Chapter 5

Discussion

5. Discussion

The proximate composition of *Lemna minor* and *Ipomoea aquatica* indicates that both species hold significant nutritional value, making them promising candidates for aquaculture feed. Previous studies have also highlighted the high protein content and balanced nutritional profiles of *L. minor* and *I. aquatica*, confirming their suitability as components of fish feed (Naseem et al., 2020; Sosa et al., 2024). The findings of the current study align with previous research, which has consistently demonstrated similar proximate composition results (Appenroth et al., 2017; Al-Snafi, 2019; Adedokun et al., 2019). However, it is important to note that these nutritional properties can vary significantly depending on growth conditions and environmental factors. This adaptability positions *L. minor* and *I. aquatica* as versatile and sustainable ingredients, capable of providing essential amino acids while serving as an affordable, eco-friendly alternative to traditional feed sources.

The amino acid profiles of *L. minor* and *I. aquatica* suggest their potential as sustainable alternatives to fish meal in animal feed. In this study, both species exhibited high levels of EAAs, including lysine, leucine, and phenylalanine, which are crucial for animal growth and health. These findings are consistent with previous research, for instance, Chakrabarti et al. (2018) reported a high EAA content in *L. minor*, while Saikia et al. (2023) documented a similar profile in *I. aquatica*. Appenroth et al. (2017) and Fiordelmondo et al. (2022) also observed significant levels of lysine, leucine, and phenylalanine in *L. minor*, further affirming the nutritional value of these plants. The high EAA content in *L. minor* and *I. aquatica* underscores their suitability as cost-effective and nutritionally rich ingredients in animal feed formulations, meeting key dietary requirements and enhancing feed sustainability.

The fatty acid profiles of *L. minor* and *I. aquatica* revealed a higher concentration of PUFA compared to SFA and MUFA. In this study, C18:3n-3 (α-linolenic acid) and C18:2n-6 (linoleic acid) were found in substantial amounts in both *L. minor* and *I. aquatica*, underscoring their potential nutritional value. Previous studies align with these findings for instance, Chakrabarti et al. (2018)

reported the presence of significant levels of α-linolenic and linoleic acids, while Saikia et al. (2023) also documented these essential fatty acids in *I. aquatica*. Similarly, Mukherjee et al. (2010) identified high concentrations of unsaturated fatty acids in *L. minor*, which are known for their health-protective properties. Appenroth et al. (2017) further supported these findings by reporting higher PUFA levels than SFA and MUFA across various duckweed species studied, including *L. minor*. Our study confirms that *L. minor* and *I. aquatica* are rich sources of unsaturated fatty acids vital for fish growth and health (Bell et al., 1986). This suggests their potential as cost-effective ingredients in fish feed formulations, offering economic and nutritional benefits.

Although aquatic plants are rich in nutrients, they also contain a variety of anti-nutritional factors (Francis et al., 2001; Tadele, 2015) that can impact feed acceptability, growth performance, and nutrient absorption in fish. In this study, the anti-nutritional compounds alkaloids, tannic acid, phytic acid, oxalate, and saponins were examined in *L. minor* and *I. aquatica*. The levels of these anti-nutritional factors in *I. aquatica* were found to be relatively low, consistent with findings from Ali and Kaviraj (2018), who also reported minimal concentrations of such compounds in this species. Similarly, the levels of anti-nutritional factors in *L. minor* were within a low range, aligning with observations from Falayae et al. (2022), who reported limited anti-nutritional content in this plant. These findings indicate that both *L. minor* and *I. aquatica* may offer viable nutritional benefits for fish feed with minimal interference from anti-nutritional factors.

Studies on replacing fishmeal with plant-based proteins in aquafeed have predominantly focused on species such as cyprinids, salmonids, and trout, with limited attention to catfish and air-breathing species (Dorothy et al., 2018; Naseem et al., 2021). However, some studies have shown positive effects on catfish growth performance when aquatic macrophytes are included in their diets (Kari et al., 2020; Naseem et al., 2021; Nandi et al., 2023). *L. minor*, known for its high nutrient content (Chakrabarti et al., 2018), has been tested in various fish species (Noor et al., 2000; Raj et al., 2001; Herawati et al., 2020; Fiordelmondo et al., 2022; Goswami et al., 2022; Irabor et al., 2022). Nevertheless, its effects on *A. testudineus* and *H. fossilis* remain unexplored. To determine the optimal inclusion level of *L. minor* in the diets of these species, feed formulations containing 0%, 5%, 10%, 15%, and 20% *L. minor* were prepared and fed over 60 days. At the end of the trial, growth performance, digestive enzyme activity, biochemical parameters, and muscle composition were comprehensively assessed.

The present study's findings indicate that dietary supplementation with *L. minor* has a significant positive impact on the growth performance of both *A. testudineus* and *H. fossilis*, underscoring its potential as a valuable feed ingredient in aquaculture. Growth performance parameters, such as FW, BWG, SGR, FE, PER, and FCR, serve as critical indicators for evaluating the effectiveness of dietary interventions. Improvements in these metrics suggest better nutrient utilisation, protein synthesis, and energy efficiency, which are vital for achieving optimal growth. Over the 60-day feeding trial, *L. minor* inclusion led to significant enhancements in all these growth parameters, consistent with previous studies on *L. minor*-based supplemented diets that support fish growth (Sosa et al., 2024). Final weight is a primary indicator of growth success, reflecting the overall response to dietary interventions. In this study, both species exhibited higher FW with *L. minor* supplementation, particularly at the 15% inclusion level (LM15), which yielded the highest ($P < 0.05$) FW in both species. This indicates that moderate inclusion of *L. minor* facilitates optimal growth, likely due to its high protein content and digestibility (Al-Snafi., 2019; Devi et al., 2022). However, a slight decline in FW was noted at the 20% inclusion level (LM20), suggesting that while *L. minor* is effective at moderate levels; excessive inclusion could result in reduced palatability or the presence of anti-nutritional factors that can limit growth (Minich & Michael, 2024). Similar trends have been observed in other studies with *L. minor*-based feeds, where optimal growth occurs within a moderate inclusion range (Asimi et al., 2018; Fiordelmondo et al., 2022).

BWG is a direct measure of weight increase and indicates the efficiency of dietary protein utilisation. BWG in both species showed a progressive increase with higher *L. minor* levels of up to 15%, demonstrating that the nutritional quality of *L. minor* supports protein synthesis and nutrient absorption. The highest ($P < 0.05$) BWG was recorded at LM15, reinforcing the effectiveness of moderate *L. minor* inclusion. However, a slight reduction in BWG was observed at LM20, indicating that nutrient imbalances or lower digestibility at higher inclusion levels could affect growth. The steady increase in BWG up to LM15 across both species supports *L. minor* as a sustainable feed ingredient that can enhance aquaculture productivity. SGR is a critical metric that measures daily growth efficiency and is influenced by dietary quality (De Silva & Anderson, 1995). The study showed a consistent increase in SGR with *L. minor* inclusion in *A. testudineus* and *H. fossilis*, peaking at LM15 for both species. This suggests that moderate levels of *L. minor* effectively enhance metabolic efficiency, promoting better growth. The decline at LM20 implies that excessive inclusion could introduce dietary elements that hinder metabolic processes and also could be related to the diet palatability that slightly decreased with the increasing *L. minor*, a common outcome with higher levels of plant-based feed ingredients (Fiordelmondo et al., 2022). The consistent improvement in SGR up to LM15 indicates that *L. minor* can optimise growth potential by improving nutrient assimilation.

FCR is a crucial measure of feed efficiency, with lower values indicating better feed utilisation (De Silva & Anderson, 1995). FCR decreased steadily with increasing *L. minor* inclusion up to LM15 in both species, reflecting improved nutrient absorption and energy conversion. The lowest ($P < 0.05$) FCR was recorded at LM15, highlighting that moderate *L. minor* inclusion enhances feed conversion efficiency. A slight increase in FCR at LM20 suggests that higher inclusion levels may lead to reduced digestibility or the presence of anti-nutritional factors that impair feed conversion efficiency (Gopan et al., 2020). FE and PER are critical indicators of how well the feed and its protein are utilised for growth (De Silva & Anderson, 1995). Both FE and PER increased with *L. minor* supplementation by up to 15%, indicating that *L. minor* promotes efficient protein conversion into body mass. The highest ($P < 0.05$) FE and PER were observed at LM15, supporting that moderate *L. minor* inclusion optimises protein utilisation. A slight decline in LM20 implies that excessive levels might introduce factors that reduce digestibility and protein assimilation. Improved growth performance is linked to better feed utilisation and increased digestive capacity in fish when fed *L. minor*. Likewise, several studies have reported positive impacts of *L. minor* on fish health and growth (Goswami et al., 2022; Irabor, 2022; Sosa et al., 2024). Survival rate is a fundamental measure of dietary safety and overall fish health. The consistent 100% survival rate across all treatments for *A. testudineus* and *H. fossilis* indicates that *L. minor* is a safe feed ingredient, even at higher inclusion levels. This aligns with previous research that highlights the safety and suitability of *L. minor* as a feed ingredient when used at appropriate levels (Noor et al., 2000; Raj et al., 2001; Asimi et al., 2018; Goswami et al., 2022; Irabor et al., 2022; Naseem et al., 2021).

Regression analysis is an essential method for identifying the optimal dose of feed additives to maximise the performance of aquatic animals (Yossa & Verdegem, 2015). Polynomial regression, specifically, is valuable for examining non-linear relationships between feed inclusion levels and growth metrics in aquaculture, helping to determine the most effective dietary formulations. In this study, polynomial regression analysis of SGR and FCR for *A. testudineus* showed a range of 16.25-17.10%, whereas *H. fossilis* showed a range of 11.89-12.30%. This method has also been proven effective in determining optimal feed levels for different species, such as sunflower inclusion at 14.3% for *H. fossilis* (Hossain et al., 2023) and caraway seed meal for *Oreochromis niloticus* (Ahmad & Abdul-Tawwab, 2011).

The proximate analysis of *A. testudineus* and *H. fossilis* fed the LM15 diet revealed significantly ($P < 0.05$) higher protein, lipid, and ash content, along with reduced moisture levels, compared to the control groups. This enhanced nutrient profile indicates that *L. minor* supplementation improves nutrient retention and elevates the overall nutritional quality of the fish, making them more valuable for aquaculture purposes (Ali & Kaviraj, 2021; Goswami et al., 2022). Elevated protein and lipid levels are particularly important as they signify improved growth performance and nutrient assimilation, reflecting effective utilisation of dietary resources. Notably, fibre content remained consistent ($P > 0.05$) across all treatment groups in both *A. testudineus* and *H. fossilis*. Similar results have been reported in *Channa striatus*-fed *L. minor* (Raj et al., 2001; Fiordelmondo et al., 2022), suggesting that these species can efficiently digest and absorb nutrients from this plant source. However, some studies have observed decreased carcass protein content in other species, such as *Clarias gariepinus* (Irabor et al., 2022) and *Barbodes gonionotus*, Bleeker (Noor et al., 2000), indicating that the effects of *L. minor* on nutrient composition may vary across different fish species.

Assessing digestive enzyme activity in fish provides insights into their digestive capacity and their ability to break down essential nutrients, such as proteins, lipids, and carbohydrates. This activity is significant because it is directly linked to fish growth and weight gain, making it a valuable measure of how effectively fish utilise their food (Gawlicka et al., 2000; Johnston et al., 2004; Bilen et al., 2018; Devi et al., 2022). Amylase is an enzyme that catalyses the hydrolysis of starch into simple sugars. In this study, *H. fossilis* displayed significantly ($P < 0.05$) increased digestive enzyme activity in specific treatments (LM10 and LM15), whereas *A. testudineus* showed higher enzyme activity in LM15 ($P < 0.05$). This suggests that *H. fossilis* and *A. testudineus* effectively utilise dietary carbohydrates when *L. minor* is included in their diet, supporting findings from similar studies on plant-based diets. Higher amylase activity, an enzyme responsible for digesting carbohydrates, is often seen in fish-consuming diets rich in plant content. For instance, *H. fossilis* exhibited elevated amylase levels when fed mulberry leaf meal (Ali et al., 2019) and a formulated diet (Khanom et al., 2022). Similarly, *A. testudineus* fed with plant extract-supplemented feed also showed increased amylase activity (Mariyam et al., 2020). Elevated amylase activity has also been recorded in *Cyprinus carpio* fed with *Anethum graveolens* (Bilen et al., 2018), in *Labeo rohita* consuming papaya leaf meal (Shanthanna & Muralidhar, 2024), in *Labeo rohita* fed *L. minor* incorporated diet (Goswami et al., 2020) and in *Labeo rajasthanicus* when *L. minor* was included in the diet, as noted by Meena et al. (2021). Some studies, however, have observed no significant difference ($P > 0.05$) in enzyme activity with specific plant diets. For instance, *A. testudineus* fed with chaya leaf meal showed no notable changes in enzyme activity (Panchan et al., 2024), suggesting that not all plant-based ingredients affect enzyme activity equally. Differences in nutrient composition or bioavailability among plants might account for this variability. The observed increase in amylase activity with plant-based diets indicates enhanced carbohydrate digestion, which could help fish adapt more effectively to diets with plant ingredients, typically high in starch.

Lipase, a crucial enzyme in lipid metabolism, breaks down dietary fats into fatty acids and glycerol, supporting energy production and growth in fish. In this study, fish-fed specific plant-based diets showed significantly higher ($P < 0.05$) lipase activity, with *A. testudineus* on the LM15 diet and *H. fossilis* on LM10 and LM15 diets exhibiting enhanced fat digestion. These results align with prior studies that demonstrate the positive effects of plant-based particularly those containing *L. minor*, on lipase activity. Studies have shown that feeding *L. minor* to *Cyprinus carpio* increases lipase activity (Goswami et al., 2022), while similar improvements were observed in *Labeo rajasthanicus* on *L. minor*-supplemented diets (Meena et al., 2021). Higher lipase activity was also observed in *H. fossilis* when fed with mulberry leaf meal (Ali et al., 2019). Further supporting these findings, *Cyprinus carpio* displayed elevated lipase levels when fed a diet containing *Spirodela polyrhiza*, another aquatic plant (Shrisvastav et al., 2022). Likewise, *A. testudineus* showed increased lipase activity when given plant extract-enriched diets (Mariyam et al., 2020). However, lipase response appears to vary depending on the type of plant source used; for instance, *A. testudineus* showed no significant change in lipase activity with a chaya leaf meal diet (Panchan et al., 2024), while *Labeo rohita* exhibited enhanced lipase levels with papaya leaf meal (Shanthanna & Muralidhar, 2024).

Trypsin is a proteolytic enzyme crucial for protein digestion, breaking down dietary proteins into smaller, absorbable peptides. In this study, fish fed the LM15 diet showed the highest trypsin activity, particularly in *A. testudineus*, while *H. fossilis* showed no significant difference ($P > 0.05$). This suggests that moderate inclusion of *L. minor* can enhance protein digestion efficiency. Similar studies report comparable findings. For instance, *Cyprinus carpio* displayed increased trypsin activity when fed diets supplemented with *L. minor* (Goswami et al., 2022). In another study, *Cyprinus carpio* also showed higher trypsin levels with diets containing *Spirodela polyrhiza* (Shrivastav et al., 2022). Likewise, increased trypsin activity was observed in *Labeo rohita* when fed an *L. minor* incorporated diet (Goswami et al., 2020).

Chymotrypsin, an essential protease working alongside trypsin, is crucial in breaking down proteins into absorbable peptides, supporting effective protein digestion and nutrient absorption. In this study, chymotrypsin activity was notably higher ($P < 0.05$) in *A. testudineus* when fed the LM15 diet, suggesting that a moderate inclusion of *L. minor* can enhance protein metabolism in this species. In contrast, *H. fossilis* showed no significant differences ($P > 0.05$) in chymotrypsin activity across dietary groups, indicating that the enzyme's response to *L. minor* may vary by species. These findings align with previous research. *Cyprinus carpio* exhibited increased chymotrypsin activity with *L. minor*-supplemented diets (Goswami et al., 2022), while *Labeo rohita* showed similar results when fed an *L. minor*-incorporated diet (Goswami et al., 2020). Furthermore, *Cyprinus carpio* also displayed elevated chymotrypsin levels with diets containing *Spirodela polyrhiza* (Shrivastav et al., 2022).

Pepsin is a crucial protease that works effectively in acidic conditions, initiating the breakdown of proteins in the stomach. In this study, pepsin activity was significantly higher ($P < 0.05$) in *H. fossilis* fed LM10, LM15, and LM20 diets, with no significant differences among these groups ($P > 0.05$). Additionally, *A. testudineus* also exhibited increased pepsin activity on the LM15 diet. These findings are consistent with research by Arriaga-Hernández et al. (2021), which indicated that incorporating plant-based ingredients like soybean meal can enhance pepsin activity. This suggests that moderate levels of *L. minor* in fish diets may positively influence protein digestion.

Total protease is a collection of enzymes responsible for breaking down proteins into amino acids, which are essential for nutrient absorption in the digestive system and can significantly enhance fish growth. In this study, the LM15 diet yielded the highest ($P < 0.05$) total protease activity in *A. testudineus*, whereas no significant difference ($P > 0.05$) was observed in *H. fossilis* fed the same diet. Prior studies support these findings by highlighting how specific plant-based ingredients in fish diets can influence protease activity. For instance, *H. fossilis* exhibited higher protease activity when fed mulberry leaf meal (Ali et al., 2019). Additionally, Khanom et al. (2022) found that *H. fossilis* fed a formulated diet showed increased protease activity. Conversely, *A. testudineus* fed a diet containing chaya leaf meal showed no significant increase in protease activity, demonstrating that not all plant ingredients produce the same effect (Panchan et al., 2024). Diets supplemented with *L. minor* were particularly effective in raising digestive enzyme activity across several fish species, potentially improving nutrient absorption and promoting growth. Studies reveal similar trends across various species. Mariyam et al. (2020) found that a plant-extract-supplemented diet increased protease activity in *A. testudineus*. Shanthanna and Muralidhar (2024) observed that *Labeo rohita* fed with papaya leaf meal also showed enhanced protease activity. Goswami et al. (2022) reported higher protease levels in *Cyprinus carpio* on an *L. minor*-supplemented diet, while Meena et al. (2021) recorded similar results in *Labeo rajasthanicus* fed with *L. minor*. Shrivastav et al. (2022) further documented increased protease activity in *Cyprinus carpio* when fed with *Spirodela polyrhiza*, another duckweed variety.

In fish-fed diets supplemented with *L. minor*, the increased activity of digestive enzymes likely enhanced their ability to utilise food effectively, leading to higher growth rates. The improved digestive enzyme activity and feed digestibility could explain the enhancement in feed utilisation and growth performance of *A. testudineus* and *H. fossilis*-fed dietary *L. minor* (Shekarabi et al., 2022). However, at a 20% inclusion level (LM20), both species showed a decline in enzyme activities. This trend aligns with other studies indicating that high levels of plant-based feeds can reduce enzyme activity and overall growth (Ali & Kaviraj, 2018; Wang et al., 2017). This may be attributed to anti-nutritional factors and increased dietary fibre in plant-based diets, which can interfere with nutrient digestion and absorption (NRC, 2011; Gopan et al., 2020). These findings emphasise the importance of keeping *L. minor* inclusion at moderate levels to maintain optimal enzyme efficiency and support healthy fish growth.

Duckweeds, particularly *L. minor*, are a rich source of fatty acids, which are essential nutrients that influence both fish health and the nutritional quality of fish as a food source for humans (Appenroth et al., 2017; Chakrabarti et al., 2018; Sharma et al., 2019). The fatty acid composition in fish is crucial for their health. It contributes significantly to their dietary value for humans and largely depends on the fish's diet, specifically on the types and proportions of fats they consume (Ackman, 2008; Zhang et al., 2020a). In this study, the inclusion of *L. minor* in the diets of *A. testudineus* and *H. fossilis* led to a significant alteration in the fatty acid profile of these fish. Specifically, increasing concentrations of *L. minor* resulted in higher levels of PUFAs, which are associated with health benefits for the fish and enhance their nutritional quality. This increase in PUFA content was accompanied by a corresponding decrease in SFA content, indicating an improvement in the overall fatty acid profile of the fish. These findings align with the findings of Herawati et al. (2020), which observed similar increases in PUFA levels in *Oreochromis niloticus* fed with fermented *L. minor*. Similarly, Shrivastav et al. (2022) reported an inverse relationship between SFA and PUFA levels in *Cyprinus carpio* with increased inclusion of greater duckweed, further supporting the potential of *L. minor* in promoting healthier fatty acid profiles in fish. The increase in PUFAs associated with *L. minor* supplementation underscores its role in enhancing fish fatty acid profiles, which is beneficial for both fish health and the dietary quality of fish. However, Fiordelmondo et al. (2022) reported contrasting results, as they did not observe significant differences in the levels of SFAs, MUFAs, and PUFAs when *L. minor* was included in the diet of *Oncorhynchus mykiss*. This discrepancy suggests that the impact of *L. minor* on fatty acid profiles may vary across fish species, potentially due to differences in species-specific dietary assimilation and metabolism.

Efficient utilisation of amino acids in animal feed is crucial for sustainable protein production and optimal growth in aquaculture species (Kaushik et al., 2010). EAAs, which cannot be synthesised by the fish and must be obtained from their diet, are particularly important for protein synthesis, immune function, and overall health. *L. minor* is a rich source of amino acids, making it a suitable candidate for aquafeed, as it contributes to protein content and a balanced amino acid profile (Chakrabarti et al., 2018). In this study, the LM15 diet resulted in the highest ($P < 0.05$) levels of total EAAs in both fish species, *A. testudineus* and *H. fossilis*. For *H. fossilis*, the LM15 diet led to significantly ($P < 0.05$) elevated total EAA levels, especially for arginine, histidine, methionine, and valine, compared to other dietary groups. Similarly, *A. testudineus* fed the LM15 diet showed the highest ($P < 0.05$) total EAA content, with peak levels of arginine, histidine, leucine, phenylalanine, threonine, and valine. This trend in both species indicates that the inclusion of *L. minor* effectively improved the amino acid profile, benefiting fish health and growth. The increased EAA levels in *H. fossilis* and *A. testudineus* align with findings by Goswami et al. (2022), where *Cyprinus carpio* fed *L. minor* showed higher EAA levels, including arginine, isoleucine, methionine, tryptophan, threonine, and valine. Enhanced amino acid profiles were also observed in *Oreochromis niloticus* fed with fermented *L. minor* meal (Herawati et al., 2020) and in *Cyprinus carpio* fed *Spirodela polyrhiza* to a certain level (Shrivastav et al., 2022). Similar improvements in amino acid content were reported in *Sparus aurata* when fed with plant protein sources (Gómez-Requeni et al., 2004). These findings suggest that *L. minor* serves not only as a valuable protein source but also enriches the amino acid composition in fish carcass, enhancing nutritional quality.

The capacity of the fish to withstand environmental stress relies on the health of their immune systems (Adel et al., 2015). The immune system of fish is crucial for ensuring their tolerance to environmental stress (Adel et al., 2016). Various studies have been reported on the effect of the dietary inclusion of plant proteins on fish growth and immune status (Kokou et al., 2015; Dossou et al., 2018). The innate immune system serves as the first line of defence in fish, offering crucial protection against various diseases and playing a more significant role in fish than in mammals (Saurabh & Sahoo, 2008). Fish skin mucus enhances this protection by containing immune components such as lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides, and IgM (Magnadottir, 2006). Immunoglobulin serves as the main antibody in fish, playing an essential role in adaptive immunity and being widely studied to assess fish health (Flajnik, 2002; Baba et al., 2021). In this study, higher total immunoglobulin was reported in the *L. minor* incorporated diet-fed fish *A. testudineus*. In the case of *H. fossilis*, there was no significant difference ($P > 0.05$) in TIg levels across all treatments, though slightly higher TIg content was observed in diets containing *L. minor* compared to the control diet. Similar findings were reported in juvenile hybrid grouper fish with increased levels of peanut meal (Ye et al., 2020). Likewise, an increase in immunoglobulin content was also noted in *Cyprinus carpio* fed with *Heracleum persicum* (Hoseinifar et al., 2016).

LYZ activity in fish is a well-known marker of innate immunity, providing an effective defence against both gram-positive and gram-negative bacteria by breaking down bacterial cell walls, which offers a rapid immune response (Saurabh & Sahoo, 2008). In this study, *A. testudineus* fed with a diet that included *L. minor* exhibited higher LYZ activity than those on a control diet, suggesting that *L. minor* may enhance immune responses in this species and potentially improve their resistance to bacterial infections. However, in *H. fossilis*, the control group (LM0) and the group receiving a 5% amount of *L.*

minor (LM5) showed similar LYZ activity levels, while other groups exhibited lower activity. This variation in response indicates that the amount or specific dietary composition of *L. minor* may have different impacts on immunity, depending on the species and dosage used. These results align with previous studies that show dietary impacts on LYZ activity in fish. For instance, studies have found that fish-fed diets enriched with soy protein concentrate displayed increased LYZ activity, supporting the idea that certain plant-based additives can improve immune markers in fish (Hoseinifar et al., 2016; Wang et al., 2017). Additionally, Gibel carp (*Carassius auratus gibelio*) fed a diet containing fermented *Moringa oleifera* leaf meal exhibited significantly higher serum LYZ activity than those on a fishmeal diet, highlighting the immune-boosting potential of specific plant ingredients (Zhang et al., 2020b).

ALP in fish skin mucus is known for its antimicrobial properties, which help defend fish against waterborne pathogens (Lalles, 2019), and it also acts as an indicator of stress in fish (Guardiola et al., 2016). In the current study, significantly ($P < 0.05$) higher ALP levels were observed in *A. testudineu* fed the LM15 diet, while no significant differences ($P > 0.05$) were seen among the other groups. For *H. fossilis*, ALP activity was significantly ($P < 0.05$) higher in the LM5 and LM10 groups, with no significant difference ($P > 0.05$) between the control group (LM0) and the LM20 group. These findings are consistent with prior studies that have also reported increased ALP activity as a response to dietary modifications (Adel et al., 2015; Zhang et al., 2020b). While the exact mechanisms behind the immune-enhancing effects of plant-based diets in fish remain unclear (Reverter et al., 2021), certain phytochemicals in plants- such as alkaloids, phenolic compounds, and steroids-are attributed to contribute to these immune benefits (Awad & Awaad, 2017).

CAT is an essential antioxidant enzyme that plays a key role in protecting the body from damage caused by reactive oxygen species, which are harmful byproducts of cellular metabolism (Machlin, 1988; Michiels et al., 1994). In this study, *A. testudineus* fed a diet containing *L. minor* showed significantly ($P < 0.05$) higher CAT activity, suggesting enhanced antioxidant defence in this group. However, in *H. fossilis*, no significant differences (*P* > 0.05) in CAT activity were observed across the different dietary groups. These findings align with previous studies that have reported increased CAT activity in fish fed various plant-based components, such as juvenile grouper fed a diet with 33% soy protein concentrate (Wang et al., 2020), juvenile Gibel carp fed fermented *Moringa oleifera* leaves (Zhang et al., 2020b), and *Pagrus major* (red seabream) fed fermented rapeseed meal (Dossou et al., 2018). This suggests that certain plant-based diets can stimulate antioxidant defences in fish, although the response may vary depending on the species and specific plant components included.

Plant-based proteins added to the fish diet impact fish development and immunological state (Dossou et al., 2018). Antioxidant defences and reactive oxygen species in animal cells were highlighted by Tocher et al. (2002), with an imbalance in ROS causing oxidative stress. Sheikhzadeh et al. (2012) established a connection between fish antioxidant defences and nutrition. In response to toxicants, the generation of reactive oxygen species increases, which is counteracted by an antioxidant enzyme system (Lushchak, 2011). The first line of protection against reactive oxygen species oxidative damage is provided by the antioxidant enzyme SOD (Fridovich, 1995). In the present study, no significant difference (*P* > 0.05) was found in SOD activity in the plant-fed fish compared to the control. Similar results were also reported in *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* fed with *L. minor* (Aslam et al., 2021). However, Wang et al. (2017) observed a noteworthy diminishing trend of SOD in *Larimichthys crocea* when fed with soy protein concentrate. The TBARS assay is commonly used to measure lipid peroxidation (Oakes et al., 2003). No significant differences (*P* > 0.05) in TBARS activity were observed compared to the control group. This result aligns with the findings by Aslam et al. (2021), which reported no significant differences in TBARS levels for *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* fed *L. minor*. These observations suggest that *L. minor* may have antioxidant properties, potentially helping to maintain cellular integrity in fish exposed to environmental stress.

Similarly, Wang et al. (2017) found no significant difference ($P > 0.05$) in TBARS levels for *Larimichthys crocea* fed a soy protein-based diet, further supporting the potential antioxidant role of plant-based diets in fish.

AST and ALT are enzymes found in various tissues, including skeletal muscles, liver, and red blood cells (Hadi et al., 2009). These enzymes serve as biomarkers, or indicators, for liver function and overall fish health. When levels of these enzymes appear in blood serum or plasma, they provide specific insights into organ health. For instance, an increase in ALT activity often signals liver disease (Shahsavani et al., 2010). In this study, *A. testudineus* and *H. fossilis* did not show significant differences ($P > 0.05$) in AST and ALT levels. This result suggests that *L. minor* does not negatively affect the liver and does not induce liver stress or dysfunction. These findings align with those of Mustofa et al. (2022), which observed no adverse liver effects in *Lates calcarifer* fed *L. minor*-based diets. Conversely, diets based on fermented soy pulp were found to increase AST and ALT levels in African catfish, suggesting that *L. minor* could be a safer option for maintaining liver health in aquaculture (Kari et al., 2020). The results of all immune parameters indicate that *L. minor* does not adversely affect the skin and tissue immune responses of *A. testudineus* and *H. fossilis* when used to replace up to 20% of animal protein in the diet. However, there are signs of potential negative effects at higher inclusion levels, likely due to the fish's limited ability to digest and metabolise large amounts of plant protein.

I. aquatica is a tropical semi-aquatic plant that grows naturally in ponds, rivers, and lowlands across Asia. *I. aquatica* is high in minerals, vitamins, and trace elements, essential and non-essential amino acids (Austin, 2007; Adedokun et al., 2019; Ramzy et al., 2019). The bioactive phytochemicals present in the plant (Saikia et al., 2023) may provide optimal growth and health benefits to aquatic animals by enhancing their defence system (Roy et al., 2022). Several studies have shown that incorporating *I. aquatica* into the diet positively affects fish growth (Odulate et al., 2013; Yousif et al., 2019). However, despite its high crude protein and lipid content, *I. aquatica* has been underutilised in fish feed formulations (Mandal et al., 2010). To date, most of the studies on *I.*

aquatica are on cyprinids (Baruah et al., 2018; Ali & Kaviraj, 2018) and tilapia (Manuel et al., 2020; Chepkirui et al., 2021), and limited works are available on carnivorous fish species (Nandi et al., 2023). This study is a first attempt to explore the potential for replacing animal-based protein in the feed of *A. testudineus* and *H. fossilis* with this freely available semi-aquatic weed. To determine the optimal inclusion level of *I. aquatica* in the diets of these species, feed formulations containing 0%, 5%, 10%, 15%, and 20% *I. aquatica* were prepared and fed over 60 days. At the end of the trial, growth performance, digestive enzyme activity, biochemical parameters, and muscle carcass composition were comprehensively assessed.

In this study, the findings indicate that supplementing *I. aquatica* in the diets of *A. testudineus* and *H. fossilis* positively influenced the growth performance of both fish species. However, results showed that the level of *I. aquatica* supplementation affected each species differently, highlighting its potential as a dietary replacement with varying impacts on different fish species. For *A. testudineus*, FW was observed in the IA15 diet group, which was significantly ($P < 0.05$) higher than in all other groups. In contrast, *H. fossilis* achieved its highest FW in the IA10 diet group, significantly ($P < 0.05$) higher than in the other groups. These results are consistent with similar studies, such as Odulate et al. (2013), which demonstrated the positive effects of *I. aquatica* supplementation in the diet of the catfish *Clarias gariepinus*. Likewise, Yousif et al. (2019) observed beneficial outcomes when *Oreochromis niloticus* was fed a diet enriched with *I. aquatica*, further supporting the positive impact of plant-based supplements on fish growth across various species.

The BWG was highest for *A. testudineus* in the IA15 diet group ($P < 0.05$), showing a 1.18-fold increase compared to the control, significantly exceeding the BWG of all other groups. However, the IA20 diet showed a slight decrease in BWG, indicating a possible threshold effect where additional supplementation no longer enhanced growth. For *H. fossilis*, the highest BWG was recorded in the IA10 diet group ($P < 0.05$), with a 1.25-fold increase over the control, while subsequent diets with higher levels of *I. aquatica* inclusion, specifically IA15 and IA20, showed a slight decrease in BWG. Both species, *A. testudineus* and *H. fossilis*, exhibited higher BWG with diets supplemented by *I. aquatica*. However, results suggest an optimal level of inclusion beyond which growth benefits start to diminish. These findings align with previous studies (Ali & Kaviraj, 2018; Ali & Kaviraj, 2021) and Baruah et al. (2018), which reported that *I. aquatica* supplementation successfully enhanced growth up to a certain level, after which the growth benefits declined. The optimal inclusion level of *I. aquatica* in the diet was higher for *A. testudineus* than for *H. fossilis*, possibly due to species-specific dietary responses.

A similar pattern was observed in the SGR in this study, with *A. testudineus* showing a significantly higher SGR in the IA15 diet group, exhibiting a 1.09-fold increase compared to the control. For *H. fossilis*, the highest SGR was recorded in the IA10 group, with a 1.14-fold increase over the control. Both species displayed a diminishing trend in SGR with *I. aquatica* supplementation beyond these optimal levels. This decline at higher inclusion levels may be due to reduced diet palatability as *I. aquatica* levels increase and certain dietary elements that may interfere with metabolic processes (Fiordelmondo et al., 2022). Lower values of FCR indicate efficient utilisation of the feeds, which may minimise production costs and make feed production a sustainable process (Martinez-Cordova et al., 2017). In the present study, lower FCR values were recorded in IA15 for *A. testduineus* and IA10 showed lower values for *H. fossilis*. A slight increase in FCR at IA20 for *A. testudineus* and IA15-IA20 for *H. fossilis* suggests that higher inclusion levels may lead to reduced digestibility or the presence of anti-nutritional factors that impair feed conversion efficiency (Gopan et al., 2020).

The FE was significantly higher in IA15, showing a 1.10-fold increase compared to the control IA0 for *A. testudineus* and a 1.16-fold increase in IA10 for *H. fossilis* compared to the control. A slight decline in FE was observed at IA20 for *A. testudineus* and between IA15-IA20 for *H. fossilis*, indicating that higher inclusion levels may introduce factors that hinder digestibility. PER was highest in IA15 and IA20 for *A. testudineus*, although a slightly lower PER was recorded in IA20; however, the difference was not statistically significant between these levels. In *H. fossilis*, the highest PER was recorded in IA10, while IA15-IA20 showed lower PER values, suggesting that higher supplementation levels may interfere with digestibility and protein absorption. The improvement in growth performance in both fish species is linked to better feed utilisation and increased digestive capacity, especially when fed diets supplemented with *L. minor*. Several studies, such as that by Naseem et al. (2021), have also reported the positive effects of an *I. aquatica*-supplemented diet on fish growth. Furthermore, the consistent 100% survival rate across all treatments for both *A. testudineus* and *H. fossilis* suggests that *I. aquatica* is a safe feed ingredient even at higher inclusion levels. This high survival rate supports the potential of *L. minor* as a reliable and safe component in the diet of these fish species, as no adverse effects were observed from its inclusion. Congruent to our observations, Nandi et al. (2023) also observed enhanced growth, reproductive parameters, and health status of *H. fossilis* when fed with a diet containing fermented water spinach. Similarly, a replacement of fishmeal by *I. aquatica* in the Nile tilapia (*O. niloticus*) diet showed no negative impact on its growth performances and nutrient utilisation (Yousif et al., 2019). Chepkirui et al. (2021) also reported the dietary incorporation of water spinach meal for improved growth performance in Nile tilapia.

However, replacing fish meal with plant sources at higher levels can often lead to poor fish growth (Abdel-Warith et al., 2013). This may be due to a deficiency of essential amino acids in most plants (El-Saidy & Gaber, 2003; Furuya et al., 2004) or increased fibres or antinutrients (NRC, 2011). Moreover, lower dietary nutrient digestibility may also result due to microbial activities for fermentation of increased fibre in the lower gut of the fish, leading to reduced growth and a higher FCR (Glencross, 2009). Similar observations were noted in our study, as the growth performance of *A. testudineus* declined at a higher inclusion level (IA20) of *I. aquatica*. This indicates the threshold level beyond which further inclusion may adversely affect the fish's growth and digestive enzyme activity. Higher inclusion levels of plant proteins can also interfere with nutrient absorption and metabolism in fish (Francis et al., 2001). Similar results were reported in *Clarias gariepinus* by Odulate et al. (2013) and in *O. niloticus* by Yousif et al. (2019) and Manuel et al. (2020) when fed with *I. aquatica* incorporated diet.

The regression analysis for SGR and FCR yielded values between 16.64 and 17.50 for *A. testudineus* and 11.73 to 11.97 for *H. fossilis*, indicating an optimal range for incorporating *I. aquatica* into their diets. This range aligns with findings from previous research, which has similarly applied regression analysis to determine optimal plant-based diet inclusion. For instance, Hossain et al. (2023) explored plant-based diet incorporation in *H. fossilis*, Ahmad and Abdul-Tawwab (2011) examined it in *Oreochromis niloticus*, and Shekarabi et al. (2022) analysed it in *Oncorhynchus mykiss*. These studies collectively support the use of regression analysis as a valuable tool in assessing dietary optimisation across various species.

The analysis of carcass proximate composition revealed no reduction in the nutritional quality of fish across all diet groups, indicating the stability of nutrient profiles even with varied levels of *I. aquatica* inclusion. In *A. testudineus*, the IA15 diet led to a significantly ($P < 0.05$) higher content of protein, lipid, and ash compared to other groups, while the IA10 group demonstrated similar nutrient enhancements. This outcome suggests an efficient utilisation and assimilation of dietary nutrients, especially in these optimal IA inclusion levels. In both *A. testudineus* and *H. fossilis*, fibre content remained consistent across treatments, showing no significant differences ($P > 0.05$), which indicates that fibre intake was stable irrespective of IA levels. However, a notable increase in carbohydrate content was observed in the IA20 group for both fish species, which may indicate a threshold effect where excessive IA inclusion leads to higher carbohydrate retention, potentially influencing overall energy metabolism. This trend aligns with previous studies, where the incorporation of *I. aquatica* enhanced protein, lipid, and ash content in various fish species (Ali & Kaviraj, 2018; Ali & Kaviraj, 2021). These findings reinforce the potential of *I. aquatica* as a beneficial dietary component, capable of improving the nutritional profile of fish without compromising carcass quality when included at optimal levels.

A key factor influencing the efficiency of nutrient uptake in fish is digestive enzyme activity, which can vary significantly based on diet composition, feeding habits, and the types of foods ingested (Almeida et al., 2018; Goswami et al., 2020; Roy et al., 2022). Our study indicates a general trend in *A. testudineus* where digestive enzyme activity (amylase, trypsin, total protease, pepsin, and lipase) increases up to 15% inclusion of *I. aquatica* (IA15), followed by a decline at higher inclusion levels (20%). This suggests IA15 represents an optimal dietary level for maximising enzyme function in *A. testudineus*. In *Heteropneustes fossilis*, enzyme activities (amylase, trypsin, chymotrypsin, pepsin, and total protease) were significantly higher in the IA10 group, while lipase activity was notably elevated ($P < 0.05$) in both IA10 and IA15 diets. This indicates that an IA10 inclusion level may be optimal for general enzyme activity in *H. fossilis*, while lipase activity can be maintained at slightly higher IA levels without adverse effects. Enhanced enzyme activity is associated with improved nutrient digestion and absorption, ultimately contributing to better growth performance. The decline in enzyme activity observed at the 20% IA inclusion level (IA20) may be attributed to increased fibre or antinutrient content, which can impede digestive efficiency, a pattern similarly reported by Manuel et al. (2020). Our growth performance results corroborate these findings, as higher enzyme activities in the IA15 group were linked to improved FCR and SGR, indicating more efficient growth. Overall, these results underscore the importance of optimising *I. aquatica* inclusion levels to balance enzyme activity and nutrient absorption for optimal growth performance in aquaculture species.

In this study, amylase activity in *A. testudineus* and *H. fossilis* increased with higher percentages of *I. aquatica* in the diet, peaking at 15% inclusion for *A. testudineus* and 10% for *H. fossilis*. Beyond these levels, amylase activity declined. Among the treatments, fish fed the IA15 diet showed the highest amylase activity ($P < 0.05$) in *A. testudineus*, while the IA10 diet yielded the highest ($P < 0.05$) activity in *H. fossilis*. This increase in amylase activity suggests that *I. aquatica* effectively stimulates amylase production, supporting more efficient carbohydrate utilisation. The enhanced enzyme activity observed in the *I. aquatica*-supplemented groups may be attributed to bioactive compounds within *I. aquatica*, which appear to promote enzyme function under optimal inclusion levels. This indicates that *I. aquatica* could play a beneficial role in improving carbohydrate digestion and overall dietary efficiency in fish diets. While Saikia et al. (2023) reported inhibitory effects of these compounds on α-amylase and α-glucosidase, their complex interactions could lead to different outcomes depending on the physiological context and dietary composition. Higher amylase activity has been associated with a higher proportion of plant materials in its diet, such as in herbivorous and omnivorous fish (Gioda et al., 2017). Better amylase activity may result in efficient utilisation of carbohydrates for protein-sparing effect, thereby making the proteins available for growth in the fish. Our results agree with that of Ali and Kaviraj (2018), where the enhanced activity of α-amylase was reported in *L. rohita* fed with a diet supplemented with 25% *I. aquatica* (fermented) leaf meal, beyond which the activity decreased at a higher level of inclusion (50 and 75%).

In this study, fish fed specific *I. aquatica* diets demonstrated significantly higher ($P < 0.05$) lipase activity, with *A. testudineus* on the IA15 diet and *H. fossilis* on IA10 and IA15 diets showing enhanced fat digestion. These findings are consistent with previous research highlighting the positive effects of plant-based diets, particularly those containing *I. aquatica*, on lipase activity. For instance, studies have shown that *Labeo rohita* fed an *I. aquatica*-incorporated diet exhibited increased lipase activity (Ali & Kaviraj, 2018). However, in contrast, *H. fossilis* did not display significant ($P > 0.05$) changes in lipase activity when fed fermented *I. aquatica* (Ali & Kaviraj, 2021). Higher lipase activity in the IA15 diet may be attributed to the essential fatty acids in *I. aquatica*, which result in the efficient utilisation of dietary fats for energy and fish growth (Gisbert et al., 2009). Similar findings were also reported in *L. rohita* when fed with *I. aquatica*-incorporated diet (Ali & Kaviraj, 2018) and in

Heteropneustes fossilis when fed with mulberry leaf by Ali et al. (2019) and soybean meal by Khanom et al. (2022). These results suggest that *I. aquatica* can enhance lipase activity in certain fish species, potentially improving lipid digestion efficiency, although effects may vary depending on processing methods and species-specific responses.

In this study, pepsin activity was significantly elevated in IA15 for *A. testudineus*, whereas it peaked in IA10 for *H. fossilis*. This increase in pepsin activity suggests enhanced protein breakdown within the stomach, leading to improved amino acid absorption (Buddington et al., 1992). Trypsin activity followed a similar pattern, with the highest levels observed in IA15 for *A. testudineus* and in IA10 for *H. fossilis*. Beyond these inclusion levels, both species showed a slight decline in trypsin activity. These findings are consistent with previous studies indicating increased trypsin activity in fish-fed macrophyte-enriched diets (Goswami et al., 2022; Shrivastav et al., 2022; Goswami et al., 2020). Chymotrypsin activity reached its peak in IA15 and IA20 for *A. testudineus*, with no significant difference between these two groups, while for *H. fossilis*, the highest chymotrypsin activity occurred in IA10. Across both species, diets supplemented with *I. aquatica* were associated with elevated chymotrypsin activity.

The activities of various proteases- total protease, serine proteases (trypsin and chymotrypsin), and acidic protease (pepsin)- showed consistent trends. The *I. aquatica*-supplemented diet boosted protease activity across these enzymes in both *A. testudineus* and *H. fossilis*, with peak activity at IA15. Beyond this level, protease activity declined at IA20 for *A. testudineus* and, notably, after IA10 for *H. fossilis*. This suggests that exceeding these inclusion levels may not yield further benefits, likely due to factors such as anti-nutritional compounds or increased dietary fibre (NRC, 2011). Reduced digestibility at higher plant inclusion levels may also result from increased ash and fibre content (Cruz-Velásquez et al., 2014) and the lack of cellulase enzymes in fish needed to break down cellulose, the main component of plant cell walls (Ray et al., 2012). Growth performance outcomes at *I. aquatica* supplemented groups were consistent with the observed enzyme activities, supporting the link between enhanced trypsin and total protease levels and improved protein digestion. Such improvements contribute to growth and muscle development (Chamchuen et al., 2014). A similar rise in protease activity was also noted in *Labeo rohita* when fed with a diet containing fermented *I. aquatica* at 50% and 75% replacement levels (Ali & Kaviraj, 2018). Goswami et al. (2022) also reported enhanced protease (total protease, trypsin and chymotrypsin activity) in *Cyprinus carpio* fed with 15-20% inclusion of *L. minor* in its diet. Similar observations were also made by Shrivastav et al. (2022) in juvenile common carp (*Cyprinus carpio*) when fed with *Spirodela polyrhiza* supplemented feed (up to 20%).

This study demonstrates that an *I. aquatica*-supplemented diet significantly altered the fatty acid profiles of *A. testudineus* and *H. fossilis*. Specifically, increasing the concentration of *I. aquatica* in the diet led to a rise in PUFAs alongside a reduction in SFAs in both species. These findings are consistent with previous studies that observed similar shifts in fatty acid composition when macrophytes were included in fish diets (Herawati et al., 2020; Shrivastav et al., 2022). In contrast, Fiordelmondo et al. (2022) reported no significant changes in SFAs, MUFAs, or PUFAs with macrophyte supplementation, suggesting that the effects of such dietary modifications may vary by species. This variability could be attributed to differences in species-specific dietary assimilation and metabolic processing of fatty acids.

The amino acid profile of *I. aquatica* highlights its potential as a valuable ingredient in aquaculture diets, offering a balanced array of essential and non-essential amino acids (Austin, 2007; Adedokun et al., 2019; Ramzy et al., 2019). The inclusion of *I. aquatica* in the diets of *A. testudineus* and *H. fossilis* was found to enhance their amino acid composition significantly. In this study, *A. testudineus* fed the IA15 diet exhibited the highest levels of essential and non-essential amino acids, suggesting that this inclusion level most effectively supports protein synthesis and overall fish health. Similarly, the IA10 diet led to the highest EAA and NEAA levels in *H. fossilis*. The presence of high-quality protein in *I. aquatica*, enriched with vital amino acids (Saikia et al., 2023), likely contributes to the improved amino acid profile in the *I. aquatica*-supplemented groups. These findings are consistent with previous studies, which indicate that plant-based diets when properly formulated, can meet the amino acid needs of fish (Yousif et al., 2019; Goswami et al., 2022). Elevated levels of specific amino acids such as arginine, leucine, and phenylalanine in *I. aquatica*-fed groups suggest its potential to enhance growth and metabolic functions. Arginine, for instance, is crucial for protein synthesis and immune support, while leucine plays a vital role in muscle protein synthesis and repair (Francis et al., 2001). However, at higher inclusion levels, a slight decline in amino acid content was observed, with *A. testudineus* showing a reduction at IA20 and *H. fossilis* at IA15–IA20. This suggests that while *I. aquatica* is beneficial up to a certain threshold. Exceeding this level may reduce amino acid bioavailability or introduce antinutritional factors that could hinder protein metabolism. Thus, optimal inclusion levels are essential to maximise the benefits of *I. aquatica* in aquaculture diets.

The biochemical analysis results indicate that incorporating *I. aquatica* into the diets of *A. testudineus* and *H. fossilis* has a positive impact on the health and physiological condition of these fish. In *A. testudineus*, an upward trend in TIg levels was observed, with a significant ($P < 0.05$) increase in the IA15 group, although a slight decline in TIg levels was noted at the IA20 concentration. Similarly, for *H. fossilis*, TIg activity was significantly ($P < 0.05$) higher in the IA10 group, while increasing the concentration of *I. aquatica* beyond this level resulted in a decrease in activity. Elevated immunoglobulin levels are typically linked to enhanced immune response, potentially contributing to improved disease resistance and overall health in fish (Ali & Kaviraj, 2018). This boost in immune function can be attributed to the rich nutritional profile of *I. aquatica*, which contains essential amino acids, vitamins, and minerals crucial for immune support and overall vitality in fish (Roy et al., 2022). LYZ functions as an enzyme that targets bacterial cell walls, aiding in the defence against bacterial infections (Chamchuen et al., 2014). In this study, *A. testudineus* fed an *I. aquatica*-incorporated diet showed higher LYZ activity, with no significant ($P > 0.05$) differences observed among the various *I. aquatica*-supplemented groups. In contrast, *H. fossilis* showed no significant ($P > 0.05$) differences in LYZ activity across treatments. These results suggest that *I. aquatica* may enhance the natural immune response in *A. testudineus* without adverse effects in *H. fossilis* when included in the diet. These findings align with previous studies, which have also reported enhanced LYZ activity with plant-based diets (Hoseinifar et al., 2016; Wang et al., 2017).

In the present study, a significantly higher ($P < 0.05$) activity of ALP was observed in *A. testudineus* fed with the IA15 diet containing *I. aquatica*, while ALP levels remained consistent across all dietary treatments for *H. fossilis* ($P > 0.05$). This suggests that *I. aquatica* may enhance the natural immune response in *A. testudineus*, with no adverse effects observed in *H. fossilis*. ALP plays an essential role in cellular signalling and immune response, which may account for this effect (Chamchuen et al., 2014). These findings align with previous studies reporting increased ALP activity in response to dietary modifications (Adel et al., 2015; Zhang et al., 2020b). SOD and CAT are key antioxidant enzymes that protect cells from damage caused by free radicals (Fridovich, 1995). In the present study, *A. testudineus* fed with an *I. aquatica*-supplemented diet showed higher CAT activity compared to the control group, although the difference was not statistically significant ($P > 0.05$). In contrast, *H. fossilis* did not display significant ($P > 0.05$) differences in CAT activity across the dietary groups. The observed increase in CAT activity in *A. testudineus* suggests an improved capacity to manage oxidative stress, which could support overall health and resilience (Francis et al., 2001). These findings align with previous studies on plant-based protein diets, which also reported enhanced CAT activity in various fish species (Wang et al., 2017; Zhang et al., 2020b; Dossou et al., 2018).

Although the *I. aquatica*-fed groups exhibited higher SOD activity in both *A. testudineus* and *H. fossilis*, these differences were not statistically significant ($P > 0.05$). Nonetheless, the elevated SOD levels in the *I. aquatica* groups indicate a potential enhancement of the fish's antioxidant defence system.

This trend aligns with findings in other species, such as *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix*, which demonstrated similar increases in SOD activity when fed with *L. minor* (Aslam et al., 2021). In contrast, research by Wang et al. (2017) reported a decrease in SOD activity in *Larimichthys crocea* when soy protein concentrate was used, suggesting that not all plant-based diets provide the same antioxidant benefits. AST and ALT enzyme levels are reliable indicators of liver health and function in fish (Kari et al., 2020). In the present study, both *A. testudineus* and *H. fossilis* exhibited similar ALT and AST levels across all dietary groups, with no significant differences ($P > 0.05$). This consistency suggests that incorporating up to 20% of *I. aquatica* in fish diets does not negatively impact liver health, thereby supporting the safety of this plant-based protein source as a dietary component (Naseem et al., 2021). The TBARS assay measures lipid peroxidation caused by free radical formation to assess oxidative stress (Oakes et al., 2003). The present study observed no significant difference ($P > 0.05$) in the TBARS among the different groups in both *A. testudineus* and *H. fossilis*. Similar results were also reported when *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* were fed *L. minor* (Aslam et al., 2021). Similarly, Wang et al. (2017) found no significant difference ($P > 0.05$) in TBARS levels for *Larimichthys crocea* fed a soy protein-based diet, further supporting the potential antioxidant role of plant-based diets in fish.