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Benefits of Pre-Digested Feed to Improve Growth Performance and Digestive Health of Juvenile Barramundi (*Lates calcarifer*)

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ABSTRACT

A 35-day experiment was conducted to analyze the effect of feed that had been predigested using rumen microbes and *Bacillus* sp. on feed efficiency, protease enzyme activity in the digestive system, intestinal histology, glycogen levels, and RNA and DNA ratios, as well as growth and survival of barramundi fish (*Lates calcarifer*). This experiment evaluated four feeding treatments based on different biomass percentages (3, 4, 5, and 6%) under controlled rearing conditions, with each treatment designed in a triplet. All four treatments were filled with barramundi (average weight: 1.08 ± 0.01 g). The results showed that fish fed 5% and 6% biomass levels exhibited significantly higher total feed consumption, protein retention, FUE, glycogen levels, RNA/DNA ratio, daily growth rate, and absolute growth (P<0.05) compared to other treatments. This treatment also exhibited a lower FCR value compared to other treatments. The overall body chemical composition of fish shows an increase in crude protein and crude fat compared to 4–3% of fish/biomass. The average length of the villi and the highest absorption area were observed at a feeding rate of 5%. In contrast, feeding with 3% and 4% biomass resulted in reduced nutrient uptake and suboptimal growth performance. These findings suggest that the addition of predigested feed with rumen microbes and *Bacillus* sp., particularly at 5–6% of the biomass, significantly improves digestive efficiency and supports optimal growth in juvenile *L. calcarifer*. This study highlights the potential for microbial fermentation in feed to produce several advantages, improving the performance of aquaculture feed through increased nutrient availability and enzymatic support.

INTRODUCTION

The increasing global market demand for barramundi (*Lates calcarifer*), a high-value aquaculture species **(Shah *et al.*, 2020; Chen *et al.*, 2024; Büthe *et al.*, 2025)**, has intensified the need for consistent and high-quality juvenile seed stocks. Traditionally, the

supply of *L. calcarifer* seeds has depended on wild capture, which is inherently unpredictable and environmentally unsustainable **(Caddy & Seijo, 2005; Balston, 2009; Hay & Probert, 2013; Goto *et al.*, 2022; Fernández *et al.*, 2023)**. To ensure the long-term viability of this industry, hatchery-based seed production systems must address key challenges related to uniformity, growth performance, and survival **(Macbeth & Palmer, 2011; Sato *et al.*, 2014; Shoko *et al.*, 2014; Thépot & Jerry, 2015; Gomes *et al.*, 2017; Domingos *et al.*, 2021; Wong *et al.*, 2023; Rey & Sahetapy, 2025)**. Although hatchery technologies have improved, achieving consistent seed quality remains a significant constraint in many regions, especially in Southeast Asia **(Robinson *et al.*, 2010; Filipe *et al.*, 2023a, b; Vieira *et al.*, 2023)**.

One persistent bottleneck in hatchery and nursery operations is the reliance on live or natural feeds, which are expensive, labor-intensive, and difficult to standardize. In response, there is a shift toward using formulated artificial diets that are nutritionally balanced and commercially scalable **(Dworjanyn *et al.*, 2007; Hamre *et al.*, 2013; Viera *et al.*, 2015; Belleggia & Osimani, 2023; Kefayat *et al.*, 2025; Mitra *et al.*, 2025; Zhang *et al.*, 2025)**. However, the prohibitive cost of premium feeds—often exceeding $45,000 USD/kg—makes them inaccessible to small-scale farmers. Consequently, cost-effective alternatives, such as protein-supplemented or enzyme-enhanced feeds, are increasingly being explored.

One promising strategy involves utilizing microbial fermentation to enhance the nutritional profile and digestibility of conventional feed. Microorganisms such as *Bacillus* spp. and various rumen microbes are rich in protein (up to 70% of dry matter) and bioactive compounds, including vitamins, antioxidants, and digestive enzymes **(Delgado *et al.*, 2013; Li *et al.*, 2025)**. These microbes produce exogenous enzymes such as amylase, protease, and cellulase, which work synergistically with endogenous enzymes to enhance nutrient absorption and feed efficiency **(Anwar *et al.*, 2023, 2024, 2025)**.

Recent studies have demonstrated that dietary probiotics, such as *Lactobacillus pentoses*, can reduce hepatic fat accumulation and enhance immune resistance in *L. calcarifer* **(Vo *et al.*, 2020; Siddik *et al.*, 2022)**. Similarly, enzyme supplementation and microbial fermentation have been associated with improved gut histology, enzyme activity, and nutrient retention in several aquaculture species **(Maas *et al.*, 2019, 2025; Mulyani *et al.*, 2023)**. Despite these advances, limited research has explored the combined use of multi-source microbes, particularly rumen fluid and *Bacillus* sp., in enhancing feed performance— specifically for the white snapper.

Rumen fluid, derived from ruminant digestive systems, contains a consortium of cellulolytic, amylolytic, proteolytic, and xylanolytic microbes that produce enzymes such as α-amylase, galactosidase, hemicellulose, and cellulase **(Ishaq & Wright, 2015; Liu *et al.*, 2021)**. *Bacillus* sp., meanwhile, is recognized for its robust enzyme secretion profile

during fermentation, including protease (1546.5 U/mL), cellulase (264 U/gds), and pectinase (1.19 IU/g) **(Pham *et al.*, 2010, 2022; Salim *et al.*, 2017; Van *et al.*, 2017)**. Additionally, *Bacillus* supplementation has been linked to increased protein content and improved feed conversion efficiency **(Chaudhary *et al.*, 2021; Mulyasari *et al.*, 2022)**.

This study addressed a key knowledge gap by evaluating the synergistic use of rumen microbes and *Bacillus* sp. in fermented feed, specifically assessing its impact on the digestive enzyme activity, gut morphology, and growth performance of *L. calcarifer* juveniles. We hypothesize that incorporating these microbes into predigested feed will enhance digestive enzyme activity and improve growth performance in the juvenile white snapper. By leveraging microbial predigestion as a sustainable feed enhancement strategy, this research contributes to the development of cost-efficient and nutritionally optimized aquafeeds tailored for barramundi hatchery systems.

MATERIALS AND METHODS

ExperimentalsSite

The study was conducted at the Takalar Brackish Water Aquaculture Fisheries Center, located in Takalar Regency, South Sulawesi, Indonesia, between coordinates 5°30' – 5°38' South Latitude and 119°22' – 119°39' East Longitude.

Duration of study

The experiment was conducted over a period of five weeks.

Sourcing and acclimation of experimental fish

A total of 120 barramundi (*L. calcarifer*) juveniles were sourced from the Takalar Brackish Water Aquaculture Fisheries Center. The fish were acclimated for one week in tanks measuring 50 × 30 × 35cm and fed commercial feed (Otohime A, Marubeni Nisshin, Japan) at a pellet size of 1.5mm, administered three times daily at 07:00, 12:00, and 18:00.

Tank preparation

Twelve plastic containers were sterilized using a water solution mixed with formalin. After sterilization, the tanks were filled with aerated water and maintained under continuous aeration for three days to ensure optimal water quality and system stabilization.

Preparation of rumen microbes and *Bacillus* sp.

The *Bacillus* sp. strain used was obtained from MSMEs Biopocall Makassar (Indonesia, NIB 0220000862736) with a density of 4.82×10^6 CFU/mL. It was activated by mixing with granulated sugar at a ratio of 1 mL:1 g and stirred for approximately one

hour following a modified method from **Anwar *et al*. (2025)**. Rumen fluid was collected from the Gowa Regency Slaughterhouse, South Sulawesi, and centrifuged at 2000 rpm for 15 minutes at the Feed Chemistry Laboratory, Hasanuddin University. This step allowed the separation of the microbial-rich supernatant, which was used as an active component.

Feed fermentation process using the predigested system

Commercial feed (PF 500 MS Prima Feed, PT Matahari Sakti) was used in this study. Fermentation involved the addition of *Bacillus* sp. and rumen fluid at a dosage of 4.5mL per 100 grams of feed for each additive **(Anwar *et al.*, 2025)**. The feed mixture was then incubated anaerobically for 24 hours at room temperature to allow microbial activity, followed by drying through aeration until reaching 99% dryness. The fermented feed was stored at room temperature until it was used.

Treatment and experimental design

The experiment followed a Completely Randomized Design (CRD) consisting of four treatment groups with three replications each: Treatment A, Feed at 3% of fish biomass; Treatment B, Feed at 4% of fish biomass; Treatment C, Feed at 5% of fish biomass; and Treatment D, Feed at 6% of fish biomass. All treatments were administered to assess the impact of different feeding rates using predigested feed enriched with *Bacillus* sp. and rumen microbes on the growth and digestive performance of the barramundi juveniles. Each group was housed in separate tanks under identical environmental and husbandry conditions to ensure experimental consistency.

Test animal maintenance

Juvenile barramundi (*L. calcarifer*) specimens were sourced from the Takalar Brackish Water Aquaculture Center. Initially, the fish were adapted to laboratory conditions for three days in holding tanks and fed commercial feed. The fish were then sorted to ensure uniformity, with an average initial body weight of 1.08 ± 0.01g and a length of 3cm. The stocking density was maintained at 10 fish per 40 liters of water. The percentage of feed given was determined by the treatment and was administered manually three times a day at 07:00, 12:00, and 17:00 local time.

Fish and feed trial

Before the experiment began, the fish were acclimated for one week and fed a commercial diet (Otohime A, Marubeni Nisshin, Japan), which contained 50% protein, 10% fat, 3% fiber, 16% ash, 2.3% calcium, and 1.5% phosphorus. Mortality was monitored, and dead fish were weighed to adjust the feed conversion ratio (FCR) calculation. Water was continuously recirculated at a rate of 3L/ min through a biofiltration system, with 20% of the water being replaced daily using aerated tap water. Water quality parameters were monitored periodically to ensure optimal conditions,

maintained throughout the experiment. At the end of the experiment, fish were fasted for 24h before measuring intestinal morphology. All samples were stored at 20°C for further biochemical analysis.

Biochemical analysis

The sampled fish were autoclaved at 120°C for 20 minutes, homogenized, and then dried at 105°C. Proximate composition analysis, including moisture, crude protein, crude lipid, ash, gross energy, and phosphorus content, was conducted according to **Helrich (1990)** standards. Crude protein was measured using the Foss 8400 Auto Kjeldahl Nitrogen Analyzer (Foss, Sweden). Crude lipid was determined via ether extraction with the SZF-06A Fat Extractor (Shanghai Xinjia Electronic Co., Ltd, China). Gross energy was assessed using a Parr 6200 Calorimeter (Parr Instrument Company, USA). These analyses ensured an accurate assessment of nutrient retention and the overall biochemical composition of the fish at the end of the feeding trial.

Protease enzyme activity and specific activity in fish digestion

Protease enzyme activity in the digestive tract of *Lates calcarifer* was measured following the method described by **Hakim *et al.* (2007)**, **Hlophe *et al.* (2014)** and **Nolasco-Soria (2021)** . The assay utilized casein as the substrate and a phosphate buffer at a pH of 7.6. Tyrosine was used as a standard, where one enzyme unit is defined as the amount of enzyme that releases 1mg of tyrosine per minute. Enzyme kinetics were monitored spectrophotometrically, ensuring repeatability using triplicate assays.

Calculation of enzyme activity

Protease activity (U/mg/min) was calculated using the following formula:

$$[(A_{ct} - A_{bl}) / (A_{st} - A_{bl})] \times P \times T^{-1}$$

Where, A_{ct} = Absorbance value of the sample A_{bl} = Absorbance value of the blank A_{st} = Absorbance value of the standard P = Dilution factor T = Incubation time (in minutes). This calculation allowed for the determination of both total protease activity and specific activity in the gastrointestinal tract, providing insight into the digestive capacity of the fish under different dietary treatments. Results were expressed in units per milligram of protein per minute (U/mg/min) **(Worthington, 1988)**.

Fish intestinal histology

The histological examination of the intestinal tissue of barramundi (*Lates calcarifer*) was performed following standard procedures as outlined by **Dawood *et al.* (2020)**. Intestinal samples were collected using a sterile scalpel and immediately fixed in 10% Buffered Neutral Formalin (BNF) for 24 hours to preserve tissue structure. Subsequent steps included paraffin embedding, microtome sectioning (5µm thickness), and hematoxylin-eosin staining, followed by microscopic observation at 400× magnification.

Measurement of glycogen levels in *Lates calcarifer*

Whole-body glycogen content in *Lates calcarifer* was assessed at both the beginning and end of the experimental period. For each time point, five fish were sampled and used for glycogen analysis. Fish samples were initially dried in an oven at 70–80°C until moisture was removed. The dried samples were then ground into a fine powder using a pestle and mortar. The powdered samples were wrapped in aluminum foil, labelled, and stored in sealed plastic bags until further analysis. This procedure ensured consistency in sample handling prior to chemical analysis.

Glycogen content was determined spectrophotometrically at a wavelength of 670nm. The following formula was used to calculate glycogen concentration:

$$\text{Glycogen (mg/g)} = \left[\left(\frac{\text{Abs}_{\text{Spl}}}{\text{Abs}_{\text{Std}}}\right) \times \text{Cons}_{\text{Std}} \times \text{DF} \times \frac{1}{1000}\right] \div \text{Sample weight (g)}$$

Where:

- **Abs_{Spl}** = Absorbance of the sample at 670 nm
- **Abs_{Std}** = Absorbance of the standard
- **Cons_{Std}** = Standard glycogen concentration (500 µg/mL)
- **DF** = Dilution factor (5×)
- **1/1000** = Conversion from micrograms to milligrams
- **Sample weight (g)** = Mass of the powdered sample analyzed

This method enabled the quantification of glycogen reserves in the fish body, providing insights into energy storage and utilization across different dietary treatments **(Carroll *et al.*, 1956)**.

RNA and DNA ratio measurement

Quantification of RNA and DNA concentrations in *Lates calcarifer* was performed using a GeneQuant spectrophotometer. For each measurement, 7µL of RNA or DNA sample was pipetted into a 5mm pathlength cuvette, with Tris-EDTA (TE) buffer used as the reference solution. All analyses were conducted in triplicate to ensure data accuracy. Absorbance readings were taken at wavelengths of 260 and 280nm (A260 and A280), and concentrations were calculated as follows **(de Silva & Anderson, 1994)**:

- **RNA concentration (µg/mL)** = A260 × 40 × Dilution Factor
- **DNA concentration (µg/mL)** = A260 × 50 × Dilution Factor

The purity of nucleic acids was assessed using the A260/A280 ratio, where values between 1.8 and 2.0 indicate acceptable nucleic acid purity. The RNA/DNA ratio was then calculated by dividing total RNA concentration by total DNA concentration. This ratio serves as an indicator of protein biosynthesis capacity and cellular metabolic activity—critical metrics for evaluating the physiological condition and growth potential of fish under various dietary treatments.

Feed conversion ratio (FCR)

Feed Conversion Ratio (FCR) was calculated to assess feed efficiency using the formula:

$$FCR=F/(Wt+D)-W0$$

Where:

- **F** = Total feed consumed (g)
- **W_t** = Final biomass of barramundi at the end of the trial (g)
- **D** = Total weight of dead fish during the trial (g)
- **W₀** = Initial biomass of barramundi at the start of the trial (g)

A lower FCR indicates better feed utilization efficiency. All values were calculated per tank and averaged across replicates.

Absolute growth

Absolute growth was measured by comparing the initial and final biomass of individual fish using the formula:

$$W=Wt-W0$$

Where:

- **W** = Absolute growth of *Lates calcarifer* (g)
- **W_t** = Final body weight (g)
- **W₀** = Initial body weight (g)

Values were recorded per fish and aggregated to calculate treatment means **(de Silva & Anderson, 1994)**.

Survival rate

Survival rate (SR) was calculated to determine the percentage of fish that survived throughout the experimental period:

$$SR\ (\%) =(Nt/N0) \times 100$$

Where:

- **N_t** = Number of fish at the end of the experiment
- **N₀** = Number of fish at the beginning of the experiment

Uniform survival across treatments indicated the non-lethality of experimental feeds **(Effendie, 1997)**.

Water quality monitoring

Water quality assessments were conducted at the Water Quality Laboratory of the Takalar Brackish Water Aquaculture Fisheries Center. Parameters monitored included temperature, salinity, pH, dissolved oxygen (DO), total suspended solids (TSS), and ammonia (NH_3). Temperature, salinity, and pH were measured twice daily, while DO levels were checked once daily. Ammonia and TSS were monitored biweekly. These

parameters were maintained within optimal ranges to ensure that environmental conditions did not confound the effects of dietary treatments on fish growth and health (Table 1). Daily logs were reviewed to ensure no deviations occurred that might influence treatment comparisons.

Table 1. Water quality during the rearing of barramundi fed with predigested feed containing rumen microbes and *Bacillus* sp.

Water quality parameter	**Observed Range**	**Recommended Range**
Temperature (°C)	27 – 29	25 – 32
pH	7.72 – 8.06	7.5 – 8.5
Salinity (ppt)	36 – 37	5 – 40
Dissolved Oxygen (mg/L)	3.30 – 3.80	≥3
Ammonia (NH_3, mg/L)	0.05 – 0.06	≤1
Total Suspended Solids (TSS, mg/L)	0.658 – 1.076	150 – 200

The observed values for all parameters remained within optimal or acceptable limits throughout the experiment, ensuring that environmental conditions did not adversely affect fish health or skew the experimental outcomes. Notably, DO levels were consistently above 3mg/ L, and ammonia levels remained well below the toxic threshold. TSS values were minimal, indicating a clean water environment and efficient filtration system.

Data analysis

The data collected from this study were analyzed using both descriptive and inferential statistical methods. Histological observations of the intestinal tissues were evaluated descriptively by comparing morphological differences across treatment groups and referencing relevant literature for interpretation. Quantitative data, including proximate body composition, protease enzyme activity, glycogen levels, RNA/DNA ratios, feed conversion ratio (FCR), absolute growth, and survival rate, were expressed as mean values. These variables were analyzed using one-way Analysis of Variance (ANOVA) to determine statistically significant differences among treatments. Normality and homogeneity of variance assumptions were tested prior to conducting the ANOVA. Post hoc comparisons were performed using Duncan's multiple range test at a 95% confidence level ($P<0.05$). Statistical analyses were conducted using SPSS software version 26.0. Water quality parameters were analyzed descriptively to ensure that environmental conditions remained within acceptable limits and did not influence the physiological or growth responses of the fish.

RESULTS

Protease enzyme activity and specific activity of fish digestion

Protease enzyme activity, specific activity and protein levels in the gastrointestinal tract of predigested fed barramundi seeds using rumen microbes and *Bacillus* sp. are shown in Table (2). These data provide insight into enzymatic efficiency across different dietary treatments.

Table 2. Protease enzyme activity, specific activity and protein levels in the gastrointestinal tract of predigested fed barramundi seeds using rumen microbes and *Bacillus* sp. in all treatments

Parameter	Treatment			
	A	**B**	**C**	**D**
Actiphytase enzyme protease (U/mL)	0.67 ± 0.01^{b}	0.67 ± 0.02^{b}	0.64 ± 0.01^{a}	0.68 ± 0.01^{b}
Specific Activities (U/mg protein)	0.22 ± 0.00^{bc}	0.23 ± 0.01^{c}	0.17 ± 0.02^{a}	0.20 ± 0.00^{b}
Protein content (mg/mL)	2.94 ± 0.02^{b}	2.88 ± 0.01^{a}	3.72 ± 0.01^{d}	3.35 ± 0.01^{c}

The results showed that protease enzyme activity tended to increase with higher biomass feeding rates. The highest enzyme activity was observed in Treatment D (6%), which also showed elevated protein levels. However, specific activity did not follow this trend, suggesting that not all measured proteins originated from active protease. Treatment A (3%) recorded lower enzyme and protein levels but a relatively high specific activity, indicating greater enzymatic efficiency relative to protein content. This finding underscores that enzyme concentration alone may not reflect digestive potential without considering specific activity.

Intestinal histopathology

The results of the intestinal histopathology of barramundi juveniles fed with predigested feed containing rumen microbes and *Bacillus* sp. under all treatments are presented in Fig. (1). These parameters provide structural insights into the digestive capacity of the fish.

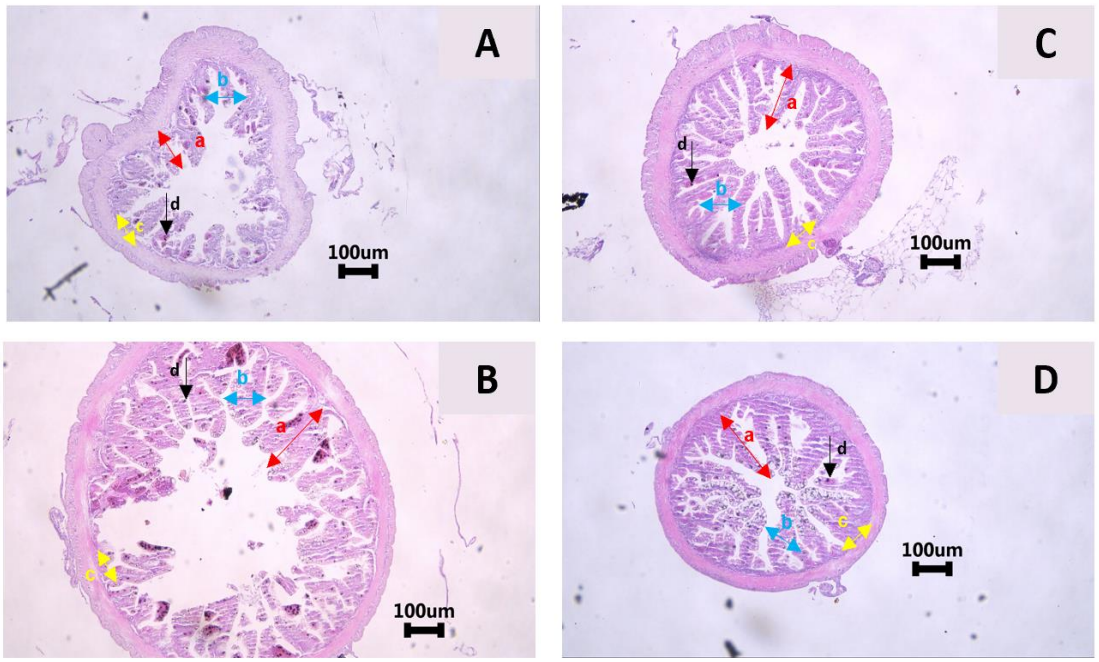

Fig. 1. Intestinal histopathology of predigested feed of predigested barramundi *fish at* all treatments. (a) Length of villi, (b) Width of apical villi (c) width of basal villi (d) Goblet cell. (H.E. Coloring; Magnification 100µm; Olympus® CX-41 microscope).

Fig. (1) illustrates that the highest average villi length (µm) was observed in Treatment C (205.20 µm²), followed by Treatment D (190.80 µm²), Treatment B (164.60 µm²), and the lowest in Treatment A (128.20 µm²). Villi width was greatest in Treatment D (52.20 µm²), followed by Treatment C (50.50 µm²), Treatment B (43.60 µm²), and again the lowest in Treatment A (43.60 µm²).

The total absorption area, a critical metric for nutrient uptake, followed a similar descending trend:

- Treatment C (8946.72 µm²)
- Treatment D (8738.64 µm²)
- Treatment B (8592.72 µm²)
- Treatment A (6474.10 µm²)

These values reflect a clear enhancement in digestive structure attributed to microbial supplementation, supporting improved nutrient assimilation.

Fish growth performance

Results on key performance indicators—including feed consumption rate, feed efficiency, body glycogen levels, daily growth rate, absolute growth, and survival rate—of *Lates calcarifer* juveniles fed with predigested feed containing rumen microbes and

Bacillus sp. are presented in Table (3). These parameters provide a comprehensive overview of nutrient utilization and physiological development across dietary treatments.

Table 3. Total Feed Consumption (g), Feed Utilization Efficiency (%), Glycogen Levels (%), Absolute Growth (g), Daily Growth Rate (%), and Survival Rate (%) of predigested fed L. calcarifer juveniles using rumen microbes and *Bacillus* sp. across all treatments.

	Feed			
Treatment Parameter	**A**	**B**	**C**	**D**
Total feed consumption (g)	86.91±0.20^{a}	87.82±0.13^{b}	89.84±0.06^{c}	89.84±0.12^{c}
Protein retention	1.65±0.01^{a}	1.72±0.01^{b}	1.91±0.01^{c}	1.92±0.01^{c}
FUE (%)	70.61±0.11^{a}	73.22±0.10^{b}	87.46±0.56^{c}	87.00±0.02^{c}
FCR	2.64±0.02^{d}	2.50±0.00^{c}	1.84 ±0.01^{a}	1.83±0.01^{a}
Glycogen content (%)	12.09±0.10^{a}	12.39±0.04^{b}	12.55±0.10^{c}	12.73±0.04^{d}
RNA/DNA Ratio (%)	0.76±0.00^{a}	0.82±0.01^{b}	0.87±0.01^{bc}	0.88±0.00^{c}
Daily Growth Rate (%)	0.35±0.03^{a}	0.46±0.03^{b}	0.67±0.01^{c}	0.67±0.01^{c}
Absolute Growth (g)	1.63±0.01^{a}	1.72±0.01^{b}	1.97±0.01^{c}	1.97±0.01^{c}
Survival rate (%)	100	100	100	100

Information: The average value in the same column with different superscript letters indicates a real difference in value (P<0.05).

The analysis of variance in feed consumption, feed efficiency, glycogen level, absolute growth, and daily growth rate of *L. calcarifer* revealed statistically significant differences among treatments. Treatments C (5%) and D (6%) demonstrated the highest performance across all indicators (P< 0.05), indicating improved nutrient utilization and metabolic health. Treatment B (4%) followed with moderate outcomes, while Treatment A (3%) recorded the lowest metrics. The survival rate, however, remained uniformly high (100%) across all treatments, confirming the safety of the feed.

Proximate level of fish bodies

Moisture content significantly increased from Feed A to Feed D (P< 0.05). Crude protein content was significantly higher in feeds C and D compared to feeds A and B, with no significant difference between feeds C and D (P> 0.05).

For crude fiber, Feed A had significantly lower levels than feeds B, C, and D, while no statistical differences were observed among feeds B, C, and D ($P > 0.05$).

Crude fat content was at its highest value in feeds C and D, both significantly different from feeds A and B ($P < 0.05$).

Ash content showed a decreasing trend from Feed A to Feed D, suggesting an inverse relationship with feed enhancement levels.

Nitrogen-Free Extract (NFE) was lowest in Feed C, followed by Feed D, and the highest in feeds A and B (Table 4).

Table 4. Proximate levels of fish body composition, including moisture, crude protein, fiber, fat, ash, and nitrogen-free extract (NFE) in *L. calcarifer* juveniles fed predigested feed containing rumen microbes and *Bacillus* sp. across all treatment groups

Treatment parameter	**Feed**			
(%)	**A**	**B**	**C**	**D**
Crude protein	72.71±0.01[a]	73.54±0.00[b]	75.09±0.08[c]	75.13±0.01[c]
Crude fiber	5.14±0.00[a]	5.18±0.00[b]	5.18±0.00[b]	5.18±0.00[b]
Crude fat	5.43±0.01[a]	5.18±0.05[b]	5.53±0.10[c]	5.51±0.08[c]
Ash	2.24±0.02[c]	2.07±0.01[b]	1.08±0.01[a]	1.07±0.10[a]
NFE	14.01±0.01[c]	14.02±0.01[c]	13.01±0.01[a]	13.09±0.01[b]

Information: The average value in the same column with different superscript letters indicates a real difference in value ($P<0.05$).

DISCUSSION

The enhancement of growth performance in juvenile barramundi (*Lates calcarifer*) using predigested feeds supplemented with rumen fluid and microbial additives, notably *Bacillus* sp., illustrates a novel nutritional approach in aquaculture. These dietary strategies have demonstrated improvements in feed conversion ratios (FCR), digestive enzyme activity, nutrient absorption, and physiological resilience **(Klanian *et al.*, 2020; Vethathirri *et al.*, 2023; Liu *et al.*, 2024)**. In this study, the highest protease activity was observed in Treatment D (6%), although specific activity levels did not increase proportionately, suggesting the presence of additional non-enzymatic proteins. Treatment A (3%) had the lowest enzyme activity but the highest specific activity, indicating greater enzymatic efficiency despite lower overall protein content **(Vinasyiam *et al.*, 2016; Vitolo, 2020)**.

These enzymatic trends were corroborated by intestinal histopathology, where treatments C and D showed marked increases in villus height and absorptive surface

area—traits directly linked to nutrient uptake efficiency **(Sawant *et al.*, 2025)**. Treatment C exhibited the highest absorption area (8946.72 μm²), suggesting enhanced epithelial development and nutrient transport potential. This is likely due to the combined effects of volatile fatty acids from rumen fluid and enzymatic contributions from *Bacillus* sp., which together stimulate mucosal growth and support microbial symbiosis **(Suzer *et al.*, 2008; Arig *et al.*, 2013; Wang *et al.*, 2024; Gresse *et al.*, 2025)**.

Improvements in gut morphology in treatments C and D were consistently reflected in growth performance indicators. Fish in these treatments exhibited significantly higher weight gain, growth rates, protein retention, and feed efficiency compared to underfed groups (Treatments A and B). These findings align with previous studies demonstrating that microbial fermentation and enzyme supplementation enhance amino acid uptake and metabolic performance **(Hugenholtz *et al.*, 2018; Diether and Willing, 2019; Ammar *et al.*, 2020; Van den Abbeele *et al.*, 2022; Li *et al.*, 2025; Patil *et al.*, 2025)**. In contrast, insufficient feeding in Treatment A resulted in suppressed enzymatic activity, lower glycogen reserves, and poor growth. Notably, no adverse effects of overfeeding were observed in Treatment D, suggesting that the increased nutrient supply was efficiently metabolized without inducing stress or nutrient waste **(Chen *et al.*, 2021; Fantatto *et al.*, 2024)**.

Elevated RNA/DNA ratios in treatments C and D further indicated intensified anabolic activity, reflecting robust protein synthesis and rapid cellular growth **(Martínez-Villaluenga *et al.*, 2006, 2007; Fernandez-Orozco *et al.*, 2007; Jawad *et al.*, 2013; Chang *et al.*, 2021)**. These biochemical indicators were supported by higher glycogen reserves and improved body composition—particularly in protein, lipid, and moisture content—highlighting enhanced energy storage and nutrient assimilation. These results underscore the importance of aligning feed quality with the digestive capacity of the species to optimize growth and reduce feed waste **(Satpathy *et al.*, 2003; Hua *et al.*, 2019; Bano *et al.*, 2023)**.

Emerging technologies such as nano-nutraceuticals—particularly selenium nanoparticles—offer promising potential to enhance antioxidant defense, immune function, and stress tolerance in *L. calcarifer* **(Dawood *et al.*, 2020; Ghaffarizadeh *et al.*, 2022; Khalil *et al.*, 2023; Mugwanya *et al.*, 2023; Fiard *et al.*, 2024; Mohtashemipour *et al.*, 2024a, b)**. Although not directly assessed in this study, the potential synergy between such compounds and microbially-supplemented diets merits further investigation as a strategy to enhance aquaculture sustainability and fish health.

The biochemical composition data supported the physiological outcomes observed. Fish in Treatments C and D, fed with higher concentrations of fermented feed, exhibited the highest levels of crude protein, fat, and moisture—indicating superior nutrient uptake and energy retention. These results affirm the value of microbial fermentation in improving feed digestibility and metabolic efficiency **(Vieira *et al.*, 2023; Anwar *et al.*, 2024)**. Conversely, the poor nutritional profile observed in Treatment A

highlights the detrimental impact of inadequate feed supplementation on growth and metabolic health **(Francis *et al.*, 2001; Kim *et al.*, 2018; Cha *et al.*, 2024; Kovalenko *et al.*, 2024)**.

Overall, the application of feed supplemented with rumen microbes and *Bacillus* sp. did not impair the physiological functions of the fish, as evidenced by the 100% survival rate of juvenile barramundi (*L. calcarifer*).

CONCLUSION

This study confirmed that the addition of rumen fluid and *Bacillus* sp. in pre-digested feed at 5–6% of biomass significantly improved growth performance and digestive health of juvenile barramundi. In contrast, feeding at 3% yielded inadequate results, highlighting the limitations of minimal supplementation. The consistent 100% survival rate further demonstrated the safety and viability of the microbial feed additive. These findings strongly support the integration of microbial fermentation as a cost-effective strategy to improve feed performance, enhance fish health, and promote aquaculture sustainability.

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