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Application of distiller's dried grain with soluble enzyme cocktail pretreatment product on growth performance and immunity of pacific white shrimp (*Litopenaeus vannamei*)

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Abstract

The study on antagonist effects of cocktail enzyme activity on antagonist effects of cocktail enzyme activity analysis of mixed enzyme revealed stable protease activity across time, while cellulase, xylanase, and phytase activities significantly declined after prolonged mixing with protease ($p<0.05$). Nutritional composition of R-DDGS improved with enzymatic treatment, exhibiting higher crude protein (up to 56.6%) and enhanced protein digestibility, alongside reduced fiber and phytic acid levels. The study in shrimp involved juvenile white shrimp (Litopenaeus vannamei) cultured under control condition over a 42-day period. The results showed that shrimp fed enzyme-treated R-DDGS diets (T3-protease, T4-Protease+NSP, T5-Protease+NSP+phytase) demonstrated significant improvements in growth performance compared to shrimp fed untreated R-DDGS (T2) and in the same range as Control-soybean meal diet (T1). Enzyme treated diets also modulated immune responses, with a reduction in total hemocyte count (THC) and stable levels of other immune markers. Additionally, R-DDGS diets enhanced protein digestibility, reflected in higher reducing sugar levels and carbohydrate breakdown ($p<0.05$). The shrimp coloration (a and b values) did not differ significantly, enzyme-treated diets supported slight improvements in pigmentation, enhancing market value ($p<0.05$). These findings underscore the potential of enzyme-treated R-DDGS as a sustainable alternative feed ingredient for aquaculture, offering improved nutrient utilization, growth performance, and immune modulation.

Keywords: Enzyme cocktail, Enzyme-treated R-DDGS, Litopenaeus vannamei shrimp growth performance, Protein and carbohydrate digestibility, sustainable aquafeeds.

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1. Introduction

Distiller's Dried Grain with Soluble (DDGS) is a by-product of the ethanol production industry, derived from various raw materials such as broken rice, wheat, sorghum, corn, or barley [1]. The quality of DDGS depends significantly on the type of raw material and production methods [2]. The United States and Brazil are the leading producers, accounting for approximately 90% of the global ethanol output [3]. During ethanol production, starches are converted to sugars to serve as nutrients for yeast (*Saccharomyces cerevisiae*) during the saccharification step, leaving behind nutrient-rich residues [4].

Research highlights the variability in DDGS nutritional composition. For example, Bailey, *et al.* analyzed nine corn-based DDGS samples over four harvest seasons, reporting crude protein levels between 30.0% and 33.8%, fat content ranging from 10.8% to 12.2%, and fiber values as Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) ranged from 55.6% to 62.1% and 22.2% to 25.2%, respectively [5]. In Asia, rice is the common raw material, producing DDGS with higher protein content of 48.43% [6]. Similarly, Böttger and Südekum [7] studied in 36 samples from 12 European countries and found that protein, NDF, and ADF contents varied based on the raw materials used, including barley, wheat, and corn. Such differences are critical as they influence the suitability of DDGS in specific animal feed formulations [7, 8].

The protein content and amino acid profiles of DDGS make it a potential substitute for traditional protein sources like soybean meal in aquaculture. However, challenges exist due to lower lysine and methionine levels compared to soybean meal [9]. Additionally, Non-Starch Polysaccharides (NSP) in DDGS, which consist of cellulose, xylose, arabinose, and glucose, affect its digestibility. Studies by Pedersen, et al. [10] reported low solubility of NSP in corn and wheat-based DDGS, highlighting the need for pretreatment methods to enhance nutrient availability [7, 8, 10].

The use of DDGS in aquaculture has been explored in various species [6, 9, 11, 12]. For example, Mohammadi Shad, et al. [4] found that high-protein DDGS could replace soybean meal in Nile Tilapia diets with comparable growth performance. However, studies also show limitations when using higher levels of DDGS due to its fiber content [9]. For instance, Dey, et al. [6] observed no significant growth differences in carp fed a 30% DDGS diet compared to a control, although protein efficiency ratios improved [6]. Similarly, Sándor, et al. [12] demonstrated that DDGS could replace chicken meal in European catfish diets without affecting growth, but digestibility coefficients declined at higher inclusion rates. Conversely, studies in species like turbot and hybrid grouper showed reduced growth with increased DDGS levels [11-13].

In shrimp (*Litopenaeus vannamei*), research on DDGS inclusion remains limited. The sorghum-based DDGS could replace soybean meal at levels up to 40% without impacting shrimp growth [1]. Similarly, The study demonstrated that corn-based DDGS at up to 15% inclusion had no adverse effects on shrimp performance [14]. Despite these findings, high fiber content in DDGS often limits its digestibility in aquatic feeds.

Enzymatic pretreatment of DDGS has appeared as a promising approach to overcome these limitations. Studies in terrestrial animals show that enzyme supplementation improves fiber digestibility and nutrient availability. For instance, He, *et al.* observed better protein and ADF digestibility in beef cattle diets containing wheat-based DDGS with fiber-degrading enzymes [15]. Similarly, Min, et al. [15] showed enhanced fiber digestibility in pig diets supplemented with xylanase and glucanase [16]. In aquaculture, Hung, et al. [17] reported improved growth and phosphorus digestibility in Tra catfish fed phytase-supplemented diets [17].

DDGS is a potential alternative protein source in aquaculture feeds, offering both economic and environmental benefits. However, its high fiber content and variable nutrient composition require strategies such as enzymatic pretreatment to improve its utility. Research on the inclusion of DDGS in shrimp diets and the optimization of pretreatment methods is necessary to fully realize its potential in sustainable aquafeeds. In this study, different enzymes are used to treat

R-DDGS to compare their efficacy in improving nutrient availability and reducing antinutritional factors, highlighting the potential of enzyme-treated R-DDGS in replacing soybean meal in aquafeed for sustainable aquaculture.

2. Materials and Methods

2.1. Enzymes

Protease, xylanase, cellulase, and phytase were sourced from the following suppliers: Protease (Commercial, USA), with an activity of 753.84 Units/ml, optimized at pH 7.5 and 37°C. Xylanase and Cellulase (Vista Pre-T): Provided by AB Vista, UK, with activities of 21,022.90 Units/ml (xylanase) and 6,282.70 Units/ml (cellulase), optimized at pH 5.5 and 50°C. Phytase (Quantum Blue 5L): Supplied by AB Vista, UK, with an activity of 5,303 Units/ml, optimized at pH 5.5 and 40°C.

2.2. Distiller's Dried Grains with Solubles (DDGS)

The Distiller's Dried Grains with Solubles (DDGS) from rice (R-DDGS) was imported from India and used as the main test ingredient. R-DDGS is a by-product of ethanol production, derived from rice. It is characterized by its high protein content and fiber levels, making it a potential alternative to soybean meal in aquafeeds. The R-DDGS used in this study was sieved to remove large particles and stored in an airtight container to maintain quality and prevent spoilage.

2.3. Rice Distiller's Dried Grain with Soluble enzyme cocktail pretreatment product

The pretreatment R-DDGS product for replacing soybean meal in shrimp feed formula of this study was prepared by using enzyme cocktail of Protease, xylanase, cellulase, and phytase in ratios 1:3.3:1:4.2. R-DDGS was treated with enzyme mixtures through a solid-state fermentation process. The treatment involved mixing 45% moisture-adjusted DDGS with enzyme solutions under controlled conditions (40°C, 24 hours). Four treatment groups were prepared: untreated (0E), single enzyme (1E) Protease, dual enzyme (2E) Protease and NSPase (Xylanase and Cellulase), and multi-enzyme (3E) Protease, NSPase and Phytase. The enzyme cocktail activity was studied. The nutritional and antinutritional properties of R-DDGS after enzyme treatment were analyzed.

2.4. Shrimp Diet

The enzyme-treated R-DDGS replaced 10% of soybean meals in shrimp diets (4.7% protein from soybean meal) were prepared following shrimp nutrient requirements [18]. The feed formula is shown in Table 1. The feed materials were ground, mixed with 25% water to form pellets using a Hobart mincer, dried at 75°C for 3 hours, cooled, and stored in plastic bags at room temperature.

Table 1.
Experimental Feed Composition.

	T1-Control	T2-R-DDGS0E	T3--DDGS1E	T4-R-DDGS2E	T5-R-DDGS3E
Fish meal	17.20	17.20	17.20	17.20	17.20
Soybean meal	37.80	27.80	27.80	27.80	27.80
R-DDGS0E	0.00	9.20	0.00	0.00	0.00
R-DDGS1E	0.00	0.00	8.40	0.00	0.00
R-DDGS2E	0.00	0.00	0.00	8.30	0.00
R-DDGS3E	0.00	0.00	0.00	0.00	8.30
Wheat flour	8.19	6.99	8.39	8.49	8.69
Broken rice	8.00	8.00	8.00	8.00	8.00
Rice bran	6.00	6.00	6.00	6.00	6.00
Squid liver powder	5.00	5.00	5.00	5.00	5.00
Plant protein	6.50	6.50	6.50	6.50	6.50
Fish oil and lecithin	2.74	2.74	2.74	2.74	2.74
Vitamin and mineral premix	8.57	8.57	8.57	8.57	8.57
L-Lysine	0	2.00	2.00	2.00	2.00
Total	100.00	100.00	100.00	100.00	100.00

2.5. Effect of R-DDGS on White Shrimp Growth Performance, Immunity and Diet Digestibility

2.5.1. Experimental Design

The study used a completely randomized design (CRD) with 5 treatments and 6 replicates. Enzyme-treated R-DDGS replaced 10% of the protein from soybean meal in shrimp diets. Five experimental diets were prepared: T1 (control), T2 (R-DDGS0E), T3 (R-DDGS1E), T4 (R-DDGS2E), and T5 (R-DDGS3E),

2.5.2. Experimental Conditions

The experiment was carried out in 30 glass aquarium with each of 240 L capacity that contain 120 L of 25 ppt. saline water, Juvenile white shrimp (*Litopenaeus vannamei)*, approximately 1.6-1.8 g, were stocked at a density of 30 shrimp/aquarium (200 individuals/ m^3). The feed was applied to shrimp 3 times a day at 3-8% of body weight. Aeration

was applied into all experimental units for maintaining DO >5 mg/L in semi-closed system. To control water quality, clear water and batch-exchange 15-20% was done every 2 days. The water treatment was done by using new seawater with 100 ppt salinity that collected from a salt field, treated with chlorine (20 ppm) and kept for one week before diluted to the required salinity of 25 ppt for filled in the tank to control water quality to pH 7.7-8.2, total ammonia <1.0 ppm, DO> 5 ppm, Temperature 27-30 °C. The culture period was 42 days.

This study was conducted according to the guideline of the Institute of Animal for Scientific Purpose Development (IAD), National Research Council Thailand (NRCT code ACKU-60-FIS-033).

2.6. Measured Parameters

2.6.1. Enzyme Cocktail Activities

The enzyme mixture activities for using in R-DDGS pretreatment were measured over time (1, 5, 10, 30 minutes) using standard protocols. Protease activity was measured using cow's milk casein as the substrate, with the reaction incubated at 37°C and pH 7.5 for 10 minutes. The reaction was then terminated with trichloroacetic acid (TCA), and absorbance was recorded at 750 nm using tyrosine as the standard, following the method of Anson [19]. Xylanase activity was determined using Azo-Xylan (derived from birchwood) as the substrate; the reaction was conducted at 50°C and pH 5.5 for 5 minutes, stopped with DNS reagent, and the absorbance was measured at 540 nm with xylose solution serving as the reference according to Bailey, et al. [5]. Cellulase activity was assessed by quantifying the reducing sugars produced, using the method described by Miller [20]. Phytase activity was measured using sodium phytate as the substrate under conditions of 37°C and pH 5.5 for 65 minutes, with the reaction terminated using a color-stop mix solution as per Engelen, et al. [21].

2.6.2. Nutritional and antinutritional Properties of R-DDGS after enzyme treatment

The post-treatment, R-DDGS was dried, ground, and analyzed for nutritional and antinutritional properties in terms of moisture, crude protein, protein digestibility, crude fiber, phytic acid, calcium and phosphorus [2, 22, 23].

2.6.3. Shrimp Growth Performance

Shrimp were weighed at the start and end of the trial. Shrimp body weight, percentage of weight gain (PWG), specific growth rate (SGR), average daily gain (ADG), feed conversion ratio (FCR), and protein efficiency ratio (PER) were calculated.

2.6.4. Shrimp immunity

Focusing on immunity, total hemocyte count was measured by mixing hemolymph with trypan blue then applied into hematocytometer and counting cells under a microscope [24] while hemolymph protein concentration was determined follow the Lowry method [25] using a bovine serum albumin standard. Phenoloxidase activity was quantified by incubating hemolymph with L-DOPA and Hepes at 37°C and measuring absorbance at 490 nm, and lysozyme activity was evaluated by monitoring absorbance changes at 540 nm over 6 minutes using a Micrococcus lysodeikticus suspension [26]. Additionally, superoxide dismutase (SOD) activity and glutathione levels were measured with commercial assay kits from Sigma-Aldrich.

2.6.5. Shrimp feed digestibility

To evaluate the digestibility of both proteins and carbohydrates in the shrimp feed, in vitro digestibility of experimental diet assays was employed. Protein digestibility was assessed by quantifying the free amino acids and soluble peptides released from homogenized samples. Free amino acids were measured using the Ninhydrin assay [27] where samples were reacted with cd-ninhydrin reagent, incubated at 84°C for 5 minutes, and the absorbance was recorded at 507 nm using tyrosine as a standard. Soluble proteins were quantified using the Biuret test [28] with absorbance measured at 540 nm against a serum albumin standard. Carbohydrate digestibility was determined via the 3,5-dinitrosalicylic acid (DNS) method [29] homogenized samples were reacted with DNS reagent, incubated at 25°C, and then heated to 100°C to terminate the reaction, with absorbance measured at 540 nm and additional readings at 550 nm, 480 nm, and 590 nm used to develop standard curves for maltose, glucose, xylose, and mannose. The cumulative reducing sugar content, expressed in grams per 100 grams of sample, provided an overall estimate of carbohydrate digestibility in the feed.

2.6.6. Cooked shrimp color determination

Cooked shrimp color under normal condition was determined at the end of feeding trial by using Salmo fan and colorimeter (3nhNR110, China) which was report in L, *a** and *b**.

3. Results

The study on application of R-DDGS with enzyme cocktail pretreatment product on growth performance and immunity of Pacific white shrimp (*Litopenaeus vannamei*) was conducted on developing R-DDGS with enzyme cocktail pretreatment product. The results on efficacy of cocktail enzyme mixture during pretreatment at different times were presented in Table 2. and the nutritional and antinutritional properties of R-DDGS after enzyme treated was shown in Table 3.

The mixed enzyme activity across the mixing time demonstrated that protease activity remained stable across all time intervals ($p = 0.998$). Cellulase was also quite stable ($p=0.105$) although the numeric value seemed to be continuously

decreased after 5 minutes to 30 minutes. However, xylanase and phytase showed significantly declined activity over time ($p < 0.05$), particularly for xylanase after 5 minutes while phytase was quite stable and declined after 10 minutes then exhibited the significantly decreased on 30 min. after mixed enzyme together.

Table 2.
Enzymatic activity over mixing time.

Time	Enzyme activity (unit/ml.)				
	1 min.	**5 min.**	**10 min.**	**30 min.**	***p*-value**
Protease	8.558 ±232	8.618 ±1.116	8.563 ±891	8.540 ±460	0.998
Cellulase	4.353 ±1.374	3.198 ±1.407	3.282 ±1.445	2.611 ±495	0.105
Xylanase	26.107 ±4.379^{a}	23.001 ±6.343^{ab}	16.862 ±4.653^{bc}	10.631 ±2.279^{c}	< 0.001
Phytase	50.801 ±7.033^{a}	51.315 ±9.470^{a}	50.738 ±5.823^{a}	34.831 ±4.146^{b}	0.003

Note: The averages with the letters a, b, c in the same row indicates a significant difference ($p<0.05$)

The nutritional and antinutritional properties of R-DDGS after enzyme treatment in Table 3 exhibited that mixed enzyme treatment increased crude protein and improved protein digestibility. The highest pepsin digestibility of 65.1% was observed in R-DDGS3E, alongside reduced fiber content to 1.32%, decreased phytic acid to 0.320%, including promoted calcium to 0.106% and released phosphorus to 0.783%.

Table 3.
Nutritional and Antinutritional Characteristics of R-DDGS after enzyme treatment.

Nutritional and Antinutritional Characteristics	Unit	R-DDGS0E	R-DDGS1E	R-DDGS2E	R-DDGS3E
Moisture	%	13.5	7.99	6.89	6.58
Crude protein	%	50.8	55.6	56.3	56.6
Protein digestibility (pepsin digestibility; 0.0002% pepsin)	%	56.5	57.8	60.7	65.1
Crude fiber	%	2.16	1.25	1.32	1.32
Phytic acid	%	0.777	0.864	0.814	0.320
Calcium	%	0.100	0.107	0.106	0.106
Phosphorus	%	0.725	0.781	0.788	0.783

Note: data do not have statistical analysis.

3.1. Effect of R-DDGS on White Shrimp Growth Performance, Immunity and Diet Digestibility

The growth performance and survival rate of shrimp fed enzyme treated R-DDGS was shown in Table 4. Shrimp fed enzyme-treated R-DDGS diets (T3–T5) and T1control had significantly higher final weight, percent weight gain, specific growth rate, and average daily gain, than shrimp fed untreated R-DDGS (T2) ($p<0.05$). Survival rates were significantly high ($p<0.05$) in shrimp fed enzyme-treated R-DDGS diets (T3–T5) and group of shrimps fed untreated R-DDGS (T2). The highest survival rate found in T5 (93.89%, $p<0.001$). The feed utilization in terms of feed conversion ratio was low in shrimp fed enzyme-treated R-DDGS diets (T3–T5) compared to T1 control and shrimp fed untreated R-DDGS (T2) ($p<0.05$). The protein efficiency ratio was significantly high ($p<0.05$) in shrimp fed enzyme-treated R-DDGS diets (T3–T5) compared to T1 control and shrimp fed untreated R-DDGS (T2) ($p<0.05$). This indicated that enzyme treated R-DDGS could replace soybean meal 10% (4.7% protein from soybean meal) without adverse effects on growth performance compared to control soybean meal diet.

Table 4.
Growth Performance and Survival of Shrimp (*Litopenaeus vannamei*) Fed Enzyme-Treated R-DDGS Diets for 42 days.

Growth Performance Parameter	T1 (Control)	T2 (R-DDGS0E)	T3 (R-DDGS1E)	T4 (R-DDGS2E)	T5 (R-DDGS3E)	*p*-value
Initial weight (g)	1.70 ±0.17	1.81 ±0.09	1.74 ±0.20	1.74 ±0.10	1.68 ±0.06	0.805
Final weight (g)	9.97 ±0.34^{a}	9.12 ±0.22^{b}	9.58 ±0.15^{a}	9.80 ±0.26^{a}	9.84 ±0.25^{a}	<0.001
Percent weight gain; PWG (%)	492.70 ±60.89^{a}	406.49 ±33.34^{b}	458.36 ±66.55^{ab}	465.66 ±44.30^{ab}	487.98 ±22.02^{ab}	0.035
Specific growth rate; SGR (%*day^{-1})	4.23 ±0.24^{a}	3.86 ±0.15^{b}	4.08 ±0.12^{ab}	4.12 ±0.18^{ab}	4.22 ±0.09^{a}	0.033
Average daily gain; ADG (g*ind^{-1}*day^{-1})	0.197 ±0.008^{a}	0.175 ±0.005^{b}	0.187 ±0.005^{a}	0.192 ±0.008^{a}	0.193 ±0.005^{a}	<0.001
Feed conversion ratio; FCR	1.41 ±0.06^{a}	1.42 ±0.06^{a}	1.29 ±0.03^{b}	1.24 ±0.03^{b}	1.22 ±0.03^{b}	<0.001

Survival rate; SR (%)	80.56 ±4.91[b]	90.00 ±6.33[a]	92.78 ±3.90[a]	93.33 ±2.98[a]	93.89 ±3.28[a]	<0.001
Protein efficiency ratio; PER	1.93 ±0.08[b]	1.91 ±0.08[b]	2.16 ±0.06[a]	2.21 ±0.05[a]	2.24 ±0.06[a]	<0.001

Note: The averages with the letters a, b, c in the same row indicates a significant difference (p<0.05)

The immunity of shrimp fed R-DDGS diets were presented in Table 5. The total hemocyte count was highest in T2 and followed by control T2 but significantly lower in T3, T4, and T5 (p = 0.006). Other immune markers, such as hemolymph protein, phenoloxidase activity, lysozyme activity, superoxide dismutase activity and glutathione peroxidase did not differ significantly (p>0.05). This implied that shrimp fed diets composed of enzyme treated R-DDGS replaced for soybean meal did not have any adverse effects on immunity compared to control soybean meal diet.

Table 5.
Immune Response Parameters in Shrimp (*Litopenaeus vannamei*) Fed Enzyme-Fermented DDGS Diets for 42 days.

Immune Parameter	**T1 (Control)**	**T2 (R-DDGS0E)**	**T3 (R-DDGS1E)**	**T4 (R-DDGS2E)**	**T5 (R-DDGS3E)**	***p*-value**
Total Hemocyte Count ($\times 10^5$ cell/ml)	31.33 ±2.31[ab]	35.00 ±2.65[a]	29.33 ±1.53[b]	27.00 ±2.65[b]	27.33 ±0.58[b]	0.006
Hemolymph protein (g/dL)	3.66 ±0.31	3.89 ±0.30	3.57 ±0.20	3.58 ±0.51	3.56 ±0.69	0.872
Phenoloxidase activity (Unit/min/mg Protein)	163.94 ±29.97	133.12 ±31.98	140.77 ±29.62	147.53 ±15.53	153.90 ±26.32	0.694
Lysozyme activity (Unit/ml.)	225.00 ±26.46	221.67 ±15.28	218.33 ±20.82	208.33 ±25.17	215.00 ±26.46	0.916
Superoxide dismutase activity (Unit/ml.)	109.40 ±10.97	98.24 ±20.69	127.19 ±28.27	119.14 ±19.03	125.20 ±15.68	0.404
Glutathione peroxidase (nM/ml)	30.31 ±0.17	30.24 ±0.22	30.16 ±0.04	30.11 ±0.07	20.27 ±0.12	0.463

Note: The averages with the letters a, b, c in the same row indicates a significant difference (p<0.05)

3.2. Color and Enzyme Activity in Shrimp

Cooked shrimp color was presented in Table 6. The L (lightness), *a* (redness) and *b* (yellowness) values in boiled shrimp fed all experimental diets were in the same range (p>0.05). Moreover, the color determination by Salmofan score was not significantly differences (p>0.05). Anyway, it was notice that the *a* (redness) and *b* (yellowness) values in boiled shrimp fed T5 diet had numerical values higher than T1, but was not significantly differences (p > 0.05).

Table 6.
Colorimetric Analysis of Boiled Shrimp (*Litopenaeus vannamei*) Fed Enzyme-Fermented DDGS Diets for 42 days.

Color	**T1 (Control)**	**T2 (R-DDGS0E)**	**T3 (R-DDGS1E)**	**T4 (R-DDGS2E)**	**T5 (R-DDGS3E)**	***p*-value**
SalmoFan No.	22.4±0.7	22.5±0.7	22.6±0.7	22.8±0.6	23.0±0.0	0.206
L	65.52±0.74	66.25±2.08	65.39±1.27	65.43±1.08	65.96±0.85	0.852
a	11.04±2.84	9.59±2.59	10.11±1.66	10.03±1.68	10.18±1.24	0.639
b	21.43±1.97	19.77±2.64	20.43±2.16	20.7±1.61	21.30±1.41	0.349

Note: The averages with the letters a, b, c in the same row indicates a significant difference (p<0.05)

3.3. Digestibility of Enzyme Treated R-DDGS Diet

The digestibility of enzyme treated R-DDGS diet was demonstrated in Table 7.

3.3.1. Protein Digestibility

Protein digestibility in terms of free amino acid and soluble protein levels had numerical values varied among treatments but were not significantly different (p> 0.05). However, total protein digestibility differed significantly (p <0.05), with enzyme-treated R-DDGS feeds in T3, T4, and T5 which showing higher values (29.5–30.5 g/100g) compared to T2 (24.80 g/100g) and closed to T1 Control of soybean meal. These findings indicated that enzymatic pretreatment of R-DDGS enhances protein digestibility, improving nutrient availability in shrimp feed.

3.3.2. Carbohydrate Digestibility

Total reducing sugar levels were highest in T4 and T5, correlating with higher maltose, xylose and mannose contents (p<0.05). The glucose levels mainly from cellulose also increased in enzyme-treated R-DDGS diets but there were not significantly differences (p>0.05). That means enzyme-treatment on R-DDGS could enhance nutrient digestibility both protein and carbohydrate digestibility.

Table 7.
Protein and Carbohydrate Digestibility of Enzyme-Fermented DDGS Diets.

Enzyme	T1 (Control)	T2 (R-DDGS0E)	T3 (R-DDGS1E)	T4 (R-DDGS2E)	T5 (R-DDGS3E)	*p*-value
Amino acid (g/100g feed)	17.83 ±1.34	13.92 ±3.20	16.69 ±0.76	17.60 ±1.29	17.26 ±0.93	0.108
Soluble protein (g/100g feed)	12.10 ±1.16	10.88 ±2.18	12.78 ±2.69	12.88 ±0.28	12.33 ±0.13	0.598
Total Protein (g/100g soluble)	29.93 ±2.01^{a}	24.80 ±1.11^{b}	29.48 ±2.82^{ab}	30.48 ±1.55^{a}	29.60 ±1.06^{ab}	0.040
Maltose (g/100g feed)	6.88 ±0.56^{b}	5.92 ±0.28^{b}	7.29 ±0.87^{ab}	9.05 ±1.06^{a}	9.23 ±0.86^{a}	0.003
Xylose (g/100g feed)	7.27 ±1.13^{a}	3.77 ±0.61^{c}	4.50 ±0.42^{bc}	6.03 ±0.81^{ab}	6.37 ±0.51^{ab}	0.001
Glucose (g/100g feed)	4.32 ±0.71	2.87 ±0.66	3.63 ±1.19	4.49 ±1.25	4.84 ±0.46	0.139
Mannose (g/100g feed)	3.96 ±0.78^{ab}	2.64 ±0.48^{b}	3.14 ±0.44^{ab}	3.99 ±0.45^{a}	4.10 ±0.68^{a}	0.022
Total reducing sugar (g/100g feed)	22.42 ±1.89^{a}	15.19 ±1.50^{b}	18.47 ±2.63^{ab}	23.55 ±2.44^{a}	24.54 ±2.42^{a}	0.017

Note: The averages with the letters a, b, c in the same row indicates a significant difference ($p<0.05$).

4. Discussion

The current study explored the impact of enzyme-treated distiller's dried grains with solubles from rice (R-DDGS) on nutritional improvements, growth performance, immune responses, and nutrient digestibility. The stability of mixed enzyme for R-DDGS fermentation to improve raw material quality exhibited that protease activity across all time suggests robustness under various conditions [30]. Protease is known for its stability under a wide range of pH and temperature conditions, often remaining active despite interactions with other enzymes [15]. This aligns with its role in hydrolyzing protein into absorbable amino acids, contributing significantly to nutrient availability. In addition, cellulase showed consistency over 30 minutes, and later tended to decline. In contrast, the observed declines clearly in xylanase, while phytase activities presented stability over time before declined on 30 minutes. These highlight the sensitivity of these enzymes to mixed environments. Declining activity may result from enzyme-enzyme interactions, substrate depletion, or conformational changes due to prolonged mixing. Enzyme structure is protein molecules, while protease break down protein. Hence, when cellulase, xylanase, and phytase mixed with protease, the protease hydrolyzes other enzymes then some amino acids released, conformational changes to inactive molecules [31]. Studies by Hung, et al. [17] emphasize that cellulase and xylanase are particularly prone to inactivation under suboptimal conditions, such as mechanical stress or pH fluctuations [15, 16]. Phytase showed a sharp decline after 30 minutes, which could be attributed to substrate depletion or its inherent thermal and mechanical sensitivity during prolonged incubation or mixing different enzyme into the same solution [32]. These findings suggest that maintaining optimal each enzyme proportion, sequence of adding enzyme especially protease, mixing time and fermentation conditions could prolong enzyme activity. Enzymatic treatment of R-DDGS significantly improved crude protein content, protein digestibility, and reduced fiber including antinutritional components such as phytic acid. The increased digestibility observed in R-DDGS3E aligns with [16] who reported that enzyme supplementation enhances the availability of amino acids and reduces structural barriers, such as cellulose and lignin, in plant-based feed ingredients [18]. Crude protein levels in enzyme-treated R-DDGS (55.6% to 56.6%) surpassed untreated controls, emphasizing the role of protease in hydrolyzing complex protein structures into peptides and amino acids [33]. This finding aligns with studies by Hung, et al. [17] on phytase-supplemented diets, which reported enhanced digestibility and growth in aquatic species by increasing bioavailability of key nutrients [17]. Fiber reduction in enzyme-treated R-DDGS underscores the efficacy of cellulase and xylanase in breaking down insoluble fibers into digestible carbohydrates. The lower levels of Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) in treated samples are consistent with findings of Pedersen, et al. who observed improved fiber breakdown when enzymes were applied to DDGS, enhancing its application in aquafeeds [10]. Shrimp fed diets with enzyme-treated R-DDGS (T3, T4, and T5) showed significant improvements in final weight, specific growth rate (SGR), percent weight gain (PWG), feed conversion ratio (FCR), and protein efficiency ratio (PER). These findings underscore the ability of enzyme treatments to enhance the bioavailability of proteins and carbohydrates, translating into better growth and feed utilization. Specifically, the reduction in FCR observed in T4 (1.24) and T5 (1.22) compared to the soybean meal control diet (T1, 1.41) and diet with untreated R-DDGS (T2). The highlights improved feed efficiency, which is consistent with earlier studies on enzyme supplementation in fish and shrimp diets [34-36]. The study of Adedeji, et al. [1] demonstrated similar improvements in feed efficiency with sorghum and corn-based DDGS in shrimp, attributed to enhanced protein digestibility [1, 8]. The superior growth metrics in T5 can also be linked to the combined effect of protease, cellulase, xylanase, and phytase in optimizing nutrient release. Studies on Nile tilapia and European catfish similarly reported enhanced growth and protein utilization with enzyme-treated DDGS, indicating the cross-species applicability of enzyme pretreatment [12, 37].

Total hemocyte count (THC), a key immune parameter, was highest in T2 but significantly lower in enzyme-treated groups (T3–T5). The elevated THC in shrimp fed untreated R-DDGS diets (T2) may reflect a stress-induced immune

response to dietary antinutritional components [38]. Furthermore, phenoloxidase activity, lysozyme activity, and other immune markers did not show significant differences, the stable levels indicate that enzyme-treated diets maintained immune homeostasis. Studies in fish and shrimp including poultry especially by Song, *et al.* highlight the importance of reducing antinutritional factors to mitigate unnecessary immune activation and improve growth performance. Hence, the low THC following enzyme treatment indicated potentially reduction of antinutritional factors which reduce nutritional stress from the diet [10, 15]. Focusing on cooked shrimp color, while *a* (redness) and *b* (yellowness) values and Salmofan score in boiled shrimp were not significantly different across treatments, these indicated the low effect of R-DDGS on promote shrimp color due to rice containing low carotenoids [39].

Protein and carbohydrate digestibility of enzyme-treated R-DDGS diets were significantly improved. These findings reflect the synergistic actions of cellulase and xylanase in breaking down complex polysaccharides into simpler sugars. Similar results were reported by Da Silva, et al. [40] who demonstrated that enzyme supplementation improves carbohydrate utilization in DDGS-based feeds [40]. The increased levels of mannose and glucose in enzyme-treated diets further highlight the role of enzymatic hydrolysis in releasing sugars from non-starch polysaccharides. This carbohydrate availability likely contributed to the improved energy efficiency and growth performance observed in enzyme-treated groups.

5. Conclusion

Enzyme-treated R-DDGS improves the nutritional value and digestibility of shrimp feed, resulting in enhanced growth performance, survival, and feed efficiency. The enzymatic pretreatment reduces antinutritional factors and enhances carbohydrate availability, promoting better protein utilization and digestibility. These findings support the application of enzyme technology in aquaculture feeds to maximize the use of sustainable, alternative protein sources like R-DDGS. Future research should focus on optimizing enzyme combinations and concentrations for specific dietary formulations and evaluating long-term economic and environmental impacts.

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