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Research article

Enhancing enzyme digestibility of red tilapia (*Oreochromis niloticus* and *O. mossambicus*) and improving water quality in fish farming using Napier grass silage

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Abstract

Importance of the work: Utilizing locally available and more affordable plant-based raw materials could mitigate the increasing costs of raw fish feed ingredients. This study should enhance understanding regarding using Napier grass (*Pennisetum purpureum* 'Pak Chong 1') as a fish feed ingredient and its effect on fish, digestive enzyme activities and water quality in aquaculture.

Objectives: To assess the efficacy of different processed forms of Napier grass as a feed ingredient for red tilapia (hybrid between *Oreochromis niloticus* and *O. mossambicus*) and efficacy of extracted tannins in reducing the ammonia content in water.

Materials & Methods: The *in vitro* digestibility was analyzed of four Napier grass variants (dried, fresh, fermented and boiled). Proteolytic, amylase and cellulase enzyme activity levels were assessed.Tannin extraction was used to evaluate the efficiency of Napier grass extracts in reducing water ammonia levels based on the microwave method, which yielded a higher tannin content than the reflux method. Then, the extracted tannins were applied to water samples to examine their efficacy in reducing the ammonia content.

Results: The red tilapia fed fermented Napier grass (SN) had the highest enzymatic activity, with trypsin activity at 0.439 µmol DL-alanine/g feed, amylase activity at 0.828 µmol maltose/g feed, while the cellulase activity was 587.123 µmol maltose/g feed ($p < 0.05$). The application of Napier grass extracts successfully reduced the ammonia content in the water samples at 0.15 mg/l NH_3-N/g dry Napier grass. Evidence for the binding of tannic acid and ammonium ion was identified.

Main finding: Napier grass, particularly in its fermented form, can serve as an effective alternative feed for aquaculture. Tannin extracted from Napier grass efficiently reduced the ammonia content in water.

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Introduction

The agricultural sector is facing challenges in terms of rising prices of raw animal feed ingredients, which poses a burden on farmers involved in fish hatcheries and aquaculture operations (Fongsamouth et al., 2022). A proactive approach has emerged to address this issue by utilizing locally available, high-protein plants as substitutes for expensive ingredients, such as soybean meal to help farmers reduce costs, promote sustainable aquaculture practices and reduce reliance on costly imported ingredients (Arriaga-Hernández et al., 2021). One such plant-based raw material that has gained popularity is Napier grass (*Pennisetum purpureum*). Napier grass is valued for its rapid growth and high yield (Dussadee et al., 2016) and its nutritional content, with a protein content range of 10.6–12.6% in its dried form (Balakrishnan et al., 2023). Apart from using Napier grass as fresh biomass for animal feed, there is potential to enhance its nutrient value through fermentation, making it more easily digestible (Hsu et al., 2013).Various fermentation methods, including urea-molasses, solid-state and enzymatic hydrolysis fermentation, have been explored to modify the properties of dried Napier grass (Camesasca et al., 2015). These offer opportunities to optimize Napier grass's nutrient profile and overall suitability as a feed ingredient, fostering its potential in agricultural systems.

Intensive fish farming involves cultivating fish at high stocking densities leading to improved fish feeding efficiency through enhanced utilization of essential digestive enzymes, such as protease, lipase and amylase (Thongprajukaew et al., 2017). For example, tilapia have various digestive enzymes in their stomach and intestines (Hangsapreurke et al., 2020; Is-haak et al., 2020). Pepsin and other enzymes are most active in highly acidic environments (Moyle and Cech, 2000). Of particular importance is the amylase enzyme, which is responsible for carbohydrate digestion. The functioning of digestive enzymes can be effectively stimulated by providing easily digestible fish feed. This, in turn, promotes better feed utilization and optimizes the nutritional intake of the fish. Adopting strategies to ensure the digestibility of fish feed not only supports the overall health and growth of the fish but also improves the efficiency and effectiveness of intensive fish farming practices.

One key aspect of waste management in aquaculture involves addressing the issue of ammonia toxicity in aquaculture ponds. Predominantly, the ammonia in these ponds originates from the protein metabolism of fish feed and is excreted through urine and the decomposition of leftover feed and waste in sediments (Tongmee et al., 2020; Tongsiri et al., 2020). In aquaculture systems, especially ones using water recirculation, the water in the fishponds often contains elevated levels of nitrogen compounds (Whangchai et al., 2022). Therefore, effective waste management strategies must be implemented to monitor and control ammonia levels, ensuring a healthy and sustainable aquatic environment for the fish.

During protein metabolism, ammonia is released and excreted as NH_4^+ and NH_3, with the combined quantity of NH_4^+ and NH_3 being referred to as total ammonia nitrogen (TAN). Water pH, temperature and salinity influence the ratio between these two ammonia forms; generally, aquatic animals excrete TAN at a rate in the range 2.0–3.5% of their feed intake (Wheaton et al., 1994). Notably, even at a concentration of just 0.1 mg/l, free ammonia can have immediate harmful effects on aquatic animals (Tan et al., 2021). There have been some reports on using forage plants as a feed additive to reduce ammonia in the urine and feces of fish. For example, *Yucca schidigera*, used as a feed additive for *Pangasianodon hypophthalmus* and *Oreochromis niloticus* (Hassan et al., 2017), resulted in a significant reduction in the ammonium nitrogen excreted into the water. In addition, livestock production controls ammonia in waste and reduces ammonia levels in fish-rearing water. For example, studies conducted by Adegbeye et al. (2019), Fayed et al. (2019) and Elabd et al. (2020) have shed light on the potential benefits of *Y. schidigera* in mitigating ammonia-related issues. Building upon this foundation, the present study aimed to ascertain the suitability of Napier grass for reducing the ammonia content within water systems and its potential as a feed source for aquatic animals. The study results should offer insights for the formulation of efficacious strategies to preserve a balanced aquatic ecosystem while harnessing Napier grass as a valuable nutritional resource.

Materials and Methods

Materials preparation and experimental procedure for Napier grass regarding in vitro digestibility and enzyme activity

This experiment was conducted at the Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai, Thailand. The study followed a completely randomized design with four treatments and three replications. The Napier grass (at 45 d after planting) used in the experiment was procured from the Uthai Thani Animal Research and

Breeding Center, Sawang Arom district, Uthai Thani province, Thailand. The Napier grass preparations were divided into four treatments with three replicates each. Treatment 1 involved dried Napier grass (DN), which was prepared by chopping fresh grass into 2.5–5 cm pieces and then drying at 60°C. Treatment 2 utilized fresh Napier grass (FN) chopped into 2.5–5 cm pieces.Treatment 3 involved fermenting dried Napier grass (SN) for 10 days. In this experiment, a 15% concentration of dried Napier grass was submerged in 5 L of distilled water. Then, the mixture was agitated to ensure uniform soaking and left to ferment at ambient temperature. In Treatment 4, the dried Napier grass underwent a boiling process at 100°C for 10 min to sterilize and eliminate some pathogens.

Sample preparation of gastrointestinal tracts of red tilapia for in vitro digestibility study

The red tilapia samples, each weighing 100 g, were dissected by opening the abdominal cavity and the small intestine were collected. Enzyme extraction was done by homogenizing the small intestine samples in 50 mM Tris-HCl buffer (pH 8) containing 200 mM NaCl with a ratio of 1:1 (weight per volume, w/v, small intestine-to-buffer). Then, the resulting extract was stored at -20°C. To further process the crude enzyme extract, the homogenized sample was crushed and immersed in an ice bath. Afterward, the samples were centrifuged at 14,000×g for 30 min at 4°C. Next, the supernatant was collected and stored at -80°C for subsequent analysis and experimentation. The digestive enzyme activity and *in vitro* digestibility were assessed for the four Napier grass treatments mentioned earlier.

Digestive enzyme activity measurement

For the trypsin activity assay, 2 µL of the crude enzyme extract was mixed with 200 µL of 1.25 mM benzoyl-L-arginine-p-nitroaniline in a 96-well plate. The enzyme activity was measured at 410 nm using a microplate reader and the reaction time was recorded. The protein content in the crude enzyme extract was estimated using the Lowry method (Lowry et al., 1951). A mixture of Lowry's reagents was prepared at an A-to-B-to C ratio of 100:1:1. Then, 100 µL of the crude enzyme extract was mixed with 3 mL of the reagent mixture and incubated for 10 min. Afterward, 300 µL of diluted Folin Ciocalteau reagent (1:11 with water) was added, and the mixture was incubated in a dark room for 30 min. The protein content was determined by measuring the absorbance at 750 nm using a spectrophotometer. The protein digestibility value was quantified as micromoles of DL-alanine equivalent, indicating the reactive amino groups released from cleaved peptides per gram of the feed sample per trypsin activity (Rungruangsak-Torrissen, 2007).

The amylase activity was determined based on Bernfeld's method (Bernfeld, 1951). In this procedure, 25 µL of 5% starch solution was mixed with 125 µL of the crude enzyme extract, 62.5 µL of buffer pH 7 and 37.5 µL of 20 mM sodium chloride. Then, each sample was incubated at room temperature for 15 min, after which 250 µL of 1% dinitrosalicylic acid (DNS) reagent was added to stop the reaction. The samples were boiled for 10 min and adjusted to a final volume of 2.5 ml with distilled water. The amylase activity was measured at 540 nm using a microplate reader, with 5% starch solution as the substrate. The amylase activity value was expressed as micromoles of maltose per gram of feed per amylase activity.

The cellulase activity was analyzed and adapted from Miller's method (Miller, 1959). Initially, 125 µL of the crude enzyme extract, 25 µL of 1% carboxymethyl cellulose (CMC), 62.5 µL of buffer pH 7, 25 µl of 5% starch solution and 37.5 µL of 20 mM sodium chloride were combined. The solutions were mixed and incubated at room temperature for 15 min. After incubation, 250 µL of 1% DNS reagent was added to stop the reaction. The samples were boiled for 10 min and adjusted to a final volume of 2.5 mL with distilled water; then, the cellulase activity was measured at 540 nm using a microplate reader, with 1% CMC as the substrate. The cellulase activity value was quantified as micromoles of maltose per gram of feed per cellulase activity.

In vitro digestibility determination

The *in vitro* digestibility of the Napier grass samples was assessed using a modified method derived from Rungruangsak-Torrissen (2007). In brief, each sample from the four Napier grass treatments was ground and approximately 5 mg of each sample was then mixed with 10 mL of 50 mM phosphate buffer (pH 7). To each sample, 50 µL of 0.5% chloramphenicol was added and the mixture was thoroughly mixed and incubated at 30°C for 24 hr. After incubation, 250 µL of each sample was combined with 250 µL of dialyzed crude enzyme extract, mixed well and incubated at 30°C for 24 hr. A 1,000 µL sample was collected, boiled for 10 min and then frozen at -80°C for further analysis.

The determination of protein digestion in the samples involved using the TNBS method to assess the levels of free amino groups in the content. The digested solution or control was mixed and then 200 µL of the resulting mixture was combined with 2 mL of 50 mM phosphate buffer (pH 8.2). Subsequently, the mixture was incubated in darkness at 60°C for 1 hr. The reaction was stopped by adding 1 mL of 1 M HCl, thoroughly mixed and left to cool to room temperature. Next, the absorbance was measured at 420 nm. The carbohydrate digestion in the samples was determined by analyzing the reducing sugar content using the DNS method. The digested solution or control was mixed and each sample was centrifuged at 10,000×g for 10 min. Then, 250 µL of the digested solution was combined with 250 µL of 1% DNS and thoroughly mixed. The samples were boiled for 10 min, allowed to cool to room temperature and then 2.5 mL of distilled water was added and mixed well. Absorbance was measured at 540 nm.

Napier grass extract using microwave and reflux extraction

The Napier grass was thoroughly washed, air-dried and then hot-air dried in an oven at 70°C for 4 hr. The resulting dried Napier grass was finely ground into powder using a 0.5 mm sieve mesh and stored in polyethylene bags at room temperature for further extraction.

Microwave extraction

A microwave extraction technique used methanol solvent to extract Napier grass compounds. The procedure involved thoroughly mixing 20 g of the ground sample with 100 mL of methanol and allowing it to soak for 15 minutes. Microwave extraction was performed at 500 W for 15 min. The extracted solution was passed through Whatman No.1 filter paper and then subjected to solvent evaporation using a rotary evaporator. The resulting crude extracts were stored at -20°C for further analysis and experimentation, following the methodology described by Senphan et al. (2021).

Reflux extraction

The reflux extraction method described in Chandrasekara and Shahidi (2015) was used, with slight modification. A sample (2 g) of dried Napier grass was mixed with 60 mL of 95% ethanol (w/v). Then, the mixture was subjected to heat reflux extraction at 95°C for 30 min to extract tannins and phenolic compounds. Next, the resulting extract was passed through Whatman filter paper No. 4 to remove impurities. Subsequently, the filtered portion was subjected to ethanol solvent evaporation in a rotary evaporator at 50°C. The obtained crude extract was further dried using a freeze dryer. To ensure preservation, the Napier grass extract was carefully packed in a tightly sealed, dark-colored bottle and stored at -20°C to maintain its quality for further analysis.

The yield of the extract from dried Napier grass was calculated based on the dry weight of extract using Equation 1:

$$\% \text{ Yield crude extract} = a / b * 100 \quad (1)$$

where a is the weight of the extracts and b is the weight of the dried Napier grass, both measured in grams.

Total phenolic content in Napier grass extract

The total phenolic content in the Napier grass extract was assessed using the Folin-Ciocalteu method, with adjustments based on the methodology described by Senphan et al. (2021). A volume of 100 µL of the Napier grass extract was mixed with Folin-Ciocalteu reagent. Following incubation for 5 min, the reaction mixture was combined with a 6% (volume per volume) Na_2CO_3 solution and allowed to incubate at room temperature for 1 hr. The absorbance of the resulting solution was measured at a wavelength of 760 nm using a spectrophotometer. The phenolic content was determined based on calculation using the standard curve of gallic acid in the range 0–0.05 mg/mL. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight of the Napier grass extract.

Efficiency of reducing ammonia in water using Napier grass tannin extract

The effectiveness was analyzed of tannins extracted from Napier grass to reduce the ammonia levels in water. The extraction process involved fermenting Napier grass at different weights (2 g, 4 g and 6 g per 1 L of water) for 7 d; then, the tannin content in the resulting extract was analyzed (adjusted from Whangchai, 2016). The Napier grass extract was filtered to remove sediments and residues from the fermentation process before the analysis. A volume of 0.2 mL of the Napier grass extract was pipetted into a test tube, adding 2.50 mL of water and 0.2 mL of Folin-Ciocalteu phenol reagent. The mixture was thoroughly mixed; then, 2 mL of 7.5% Na_2CO_3 solution was added and shaken well. The test tube was incubated in darkness at room temperature for 60 min, and the absorbance was measured at 760 nm. This allowed the determination of

the tannin content in the Napier grass extract by comparing its absorbance with the standard curve of a tannic acid solution.

The effect of the tannins extracted from Napier grass on reducing the ammonia content in water was assessed at intervals of 20 min for 80 min. This analysis aimed to evaluate the efficiency of the Napier grass tannin extract on reducing the ammonia levels over time, considering other reports regarding the effectiveness of crude tannins in reducing ammonia in water (Whangchai, 2016; Arismendi et al., 2018). Standard methods (American Public Health Association, 1980) were used to analyze the total ammonia-nitrogen (TAN) in the water.

Formation of tannic acid and ammonium ion prediction using Fourier transform infrared spectroscopy

The assessment of ammonia nitrogen reduction in this study involved analyzing the formation of tannic acid, and prediction of ammonium ions using Fourier transform infrared spectroscopy (FTIR). An ammonium solution (100 mg/L) was prepared by dissolving 0.0297 g of ammonium chloride (Ajex Finechem) adjust to 100 mL of deionized water. Next, 0.9950 g of tannic acid was dissolved in 2.5 mL of the 100 mg/L ammonium solution and the mixture was stirred until homogeneous. Attenuated total reflectance-FTIR (ATR-FTIR) was used to analyze the solution using a spectrum RX instrument (PerkinElmer). The analysis involved 32 scans with a resolution of 4 cm^{-1}, covering the spectral range 4000–600 cm^{-1}. This allowed for the characterization and examination of the solution's molecular composition and structural properties.

Statistical analysis

Analysis was conducted using one-way analysis of variance, with the means compared using Duncan's new multiple range test at the 95% confidence level. The analysis was performed using the SPSS for Windows software, version 15.0 (SPSS Inc.). A t test was used to compare between the yields of Napier grass extract obtained using the microwave and reflux extraction methods. Linear regression was used to evaluate effects of different tannin extracts on the percentage of ammonia removal. Results were presented as mean ± SD values.

Ethics statement

This study was approved by the Ethical Committee of Maejo University, Chiang Mai, Thailand (Approval No. MACUC005F/2566).

Results and Discussion

Evaluation of Napier grass digestion for aquatic animal feed

This study aimed to investigate in detail the impact of Napier grass on the enzyme digestibility of red tilapia, explicitly focusing on the *in vitro* protein digestibility. Most studies on fish growth have centered around the digestibility of proteins and fats, somewhat sidelining the contribution of carbohydrates (Haidar et al., 2016; Jongjaraunsuk and Taparhudee, 2022). Despite being a cost-effective energy source suitable for various organisms, including fish, the dietary guidelines on carbohydrate intake need to be adequately delineated. Experts advise a calculated inclusion of carbohydrates in fish feed to bolster energy (Azaza et al., 2020). However, this strategy demands nuanced application, particularly in carnivorous species, such as trout and salmon, that demonstrate limited ability to metabolize complex carbohydrates (Kaushik and Oliva-Teles, 2013). Facilitating a diet rich in assimilable carbohydrates prevents the excessive breakdown of proteins and lipids and supports a nutrient-balanced growth trajectory in fish (Klahan et al., 2023). Enzymes play a vital role in breaking down food molecules into easily absorbable components during digestion (Medina-Félix et al., 2023).

The present study assessed four different processed types of the Pakchong 1 strain of Napier grass: dried (DN), fresh (FN), fermented (SN) and boiled for 10 min (BN). Trypsin enzymes were extracted from the small intestines of red tilapia samples to measure *in vitro* protein digestibility. The results showed that the highest protein digestibility was in the SN treatment (0.439±0.015 µmol DL-alanine/g feed/trypsin activity). This was followed by FN and DN, with protein digestibility values of 0.376±0.004 µmol DL-alanine/g feed/trypsin activity and 0.240±0.030 µmol DL-alanine/g feed/trypsin activity, respectively. On the other hand, BN had the lowest ($p < 0.05$) protein digestibility (0.142±0.025 µmol DL-alanine/g feed/trypsin activity), as shown in Fig. 1. These findings demonstrated that the fermentation process significantly enhanced the protein digestibility of Napier grass, as reflected by the highest digestibility observed in the fermented samples.

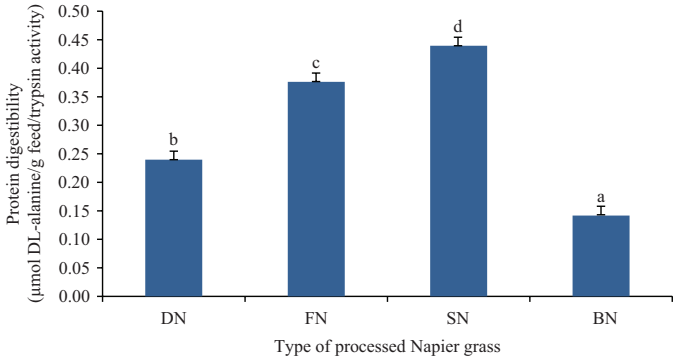

Fig. 1 Protein digestibility by enzyme trypsin from intestine of red tilapia of Napier grass prepared in different forms, where error bars indicate SD, different lowercase letters above bars indicate significant ($p < 0.05$) differences; DN = dry Napier grass, FN = fresh Napier grass, SN = Napier grass silage and BN = boiled Napier grass

In addition, the present study examined the *in vitro* carbohydrate digestibility of Napier grass using the amylase enzyme extracted from the small intestine of red tilapia. The results revealed notable differences in carbohydrate digestibility among the different treatments. The highest carbohydrate digestibility was in the SN treatment (0.828±0.015 µmol maltose/g feed/amylase activity), followed by the FN and BN treatments, with values of 0.734±0.165 µmol maltose/g feed/amylase activity and 0.621±0.059 µmol maltose/g feed/amylase activity, respectively. On the other hand, the DN treatment had the lowest carbohydrate digestibility (0.508±0.131 µmol maltose/g feed/amylase activity), as shown in Fig. 2.

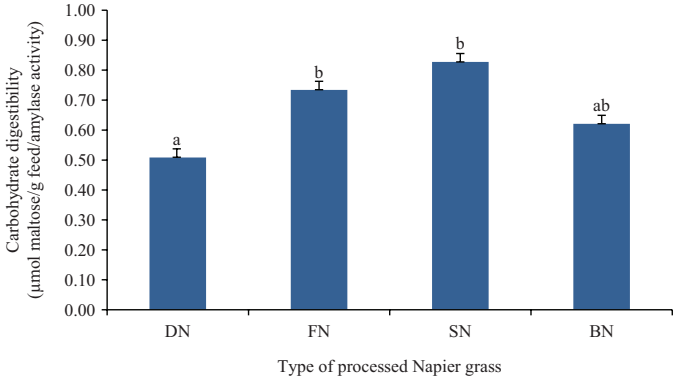

Fig. 2 Carbohydrate digestibility by enzyme amylase from intestine of red tilapia of Napier grass prepared in different forms, where error indicate SD, different lowercase letters above bars indicate significant ($p < 0.05$) differences; DN = dry Napier grass, FN = fresh Napier grass, SN = Napier grass silage and BN = boiled Napier grass

The study also investigated *in vitro* carbohydrate digestibility based on the cellulase enzyme. The results were the same as for the amylase activity, with the highest carbohydrate digestibility in the SN treatment (587.123±10.575 µmol maltose/g feed/cellulase activity), followed by the FN and BN treatments, though not significantly different from each other (520.806±117.012 µmol maltose/g feed/cellulase activity and 440.534±41.773 µmol maltose/g feed/cellulase activity, respectively). On the other hand, the DN treatment had the significantly lowest carbohydrate digestibility (360.596±92.702 µmol maltose/g feed/cellulase activity), as shown in Fig. 3.

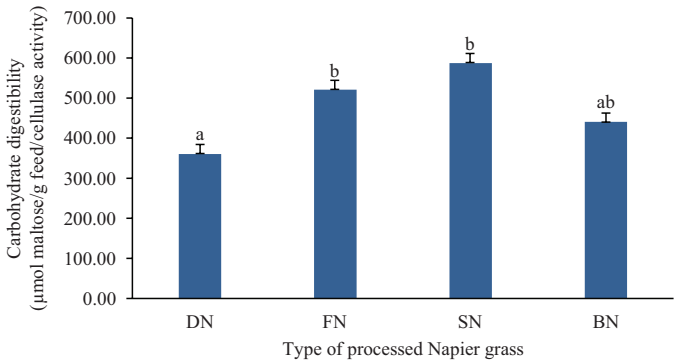

Fig. 3 Carbohydrate digestibility by enzyme cellulase from intestine of red tilapia of Napier grass prepared in different forms, where error bars indicate SD, different lowercase letters above bars indicate significant ($p < 0.05$) differences; DN = dry Napier grass, FN = fresh Napier grass, SN = Napier grass silage and BN = boiled Napier grass

These findings demonstrated the influence of different Napier grass treatments on carbohydrate digestibility in red tilapia. Processing methods, such as fermentation and boiling, are crucial in improving carbohydrate digestibility as a feed ingredient for red tilapia (Klahan et al., 2023). Notably, the 10 d fermentation process increased carbohydrate digestibility, indicating improved breakdown of cellulose into maltose. Incorporating fermented or properly cooked Napier grass into the tilapia diet optimized cellulose digestion and maximized nutrient utilization. The present study has provided valuable insights into the impact of Napier grass on cellulose digestibility in red tilapia, offering strategies to optimize feed formulations and enhance nutritional utilization in aquaculture practices.

Overall, these findings revealed that SN had the highest enzyme activity across all three enzymes namely trypsin (proteolysis), amylase (carbohydrate digestion), and cellulase (cellulose digestion), underscoring its potential to enhance enzyme digestibility (Fig. 1 and Fig. 2). This discovery has

important implications for enhancing the nutritional profile and digestibility of red tilapia. Furthermore, there is supporting evidence from the study by Chukwannuan and Tongsiri (2017) who showed that all three types of Napier grass, particularly Napier grass silage fermented in the cow stomach, exhibited significant potential as animal feed raw materials. Combined, the present and these other results highlight the suitability and viability of Napier grass silage in animal feed formulations.

Bureenok et al. (2012) explored the impact of silage supplementation on the visual attributes and nutritional composition of Napier grass silage and concluded that the fermentation process of Napier grass resulted in a pH reduction owing to acid production. This acidic environment facilitated softening of the Napier grass structure and amplified enzymatic activities beneficial for fish. Microbial fermentation promotes the growth of bacteria, particularly *Bacillus* sp., *Pseudomonas* sp. and *Clostridium* sp. (Swindoll et al., 1988), which are known to produce protease and amylase enzymes. The protease enzymes break down proteins into shorter-chain peptides or free amino acids, while the amylase enzymes hydrolyze the 1,4-glycosidic bonds in starch, converting them into smaller sugar molecules (Champasri et al., 2021). Some bacterial and fungal species synthesize cellulolytic enzymes, such as cellulases, hemicellulases and ligninases. Among these, cellulases are specialized enzymes that specifically act on cellulose molecules, cleaving the β-1,4 glycosidic bonds that link glucose units within the cellulose structure (Bayer et al., 2004). This process results in enhanced digestibility of protein and carbohydrates in the fermented Napier grass treatment. Similar results have been observed in studies on solid-state fermentation of Wolly Grass (*Imperata cylindrica*), potato peel and soybeans by *Bacillus subtilis* strains (Mukherjee et al., 2008; Akcan and Uyar, 2011).

Fermentation processes have been widely used to improve the nutritional value of feed crops and to preserve traditional food (Ndelekwute et al., 2018). The advantages of fermentation include the removal of antinutrients from feed crops and raw feed materials. Microorganisms living in feed crops carry out the fermentation process under anaerobic conditions, resulting in a decrease in pH through the production of lactic acid by lactic acid bacteria such as *Lactobacillus* spp. These bacteria digest carbohydrates from feed crops and eliminate anti-nutritional compounds in forage crops (Samtiya et al., 2020).Therefore, fermented Napier grass is a suitable feed option for aquatic animals.

Effect of Napier grass extract on ammonia reduction

Yield of Napier grass extract using microwave and reflux extraction

The total phenolic content, measured in milligrams of tannic acid per gram of extracts, varied between the microwave-assisted and reflux extraction methods (Fig. 4). The microwave-assisted extraction method yielded a higher ($p < 0.05$) total phenolic content (3.22 mg tannic acid/g extracts) than the reflux extraction method (3.01 mg tannic acid/g extracts). It has been widely acknowledged that microwave-assisted extraction surpasses the conventional solvent extraction method based on its advantages, including a shorter extraction time, a higher extraction rate and less solvent required. The higher total phenolic content obtained using microwave-assisted extraction suggests its suitability for extracting phenolic compounds from Napier grass.

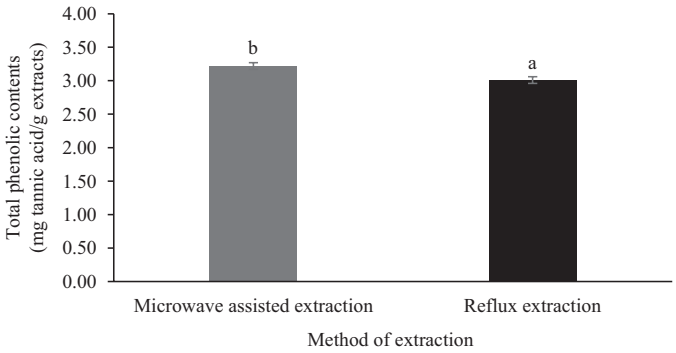

Fig. 4 Mean total phenolic contents of Napier grass extracts using microwave-assisted or reflux extraction methods, where error bars represent SD and different lowercase letters above bars indicate significant ($p < 0.05$) differences.

Effect of tannin extract from Napier grass on reducing ammonia in water

The efficiency of extraction from fermented Napier grass in reducing ammonia was observed in standard ammonia-nitrogen samples at a concentration of 1.63 mg/L for 80 min. It was found that 6 g/L of Napier grass extracts could reduce the ammonia content by as much as 0.48 mg/mL, followed by 4 g/L and 2 g/L of Napier grass extracts, with values of 0.38 mg/L and 0.18 mg/L, respectively. Furthermore, 6 g/L of Napier grass extracts could reduce ammonia by as much as 29.45% at 80 min, followed by 4 g/L and 2 g/L of Napier grass extracts, with the values of 23.31% and 11.04%, respectively (Fig. 5 and Table 1).

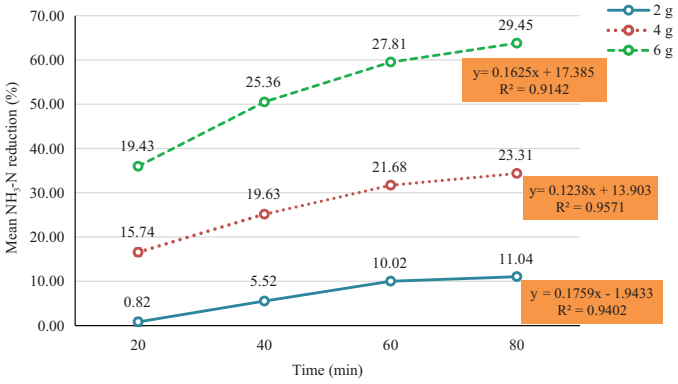

Fig. 5 Regression analysis of ammonia-nitrogen reduction resulting from the addition of varying amount of tannin extract (2, 4 and 6 g) during treatments lasting 20 to 40 min, applied to each standard ammonia-nitrogen sample, where R^2 = coefficient of determination

Table 1 Percentage of ammonia-nitrogen reduction resulting from the addition of Napier grass tannin extraction at 2, 4 or 6 g

Treatment	Time (min)			
	20	40	60	80
2 g	0.82±0.94[a]	5.52±0.61[a]	10.02±0.35[a]	11.04±0.00[a]
4 g	15.74±0.94[b]	19.63±0.62[b]	21.68±0.36[b]	23.31±0.62[b]
6 g	19.43±0.71[c]	25.36±0.36[c]	27.81±0.35[c]	29.45±0.62[c]

Different lowercase superscripts within each column indicate significant ($p < 0.05$) differences among means.

Tannin-rich extracts derived from specific plants have demonstrated the capability to effectively bind ammonia, thereby mitigating its toxicity and consequently enhancing overall water quality. For example, Suwanpakdee et al. (2016) documented the efficacy of tannin extracts obtained from fermented rice straw, demonstrating a notable reduction of ammonia levels in water samples at a concentration of 0.1 mg/L. Yang et al. (2015), Adegbeye et al. (2019) and Paray et al. (2021) reported that *Yucca schidigera* and its extracts have the potential to enhance water quality by lowering the levels of total ammonia nitrogen and nitrate in both freshwater and marine environments that are utilized in aquaculture. Notably, concentrations of 0.25 mg/L and 0.75 mg/L of Yucca extract effectively reduced the levels of ammonia, nitrate and nitrite within a water recirculating system. Additionally, this treatment had a positive effect by improving the survival rates of juvenile fish (Vargasmachuca et al., 2015).

The present study tested Napier grass extract rich in tannins on water samples and demonstrated effective ammonia reduction at 0.15 mg/L NH_3-N/g dry Napier grass. The reduction of ammonia may have been caused by the binding of tannic acid with ammonia, as suggested by the evidence presented in the following part of this study. Another essential aspect of reducing the ammonia content in Napier grass extract is the conversion of ammonia to nitrite or nitrate by certain microorganism groups, particularly bacteria found in silage (Klahan et al., 2023). Franco et al. (2022) highlighted that plant materials harbor a variety of epiphytic microorganisms, such as bacteria, yeasts and molds, with silage fermentation resulting from bacterial community activity. These bacteria could facilitate the transformation or reduction of ammonia compounds in Napier grass extract.

Binding of tannic acid and ammonium ion predicted using Fourier transform infrared spectroscopy

To predict the cohesion of tannic acid and ammonium ions in water, the reaction of tannic acid and NH_4Cl 100 mg/L was measured using ATR-FTIR (PerkinElmer). Fig. 6 presents the characteristic bands of NH_4Cl: 3117 cm^{-1}, 3014 cm^{-1}, 2802 cm^{-1} and 679 cm^{-1}. The band at 1388 cm^{-1} could be assigned to the deformation of NH_4^+ in NH_4Cl (Borisov et al., 2011). The dominant band of tannic acid was 1602 cm^{-1} that was assigned to stretching the C=C aromatic ring (Falcão and Araújo, 2014). Tannic acid dissolved in 100 mg/l of NH_4Cl solution was investigated and the results are shown in Fig.7. The vibration band at 1619 cm^{-1} was assigned to stretching of the C=C aromatic ring (Sepperer et al., 2020). The band at 1449 cm^{-1} of tannic acid was shifted to 1454 cm^{-1}. There was a change in the C=C aromatic ring of tannic acid, with the bond distance of the C=C in the aromatic ring perhaps being longer due to the positive charge of the NH_4^+ bonded with π-e^- of the C=C aromatic ring. The band at 1345 cm^{-1} appeared in tannic acid in an ammonium solution and may have been a compound of ammonium and tannic acid (Akter et al., 2016).

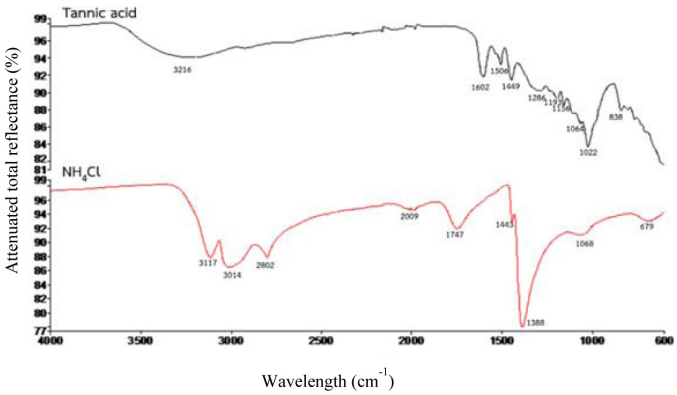

Fig. 6 Attenuated total reflectance-Fourier transform infrared spectrometry spectra of tannic acid and ammonium chloride solution

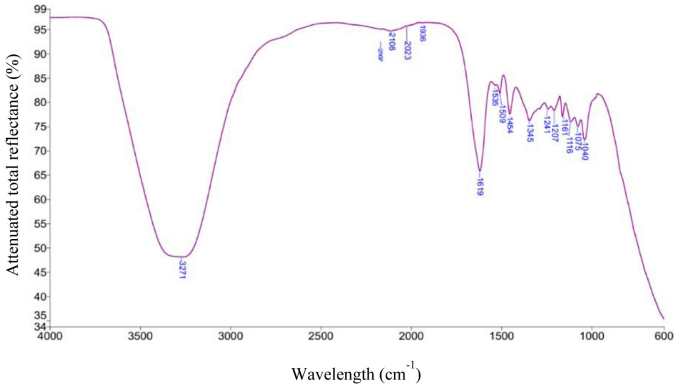

Fig. 7 Attenuated total reflectance-Fourier transform infrared spectrometry spectrum of tannic acid in 100 mg/l of NH_4Cl

The flocculant of tannic acid bonded with ammonium when the solution was incubated at room temperature. The prediction of the interaction of NH_4^+ and the C=C aromatic ring of tannin is shown in Fig. 8.

Tannins are diverse group of substances with complex chemical structures containing polyphenols that have been increasingly used in agriculture for their protein coagulation properties (Arismendi et al., 2018). They form complex compounds and easily precipitate or flocculate due to the presence of side chains (Adamczyk et al., 2017). In intensive aquaculture systems, high ammonia concentrations can be detrimental to fish (Hassan et al., 2017). The present study found that the tannins in the Napier grass extract effectively reduced the ammonia content in water. Other studies by Hashida et al. (2009) and Braghiroli et al. (2013) concluded that that the reduction of ammonia may be caused by the binding of tannins with ammonia to form a precipitate, as observed in the flocculation of tannic acid with ammonium during incubation at room temperature.

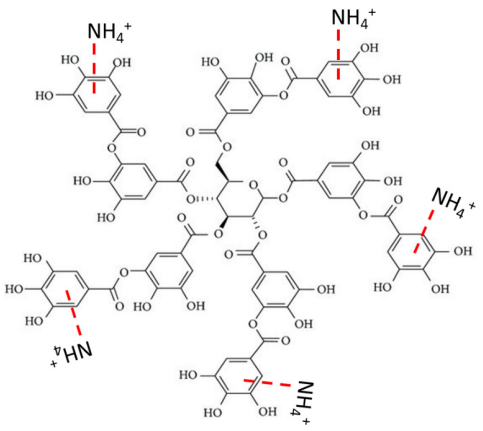

Fig. 8 Predicted interaction of NH_4^+ and C=C aromatic ring of tannin

Conclusion

The influence was investigated of Napier grass on the enzyme digestibility of red tilapia and its potential to reduce ammonia levels in aquaculture ponds. Fermented Napier grass demonstrated the highest proteolytic capacity, carbohydrate digestion and cellulase enzyme activity. The overall effect of fermentation led to an enhancement in the nutritional quality of silage, rendering it a viable and beneficial dietary option for aquatic animals. Napier grass extract obtained using microwave extraction, showed promise in reducing the ammonia content in water. These findings have important implications for the aquaculture industry, highlighting Napier grass silage as a cost-effective feed option that can reduce food costs for aquatic animals.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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