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Effective Microorganisms as a Probiotic Strategy to Improve Performance and Reduce Ammonia Emissions in Guinea Pigs *(Cavia porcellus)*

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ABSTRACT

In Andean production systems, guinea pigs (Cavia porcellus) are a key source of animal protein for food security; however, their productivity is limited by inefficient nutrient use, antimicrobial misuse, and gut microbiota imbalances. These factors reduce performance and increase nitrogen emissions (NH_3), affecting system sustainability. This study evaluated the effect of dietary supplementation with effective microorganisms (EM) on productive performance, intestinal morphometry, and ammonia emissions during the fattening phase. A total of 120 animals (60 males and 60 females) were assigned to a completely randomized bifactorial design with four EM levels (0, 100, 150, and 200 mL) and sex. Productive variables were analyzed using generalized linear models, intestinal morphometry by ANOVA, and ammonia descriptively. Significant effects ($p < 0.05$) were observed for all productive parameters. The 100 mL dose showed the best performance, with higher weight gain (607.1 g), final weight (1052.8 g), carcass yield (71.9%), and improved feed conversion (3.94). Males outperformed females. EM increased villus length and width without affecting crypt depth, suggesting improved absorption. Ammonia levels decreased and stabilized, with no association with weight gain. EM improves productivity and environmental conditions, with intermediate doses being optimal.

Keywords: *Productive performance, Feed efficiency, Intestinal morphometry, Villus development, Ammonia emissions, Sustainable livestock*

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INTRODUCTION

The guinea pig *(Cavia porcellus)* is a species of high productive, social, and cultural relevance in South America, where its production represents an important source of animal protein due to its high nutritional value, constituting a strategic activity for food security and the economic sustainability of rural communities (Camacho & Patiño, 2022; Kariri *et al*., 2024; Donoso *et al*., 2025).

Globally, the increasing demand for animal protein, driven by population growth and changes in consumption patterns, has intensified pressure on traditional livestock systems, highlighting the need for more efficient and sustainable production alternatives (FAO, 2022; Tam *et al*., 2023). In this context, minor livestock species such as guinea pigs represent a viable option within resilient food systems, due to their high feed conversion efficiency and lower environmental footprint compared to larger livestock species (Herrero *et al*., 2023; Singh *et al*., 2023).

In Andean regions, guinea pig production offers several advantages, including high reproductive rates, low space requirements, and adaptability to family-based production systems, facilitating access to animal protein in rural populations (Carcelén *et al*., 2020, Buela *et al*., 2022). In Ecuador, this activity involves approximately 21 million animals, with an annual production of around 47 million guinea pigs, mainly under semi-intensive and smallholder systems characterized by limited adoption of nutritional technologies and frequent use of antibiotics as an empirical health management strategy (Ma *et al*., 2021; Chavez and Avilés, 2022; MAG, 2022).

Under these conditions, animal nutrition has become a key strategy to improve productive performance and system sustainability, promoting the use of functional feed additives that enhance nutrient utilization and reduce dependence on antibiotics (Kogut & Arsenault, 2016; Gadde *et al*., 2017; Alagawany *et al*., 2018; Carcelén *et al*., 2021; Islam *et al*., 2025), without compromising product safety or environmental balance (FAO, 2022; Córdova *et al*., 2024; Hashem *et al*., 2024).

Among these alternatives, effective microorganisms (EM) have emerged as a promising technology based on microbial consortia including lactic acid bacteria, photosynthetic bacteria, and yeasts, capable of modulating the gut microbiota and promoting beneficial fermentative processes (Domínguez-Bello *et al*., 2019; Morocho & Leiva-Mora, 2021; Guzek *et al*., 2023; Csep *et al*., 2024).

Dietary supplementation with EM has been associated with improvements in feed conversion efficiency, weight gain, and metabolic performance by promoting a balanced and functionally active gut microbiota (Zhu *et al*., 2022; Cissé *et al*., 2024; Zhao *et al*., 2024). Additionally, these microbial consortia have been reported to reduce undesirable metabolites, such as ammonia, by optimizing fermentation processes and decreasing protein deamination both in the gastrointestinal tract and in the production environment (Pereira *et al*., 2022; Chen & Chen, 2023; Liu *et al*., 2023; Mingcheng *et al*., 2025; Song *et al*., 2025). Previous studies in other animal species have demonstrated that EM supplementation can improve feed efficiency, growth performance, and intestinal health, as well as reduce ammonia emissions through enhanced fermentation and reduced nitrogen losses (Carcelén *et al*., 2021; Salikhova *et al*., 2023; Pinchao *et al*., 2024; Zhao *et al*., 2024; Goicochea-Vargas *et al*., 2025). However, in guinea pigs, scientific evidence remains limited, particularly regarding the integrated effects of EM on productive performance, intestinal morphometry, and ammonia emissions.

Therefore, this study aimed to evaluate the effects of dietary supplementation with effective microorganisms in guinea pigs during the fattening phase, assessing their influence on productive performance, intestinal morphometry, and ammonia emissions, in order to generate scientific evidence that contributes to improving productivity and sustainability in guinea pig production systems.

MATERIALS AND METHODS

Study area

The study was conducted in Tungurahua Province, Cevallos canton (Ecuador), located at an altitude of approximately 2894 m above sea level (1°21′00″ S, 78°37′00″ W). The area is characterized by a temperate climate, with temperatures ranging from 13 to 16 °C and annual precipitation between 200 and 500 mm.

Procedures

Sample size

A total of 160 guinea pigs (80 males and 80 females) were used, weaned at an average initial body weight of 460 ± 25 g, and clinically healthy. Animals were selected based on physiological uniformity and health status. Prior to the experiment, all animals underwent an adaptation period to the experimental diet to minimize stress and gastrointestinal adjustment effects. The farm operated under a semi-intensive production system.

Effective microorganisms

A commercial formulation of effective microorganisms (EM-1®), composed mainly of lactic acid bacteria, photosynthetic bacteria, and yeasts, was used. Activation was performed by mixing 5% EM-1®, 5% molasses, and 90% water, followed by anaerobic fermentation in sealed containers for 5–7 days until the pH reached ≤ 3.5, indicating adequate fermentation and microbiological stability.

Experimental design and treatments

Improved Type 1 guinea pigs were used, and a completely randomized bifactorial design was applied, considering EM inclusion level and sex as main factors. Three experimental treatments and one control group were established. The control treatment (T0) included male and female guinea pigs with two replicates per sex and received 0 mL of effective microorganisms (EM). Likewise, treatments T1, T2, and T3 included both sexes with two replicates per treatment–sex combination and received 100, 150, and 200 mL of EM, respectively. Supplementation was administered twice per week by incorporating EM into the basal diet formulated for the animals.

Animal management

Animal management followed Good Livestock Practices established by AGROCALIDAD (2023), ensuring adequate biosecurity, hygiene, and animal welfare conditions. All animals received veterinary care, including pre-experimental deworming with ivermectin (two drops per animal), according to standard guinea pig management guidelines (Vivas, 2013; Nyamagoud *et al*., 2024; Petronis *et al*., 2025).

Feeding was controlled, with a basal diet consisting of forage and concentrate in an 80:20 ratio, provided twice daily (08:00 and 16:00 h), meeting the minimum nutritional requirements for guinea pigs during the fattening phase (FAO, 2016). Animals were housed in cages (1.5 × 1.0 m) at a stocking density of 10 animals per cage.

Slaughter and post-mortem procedures

At the end of the experimental period (77 days), animals were slaughtered following technical and ethical protocols to ensure tissue integrity and reliability of subsequent measurements, particularly for intestinal histomorphometry and carcass evaluation (Underwood & Anthony, 2020; Céspedes *et al*., 2023) Four animals per treatment were randomly selected and subjected to a 12 h fasting period to minimize variation in gastrointestinal content (Grandin & Cockram, 2020; Çınaroğlu *et al*., 2023; Cakmak *et al*., 2024; Manfredini *et al*., 2024). Subsequently, animals were euthanized according to established recommendations for laboratory rodents (Gimeno *et al*., 1990), as an alternative to procedures described in the Mexican Official Standard NOM-033-SAG/ZOO-2014 (2015); Leadbeatter and Tjaya (2024).

Evaluated parameters

Intestinal sampling and measurements

Evisceration was performed by laparotomy, allowing complete removal of the small intestine (duodenum) and ensuring accurate anatomical identification (Kim & Layton 2019). Samples were washed with sterile distilled water to remove residual feed that could interfere with histological evaluation (Bratt & Naimi-Akbar, 2023; Isaac *et al*., 2023).

Productive parameters

Animals were weighed weekly, and the following variables were evaluated: weight gain (g), feed conversion ratio, final weight (g), mortality (%), and carcass yield (%). Mortality was recorded daily and expressed as a percentage relative to the total number of animals per treatment. Carcass yield (%) was calculated as (carcass weight/final live weight) × 100 after slaughter and evisceration, excluding viscera and non-edible components.

Histological parameters

Histological analysis of the small intestine followed a previously described protocol (Arce, 2017), applied under similar conditions by Masaquiza *et al*. (2025); Muthanandam *et al*. (2024). A 2 cm segment of the duodenum was collected, washed with saline solution, and fixed in 10% formalin for 24 h. Samples were dehydrated in a graded alcohol series, cleared in xylene, and embedded in paraffin. Sections (10 µm) were obtained using a rotary microtome and stained with hematoxylin–eosin for microscopic evaluation. Villus height, villus width, and crypt depth were measured using optical microscopy. The representative histological images used for intestinal morphometric assessment were deposited in Zenodo and are publicly available at: doi:10.5281/zenodo.19541885

Ammonia evaluation

The experiment was conducted in a conditioned barn divided into two independent sections: one for the control group and one for EM-treated animals, ensuring homogeneous management and environmental conditions. Ammonia (NH_3) concentration was recorded weekly using a portable digital detector (BTMETER BT-5800G), with measurements taken at animal height.

Statistical analysis

Productive variables were analyzed using generalized linear models (GLM) with a Gamma distribution and log link function (GENLIN procedure, SPSS). The model included treatment, sex, and their interaction, with initial body weight incorporated as a covariate when appropriate. Statistical significance was assessed using Wald tests (α = 0.05), and estimated marginal means were compared using Bonferroni-adjusted pairwise comparisons.

Normality of histological variables was assessed using the Shapiro–Wilk test, confirming a normal distribution. Subsequently, a factorial ANOVA was applied to evaluate treatment, sex, and their interaction. When significant differences were detected ($p < 0.05$), Tukey's multiple comparison test was performed.

Ammonia concentrations were analyzed descriptively to identify temporal trends and differences between treatments. Additionally, Spearman's correlation analysis was performed to assess relationships between productive and histological variables, as well as between weight gain and ammonia concentration.

RESULTS AND DISCUSSION

Productive parameters

The analysis of productive variables revealed a significant effect of treatment **(Table 1)**, sex, and their interaction across all evaluated indicators ($p < 0.05$), confirming that supplementation with effective microorganisms integrally modulated the productive response of the animals.

During the growth phase, weight gain was significantly higher in T1 (607.1 g), followed by T3 (576.1 g) and T2 (567.5 g), while the control group showed the lowest value (539.4 g). A similar pattern was observed for final body weight, with T1 reaching the highest value (1052.8 g), exceeding T3 (1025.4 g) and T2 (1015.2 g), whereas T0 (981.7 g) was lower. These results indicate that the inclusion of effective microorganisms enhances growth performance; however, this response was not proportional to increasing dosage, suggesting an optimal effect at intermediate inclusion levels.

Table 1. Estimated means of productive variables as affected by treatment and sex

Variable	Treatments				Sex		*p-value*		
	T0	T1	T2	T3	F	M	Treat	Sex	T×S
Wg (g)	539.4±9.9 **d**	607.1 ± 5.1 **a**	567.5 ± 13 **c**	576.1 ± 23 **b**	561.9 ± 12.5	583.1 ± 12.5	<0.001	<0.001	<0.001
Fc	4.28 ± 0.03 **a**	3.94 ± 0.03 **c**	3.93 ± 0.02 **c**	4.14 ± 0.07 **b**	4.07 ± 0.07	4.08 ± 0.06	<0.001	<0.001	<0.001
Cy	65.9 ± 1.2 **d**	71.9 ± 0.5 **a**	66.2 ± 1.2 **c**	68.0 ± 1.8 **b**	67.0 ± 1.1	69.0 ± 1.3	<0.001	<0.001	<0.001
Fw (g)	981.7 ± 9.4 **d**	1052.8 ± 3.3 **a**	1015.2 ± 2.6 **c**	1025.4 ± 16.9 **b**	1007.1 ± 11.4	1030.4 ± 10.0	<0.001	<0.001	<0.001

Wg: Weight gain (g). Fc: Feed conversion. Cy: Carcass yield (%). Fw: Final weight (g). F: Female, M: Male

Similarly, carcass yield followed a pattern consistent with growth-related variables, reaching its highest value in T1 (71.9%), followed by T3 (68.0%) and T2 (66.2%), while the control group showed the lowest yield (65.9%). This behavior suggests that productive improvements are not limited to live weight but also translate into greater efficiency in the deposition of edible tissue.

Regarding feed conversion ratio, significant improvements were observed in the supplemented treatments, particularly in T1 (3.94) and T2 (3.93), compared with the control (4.28). However, T3 showed a slight deterioration (4.14), reinforcing evidence of a non-linear response, where higher inclusion levels do not necessarily optimize feed efficiency. Overall, these results indicate that the intermediate dose (T1) simultaneously maximizes growth, efficiency, and productive performance.

No mortality was recorded in any of the treatments, indicating that the experimental conditions did not generate adverse effects on animal survival and supporting the biological validity of the productive results obtained.

The effect of sex was consistent across all variables, with higher values observed in males compared to females in weight gain (583.1 vs. 561.9 g), final weight (1030.4 vs. 1007.1 g), and carcass yield (69.0% vs. 67.0%), indicating a greater capacity for growth and tissue deposition in this group. This pattern is consistent with previous reports showing that males exhibit superior productive performance due to physiological differences associated with growth.

Finally, the treatment × sex interaction was significant, indicating that the response to supplementation depends on the sex of the animals. Although T1 maintained the best performance in both groups, differential responses were observed in intermediate treatments, suggesting that sex-related biological factors modulate the efficiency of effective microorganism utilization. This finding underscores the importance of accounting for sex when optimizing nutritional strategies.

Histological parameters

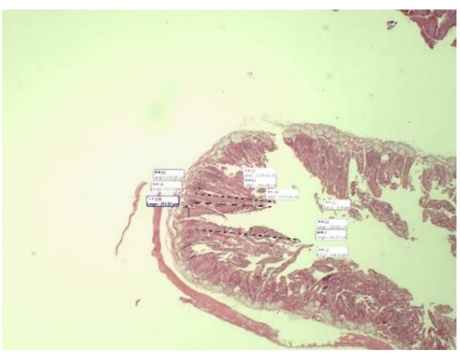
Morphometric variables showed significant differences in villus length and width ($p < 0.05$), whereas crypt depth did not vary among treatments **(Table 2)**. Overall, treatments T1 and T2 exhibited the highest values compared to the control group, while T3 showed an intermediate response.

Table 2. Effect of effective microorganism supplementation on small intestinal histological parameters in guinea pigs.

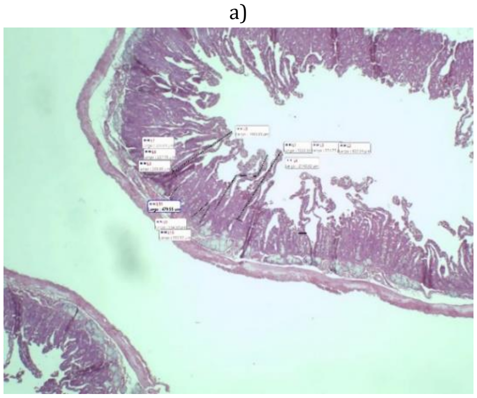
Item (µm)	Treatments				Sex		*p-value*		
	T0	**T1**	**T2**	**T3**	**Female**	**Male**	**Treat**	**Sex**	**T × S**
Vl	1592 ± 77 b	2025 ± 98 a	2033 ± 98 a	1964 ± 95 ab	1804.13 ± 223.62 b	2022.53 ± 234.36 a	0.002	0.043	0.332
Vw	155 ± 11.6 b	240.9 ± 18 a	233 ± 17.4 a	213 ± 15.9 ab	190.97 ± 31.63 b	232.64 ± 49.16 a	0.028	0.446	0.488
Cd	686 ± 60.9 a	496 ± 44 a	531 ± 47.1 a	618 ± 54.9 a	559.31 ± 128.39 a	614.79 ± 110.42 a	0.076	0.978	0.050

Vl: Villus length. Vw: Villus width. Cd: Crypt depth. T: Treatment. S: Sex.

Supplementation with effective microorganisms primarily promoted the development of intestinal villi, without inducing proportional changes in crypt depth. From a functional perspective, this pattern suggests an expansion of the absorptive surface, potentially enhancing nutrient utilization rather than increasing intestinal epithelial turnover, indicating a structural response oriented toward improved absorptive capacity. This effect was also evident at the microscopic level **(Figures 1a–1c)**, where the evaluated structures showed a comparable organization across treatments, with variations mainly associated with villus development.



a)



b)

c)

Figure 1. Histological sections of the duodenum showing measurements of villus height, villus width, and crypt depth in guinea pigs supplemented with effective microorganisms. (a) Treatment 1 (male): L4–L6, villus height; L8–L10, villus width; L1–L3, crypt depth. (b) Treatment 2 (female): L1–L5, villus height; L6–L8, villus width; L9–L11, crypt depth. (c) Treatment 3 (male): L1–L5, villus height; L6–L8, villus width; L9–L11, crypt depth. The full image dataset is available in Zenodo: doi:10.5281/zenodo.19541885.

Regarding sex, only villus length showed significant differences, with higher values observed in males. No differences were found in villus width or crypt depth, nor was there a significant interaction between treatment and sex, indicating that the histological response was consistent across both groups. This suggests that intestinal morphometry shows lower sex-related variability than productive parameters.

Ammonia measurements

Ammonia (NH_3) concentration showed clear differences between treatments with and without effective microorganism supplementation, indicating a treatment effect on the dynamics of this gas within the system **(Figure 2)**. During the initial phase, both groups exhibited comparable baseline conditions; however, from the second week onward, a progressive divergence in their trajectories was observed.

The control group exhibited a more pronounced and sustained increase in NH_3 over time, with intermediate fluctuations but maintaining an overall upward trend. This pattern culminated in a marked increase by week 11, reaching concentrations close to 50 ppm, suggesting an accelerated accumulation of potentially harmful nitrogenous compounds.

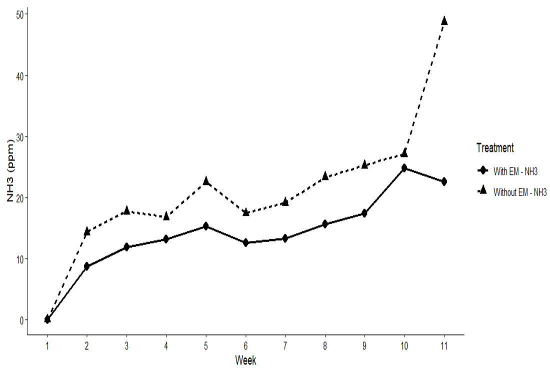


Figure 2. Weekly ammonia concentration under treatments with and without effective microorganisms (EM).

In contrast, the treatment with effective microorganisms exhibited a more moderate and stable increasing pattern, maintaining consistently lower NH_3 concentrations compared to the control group. Additionally, toward the final weeks, a stabilization trend was observed, which may indicate a balance in nitrogen transformation processes within the system.

Correlation between productive and histological parameters

Correlation analysis revealed strong relationships among productive variables, highlighting a close positive association between weight gain, final weight, and carcass weight ($r = 0.93$–0.98), indicating that body growth is the main determinant of productive performance **(Figure 3)**.

Conversely, feed conversion ratio showed negative correlations with productive variables, particularly with weight gain ($r = -0.66$), final weight ($r = -0.69$), and carcass yield ($r = -0.77$), indicating that lower feed conversion values (i.e., higher efficiency) are associated with improved productive outcomes.

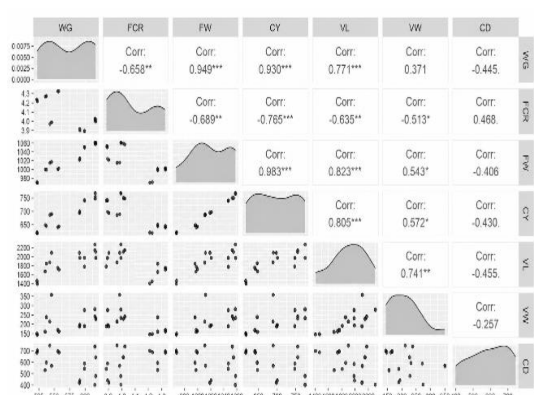


Figure 3. Pairwise correlation matrix and scatterplot relationships among feed conversion ratio (FCR), weight gain (WG), final weight (FW), carcass yield (CY), villus length (VL), villus width (VW), and crypt depth (CD) in experimental animals. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

With respect to morphometric variables, villus length showed positive associations with weight gain ($r = 0.77$), final weight ($r = 0.82$), and carcass yield ($r = 0.80$), highlighting its relationship with productive development. Villus width exhibited moderate correlations with final weight ($r = 0.54$) and carcass yield ($r = 0.57$), whereas crypt depth showed negative relationships with most productive variables ($r = -0.26$ to -0.45), suggesting a lower direct contribution to performance.

Correlation between weight gain and ammonia emissions

The relationship between ammonia (NH_3) concentration and weekly weight gain showed variable patterns depending on treatment and sex. In the treatments supplemented with effective microorganisms, correlations were weak in both females and males, with an almost null relationship observed in females ($\rho = -0.03$; $p = 0.946$) and a weak positive correlation in males ($\rho = 0.15$; $p = 0.654$). Similarly, in the control treatment, females showed no association ($\rho = -0.03$; $p = 0.925$), whereas males exhibited a moderate positive correlation ($\rho = 0.50$; $p = 0.120$). However, none of these relationships reached statistical significance.

Productive parameters

These findings partially agree with those reported by Carcelén *et al*. (2020), who found no significant changes in weight gain when increasing probiotic levels from 0 to 3 mL, although improvements in feed conversion ratio were observed. Similarly, Carcelén *et al*. (2021) reported slight increases in final weight and weight gain, accompanied by a reduction in feed conversion ratio.

Likewise, *et al*. (2022) reported improvements in productive parameters and carcass yield with probiotic supplementation. However, the carcass yield obtained in T1 (71.9%) was higher than that reported in these studies, suggesting a potential influence of dosage, microbial consortium composition, and management conditions on the magnitude of the response.

Moreover, this finding suggests that supplementation with effective microorganisms did not compromise animal health, in agreement with studies reporting the safety and beneficial effects of these additives on microbial balance and physiological responses in animal production systems (Kogut & Arsenault, 2016; Gadde *et al*., 2017; Çınaroğlu *et al*., 2023).

Figueiredo *et al*. (2020) also reported higher values in male guinea pigs for slaughter weight, total weight gain, carcass weight, and meat yield compared to females. Therefore, the productive superiority of males does not represent an isolated finding but rather a consistent biological response in fattening guinea pigs, associated with greater tissue deposition and growth efficiency.

Histological parameters

These results are consistent with those reported by Cáceres *et al*. (2021), who observed increases in villus length and width without significant changes in crypt depth in guinea pigs supplemented with functional additives. Similarly, the use of organic acids has been associated with enhanced intestinal villus development, indicating that such additives may induce structural modifications at the level of the intestinal mucosa, particularly through expansion of the absorptive surface (Masaquiza *et al*., 2025).

Furthermore, Cuenca-Condoy *et al*. (2025) reported that intestinal responses depend on inclusion level, showing that intermediate doses can optimize villus development without inducing proportional changes in other structures. Collectively, these findings support a differential and non-linear response of intestinal tissue to supplementation.

Ammonia measurements

The observed trend is consistent with recent studies reporting that microbial additives can reduce ammonia volatilization, although the magnitude of the effect varies depending on the system and environmental conditions. Within this comparative framework, the non-EM group not only showed a more pronounced upward trend but also reached levels that are considered unfavorable in other intensive production systems, whereas the EM-treated group remained closer to acceptable thresholds throughout most of the monitoring period (Costantino *et al*., 2020; Swelum *et al*., 2021).

Similarly, in swine manure systems, Terrero *et al*. (2024) reported reductions of up to 59% under favorable conditions, highlighting a strong dependence on storage context. Likewise, El Bied *et al*. (2024) documented reductions of up to 90% through the use of biological additives, particularly when combined with complementary strategies such as aeration.

Compared with these findings, the results of the present study confirm that effective microorganisms can mitigate ammonia accumulation and stabilize its temporal dynamics; however, the magnitude of the observed effect was more moderate, suggesting a significant influence of factors such as substrate type, nitrogen load, and environmental management conditions on NH_3 dynamics.

Correlation

Similarly, Cáceres *et al*. (2021) reported improvements in intestinal morphology with the use of inulin, without significant effects on weight gain. Likewise, Cuenca-Condoy *et al*. (2025) observed that moderate probiotic doses optimize intestinal histomorphometry, whereas higher inclusion levels do not necessarily enhance productive performance.

Overall, these results indicate that body growth, represented by weight gain and final weight, constitutes the primary driver of productive performance, while feed efficiency acts as an inversely related factor, and morphometric variables contribute in a complementary manner.

NH_3 is recognized as a potentially harmful gas for respiratory health and animal welfare, capable of affecting feed intake and growth when present at elevated concentrations (Groot *et al*., 1998; Miles *et al*., 2004). However, its impact depends on the magnitude and duration of exposure, as well as the physiological adaptive capacity of the (Naseem & King, 2018; Zhou *et al*., 2020). In this context, it is likely that the recorded concentrations did not exceed the critical thresholds required to induce negative metabolic effects, which may explain the absence of a clear relationship with weight gain.

Likewise, the high variability observed in the data suggests that weekly growth was primarily influenced by other factors, such as dietary nutritional quality, management conditions, and physiological differences among individuals. Consequently, ammonia may have acted more as an environmental indicator of the system rather than a direct limiting factor of productive performance in this study, in agreement with previous reports in animal production systems (David *et al*., 2015).

CONCLUSION

Supplementation with effective microorganisms significantly improved productive performance in guinea pigs, as evidenced by increased weight gain, final weight, carcass yield, and improved feed conversion ratio. This effect was associated with enhanced development of intestinal villi, suggesting improved nutrient absorption capacity. However, the response was not proportional to dosage, with intermediate inclusion levels identified as the most efficient. Additionally, the absence of mortality across all treatments confirms that supplementation did not compromise animal health or welfare under the conditions of this study.

The application of effective microorganisms contributed to reducing and stabilizing ammonia concentrations within the system; however, no statistically consistent relationship was observed between NH_3 and weight gain. This indicates that, under the conditions of this study, ammonia did not act as a direct limiting factor for growth, with nutrition and management playing a more relevant role in determining productive performance.

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ETHICS STATEMENT: All procedures involving animals were conducted in accordance with institutional and national ethical guidelines for animal experimentation, ensuring appropriate standards of animal welfare, handling, and care throughout the study.

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