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CARRERA DE MEDICINA VETERINARIA

SIMBIÓTICOS REFORZADOS CON ENZIMAS: UNA ALTERNATIVA SOSTENIBLE A LOS ANTIBIÓTICOS PROMOTORES DE CRECIMIENTO EN POLLOS BROILER

TRABAJO DE TITULACIÓN PREVIO A LA OBTENCIÓN DEL TÍTULO DE MEDICO VETERINARIO.

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CUENCA- ECUADOR

2025

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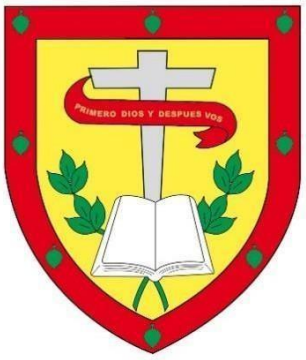

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ERICK SAUL ANDRADE MUYULEMA portador de la cédula de ciudadanía Nº **0302330196, BISMARCK NEY RAMIREZ GALLARDO** portador de la cédula de ciudadanía Nº **0705109957.** Declaramos ser los autores de la obra: "**Simbióticos reforzados con enzimas: una alternativa sostenible a los Antibióticos Promotores de Crecimiento en pollos Broiler"** sobre la cual nos hacemos responsables sobre las opiniones, versiones e ideas expresadas. Declaramos que la misma ha sido elaborada respetando los derechos de propiedad intelectual de terceros y eximo a la Universidad Católica de Cuenca sobre cualquier reclamación que pudiera existir al respecto. Declaramos finalmente que nuestra obra ha sido realizada cumpliendo con todos los requisitos legales, éticos y bioéticos de investigación, que la misma no incumple con la normativa nacional e internacional en el área específica de investigación, sobre la que también nos responsabilizamos y eximimos a la Universidad Católica de Cuenca de toda reclamación al respecto.

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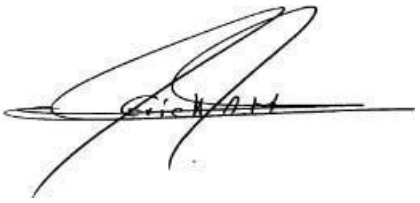

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Certificación

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Enzyme-Enhanced Symbiotics: A Sustainable Alternative to Antibiotic Growth Promoters in Broiler Chickens

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This study evaluated the effect of dietary supplementation with enzyme-enhanced symbiotic on productive, immunological, digestive, and histo-morphological parameters in broiler chickens. The research was conducted under commercial conditions in the Balsas canton, El Oro province (Ecuador), using a longitudinal experimental design. A total of 84,000 birds were randomly allocated to four treatments: T0 (control), T1 (0.01%), T2 (0.03%), and T3 (0.05%) symbiotic, all combined with 0.01% protease enzymes. Productive performance, apparent digestibility, intestinal morphometry, and serum immunoglobulins (IgY, IgM, IgA) were assessed on days 1 and 21 of age. Treatment T2 (0.03%) yielded the best zootechnical performance, with greater weight gain, more efficient feed conversion, and enhanced intestinal mucosal development, as evidenced by increased villus length and density. Additionally, T2 improved crude protein and fat digestibility and significantly stimulated IgM production, suggesting more effective enteric immune activation. In contrast, T3 (0.05%) exhibited negative effects on intestinal morphology and weight gain, indicating that higher concentrations may cause physiological interference. No clear dose-dependent trend was observed for crude fiber and dry matter digestibility. Statistical analysis confirmed significant differences ($p < 0.05$) among treatments. In conclusion, supplementation with 0.03% symbiotic enhanced with enzymes proved to be the most effective strategy for improving intestinal health, nutrient utilization, and productive performance in broiler chickens, establishing itself as a promising functional alternative for intensive poultry systems.

Keywords: Synbiotics; enzymes; broiler chickens; productive parameters; intestinal morphometry; immunoglobulins; digestibilit.

1. Introduction

The poultry industry plays a key role worldwide in the production of animal-based protein. The United States leads global chicken meat production with approximately 21 million metric tonnes, followed by Brazil and China, each with over 14 million metric tonnes (Orúz, 2023). In the regional context, Ecuador produced 495,000 tonnes of chicken meat in 2022 (Corporación Nacional de Avicultores del Ecuador – CONAVE, 2021), and began exporting in 2023 with a shipment of 28 tonnes to the Bahamas (Ministerio de Agricultura y Ganadería – MAG, 2023). Within the country, the provinces of Guayas, Manabí, and El Oro stand out as the main producing areas in the coastal region (Instituto Nacional de Estadística y Censos – INEC, 2022).

Poultry farming in Ecuador represents a fundamental pillar of the national economy, contributing 23% to the agricultural Gross Domestic Product (GDP) and 3% to the national GDP. It generates over USD 3.5 billion annually and provides more than 300,000 direct and indirect jobs (CONAVE, 2021). Per capita chicken meat consumption reached 32 kg/person/year in 2020 (Navarrete, 2020) and was estimated at 28 kg in 2023 (Jaramillo, 2023), highlighting its importance in the national diet and food security.

Across Latin America, chicken meat and eggs show the highest per capita consumption among animal protein sources, averaging 33.67 kg of meat and 230 eggs per person per year (Ruíz, 2023). This reality positions poultry farming as a strategic component of global human nutrition. However, the sustainability of the sector faces growing challenges related to production efficiency, intestinal health of birds, and responsible use of zootechnical inputs.

Factors such as the high cost of imported raw materials (Gutiérrez, 2018; Pomboza et al., 2018), intestinal dysbiosis, and the risks of mycotoxicosis (Uculmana, 2019) undermine production profitability, particularly in areas such as Balsas canton, El Oro province. Despite a 400% growth in production since the 1980s, this area faces structural limitations such as limited access to capital, outdated production models (González et al., 2020), lack of organization among poultry producers, and scarce applied local research (Cueva & Maldonado, 2011), as well as meat oversupply leading to price drops below production costs (Romero, 2017; Romero, 2018; Nuñez, 2018).

Simultaneously, the restriction on the use of antibiotic growth promoters (AGPs) and prophylactics in commercial poultry farming—driven by scientific and regulatory pressures due to increasing antimicrobial resistance (Cancho & Simal, 2000; Torres & Zarazaga, 2002; Santovito et al., 2018)—has triggered a global crisis with repercussions for both human and

animal health (Espinosa et al., 2019; González et al., 2020). This situation has encouraged the scientific community to explore viable alternatives that do not leave residues in poultry products or cause side effects for consumers.

In this context, replacing AGPs has become a priority in animal production, leading to the development of nutritional strategies based on functional additives such as probiotics, prebiotics, symbiotic, enzymes, and phytobiotics (Cepero, 2013; Gutiérrez, 2013). These alternatives have proven effective in improving digestibility, enhancing immune function, and optimizing the productive performance of birds without compromising food safety (Castro & Rodríguez, 2005; Blajman et al., 2015; Álvarez et al., 2023; Jurado et al., 2021).

Probiotics are live microorganisms that support digestion, maintain gut microbiota balance, and promote bird growth (Blajman et al., 2015), while prebiotics act as non-digestible substrates that selectively stimulate beneficial microbiota (Castro & Rodríguez, 2005). Their synergistic combination forms symbiotic, which have shown positive effects on zootechnical performance (Dong et al., 2024). Among the most commonly used symbiotic is the association of Lactobacillus spp. and *Saccharomyces cerevisiae*—the latter a yeast regarded as safe by the FDA, with high protein value and significant benefits for intestinal morphometry and productive parameters (Cuenca et al., 2022; Jurado et al., 2021; Jurado & Zambrano, 2020; Yin et al., 2023; Rodríguez & Moreno, 2016; Seminario & Cuenca, 2018; Toalombo et al., 2021).

Additionally, the use of enzymes in poultry diets has shown improvements in feed conversion and weight gain (Cortés et al., 2002), suggesting that combining symbiotic with enzymes could enhance the mechanisms of action of both additives, thereby increasing their effectiveness as alternatives to antibiotics.

Therefore, the present study aims to evaluate the impact of enzyme-enhanced symbiotic on the productive performance of broiler chickens, as a sustainable strategy that meets current market demands and global sanitary restrictions, particularly in local contexts such as Balsas canton.

2. Materials and Methods

2.1 Study Locationand Design

This study was conducted in Balsas canton, El Oro province, Ecuador, in commercial poultry facilities operating under an intensive production system. The region is characterized by a dry tropical climate, with an average annual temperature of 26 to 28 °C and relative humidity ranging from 70% to 80% (Romero, 2015); conditions which significantly influence the development, performance, and physiological adaptation of the birds under study.

The experimental design was observational, experimental, and longitudinal. The effect of dietary supplementation with symbiotic enhanced with protease enzymes on broiler chicken performance was evaluated. Three symbiotic levels – Lactopharm at 0.01%, 0.03%, and 0.05% – were combined with a fixed concentration of protease enzymes (0.01%) per tonne of feed. The symbiotic product contained Lactobacillus acidophilus, *Streptococcus faecium*, *Saccharomyces cerevisiae*, vitamins (A, D3, E, B1, B2, B6, B12), niacinamide, pantothenic and citric acids, glucose, and potassium chloride.

A total of 84,000 one-day-old broiler chickens were randomly assigned to four experimental treatments: T0 (control, no additives), T1 (0.01% symbiotic + 0.01% protease enzymes), T2 (0.03% symbiotic + 0.01% protease enzymes), and T3 (0.05% symbiotic + 0.01% protease enzymes). Each treatment consisted of four replicates of 5,250 birds each, all maintained under uniform management, feeding, and biosecurity conditions across experimental units.

2.2 Sample Collectionand Euthanasia of Birds

Serum levels of immunoglobulins IgA, IgG, and IgM were analyzed in broiler chickens on days 1 and 21 of age to assess the humoral immune response to the treatments. On day 1 (24 hours' post-hatch), blood samples were collected from 4 birds per treatment without prior access to feed, under the assumption that baseline immunoglobulin levels were homogeneous across groups. On day 21, representative samples of 20 birds per treatment were analyzed, with 5 birds randomly selected from each replicate, taking into account the accumulated effect of the symbiotic and enzymes administered via the diet.

Blood samples were collected via brachial vein puncture (wing vein) using a sterile syringe, extracting 2 mL per bird. Samples were deposited into pre-labelled, anticoagulant-free tubes (red-top) and transported under controlled conditions to the laboratory for serological and immunological analysis.

For evaluation of intestinal morphometric variables—including villus height and width, number of villi per field, and depth of the crypts of Lieberkühn—5 cm tissue segments were collected from the duodenum, jejunum, and ileum. Samples

were gently flushed with sterile saline solution (0.9% NaCl) to remove luminal contents and fixed in 10% formalin in properly labelled containers for subsequent histological processing.

Faecal samples for digestibility analysis were individually collected after birds were placed in metabolic cages. The amount of feed offered and faecal output were precisely recorded to calculate the apparent digestibility coefficient.

Bird euthanasia was performed in accordance with the ethical guidelines of the European Union's animal welfare regulations (Majó & Dolz, 2011). One-day-old chicks were euthanized by atlanto-occipital dislocation. In contrast, 21-day-old birds were euthanized via intravenous administrationof sodiumpentobarbital at a dose of 80 mg/kg body weight.

2.3 Variables Evaluated

Four main groups of variables were evaluated during the study: productive, immunological, intestinal morphometric, and digestibility parameters.
Productive variables included: feed intake (g/bird), weight gain (g/bird), feed conversion ratio (feed consumed/weight gain), and mortality rate (%). These were recorded weekly from day 1 to day 42 of age, using a sample of 1,000 randomly selected birds, grouped in batches of 50 per group.
Immunological variables included serum concentrations of IgA, IgG, and IgM, quantified using indirect ELISA on days 1 and 21 of age.

Intestinal morphometric variables involved measuring villus height and width, number of villi per field (400x), and depth of the crypts of Lieberkühn in the duodenum, jejunum, and ileum. Samples were fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin–eosin for microscopic examination. These variables were analizad in days 1 and 21 of age.
Finally, digestibility variables were determined through the analysis of faeces collected in metabolic cages. The apparent digestibility coefficient of feed was calculated on day 21, based on actual feed intake and dry weight of excreta, using the formula proposed by Dwain & Vallentine (2011):

$$\text{Apparent digestibility} = \frac{\text{Nutrient consumed} - \text{Nutrient in feces}}{\text{Nutrient consumed}}\ x\ 100$$

2.4 Statistical Analysis

The data obtained were analyzed using InfoStat statistical software, version 2020. Prior to analysis, assumptions of normality and homogeneity of variances were tested using the Kolmogorov–Smirnov test, the modified Shapiro–Wilk test, and Levene's test, respectively. Variables that met the assumptions required for parametric analysis were assessed using ANOVA, while those that did not meet these assumptions were analysed using non-parametric tests (Kruskal–Wallis). Both types of analysis were conducted at a significance level of $p < 0.05$ to comparedifferences among treatments.

3. **Results**

3.1. Analysis of Productive Variables

Table 1 presents the weekly productive performance of broiler chickens subjected to different concentrations of symbiotic (0.01%, 0.03%, and 0.05%), all supplemented with 0.01% protease enzymes, compared with a control group (T0). Feed intake (FI), weight gain (WG), and feed conversion ratio (FCR) were evaluated, showing statistically significant effects ($p < 0.0001$) depending on both treatment and time across all weeks and variables.

T2 (0.03% symbiotic + 0.01% enzyme) exhibited the most balanced productive performance throughout the six-week period, achieving high weight gains with moderate feed intake and acceptable feed conversion ratios. Although T3 (0.05% symbiotic + 0.01% enzyme) demonstrated good feed efficiency during certain weeks, it showed reduced weight gain, suggesting an inhibitory effect due to overdosing. This indicates that higher concentrations may be counterproductive. T1 (0.01%) showed variable performance—outstanding in feed efficiency during the early weeks but declining in the final stages. The control group (T0), while showing occasional high weight gains, maintained the poorest feed conversion rates, indicating less efficient nutrient utilization.

Overall, these results demonstrate that enzyme-enhanced symbiotic supplementation had dose-dependent positive effects on the productive parameters evaluated. T2 (0.03% symbiotic + 0.01% enzyme) emerged as the most efficient and balanced combination, achieving sustained improvements in zootechnical performance without compromising feed efficiency. These findings highlight the importance of establishing optimal dosages that synergistically enhance digestive physiology and nutrient utilization in broiler chickens.

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Table 1. Analysis of the Inclusion of Enzyme-Enhanced Symbiotic in the Weekly Productive Parameters of Broiler Chickens.

Week	Productive Parameter	T0	T1	T2	T3	p value
1	FI (g)	142.53[c]	131.73 [a]	136.00 [b]	130.95 [a]	<0.0001
	WG (g)	159.87[b]	156.15 [a]	176.24 [c]	174.33 [c]	<0.0001
	FCR	0.89[d]	0.84 [c]	0.77 [b]	0.75 [a]	<0.0001
2	FI (g)	304.02 [c]	272.44 [b]	316.00 [d]	231.06 [a]	<0.0001
	WG (g)	296.97 [b]	350.01 [c]	359.73 [d]	274.27 [a]	<0.0001
	FCR	1.02 [d]	0.78 [a]	0.88 [c]	0.84 [b]	<0.0001
3	FI (g)	387.84 [c]	379.06 [b]	400.30 [d]	369.28 [a]	<0.0001
	WG (g)	542.96 [d]	536.49 [c]	512.15 [a]	520.26 [b]	<0.0001
	FCR	0.71 [a]	0.71 [a]	0.78 [b]	0.71 [a]	<0.0001
4	FI (g)	553.45 [c]	548.19 [b]	565.54 [d]	507.95 [a]	<0.0001
	WG (g)	754.00 [c]	728.29 [a]	755.02 [c]	740.03 [b]	<0.0001
	FCR	0.73 [b]	0.75 [c]	0.75 [c]	0.69 [a]	<0.0001
5	FI (g)	786.83 [b]	820.90 [c]	820.83 [c]	773.85 [a]	<0.0001
	WG (g)	688.83 [d]	619.48 [a]	665.68 [b]	671.25 [c]	<0.0001
	FCR	1.14 [a]	1.33 [d]	1.23 [a]	1.15 [b]	<0.0001
6	FI (g)	1013.87 [b]	1026.52 [c]	1146.18 [d]	983.56 [a]	<0.0001
	WG (g)	590.42 [b]	565.28 [a]	691.97 [c]	690.50 [c]	<0.0001
	FCR	1.72 [c]	1.82 [d]	1.66 [b]	1.42 [a]	<0.0001

FI (Feed Intake), WG (Weight Gain), FCR (Feed Conversion Ratio).
Means sharing a common letter are not significantly different (p > 0.05).

3.2. *Analysis of Intestinal Morphometry Variables*

On day 1, prior to the administration of enzyme-enhanced symbiotic, intestinal morphometry was evaluated to verify the initial homogeneity of the experimental groups. Although most parameters showed no statistically significant differences, spontaneous morphometric variations were observed in some variables, which challenge the assumption of baseline uniformity.

In the duodenum, villus length was significantly greater in groups T1 (115.60 µm) and T2 (105.60 µm) compared to the control group T0 (99.24 µm) ($p = 0.0198$). In the jejunum, crypt depth was higher in T3 (21.02 µm) compared to T0 and T1 ($p = 0.0100$). Likewise, villus density was higher in T2 in both the duodenum (35) and jejunum (36) ($p = 0.0045$ and 0.0127, respectively) compared to T0 and T3. These differences, not attributable to treatment, reflect some initial biological variability that should be considered when interpreting subsequent results.

At 21 days, following continuous supplementation, dose-dependent morphological changes were evident, with group T2 (0.03%) exhibiting the most favourable values across multiple variables.

Villus length: In the duodenum, T2 showed the greatest length (870.60 µm), significantly higher than the other treatments ($p = 0.0126$). In the ileum, marked differences between treatments were observed ($p < 0.0001$), with higher values in T1 (377.20 µm) and T2 (351.72 µm), and a notable decrease in T3 (290.58 µm), suggesting an adverse effect due to overdosing.

Villus density: This parameter increased significantly in all intestinal segments of T1 and T2, with T2 recording the highest density in the duodenum (54.53), jejunum (56.07), and ileum (53.13) ($p < 0.0001$ for duodenum and ileum; $p = 0.0142$ for jejunum). T3 exhibited the lowest values across all segments, possibly due to saturation effects or interfere nce with symbiotic action.

Villus width and crypt depth: Width was greater in the control group in both duodenum ($p < 0.0001$) and jejunum ($p = 0.0071$), potentially as a compensatory response to reduced functional surface area. Regarding crypt depth, the duodenum of T3 showed significantly lower values (43.16 µm; $p = 0.0011$), which may indicate reduced epithelial turnover or proliferative inhibition at that concentration.

In summary, administration of enzyme-enhanced symbiotic for 21 days promoted positive structural changes in the intestinal mucosa, especially in group T2 (0.03%), which significantly improved villus length and density without compromising crypt depth. These effects could translate into increased absorptive capacity and digestive efficiency. In

contrast, group T3 (0.05%) not only failed to improve the evaluated parameters but also showed reductions in several, indicating that higher concentrations do not guarantee better effects and may be detrimental.

These findings underscore the importance of optimizing symbiotic dosage, considering its interaction with intestinal physiology. The structural stimulation observed at 21 days supports its potential as a functional alternative to enhance intestinal development in broiler chickens. See Table 2.

Table 2. Analysis of the Inclusion of Enzyme-Enhanced Symbiotic in Intestinal Morphometric Parameters of Broiler Chickens (Days 1 and 21).

Day 1						
Variables	Segment	T0	T1	T2	T3	p Value
	Duodenum	99.24 ^{a}	115.60 ^{b}	105.60 ^{b}	105.10 ^{ab}	0.0198
	Jejunum	106.07 ^{a}	101.53 ^{a}	98.14 ^{a}	101.61 ^{a}	0.3174
Length µM	Ileum	99.82 ^{a}	98.68 ^{a}	104.70 ^{a}	100.36 ^{a}	0.1119
	Duodenum	25.97 ^{a}	24.67 ^{a}	25.20 ^{a}	26.11 ^{a}	0.8541
	Jejunum	22.86 ^{a}	25.27^{a}	23.83 ^{a}	25.86 ^{a}	0.4059
Width µM	Ileum	25.14 ^{a}	26.45 ^{a}	23.31 ^{a}	24.94 ^{a}	0.5736
	Duodenum	18.72 ^{a}	17.29 ^{a}	18.25 ^{a}	19.44 ^{a}	0.5653
	Jejunum	18.55 ^{a}	17.56 ^{a}	19.11 ^{ab}	21.02 ^{b}	0.0100
Depth µM	Ileum	20.48 ^{a}	20.35 ^{a}	20.35 ^{a}	21.65 ^{a}	0.5689
	Duodenum	31.00 ^{a}	31.00 ^{a}	35.00 ^{b}	29.00 ^{a}	0.0045
	Jejunum	34.00 ^{ab}	33.00 ^{a}	36.00 ^{b}	32.00 ^{a}	0.0127
Density(4x)	Ileum	33.00 ^{a}	36.00 ^{a}	30.00 ^{a}	31.00 ^{a}	0.0028
Día 21						
	Duodenum	780.10 ^{a}	736.63 ^{a}	870.60 ^{b}	754.71 ^{a}	0.0126
	Jejunum	434.04 ^{a}	475.02 ^{a}	434.38 ^{a}	428.36 ^{a}	0.0715
Length µM	Ileum	341.63 ^{b}	377.20 ^{c}	351.72 ^{bc}	290.58 ^{a}	<0.0001
	Duodenum	69.99 ^{b}	46.79 ^{a}	45.70 ^{a}	51.22 ^{a}	<0.0001
	Jejunum	55.86 ^{b}	48.05 ^{a}	46.02 ^{a}	48.81 ^{a}	0.0071
Width µM	Ileum	53.79 ^{c}	42.91^{a}	52.00 ^{bc}	45.99 ^{ab}	0.0007
	Duodenum	54.45 ^{b}	56.31 ^{b}	55.59 ^{b}	43.16 ^{a}	0.0011
	Jejunum	47.74 ^{a}	47.16 ^{a}	51.28 ^{a}	52.25 ^{a}	0.5538
Depth µM	Ileum	50.04 ^{a}	48.09 ^{a}	50.87 ^{b}	47.17 ^{a}	0.4723
	Duodenum	46.67 ^{b}	50.93 ^{bc}	54.53 ^{c}	37.47 ^{a}	<0.0001
	Jejunum	55.47 ^{ab}	58.93 ^{b}	56.07 ^{ab}	52.00 ^{a}	0.0142
Density(4x)	Ileum	56.07 ^{c}	50.00 ^{ab}	53.13 ^{bc}	47.27^{a}	<0.0001

Means sharing a common letter are not significantly different (p > 0.05).

3.3. Bromatological Composition of the Balanced Feed and the Effect of Symbiotic on Apparent Digestibility in Broiler Chickens

The bromatological analysis of the balanced feed used in the present study (Table 3) reveals a nutritionally adequate formulation for broiler chickens, with a dry matter content of 90.02% and a protein concentration of 22.07%, determined by the Kjeldahl method (AOAC 2001.11). Crude fiber content was 4.21%, while the lipid fraction represented 4.98%, quantified by the Goldfish method (AOAC 920.39). Ash content reached 6.51%, which, when subtracted from the total, resulted in an organic matter content of 93.49%.

Following the inclusion of an enzyme-enhanced symbiotic at different levels (0.01%, 0.03%, and 0.05%), the apparent digestibility of nutrients was evaluated (Table 4). Statistically significant differences ($p < 0.0001$) were observed in crude protein digestibility, with a progressive increase from the control group (54.60%) to the group supplemented with 0.03% (64.95%), demonstrating a substantial improvement in protein utilization efficiency. This trend suggests a possible synergy between the symbiotic components and the added enzymes, promoting greater availability of amino acids in the intestinal lumen.

Regarding fat digestibility, significant improvements ($p < 0.0001$) were observed, with the highest value recorded in group T3 (0.05% symbiotic) at 57.60%, compared to 51.01% in the control group. This finding could be related to enhanced emulsification and lipid absorption, facilitated by lipolytic enzyme activity or changes in the intestinal microbiota that optimize the digestive environment.

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For crude fiber digestibility, moderate differences were recorded (p = 0.0109). Although the lowest value was found in T1 (10.26%) and the highest in T0 (11.88%), the differences did not follow a clear dose-dependent pattern, suggesting that the response to symbiotic inclusion on the fiber fraction may depend on more complex interactions between fiber type, intestinal microbiota, and supplemented enzymatic activity.

Finally, dry matter digestibility showed significant differences (p < 0.0001), although without a clear increasing trend with symbiotic inclusion. Group T1 exhibited the highest value (79.04%), statistically superior to T2 (76.43%) and T3 (77.52%), but comparable to the control group (78.90%), indicating that symbiotic addition did not consistent ly affect overall feed digestibility but selectively improved key nutrients such as protein and fat.

Table 3. Bromatological Analysis of the Balanced Feed Provided to the Chickens

Parameter %	Result (PS) %	Method/Standard
Total Moisture	9.98	AOAC/Gravimétrico/ AOAC 925.10
Dry Matter	90.02	Calcularion
Protein	22.07	AOAC/kjeldahl /AOAC 2001.11
Fiber	4.21	AOAC/Gravimétrico/ AOAC 930.15
Fat	4.98	AOAC/Goldfish/ AOAC 920.39
Minerals	6.51	AOAC/Gravimétrico/ AOAC 923.03
Organic Matter	93.49	Calculation

Table 4. Analysis of the Effect of the Enzyme on Nutrient Digestibility

	Digestibility				
Parameters	T0	T1	T2	T3	p Value
Dry Matter	78.90 ^{b}	79.04 ^{b}	76.43 ^{a}	77.52 ^{a}	<0.0001
Protein	54.60 ^{a}	62.22 ^{b}	64.95 ^{c}	63.03 ^{bc}	<0.0001
Fiber	11.88 ^{b}	10.26 ^{a}	11.54 ^{ab}	10.83 ^{ab}	0.0109
Fat	51.01 ^{a}	53.61 ^{b}	56.41 ^{c}	57.60 ^{c}	<0.0001

Means sharing a common letter are not significantly different (p > 0.05).

3.4. Analysis of Immune System Variables

Serum immunoglobulin levels evaluated on day one of age reflect the basal immunological status of the chicks, primarily influenced by the passive transfer of maternal antibodies through the yolk. At this point, supplementation with enzyme - enhanced symbiotic had not yet commenced, so the differences observed between treatments are attributable to natural variability among the groups.

For IgY, the highest values were recorded in T0 (3.47 g/l) and T3 (3.28 g/l); meanwhile, for IgM, groups T0, T2, and T3 showed similar concentrations that were significantly higher than T1 (0.55 g/l). Finally, IgA concentrations were statistically higher in T0, T1, and T3, while T2 presented the lowest value (1.59 g/l). This pattern suggests that some groups started with a lower maternal antibody load, which could influence their subsequent immune response. Overall, these initial differences were taken into account when interpreting treatment effects in later days, as the immunological starting point may condition individual responses to supplementation.

At 21 days, IgY levels progressively increased according to the symbiotic dose. T3 reached the highest value (3.00 g/l), significantly exceeding all other treatments, suggesting a positive cumulative effect of the enzyme-enhanced symbiotic on secondary humoral immunity activation. This result aligns with other studies indicating that symbiotic and enzymes can act synergistically by improving the absorption of key nutrients necessary for immunoglobulin synthesis and immune system maturation.

Regarding IgM, T2 (2.60 g/l) showed a highly significant increase compared to other groups, indicating that the 0.03% dose was particularly effective in stimulating IgM production at this age. This increase could be associated with a more efficient activation of the immune system against enteric antigens, mediated by a more established microbiota.

Finally, IgA values in T3 (2.07 g/l) were significantly higher, whereas no statistical differences were observed in the other treatments. This finding supports the hypothesis that the 0.05% symbiotic combined with enzymes favors the maturation of intestinal immunity, possibly by improving mucosal integrity and stimulating the activity of IgA-producing plasma cells. See Table 5.

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Table 5. Effect of Symbiotic Plus Enzymes on Serum Immunoglobulin Concentrations.

Day	Inmunoglobulin	T0	T1	T2	T3	p Value
1	IgY (g/l)	3.47 ^{b}	3.00 ^{a}	2.58 ^{ab}	3.28 ^{b}	0.0031
	IgM (g/l)	1.54 ^{b}	0.55 ^{a}	1.52 ^{b}	1.35 ^{b}	0.0003
	IgA (g/l)	2.41 ^{b}	2.55 ^{b}	1.59 ^{a}	2.19 ^{b}	0.0009
21	IgY (g/l)	2.47 ^{c}	1.53 ^{a}	2.07 ^{b}	3.00 ^{d}	<0.0001
	IgM (g/l)	1.41 ^{a}	1.49 ^{a}	2.60 ^{b}	1.57 ^{a}	<0.0001
	IgA (g/l)	1.02 ^{a}	0.96 ^{a}	1.07 ^{a}	2.07 ^{b}	0.0001

Means sharing a common letter are not significantly different ($p > 0.05$).

Discussions

The productive parameters evaluated in the present study demonstrate that the inclusion of enzyme-enhanced synbiotics exerts positive effects on the zootechnical performance of broiler chickens. These findings align with those reported by Attia et al. (2023), who observed significant improvements in productive performance and economic profitability when supplementing diets with 2 g/kg of *Saccharomyces cerevisiae*. Such beneficial effects are partly attributed to the yeast's ability to modulate intestinal microbiota, optimise nutrient digestion, and strengthen the intestinal barrier, potentially translating into greater feed efficiency. Additionally, the synbiotic's capacity to compete with pathogenic microorganisms and produce beneficial metabolites offers advantages for consumers, positioning it as a viable alternative to antibiotic growth promoters (Dong et al., 2024).

These results are further supported by Awais et al. (2019), who demonstrated that the combined addition of Lactobacillus and *Saccharomyces* to broiler diets significantly increased body weight gain, improved feed conversion ratio, and enhanced immune function. The authors attributed these effects to increased stability of beneficial microbiota, production of antimicrobial organic acids, and improved intestinal functionality. Rodríguez et al. (2020) evaluated the effect of the PROBIOLEV® symbiotic (*Saccharomyces cerevisiae* plus *Bacillus subtilis* E44) at 75 ml/kg of feed offered to mature laying hens (162–252 days), reporting increased laying rate, higher proportion of hens entering reproduction, improved feed conversion ratio, decreased mortality, and enhanced viability in supplemented birds.

However, some studies report contrasting results. Da Silva et al. (2018), using a symbiotic combination of Protexin Concentrate® and BioMos® (based on mannan-oligosaccharides derived from *S. cerevisiae* cell walls), found no significant improvements in productive parameters or carcass quality compared to controls. This discrepancy may be explained by factors such as variability in probiotic strains, administered dose, delivery method, environmental conditions during trials, animal health status, or basal diet composition (Díaz et al., 2017). The efficacy of a symbiotic likely depends on an appropriate dose-response relationship and synergy with specific enzymes, which act on structural feed substrates to enhance nutrient release and utilization.

Regarding intestinal morphometry, the study recorded positive structural changes in the intestinal mucosa, particularly with 0.03% enzyme-enhanced symbiotic inclusion. Similar results were reported by Santos et al. (2016), who evaluated the impact of undefined flora probiotics and symbiotic in Coob 500 pullets and observed improvements in intestinal morphology, notably an increased villus-to-crypt ratio in the duodenum and jejunum. Similarly, Çalık et al. (2020) found that adding *Paenibacillus xylanexedens* combined with 1% inulin and 0.5% lactulose in Ross 308 broiler diets increased ileal villus height at day 42. Tellez et al. (2010) demonstrated that *Aspergillus* meal increased villus height and surface area in the duodenum and ileum of neonatal turkeys.

In the present study, at 21 days of age, significant differences were observed in serum concentrations of immunoglobulins (IgY, IgM, and IgA) among treatments, indicating an immunomodulatory effect attributable to enzyme-enhanced symbiotic supplementation. Similar effects were reported with the inclusion of 1 kg yeast (Saccharomyces cerevisiae) plus 200, 150, 100, and 50 mg/kg camu camu (Myrciaria dubia) in broiler diets, showing improvements in immune function at constant or decreasing doses (Paredes and Mantilla, 2024). This effect is due to most probiotics' ability to balance and maintain intestinal microflora in avian species (Edens, 2003). Ding et al. (2018) reported that dietary supplementation with xylo-oligosaccharides (XOS) in laying hen diets increased villus height, villus-to-crypt ratio, and relative jejunal length; plasma levels of IgA, IgM, IL-2, and TNF-α were also elevated, indicating enhanced immunity.

Kridtayopas et al. (2019) supplemented Arbor Acres broilers with mannan-oligosaccharide prebiotics (*Bacillus subtilis, Bacillus licheniformis*) and symbiotic at 0.1%, observing improved epithelial barrier function and increased colonisation by Bac*illus, Lactobacillus*, and *Clostridium spp*. in the intestine. They concluded these feed additives, especially symbiotic, promote recovery of intestinal function and health after stress. This view is supported by Toumi et al. (2021), who reported these products enhance dysregulated immune responses and prevent intestinal dysbiosis.

Mazur et al. (2024) supplemented commercial turkey diets contaminated with ochratoxin A with probiotics and symbiotic based on lactic acid bacteria strains, inulin, and Saccharomyces cerevisiae, observing improved health status and reduced mycotoxin accumulation in tissues. Thus, using these microorganisms as feed additives offers an alternative to mitigate or eliminate mycotoxin toxicity in animals (Zoghi et al., 2022; Arsène et al., 2021; Angelakis, 2017; Jalantha et al., 2024), promoting animal welfare and reducing antibiotic use (Chun et al., 2023; Torres et al., 2024).

This study also showed that inclusion of an enzyme-enhanced symbiotic significantly improved digestibility of key nutrients such as crude protein and fat in broilers, suggesting a synergistic effect of symbiotic and exogenous enzymes in optimizing macronutrient degradation and absorption in the gastrointestinal tract. However, crude fiber digestibility exhibited variable responses without a clear trend, possibly due to the structural complexity of non-starch polysaccharides and the limited enzymatic specificity of the supplement. Dry matter digestibility showed no consistent improvement, in agreement with Giménez and Calvo (2024), who reported that exogenous proteases improve ileal nitrogen and essential amino acid digestibility but do not significantly affect dry matter digestibility.

Similar findings were described by Cortés et al. (2002), who documented that inclusion of an enzymatic complex composed of alpha-amylases, xylanases, and proteases in conventional diets and formulations with reduced crude protein and metabolizable energy, based on maize, sorghum, and soybean meal, significantly enhanced broiler performance. Juárez et al. (2020) demonstrated that adding a multienzyme complex (amylases, proteases, xylanases) combined with probiotics (Bacillus subtilis) to diets based on sorghum, soybean, and canola improved metabolizable energy, crude protein, and essential amino acid digestibility (lysine, methionine), enabling reduction in crude protein and energy levels without compromising productive performance in Bovans White hens aged 42–54 weeks.

These data are reinforced by Vásconez et al. (2020), who highlighted that strategic use of exogenous enzymes under controlled conditions and proper nutritional management increases weight gain and profitability in poultry systems by improving nutrient utilization efficiency and carcass yield.

However, not all studies agree with these benefits. Contrastingly, Zheng et al. (2023), in an in vitro study, reported that addition of four proteases (AcP from *Aspergillus niger*, AlP and NeP from *Bacillus subtilis*, and Ker from *Bacillus licheniformis*), individually or combined, significantly reduced apparent ileal crude protein digestibility. The authors suggested this effect could result from high protease doses interfering with endogenous enzyme activity (trypsin, chymotrypsin), especially under suboptimal pH or enzymatic competition in the small intestine. Although body weight gains improved, ileal crude protein digestibility was compromised in vivo.

Thus, it is evident that exogenous protease efficacy depends on multiple factors, including administered dose, dietary protein quality and level, physiological status of the bird, and production environment conditions. As emphasised by several authors (Aguilera, 2018), no enzyme produces a universally positive effect, as in vivo physiological responses are influenced by many variables that must be carefully controlled in experimental designs. A robust empirical database and controlled trials are therefore required to understand the kinetic action of these enzymatic additives, aiming to formulate high-precision nutritional diets that maximize nutrient utilization without compromising intestinal health or productive performance.

Conclusion

Supplementation with 0.03% enzyme-enhanced symbiotic optimized productive performance, intestinal structure, nutrient digestibility, and immune response in broiler chickens, establishing itself as the most efficient dose evaluated in this study. The findings support the potential of enzyme-enhanced symbiotic as functional tools to improve intestinal health, nutritional utilization, and immunological response in broilers. However, it is essential to define optimal dosing windows and consider the complex interactions among microbiota, enzymes, and intestinal physiology to maximize benefits without compromising animal welfare or production efficiency. Future research should focus on elucidating the underlying molecular mechanisms and assessing long-term responses under commercial conditions.

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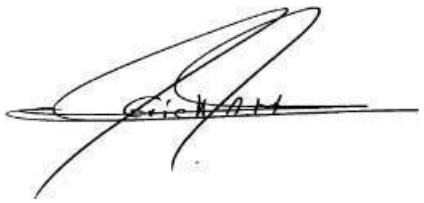

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