison with the peak areas of the corresponding internal standard of 0.5 (heptadecanoic acid-C17:0, 10 mg/ml made up in C: M (2:1) (Sigma Aldrich Inc, St. Louis, USA, Cat. H3500, 98%). Thereafter, converting each FA into a percentage of the total FA quantified (Jabeen & Chaudhry, 2011). Table 4.3 shoes the dietary FA content.

Table 4.1: Saturated, mono-saturated, and poly-unsaturated fatty acid content (%) of control and black mulberry-containing diets and absolute BMFP

Fatty acid	**Diets[1]**				
	BMFP0	**BMFP5**	**BMFP10**	**BMFP12**	**BMFP**
Saturated					
Myristic acid (C14:0)	7.44	7.52	7.13	6.90	2.402
Palmitic acid (C16:0)	54.40	60.88	58.41	59.76	68.075
Heptadecanoic acid (17:0)	1.92	1.11	1.13	1.10	0.459
Arachidic acid (C20:0)	0.07	1.28	0.17	0.01	2.419
Stearic acid (C18:0)	23.49	18.88	18.14	4.29	10.628
Behenic acid (22:0)	0.07	0.05	0.17	0.15	0.008
Lignoceric acid (24:0)	0.00	0.02	0.53	0.29	0.198
Mono-unsaturated					
Palmitoleic acid (C16:1)	0.33	0.30	0.34	0.31	0.004
Oleic acid (C18:1)	0.33	0.30	0.53	19.18	0.344
Nervonic acid (24:1)	0.00	0.11	0.01	0.01	0.102
Poly-unsaturated					
γ-linolenic acid (C18:3 n-6)	0.24	0.28	0.48	0.39	0.459
α-linolenic acid (C18:3 n-3)	0.13	0.06	0.23	0.19	0.120
Eicosenoic acid (C20:1)	0.04	0.10	1.53	1.41	0.054
Eicosadienoic acid (C20:2)	0.14	0.04	0.23	0.21	0.184
Homo-gamma-linolenic acid (20:3)	0.49	0.35	0.65	0.79	0.185
Erucic acid (C22:1)	0.04	0.01	1.05	1.77	1.774
Docosahexaenoic acid (C22:6)	0.09	0.00	0.00	0.00	0.003

[1] Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg.

4.3 Statistical analysis

Primary data were subjected to normality and homogeneity using the NORMAL option and Levene's test before applying the General Linear Model (GLM) procedure (SAS, 2010). Statistical analysis was carried out using the GLM procedure and response surface egression (RSREG) using the Statistical Analysis System (SAS, 2010), as already described in chapter 3. Statistical significance was declared at $p \leq 0.05$. Mean separation was performed using the PDIFF option of the LSmeans statement.

4.4 Results

4.4.1 Fillet proximate composition

Table 4.2: Proximate composition of Dusky kob fillets in response to black mulberry fruit powder-containing diets

Proximate	**Diets**[1]					**Significance**[3]		
	BMBP0	**BMBP5**	**BMBP10**	**BMBP12**	**SEM**[2]	**GLM**[4]	**Linear**	**Quadratic**
Dry matter	513.98	493.15	451.28	494.88	0.312	0.312	NS	NS
Moisture	486.03	506.85	548.73	505.13	0.312	0.312	NS	NS
Ash	75.40	81.85	104.98	99.00	0.231	0.231	NS	NS
Crude protein	274.50	289.43	271.10	241.23	0.116	0.116	NS	NS
Crude fat	79.55	85.93	70.03	69.35	0.101	0.101	NS	NS
Gross energy	2198.29	2472.32	2254.81	2607.15	0.317	0.316	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet; [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [4]GLM; General Linear Model.

The GLM model did not show any significance ($p > 0.05$) dietary effect on moisture content, dry matter, crude protein, crude fat, gross energy, and ash content of Dusky kob fillets (Table 4.3). Similarly, the RSREG analysis did not show any significant linear or quadratic trends of proximate components in response to incremental levels of BMFP.

4.4.2 Amino acids

The GLM procedure showed a significant ($p < 0,05$) dietary effect on lysine and phenylalanine content, but not ($p > 0,05$) for serine, isoleucine, leucine, tryptophan, tyrosine, cysteine, aspartic acid, glutamine, proline, hydroxyproline, methionine, histidine, glycine, arginine, valine, and alanine (Table 4.4). The RSREG analysis revealed positive quadratic patterns ($p < 0.05$) for lysine and phenylalanine in response to graded dietary levels of BMFP. Quadratic trends were observed for lysine [$y = 2.37(\pm 0.139) + 0.19(\pm 0.059)x - 0.02(\pm 0.005)x^2$; $R^2 = 0.05710$; $p = 0.0060$] as shown in Figure 4.1, and phenylalanine [$y = 1.34(\pm 0.100) + 0.07(\pm 0.042)x - 0.01(\pm 0.003)x^2$; $R^2 = 0.2366$; $p = 0.0367$] as depicted in Figure 4.2.

Dietary effects on lysine and phenylalanine deposition in kob fillets varied ($p < 0.05$). Fish reared on BMFP5 had similar lysine levels as those on BMFP10, while the effect of BMFP5 on lysine deposition differed compared to the control diet and BMFP12. Fillets from fish reared on BMFP5 (2.80 g/100 g DM) had higher lysine deposits compared to those from counterparts fed the control diet (2.40 g/100 g DM) and those fed BMFP12 (2.11 g/100 g DM). The lysine content of fillets from fish fed BMFP12 differed to that of fillets from fish fed BMFP10 and BMFP12 but was similar to that from counterparts fed the control diet. The phenylalanine content for fish reared on BMFP5 differed with those reared on BMFP10 and BMFP12 but was similar with the control diet. Dusky kob fillets from fish reared on BMFP5 had the higher ($p < 0.05$) phenylalanine content (1.47 g/100 g DM) compared to BMFP10 and

BMFP12. However, Fish reared on BMFP10 (1.13) and BMFP12 (0.93 g/100 g DM) promoted the lower phenylalanine content in fish tissue compared to BMFP5.

Lysine and phenylalanine levels were enhanced in response to incremental levels of BMFP (Figures 4.1 and 4.2). Positive quadratic trends were observed for both lysine and phenylalanine content in fish fillets in response to incremental dietary levels of BMFP. The quadratic response revealed that 5.6 and 3.9% were the optimum inclusion levels of BMFP that maximized lysine and phenylalanine in juvenile Dusky kob tissue, respectively. The supplementation of BMFP above 6% in a commercial Dusky kob diets resulted in a decline of these two essential amino acids.

Table 4.3: Amino acid content (g/100 g DM) of Dusky kob fillets in response to the inclusion of black mulberry fruit powder in experimental diets

Amino acids	**Diets[1]**					**Significance[3]**		
	BMFP0	**BMFP5**	**BMFP10**	**BMFP12**	**SEM[2]**	**GLM[4]**	**Linear**	**Quadratic**
EAA[3]								
Lysine	2.40^{ab}	2.80^{c}	2.77^{bc}	2.11^{a}	0.122	0.0112	NS	*
Methionine	0.70	0.76	0.82	0.67	0.077	0.5173	NS	NS
Valine	1.53	1.60	1.57	1.34	0.086	0.2160	NS	NS
Histidine	1.40	1.43	1.58	0.95	0.162	0.2418	NS	NS
Leucine	1.92	2.10	2.15	1.79	0.126	0.2418	NS	NS
Isoleucine	1.28	1.38	1.37	1.13	0.077	0.1749	NS	NS
Arginine	1.92	2.01	2.41	2.05	0.228	0.4831	NS	NS
Phenylalanine	1.34^{bc}	1.47^{c}	1.13^{ab}	0.93^{a}	0.106	0.0313	*	*
Threonine	1.37	1.44	1.45	1.25	0.081	0.3543	NS	NS
NEAA[4]								
Tyrosine	0.73	0.91	0.43	0.69	0.145	0.2179	NS	NS
Proline	1.54	1.71	1.93	1.70	0.177	0.5201	NS	NS
HO-Proline	0.59	0.65	0.88	0.77	0.115	0.3398	NS	NS
Serine	1.20	1.29	1.46	1.24	0.132	0.5544	NS	NS
Aspartic acid	2.50	2.83	2.88	2.42	0.205	0.3430	NS	NS
Alanine	2.11	2.19	2.10	1.83	0.161	0.4607	NS	NS
Glycine	2.86	3.16	3.52	3.12	0.346	0.6248	NS	NS
Glutamic acid	3.97	4.30	4.76	4.06	0.285	0.2795	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet; [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [4]GLM: General Linear Model; [a,b,c]In a row, means with similar subscripts do not differ (p > 0.05).

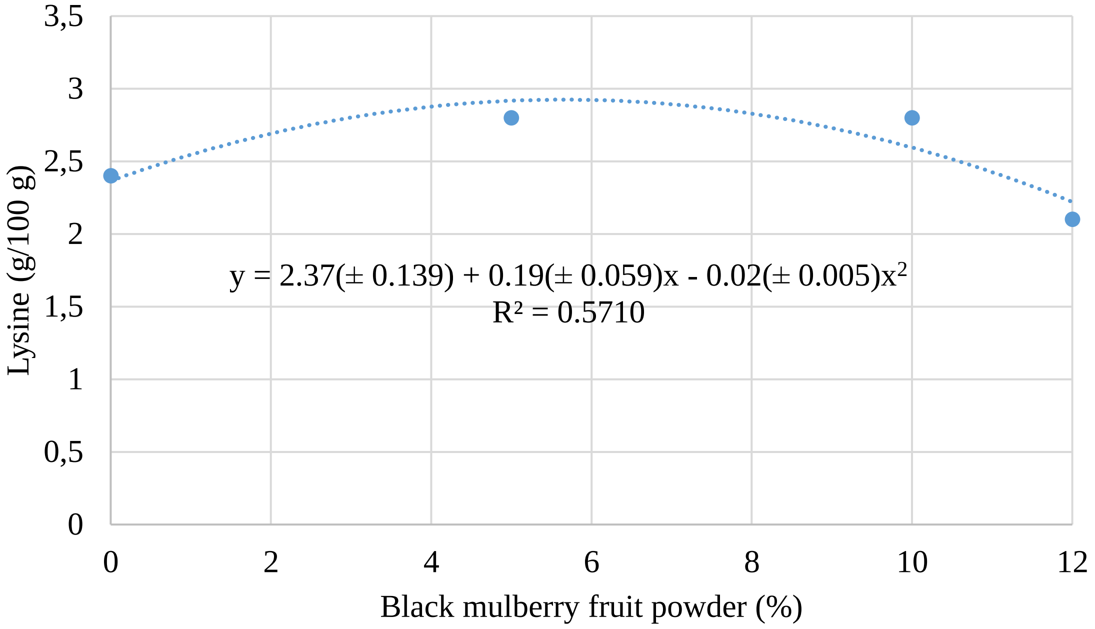

Figure 4.1: Lysine levels in kob fillet in response to incremental levels of dietary black mulberry fruit powder.

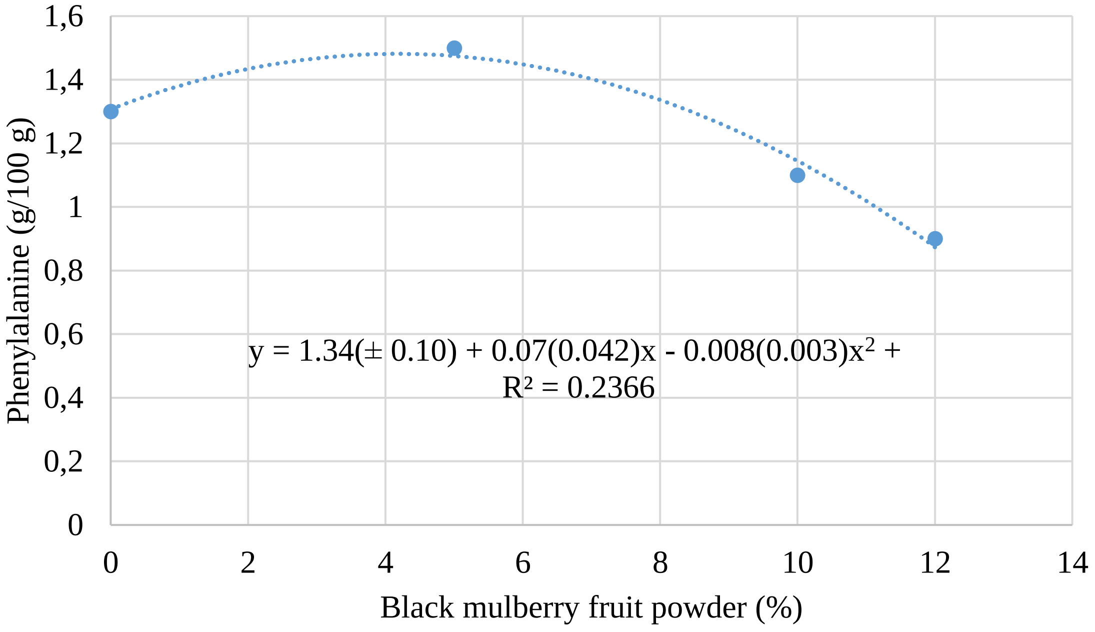

Figure 4.2: Phenylalanine levels in kob fillet in response to incremental levels of dietary black mulberry fruit powder

4.4.3 Mineral composition

A total of 9 minerals were assayed in Dusky kob fillets. Four trace minerals, which included iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn), and five macro minerals: magnesium (Mg), potassium (K), calcium (Ca), sodium (Na), and phosphorus (P) were assayed in kob fillet. The GLM procedure showed a significant ($p < 0.05$) dietary effect on Mg, Mn, and Zn deposition levels in kob fillets but not ($p > 0.05$) for Fe, Ca, Cu, P, Na, and K (Table 4.5). However, the RSREG analysis did not reveal any significant linear or quadratic trends for Mg, Mn, and Zn levels in kob fillets in response to incremental dietary levels of BMFP.

The control diet and BMPF10 had similar effects on Mg deposition in kob filles $p > 0.05$). However, fish offered BMFP10 (1.25 mg/L) and BMFP0 (0.93 mg/L) had the higher fillet Mg levels compared to those reared on BMFP5 (0.44 mg/L) and BMFP12 (0.48 mg/L). Fish reared on BMFP10 (0.28 mg/L) had the highest level of Mn compared to BMFP5 (0.24 mg/L), BMFP0 (0.21 mg/L), and BMFP12 (0.19 mg/L) and BMFP12 promoted the lowest Mn levels (Table 4.4). The diet BMFP10 promoted the higher levels of Zn (0.46 mg/L) in kob filles tissue, while the control diet (0.22 mg/L), BMFP5 (0.16 mg/L) and BMFP12 (0.13 mg/L) promoted the lower levels. A notable decline in Mg, Mn, and Zn was observed in kob fillets when BMFP was included above 10%.

Table 4.4: Mineral composition (mg/L) in juvenile Dusky kob fillets in response to incremental levels of black mulberry fruit powder

	Diets[1]					Significance[3]		
Mineral	**BMFP0**	**BMFP5**	**BMFP10**	**BMFP12**	**SEM[2]**	**GLM[4]**	**Linear**	**Quadratic**
Ca	0.77	1.63	7.43	0.58	3.043	0.4345	NS	NS
Cu	0.58	0.49	0.63	0.45	0.097	0.5881	NS	NS
Fe	0.15	0.18	0.25	0.40	0.061	0.1469	NS	NS
K	8.78	7.53	11.45	7.93	2.255	0.6414	NS	NS
Mg	0.93^b	0.44^a	1.25^b	0.48^a	0.126	0.0285	NS	NS
Mn	0.21^b	0.24^c	0.28^d	0.19^a	0.007	0.0030	NS	NS
Na	1.65	1.91	4.19	2 .24	0.945	0.3460	NS	NS
P	2.10	1.90	5.53	1.81	1.008	0.1608	NS	NS
Zn	0.22^a	0.16^a	0.46^b	0.13^a	0.051	0.0323	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet; [a,b,c]Within row means with different subscripts do not differ (p > 0.05); [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [4]GLM; General Linear Model.

4.4.4 Fatty acids

Fatty acid profile (a total of 20 FA's) was assayed in juvenile Dusky kob fish fillets, which included seven saturated fatty acids (SFA), ten long chain poly-unsaturated fatty acids (LC-PUFA), three mono-saturated fatty acids (MUFA). There was a dietary effect ($p < 0.05$) on stearic acid content in kob fillets, but not ($p > 0.05$) for the other 19 Fatty acids. There was no significant trend ($p > 0.05$) observed for stearic acid in kob fillets as dietary BMFP levels increased (Table 4.6). The stearic acid content of Dusky kob fillets from fish fed BMFP5 differed to that of counterparts fed BMFP10 ($p < 0.05$) BMFP10 (18%) resulted in lower stearic acid content compared to BMFP5 (21.32 %).

Table 4.5: Saturated, poly-unsaturated, and mono-saturated fatty acid content (%) of control and black mulberry containing diets values are expressed as weight percentage (Mass of fatty acid/mass of oil in the sample)

Fatty acid	**Diets[1]**					**Significance[3]**		
	BMFP0	**BMFP5**	**BMFP10**	**BMFP12**	**SEM[2]**	**GLM[4]**	**Linear**	**Quadratic**
Saturated								
Myristic acid (C14:0)	5.23	5.32	5.19	5.59	0.40	0.895	NS	NS
Palmitic acid (C16:0)	56.59	55.73	49.94	54.51	2.55	0.294	NS	NS
Heptadecanoic acid (17:0)	1.27	1.49	1.48	1.31	0.19	0.772	NS	NS
Arachidic acid (C20:4)	0.16	0.15	0.53	0.30	0.18	0.426	NS	NS
Stearic acid (C18:0)	20.90^{ab}	21.32^{b}	18.33^{a}	20.35^{ab}	0.712	0.041	NS	NS
Behenic acid (22:0)	1.14	0.91	0.91	0.66	0.30	0.744	NS	NS
Lignoceric acid (24:0)	0.04	0.10	0.02	0.03	0.04	0.525	NS	NS
Mono-unsaturated								
Palmitoleic acid (C16:1)	0.51	0.75	0.83	0.45	0.15	0.255	NS	NS
Oleic acid (C18:1)	0.14	0.19	0.23	0.25	0.05	0.397	NS	NS
Nervonic acid (24:1)	0.02	0.03	0.04	0.03	0.12	0.904	NS	NS
Poly-unsaturated								
γ-linolenic acid (C18:3n-6)	0.46	0.48	0.57	0.40	0.10	0.690	NS	NS
α-linolenic acid (C18:3 n-3)	0.15	0.19	0.61	0.49	0.18	0.237	NS	NS
Eicosanoic acid (C20:0)	1.52	1.12	1.83	1.43	0.27	0.341	NS	NS
11-Eicosenoic acid (C20:1)	0.11	0.32	0.14	0.14	0.12	0.600	NS	NS
11-14-Eicosadienoic acid (C20:2)	0.40	0.23	0.51	0.34	0.09	0.187	NS	NS
Homogamma-linolenic acid (20:3)	0.49	0.35	0.81	0.64	0.17	0.294	NS	NS
Erucic acid (C22:1)	0.05	0.23	0.08	0.03	0.10	0.616	NS	NS
Decosadienoate (22:2)	0.13	0.10	0.11	0.03	0.04	0.368	NS	NS
Decosatetraenoate (22:4)	0.03	0.03	0.02	0.24	0.11	0.464	NS	NS
Docosahexaenoic acid (C22:6)	0.14	0.12	0.20	0.18	0.07	0.844	NS	NS

[1]Diets: Formulated by including black mulberry fruit powder in a commercial Dusky kob diet at 0, 50, 100, and 120 g/kg; [2]SEM: Standard error mean of each diet. [3]Significance: NS = $p \geq 0.05$; * = $p \leq 0.05$; [4]GLM: General Linear Model; [a,b,c]In a row, means with similar subscripts do not differ ($p > 0.05$)

4.5 Discussion

4.5.1 Fillet proximate composition

The current study investigated, for the first time, the effects of fortifying a commercial Dusky kob fish diets with BMFP on the proximate and mineral content and the, amino acid, and fatty acid profiles of fillets of juvenile Dusky kob. The mineral, amino acid, lipid and LC-PUFA components are the main nutrients found in fish meat (Jiang *et al.,* 2016). The potential use of BMFP as a sustainable natural feed additive in kob diets is based on the presence of bioactive compounds (Table 3.2), which could potentially enhance stability from oxidative depreciation and nutrition of kob fillets (Wen *et al.,* 2019; Khalifa *et al.,* 2018; Sânchez-Salcedo *et al.,* 2015; Ercisli & Orhan, 2006). Fish meat is rich source of essential nutrients including the essential amino acids lysine and methionine.

The current study attempted to demonstrate the benefits of incorporating BMFP into Dusky kob diets by measuring changes in fish fillet nutrient composition. No dietary effects on dry matter, moisture, ash, crude protein, crude fat, and gross energy were observed (Table 4.3). These results are not consistent with those obtained by Shekarabi *et al.* (2019), who reported that ash, crude protein, and moisture content were increased ($p < 0.05$) in rainbow trout fish fed mulberry juice powder at 0.5 and 0.75%. The discordance in the results in comparison between previous studies with the current study can be attributed to the use of BMFP powder in the current study, which could have affected the bioavailability of unique phytochemicals differently in kob fish fillets. . Another cause may be due to the differences in the fish type in Shekarabi *et al.* (2019) study and in the current study. Although the evidence suggests that BMFP-containing diets did not enhance any of the six proximate components, it is essential to note that supplementation with BMFP at higher concentrations did not compromise any of the proximate components in fish fillets.

4.5.2 Amino acids

Amino acids are essential indicators of the nutritive value of food resources (Long, Dong, Tan, Zhang, Chi, Yang, Liu, Xie, Deng, Yang & Zhang, 2022). The amino acid content, proportion and type are some of the key determinants of the nutritional value and quality of fish fillets and influence taste and flavour. More than 50% of amino acids uptake by fish can be deposited into body protein (Andersen, Waagbø & Espe, 2016). In fish, between fifty to eighty per cent of non-protein nitrogenous compounds are in the form of amino acids, in which significant quantities of these include lysine, alanine, proline, arginine, histidine, glutamic acid, and taurine (Doĝan & Ertan, 2017). Traditionally, amino acids are categorised into two groups: the essential amino acids valine, histidine, lysine, methionine, leucine, isoleucine, arginine, phenylalanine, threonine and cysteine and non-essential amino acids tyrosine, proline, alanine, glycine, aspartic acid and glutamic acid (Mertz, Beeson & Jackson, 1952). Essential amino acids have to be supplied in the diet as fish cannot synthesise them de novo (Andersen, Waagbø & Espe, 2016). Mulberry fruits are considered an important source of about eighteen amino acids, including the essential amino acids (Herman *et al.*, 2022) that are required for normal human foetal growth and development and neonatal growth (insert relevant reference). The amino acids glutamic acid, aspartic acid, alanine and glycine have been shown to influence fillet taste and flavour (Doĝan & Ertan, 2017). Findings from the current study show that fortifying a commercial Dusky kob diet with BMFP did not enhance the content of these amino acids in the kob fillets. This could be due to the presence of bioactive compounds that affect their deposition in fish muscle.

Table 3.3 shows that lysine (2.49 g/ 100g DM) and phenylalanine (2.09 g/ 100g DM) content in mulberry fruit powder was lower compared to control diet, which had 3.33 and 2.34 g/100 g DM of lysine and phenylalanine, respectively. Consequently, a decrease in these essential amino acids in response to incremental levels of BMFP-containing diets was expected. From the quadratic responses it was calculated that 5.6 and 3.9% were the optimum inclusion levels of BMFP that maximized lysine and phenylalanine in juvenile Dusk kob fillet, respectively. The supplementation of BMFP above 6.0% in kob diets resulted in a decline of these two essential amino acids (Figures 4.1 and 4.2). A possible cause in the decline of these two essential amino acids in response to inclusion levels of BMFP above 6% may be due to an increased presence of anti-nutrients that may have limited bioavailability of lysine and phenylalanine.

Lysine is reported as the first limiting essential amino acid (Abboudi, Mambrini, Ooghe, Larondelle & Rollin, 2006) in several protein-based feed sources available for fish feed manufacturers, especially plant-based protein sources (Hauler & Carter, 2010). It is considered as one of the essential dietary nutrients necessary for realising optimal feed utilization and improving growth performance, preventing fin rotting and mortality (Hamid, Abdullah, Zakaria, Yusof & Abdullah, 2016). As reported in chapter 3, while BMFP-containing diets did not influence feed intake and SGR it however improved weight gain in weeks 2 and 4 and FCR in week 4. In the current study, a positive quadratic response of lysine in kob fillets was observed in response to the supplementation. This is in line with the weight gain and feed conversion ratio reported in chapter 3. The possible increase in lysine deposition in kob tissue could have translated into enhanced growth performance (for weeks 2 and 4), although this was not maintained in other feeding weeks. The optimal inclusion level of BMFP was calculated at 5.64 % for maximum lysine deposition in the fish fillet. It was revealed that black mulberry concentrations above 6% resulted in a decline of lysine deposition in kob tissue. A possible cause in the reduction of lysine deposition in fish tissue at higher BMFP levels could be the presence of phytochemicals that may suppress lysine metabolism and not due to a deficiency of lysine in diets, as BMFP had high lysine content (Table 3.3). However, there is limited research on phytochemicals that may potentially have a negative effect on lysine deposition in fish fillets. Phenylalanine is an important amino acid in fish growth (Feng, Kang, Wang, Ding, Zhu & Hang, 2019). The deficiency of phenylalanine could cause a reduction in growth and impaired intestinal structural integrity of juvenile grass carp (Sayed & Ahmed, 2022). The optimal dietary requirement of phenylalanine is between 0.5 to 1.7% in selected fish species including Nile tilapia (*Oreochromis niloticus*), Chinook salmon (*Oncorhynchus tshawytscha*), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), and rohu carp (*Labeo rohita*) (Kim, Rahimnejad, Song & Lee, 2012). In chapter 3, amino acid profile reveal that diets contained higher levels of phenylalanine above 2% across all treatment groups, which was beyond the normal range of dietary requirement for phenylalanine. However, phenylalanine content in fish fillets fell within the normal range between 0.93 and 1.47%. Findings from the current study suggest that fish growth performance was not negatively affected by the supplementing BMFP in kob diets.

4.5.3 Minerals

Minerals are inorganic elements present as macronutrients and micronutrients in feed and food (Chanda, Paul, Ghosh & Giri, 2015). In aquatic organisms such as fish, mineral composition depends on the species, dietary source, environment, stages of development, and physiological status (Chanda *et al.,* 2015). Fish diets are regarded as the main source of minerals for metabolic function, but fish can also absorb minerals from the surrounding ambient (fresh and or marine) water (Watanabe, Kiron & Satoh, 1997). In vivo mineral function is categorised into four key areas: catalytic, structural, physiological, and metabolic regulation (Lall & Kaushik, 2021). Consequently, mineral deficiencies can lead to anatomical, biochemical and functional pathologies as influenced by the duration and degree of mineral deprivation (Watanabe *et al.*, 1997).

Findings for the current study showed that diets influenced Mn, Mg, and Zn deposition levels in kob fillets but not Fe, Ca, Cu, P, Na, and K levels (Table 4.5). Manganese is an essential trace element in fish associated with the development, growth, and modulation and regulation of energy metabolism (Jiang *et al.*, 2016; Wang, Kuntke, Saakes, van der Weijden, Buisman & Lei, 2022). The diet is the primary source of Mn supply and the entro-hepato-biliary system which plays a crucial role in regulating the absorption and excretion of Mn in vertebrates (Jesu Prabhu, Silva, Kröeckel, Holme, Ørnsrud, Amlund, Lock & Waagbø, 2019). Mulberry fruits have been reported as an important source of potassium, iron, sodium, copper, zinc, magnesium, calcium, and manganese (Rohela *et al.*, 2020). Moreover, BMFP-containing diets positively influenced mineral composition in juvenile Dusky kob fish, although no trend was observed across all experimental diets.

4.5.4 Fatty acids

The fatty acid (FA) profile of fish muscle usually reflects the proportions contained in consumed dietary treatments (Mdhluvu *et al.*, 2021). The diet strongly affects the FA profile in fish tissues (Sanchez-Salcedo *et al.*, 2016). In addition, FA absorption is strongly influenced by both the chemical composition and physicochemical properties of the diet (Xu *et al.*, 2020). The absorption of FA acids from hydrolysed dietary fat is is the first direct step affecting their incorporation into fish tissues (Xu *et al.*, 2020). In the current study experimental diets influenced ($p < 0.05$) on the stearic acid content of the kob fillet. Stearic acid content in fish fillets did not reveal any significant pattern as the level of BMFP increased.

Essential fatty acids are long-chained PUFA synthesised from oleic, linoleic, and linolenic acids (Abedi & Ali Sahari, 2014). Polyunsaturated fatty acids are integral components of healthy and functional cell membranes, are key substrates for synthesis of leukotrienes, prostaglandins, thromboxane and eicosanoids and required for the development and normal function of the central nervous system (Ercisli & Orhan, 2006). Mulberries have been reported to contain high concentrations levels of myristic acid, linoleic acid, stearic acid and palmitic acid (Rohela *et al.*, 2020.) Findings from the current study are consistent with those of Rohela *et al.* (2020): myristic acid, stearic acid, and palmitic acid were found in higher concentrations in BMFP-based diets. On the other hand, linolenic acid was found in very low concentrations. The PUFA levels were higher than that of MUFA and SFA in mulberry fruits as found by Rohela *et al.* (2020). However, based on findings from the current study (Table 4.1), this was not the case as SFA levels were greater than MUFA and PUFA.

The results show that PUFA were not significantly ($p > 0.05$) influenced by BMFP-containing diets. However, PUFA content of the fish fillets was not negatively affected by fortifying the commercial Dusky kob diet with BMFP. Results from the current study reveal that BMFP-containing diets did not enhance FA in fish fillets, but most importantly, PUFA composition levels were not compromised. According to Xu *et al.* (2020), fish products provide protein and n-3 LC-PUFA that are crucial for maintenance of human health. Long-chain polyunsaturated fatty acids are biologically important compounds that confer protection against diseases, including cardiovascular diseases and rheumatoid arthritis, and they also display anticarcinogenic properties and support the immune system and normal brain physiology (Xu *et al.*, 2020). The FA profile of fish meat is an essential characteristic for consumers because of the potential health benefits associated with MUFA and PUFA (Baldissera *et al.,* 2020). Eicosapentaenoic (EPA; C20:5 n-3) and docosahexaenoic (DHA; C22: 6 n-3) are key diet-derived nutrients that are found in high concentration in fish products (Jiang *et al.,* 2016). These long chain PUFAs have multiple health benefits in humans: enhancement of cognitive development during the first five years of life and mitigation of neurodegenerative and coronary heart disease (Montenegro, Descalzo, Rizzo, Rossetti, García & Perez, 2022). In the current study, EPA was not found in fish fillets, however, DHA was present and did not respond negatively following the supplementation of BMFP.

4.6 Conclusion

Black mulberry fruit powder has been reported to possess essential nutrients that could potentially enhance the nutrient composition of juvenile Dusky kob fish fillets. Findings from the current study suggest that fortifying a commercial kob fish diet with BMFP enhanced amino acid content (lysine and phenylalanine) and mineral composition (Mg, Mn, and Zn) as expected. In conclusion, the optimum BMFP dietary inclusion levels for lysine and phenylalanine were found to be 5.6 and 3.9%, respectively.

4.7 References

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5 CHAPTER FIVE: GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

5.1 General discussion

In South Africa, efforts to expand Dusky kob aquaculture production is faced with several aquafeed-related challenges that need to be overcome to ensure a sustainable enterprise. Consequently, there is need for aquafeed manufacturers to engineer socially, economically, and environmentally sustainable diets (Craig, Helfrich, Kuhn & Schwarz, 2017). Most fish products consumed by humans and animals (fishmeal and fish oil) come from captured wild fish, resulting in the over-exploitation of wild fish stocks. In addition to overfishing, fish stocks have been depleted due to toxic contaminants, habitat depletion, and climate change (Caipang, Mahuhay-Omar & Gonzales-Plasus, 2019). Other pressing challenges associated with aquaculture production include high cases of disease infestation, use of synthetic prophylactic agents as pre-emptive antibiotics and chemotherapeutics in fish diets to forestall the proliferation of fish pathogens resulting in the emergence of drug-resistant bacteria (Tang, Cai, Liu, Wang, Lu, Wu & Jian, 2014), overreliance on expensive fishmeal and fish oil products as aquafeeds, and reduced fillet shelf-life (Citarasu, 2009). Thus, this current study evaluated the effectiveness of supplementing black mulberry fruit powder (BMFP) (*Morus nigra*) at incremental levels to test physiological and tissue nutrient responses to determine an optimal dietary inclusion levels of BMFP in juvenile Dusky kob diets. The aim of this investigative study was to evaluate the utility of BMFP as a nutraceutical source in juvenile Dusky kob fish diets. This final discussion summarises each experimental study (chapters 3 – 4) and brings to the fore the potential utility of plant-based feed additives in juvenile Dusky kob.

The current study is divided into two experimental chapters that evaluate the utility of BMFP-containing diets.. Chapter 3 evaluated the effectiveness of BMFP-containing diets on feed utilization, growth performance, chemical nutrient and phytogenic content and haemo-biochemical in juvenile Dusky kob. On the other hand, chapter 4 evaluated the dietary effects of BMFP-containing diets on Dusky kob fillet nutrient (proximate, mineral, amino acid and fatty acid) response to dietary supplementation with BMFP. Black mulberry fruit powder has special bioactive compounds with putative antioxidant, immunostimulant, antimicrobial, anti-inflammatory, appetite stimulation, and growth-promoting properties (Reverter, Bontemps, Lechinni, Banaigs & Sasal, 2014; Khalid *et al.*, 2011; Li, Lu, Wu, Xiong, Luo, Ma & Liu, 2020; Shekarabi, Omidi, Dawood, Avazeh & Heidari, 2019; Yilmaz, Ergon, Yigit, Yilmaz & Ahmadifar, 2020; Sharif & Pirsa, 2021; Jan, Parveen, Zahiruddin, Khan, Mohapatra & Ahmad, 2021). However, despite the potential utility of this natural nutraceutical source, its efficacy as a dietary supplement in Dusky kob fish diets had not be interrogated. The investigation was carried out using four isonitrogenous and isoenergetic experimental diets were formulated by including BMFP in a Dusky kob commercial diet at 0 (BMFP0), 5 (BMFP5), 10 (BMFP10), and 12% (BMFP12) w/w. Findings from the current study suggest that dietary BMFP did not compromise feed intake and utilization efficiency instead improved weight gain in weeks 2 and 4 of the feeding trial. In the case of FCR, current stud findings show that the fish showed differences but age-dependent response BMFP-containing diets. It was only in week four that FCR responded to diets suggesting that prolonged use of BMFP-containing diets resulted in juvenile kob being adapted to the diets as evidenced by better feed utilization efficiency.

Black mulberry fruit powder-containing diets influenced lymphocytes, monocytes, and eosinophils counts, but not haematocrit, thrombocytes, neutrophils, and basophils. Quadratic trends were observed for lymphocytes, and monocytes, whereas a positive linear trend was observed for eosinophil counts in response to incremental levels of BMFP. The results suggest that the inclusion of BMFP in kob diets boosted the fish immune system by enhancing blood leukocyte counts, which was expected given the array of potentially immune-stimulating bioactive compounds detected and quantified in BMFP. From the quadratic responses observed for lymphocytes, monocytes and blood urea, it was calculated that the BMFP inclusion levels of 6.1, 8.4, and 7.5%, respectively, would optimize the levels of these blood parameters. Overall, it is recommended from these quadratic responses observed for lymphocytes, monocytes, and blood urea that the optimum inclusion level of BMFP in juvenile Dusky kob should not exceed 10%.

Chapter 4 assessed the effect of BMFP-containing diets on fish fillet proximate, mineral amino acid, and fatty acid (FA) content. The fillet analysis of juvenile Dusky kob revealed that amino acid and mineral content were significantly enhanced. Lysine and phenylalanine were significantly improved in response to BMFP-containing diets. From the quadratic responses, it was calculated that 5.6 and 3.9% were the optimum inclusion levels of BMFP that maximized lysine and phenylalanine in juvenile Dusk kob fillets, respectively. The supplementation of BMFP above 6.0% in kob diets resulted in a decline of these two essential amino acids. Regarding the mineral content of fish fillets, current study findings revealed that Mn, Mg, and Zn deposition were significantly influenced by dietary BMFP. There was no significant trend observed for these minerals. However, experimental diets did not significantly affect Fe, Ca, Cu, P, Na, and K. The evidence suggested that BMFP10 promoted the highest Mn, Mg, and Zn deposition in the fish fillets. Therefore, BMFP-containing diets had a positive influence on the fillet mineral content. The results revealed that BMFP-containing diets influenced the fish fillet fatty acid content. However BMFP10 was the only diet that significantly suppressed stearic acid in kob fish fillet.

5.2 Conclusions and recommendations

In conclusion, the use of black mulberry fruit powder as a nutraceutical source in juvenile Dusky kob fish is a viable nutritional strategy to enhance the sustainability of the kob aquaculture industry. This is because BMFP has been shown to possess beneficial bioactive compounds that can boost appetite, immunostimulation, anti-microbial properties, growth, stability, and fish health. Observed results suggest that dietary inclusion of BMFP did not enhance feed utilization as expected, but it had beneficial effects on weight gain and some haemo-biochemical parameters (lymphocytes, monocytes, and blood urea) in juvenile Dusky kob. It is recommended that the optimal levels of BMFP inclusion for optimal lymphocytes, monocytes, and blood urea levels be capped at 6.1, 8.4 and 7.5%, respectively. Black mulberry-containing diets positively influenced lysine and phenylalanine deposition in fish fillets. Based on quadratic analyses, it was calculated that 5.6 and 3.9% be the optimal inclusion levels of BMFP for maximisation of the deposition of lysine and phenylalanine deposition in Dusky kob fillets, respectively. Dietary BMFP inclusion levels above 6.0% would cause significant decline in lysine and phenylalanine depositions. It is recommended that future studies evaluate the potential of individual phytochemicals identified as present in the BMFP to enhance fish growth performance and health and meat nutrient content and quality and fillet shelf life.

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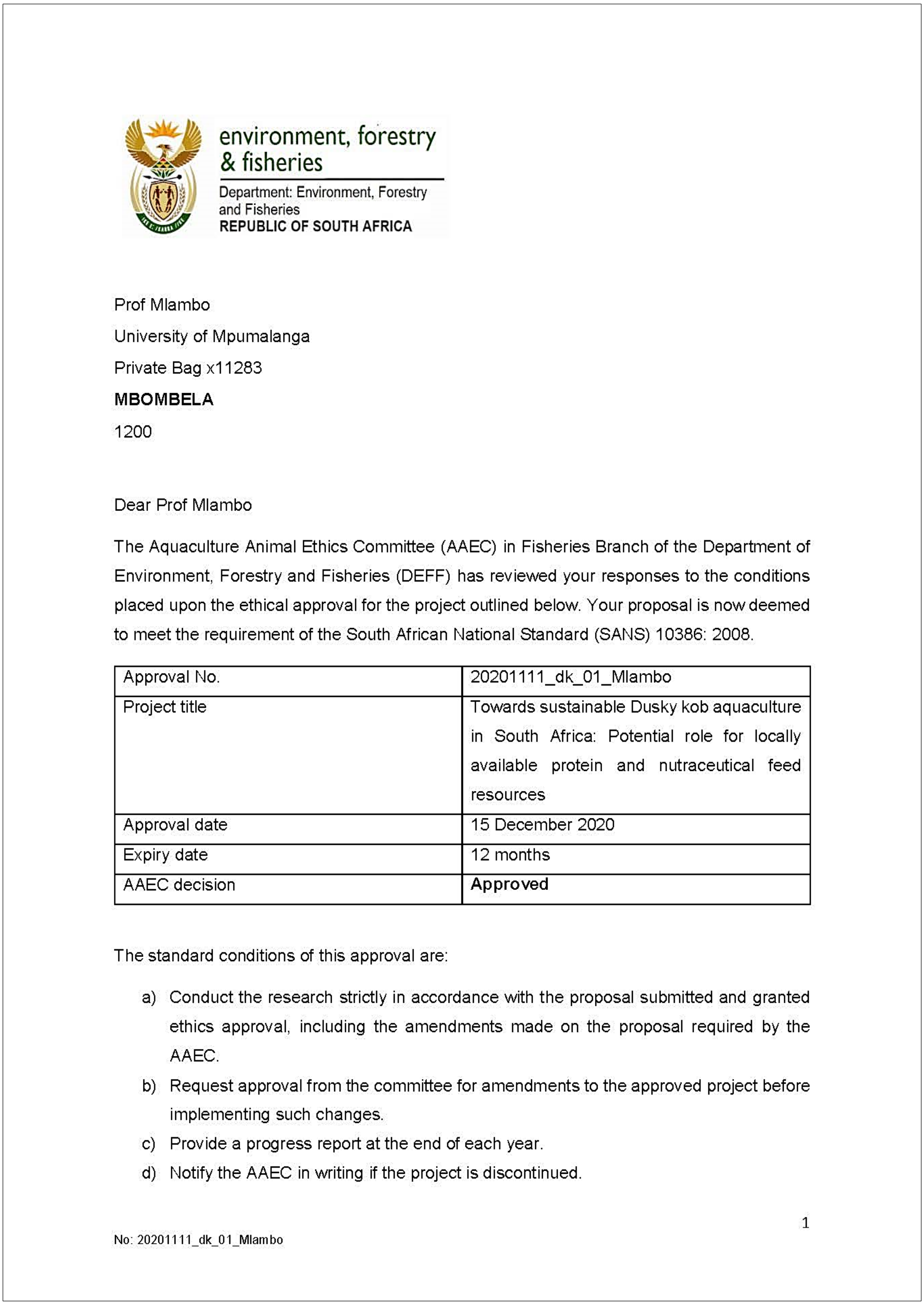
APPENDICES

Appendix 1: Ethics approval by the Aquaculture Animal Ethics Committee (AAEC)



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Prof Mlambo
University of Mpumalanga
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1200

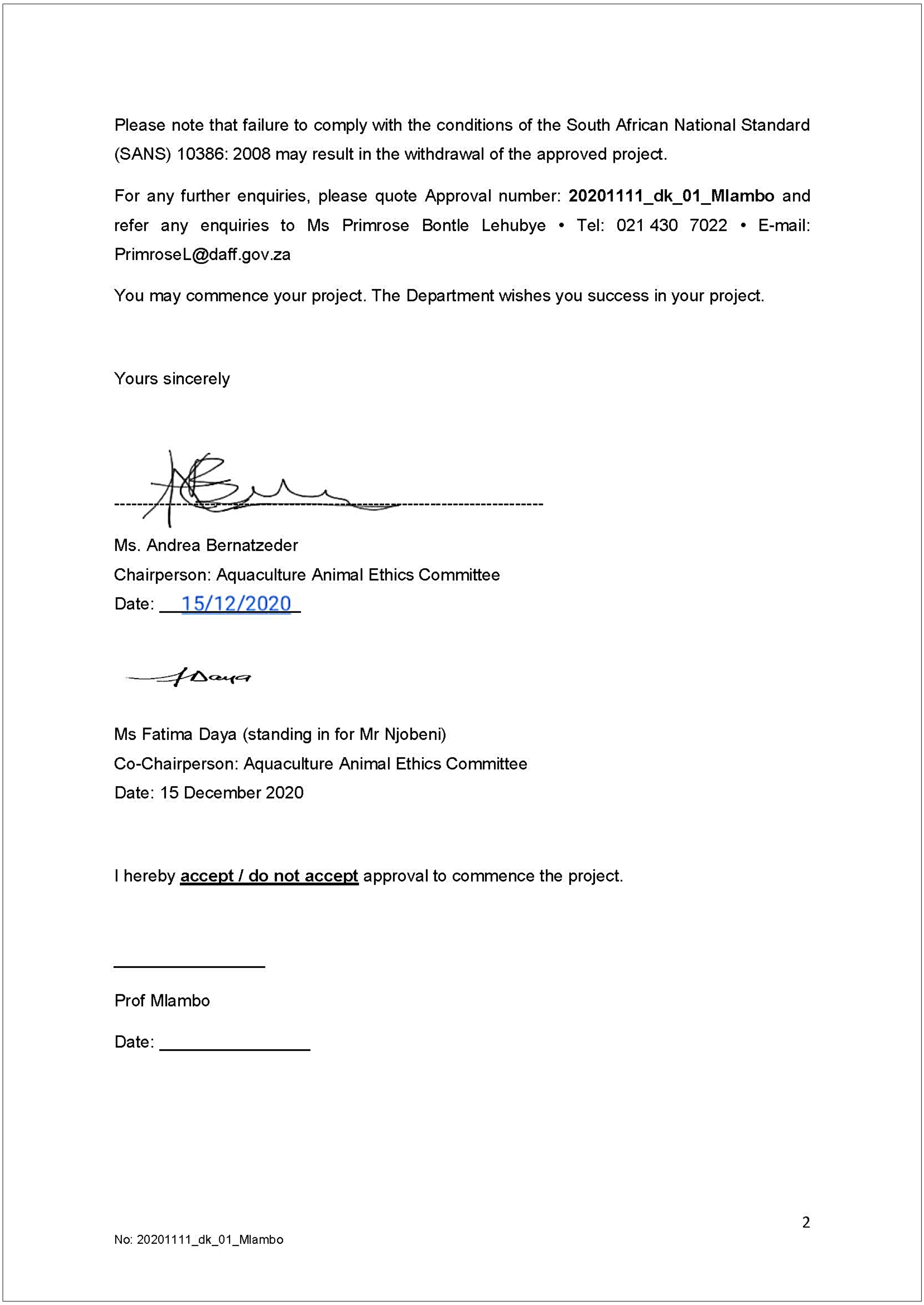
Dear Prof Mlambo

The Aquaculture Animal Ethics Committee (AAEC) in Fisheries Branch of the Department of Environment, Forestry and Fisheries (DEFF) has reviewed your responses to the conditions placed upon the ethical approval for the project outlined below. Your proposal is now deemed to meet the requirement of the South African National Standard (SANS) 10386: 2008.

Approval No.	20201111_dk_01_Mlambo
Project title	Towards sustainable Dusky kob aquaculture in South Africa: Potential role for locally available protein and nutraceutical feed resources
Approval date	15 December 2020
Expiry date	12 months
AAEC decision	**Approved**

The standard conditions of this approval are:

a) Conduct the research strictly in accordance with the proposal submitted and granted ethics approval, including the amendments made on the proposal required by the AAEC.
b) Request approval from the committee for amendments to the approved project before implementing such changes.
c) Provide a progress report at the end of each year.
d) Notify the AAEC in writing if the project is discontinued.



Please note that failure to comply with the conditions of the South African National Standard (SANS) 10386: 2008 may result in the withdrawal of the approved project.

For any further enquiries, please quote Approval number: **20201111_dk_01_Mlambo** and refer any enquiries to Ms Primrose Bontle Lehubye • Tel: 021 430 7022 • E-mail: PrimroseL@daff.gov.za

You may commence your project. The Department wishes you success in your project.

Yours sincerely

Ms. Andrea Bernatzeder
Chairperson: Aquaculture Animal Ethics Committee
Date: 15/12/2020

Daya

Ms Fatima Daya (standing in for Mr Njobeni)
Co-Chairperson: Aquaculture Animal Ethics Committee
Date: 15 December 2020

I hereby **accept / do not accept** approval to commence the project.

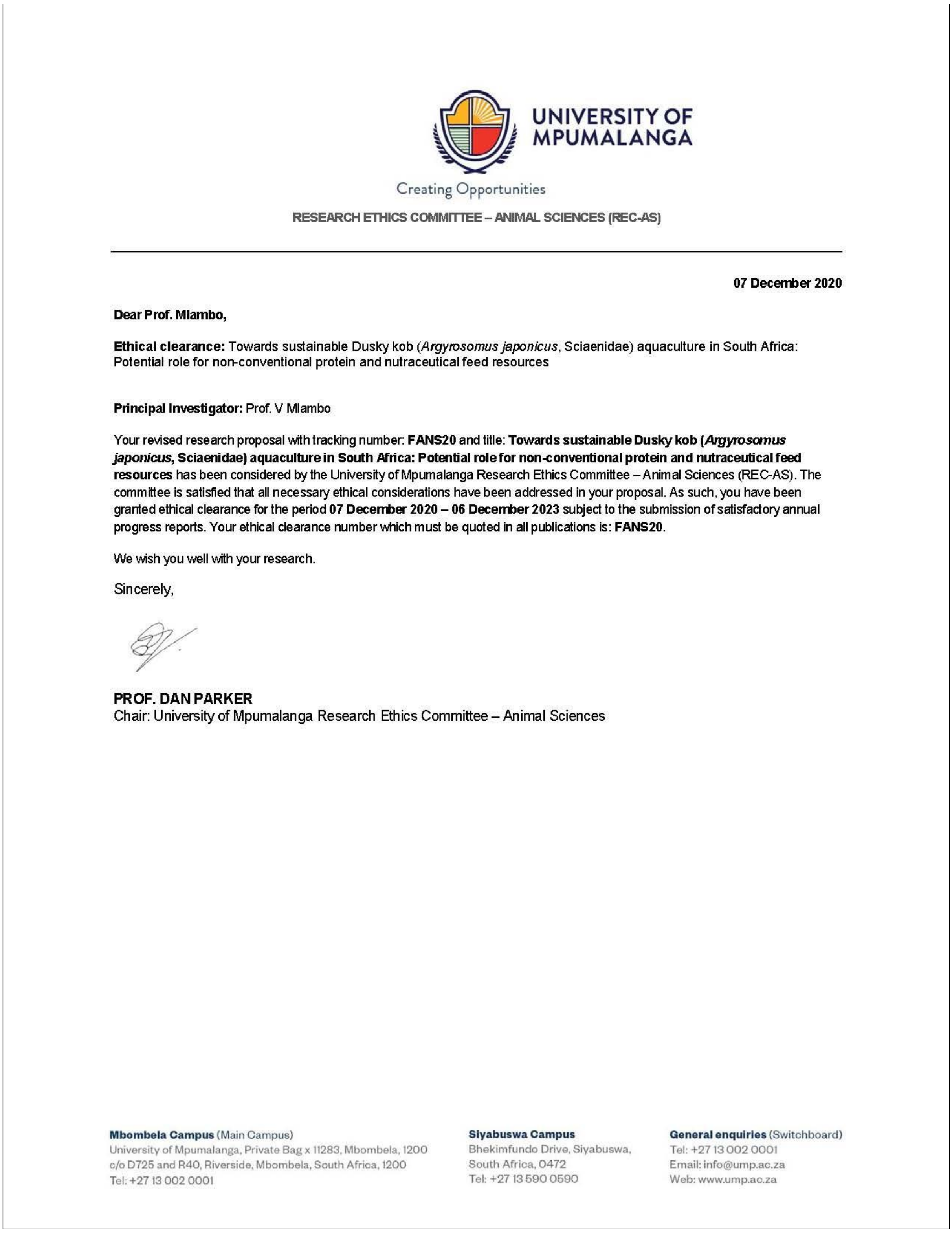
____

Prof Mlambo

Date: ________________

,

Appendix 2: Ethics approval by the Animal Science Ethics Committee at the University of Mpumalanga



UNIVERSITY OF MPUMALANGA
Creating Opportunities
RESEARCH ETHICS COMMITTEE – ANIMAL SCIENCES (REC-AS)

07 December 2020

Dear Prof. Mlambo,

Ethical clearance: Towards sustainable Dusky kob (*Argyrosomus japonicus*, Sciaenidae) aquaculture in South Africa: Potential role for non-conventional protein and nutraceutical feed resources

Principal Investigator: Prof. V Mlambo

Your revised research proposal with tracking number: **FANS20** and title: **Towards sustainable Dusky kob (*Argyrosomus japonicus*, Sciaenidae) aquaculture in South Africa: Potential role for non-conventional protein and nutraceutical feed resources** has been considered by the University of Mpumalanga Research Ethics Committee – Animal Sciences (REC-AS). The committee is satisfied that all necessary ethical considerations have been addressed in your proposal. As such, you have been granted ethical clearance for the period **07 December 2020 – 06 December 2023** subject to the submission of satisfactory annual progress reports. Your ethical clearance number which must be quoted in all publications is: **FANS20**.

We wish you well with your research.

Sincerely,

PROF. DAN PARKER
Chair: University of Mpumalanga Research Ethics Committee – Animal Sciences

Mbombela Campus (Main Campus)
University of Mpumalanga, Private Bag x 11283, Mbombela, 1200
c/o D725 and R40, Riverside, Mbombela, South Africa, 1200
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Siyabuswa Campus
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South Africa, 0472
Tel: +27 13 590 0590

General enquiries (Switchboard)
Tel: +27 13 002 0001
Email: info@ump.ac.za
Web: www.ump.ac.za

Appendix 3: Certificate of approval of research proposal

UNIVERSITY OF
MPUMALANGA

FACULTY OF AGRICULTURE AND NATURAL SCIENCES

Postgraduate Studies Committee

Certificate of Approval – Research Proposal

Date of this Approval:	**01 October 2021**

Student Details

1	Student Name:	T MASHILOANE
2	Student Number:	201736802
3	School	School of Agricultural Sciences
4	Degree Registered for:	Master of Agriculture
5	Date of First Registration:	February 2020
6	Supervisor(s):	Prof. V.Mlambo

The research proposal entitled **Black mulberry fruit as a nutraceutical source for juvenile dusky kob: Physiological and tissue nutrient responses** has been evaluated and approved by the Postgraduate Studies Committee of the Faculty of Agriculture and Natural Sciences.

Chairperson: Prof. Victor Mlambo

UNIVERSITY OF MPUMALANGA
06 -10- 2021
FACULTY OF AGRICULTURE
AND NATURAL SCIENCES

Signature:

Date & Official Stamp

Appendix 4: MSc. Oral research presentation at the University of Mpumalanga

FACULTY SEMINAR SERIES

You are invited to the Faculty of Agriculture and Natural Sciences Research Seminar Series. The seminar will be delivered by ***Prof. Hossana Twinomurinzi*** - *[Placing 4IR in Context]* and ***Mr Thabang Mashiloane*** - *[Black mulberry fruit powder as a nutraceutical source for juvenile dusky kob: Physiological responses].*

DATE: 12 October 2022
TIME: 11hrs – 14hrs00
VENUE: Auditorium 6

ABOUT THE SPEAKERS

Prof Hossana Twinomurinzi BSc Hons (Mathematics), Masters (IT), PhD (IT) is a C2 South Africa NRF Rated Researcher and 4IR Professor with the Department of Applied Information Systems, University of Johannesburg. He is currently the Head of the Centre for Applied Data Science which seeks to infuse data science efforts in the College of Business and Economics. He is an Associate Editor for the African Journal of Information and Communication and has previously served as Director for the NEMISA Digital Skills Research Unit, Associate Editor at the African Journal of Information Systems, Chairperson for the ICT4D Flagship at Unisa, and Secretary for SAICSIT. His primary research interests are in Applied Data Science, Digital Skills, Digital Government, Digital Innovation and ICT for development. He has supervised several Masters and Doctoral students in the areas of data analytics, digital government and ICT for development. He has extensive executive and management experience in the IT sector.

Mr Mashiloane holds a BSc. in Agriculture degree from the University of Mpumalanga. He is a former recipient of the Vice Chancellor's Scholarship for three consecutive years (2018 – 2020). As a result, he was nominated to participate in a Leadership and Development programme in 2019. He has also been a recipient of three consecutive Academic Merit awards (2017 – 2019). In 2019, he attended the International Summer Institute programme at Dalhousie University in Canada. He also had the privilege to participate in the Virtual Mobility of Sustainable Development and One Health Initiative programme offered by the University of Bolonga (Italy) in 2021. He has worked as an Examination Assistant at the Department of Higher Education and Training (2018). He is currently enrolled for an MSc degree in Agriculture specializing on the nutrition of the dusky kob (Argyrosomus japonicus), an economically important fish for South Africa's aquaculture industry.

Appendix 5: MSc. Oral research presentation at the Aquaculture Association of South Africa 2022, University of Stellenbosch

Aquaculture Association of Southern Africa

AQUACULTURE ASSOCIATION OF SOUTHERN AFRICA CONFERENCE 2022

11-15 July 2022 | Stellenbosch | South Africa

Aquaculture - Inspire / Accelerate / Impact

Dear Thabang Mashiloane

On behalf of the AASA2022 Scientific Committee we are pleased to confirm that your abstract titled:

Black mulberry fruit powder as a nutraceutical source in juvenile dusky kob fish: Physiological and tissue nutrient response

has been accepted as an **ORAL PRESENTATION** and will be included in the official conference programme. Please note the following **IMPORTANT** information to ensure that your oral presentation will be included in the programme:

1. Please confirm by no later than **20 May 2022** that you have received our email and that you will be presenting your oral.
2. No oral presentation(s) will be included in the official conference programme unless the author has registered **and paid their registration fees by 10 June 2022.** Please visit the website https://www.aasa-aqua.co.za/conferences/ to register online.
3. The early bird registration fee will only be valid for all presenting authors until 13 May 2022. To qualify for the early bird registration, payments must be received on or before this date.
4. Correspondence containing details on the time of your presentation other relevant Conference Programme information will follow shortly.

Guidelines for Oral Presentations

- We have allowed 15-minute slots for all presentations. This includes time for questions, and we will only accommodate questions if time allows it.
- Presentations must be prepared in PowerPoint (PP). To ensure compatibility, save your presentations in both newer (pptx) and older (ppt) file formats.
- All presenters must make their presentations available to the conference technical assistant at least one session prior to your session.

Please contact the Conference Administrator at conferences@aasa-aqua.co.za if you require any assistance.