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Synbiotic-Enriched *Artemia* as a Functional Feed for Enhancing Immune Response, Gut Health, and Survival in Glass Eel (*Anguilla bicolor*)

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ABSTRACT

This research examined the functional advantages of synbiotic-enriched *Artemia*, which includes *Lactobacillus* sp. and inulin, as a live feed for glass eel (*Anguilla bicolor*) during the seed stage. The experiment spanned eight weeks and employed a completely randomized design featuring four treatments: control, probiotic-enriched, prebiotic-enriched, and synbiotic-enriched *Artemia*. The evaluated parameters comprised haematological indicators of immune response, intestinal histology, gut microbiota composition, survival rate, and specific growth rate. The synbiotic treatment achieved the highest survival rate ($70.6 \pm 5.6\%$) and lactic acid bacteria count (19.76×10^5 CFU/g), alongside notable enhancements in intestinal morphology and immune cell activity relative to other groups ($P < 0.05$). Probiotic supplementation increased growth rate; however, only synbiotic and prebiotic treatments enhanced significantly the immune parameters and gut health. The findings indicated that synbiotic-enriched *Artemia* improved significantly the disease resistance, gut integrity, and survival rates in *A. bicolour* during the initial stages of aquaculture, thereby promoting more sustainable seed production methods. This study offers new perspectives on the incorporation of synbiotics in live feed to enhance larval fish health and resilience within intensive aquaculture systems.

INTRODUCTION

The cultivation of *Anguilla bicolor*, a tropical eel species of considerable economic importance, has garnered heightened interest owing to its substantial market demand and its contribution to sustainable fisheries **(Arai *et al*., 2020; Shanmughan *et al*., 2022)**.

Enhancing the survival and health of glass eel larvae is essential for the success and sustainability of eel farming operations **(Putra *et al*., 2023)**. Probiotics and prebiotics are gaining recognition as effective methods to improve host immunity, gut health, and overall resilience in aquaculture species **(Hagihara *et al*., 2018; Mugwanya *et al*., 2022; Abd El-Hamid *et al*., 2024)**. Synbiotics influence intestinal microbiota, enhance immune response, and increase resistance to pathogens, providing critical advantages during the early life stages of fish **(Politis *et al*., 2023; Linda *et al*., 2025)**. Multiple studies indicate that dietary synbiotics positively influence growth, survival, and immune responses in various aquaculture species, such as tilapia, catfish, and prawns **(Chouayekh *et al*., 2023; Wang *et al*., 2024)**.

Despite advancements, research focusing on the enrichment of live feeds like *Artemia* with synbiotics for glass eel (*A. bicolor*) is still limited **(Safari *et al*., 2021; Setiadi *et al*., 2023)**. *Artemia* serves as a primary live feed that effectively facilitates the delivery of functional additives, promoting efficient uptake and utilization by larval fish **(Samat *et al*., 2020)**. The synergistic effects of combining probiotics, such as *Lactobacillus* sp., and prebiotics, such as inulin, in *Artemia*-based feeds, along with their impacts on the immune response, gut histology, and survival of glass eel, remain understudied. Addressing this research gap is critical, considering the escalating pressures on natural eel populations resulting from overfishing and environmental degradation **(Wibowo *et al*., 2021; Shanmughan *et al*., 2022; Putra *et al*., 2023)**. Investigating the function of enriched synbiotic feed in enhancing fish health, bolstering immune responses, and maximizing survival rates in early rearing stages is crucial for conservation and aquaculture efficiency **(Singh *et al*., 2015; Hagihara *et al*., 2018)**. This study evaluated the effects of synbiotic-enriched *Artemia* on immune parameters, gut microbial composition, intestinal histology, growth, and survival of *Anguilla bicolor* at the glass eel stage. This research aimed to offer novel insights for the development of functional live feeds and to advance sustainable and resilient eel aquaculture.

MATERIALS AND METHODS

Design of experiment

This research used a completely randomized design (CRD) featuring four treatments and three replicates: P0 (control, no enrichment), P1 (*Artemia* enriched with *Lactobacillus sp*. at 700mg/ L), P2 (*Artemia* enriched with inulin at 100mg/ L), and P3 (*Artemia* enriched with synbiotics: *Lactobacillus* sp. at 700mg/ L and inulin at 100mg/ L). The metrics analyzed included immune response, as indicated by haematologic factors and intestinal tissue histology, and gut health indicators such as total bacteria and lactic acid bacteria, along with the seed survival rate at the end of the trial.

Hatching and cultivation of *Artemia*

Artemia cysts (Sanders Great Salt Lake *Artemia*, KKP RI AR 2619012022, Indonesia) were subjected to decapsulation and subsequently incubated in sterile saltwater, with a salinity maintained at 30 ± 1ppt and a temperature of 28 ± 1°C. The sterilization

procedure utilized calcium hypochlorite at a concentration of 30ppm and underwent filtration through a 1.0µm filter, along with continuous aeration. The hatching protocol is derived from the research conducted by **Leger *et al*. (1987)**. Hatched *Artemia nauplii* are distributed within a square fibertank with dimensions of 1 × 0.4m, ensuring a density of 1.5–3 individuals/mL to facilitate biomass production **(Azimirad *et al*., 2016; Planas *et al*., 2017)**. Spirulina flour was provided to *Artemia* at a dosage of 25 to 50mg/ L per day, administered 3 to 4 times daily **(Azimirad *et al*., 2016; Van Hoa *et al*., 2021)**. The cultivation system employs a biofloc methodology, integrating a carbon source derived from modified cassava flour (MOCAF).

Enhancement of *Artemia*

The enrichment process entails utilizing an emulsion suspension that consists of lecithin, warm water, and coconut oil in a defined ratio of 0.1:10:1. A total of 150mL of suspension was employed as the mixing medium for probiotics, prebiotics, and synbiotics, which were homogenized using a mixer **(Ghaderpour *et al*., 2013)**. *Artemia nauplii* individuals were enriched in a 3-liter container at a density of 100 individuals per milliliter. In comparison, *Artemia* biomass was enriched in the same container at a density of 2,000 individuals per milliliter for a duration of 3 hours. After enrichment, *Artemia* underwent filtration through a mesh with a size of 300µm, followed by immersion in a saline solution with 0.1% benzalkonium chloride for 60 seconds, and were then rinsed with sterile water **(Azimirad *et al*., 2016)**.

Maintenance of glass eels

The glass eel stage of *Anguilla bicolor* was obtained from fishermen's catches in Poso Regency, Central Sulawesi, exhibiting an average weight of 0.126 ± 0.010g per individual and a length of 49.87 ± 1.25mm. Sixty glass eels were introduced into each 20-liter plastic container, leading to a density of three individuals per liter **(Muchlisin *et al*., 2021)**. Adaptation took place over a span of 7 days using brackish water media characterized by a salinity range of 1- 2ppt. *Artemia* was enhanced in alignment with the provided feed treatment. During the first 20 days, *Artemia nauplii* were provided at a density of 5 individuals per milliliter. Following this, *Artemia* biomass, aged 2 weeks, was administered daily at a rate of 10% of the total fish biomass, with a larger portion allocated for the afternoon **(Abdel-Hay *et al*., 2019)**. The adjustment of the feed quantity depends on the biomass obtained from biweekly growth sampling conducted during the 8-week maintenance phase. Water underwent replacement every three days, while the evaluation of water quality parameters, including temperature, dissolved oxygen, pH, and ammonia, was assessed every week.

Phagocytosis activity

Phagocytosis activity was assessed following the methodology of **Hamczyk *et al*. (2015)**, which includes the preparation of smears and subsequent staining using Giemsa. The quantification of phagocytic activity was expressed as the percentage of haemocyte

cells involved in the phagocytosis of microorganisms.

Total erythrocyte count

The measurements were conducted using the Neubauer haemocytometer technique **(Esmaeili, 2021)**. The enumeration of erythrocytes was conducted on the haemocytometer grid following the dilution of the blood sample.

Analysis of leukocyte differential count

Enumeration of leukocyte differential count was conducted utilizing Giemsa-stained blood smears. The enumeration of lymphocytes, neutrophils, and monocytes was conducted using a microscope with a magnification of 1000× **(Fazio, 2019)**. The total leukocyte count was employed to determine percentages.

Examination of intestinal tissue at the histological level

The histology method is credited to **Ratucoreh and Retnoaji (2018)**. The intestines of elver eels were meticulously sectioned into segments measuring 0.5 to 1.0cm, subsequently washed with a 0.9% sodium chloride solution, and preserved in a 10% neutral buffered formalin solution for a duration of 24 to 48 hours. Dehydration was performed utilizing graded ethanol concentrations ranging from 70 to 100%, subsequently followed by a cleaning process with xylene and infiltration in liquid paraffin maintained at a temperature of 58– 60°C. The tissue was sectioned with a microtome to a thickness of 4–6µm and was subsequently mounted onto a glass slide. The staining procedure was performed utilizing haematoxylin-eosin. The specimen underwent a dewaxing process utilizing xylene, followed by rehydration with graded ethanol. It was then stained with haematoxylin for a duration of 5 to 10 minutes and subsequently differentiated using acid-alcohol, ammonia solution, and eosin for 1 to 2 minutes. After the processes of rehydration and clarification, the specimen was mounted using balsam and a coverslip. Observations were conducted utilizing a light microscope at magnifications of 40×, 100×, and 200×, and the results were documented. While quantitative histological scoring was not conducted, representative sections from each treatment were qualitatively assessed based on villus height, epithelial integrity, goblet cell density, and mucosal folding. The observations indicated distinct structural differences among treatments, as described according to established histological evaluation criteria **(Vatsos, 2021; De Marco *et al.*, 2023)**.

Examination of the gastrointestinal tract microbiota

After the experiment, intestinal specimens were collected from five fish in each container. A total of 100 microliters of intestinal homogenate was inoculated onto nutrient (NA) agar medium for the enumeration of total bacteria and onto MRS medium for the isolation of lactic acid bacteria. The samples were subsequently incubated at 30°C for 24 to 48 hours. The enumeration of colonies was conducted on plates, revealing a colony count that varied between 30 and 300 **(Hoseinifar *et al.*, 2015)**.

Survival rate

The survival rate (SR) was determined at the conclusion of the rearing period, utilizing the formula outlined by **Zainuddin *et al.* (2019):**

$$SR\ (\%) = \frac{N_t}{N_o} \times 100\%$$

Description:
SR = Survival rate (%)
N_t = number of fish at the end of the experiment (individuals)
N_o = Number of fish at the beginning of the experiment (individuals)

Specific growth rate

The SGR was determined by calculating the average initial and final weights following an 8-week rearing period, utilizing the formula provided by **Xue *et al*. (2021):**

$$SGR = \frac{\ln W_t - \ln W_o}{t} \times 100\%$$

Description:
SGR = Specific growth rate (%/day)
W_t = Final average fish weight (g)
W_o = Initial average fish weight (g)
t = Duration of rearing (day)

Statistical analysis

The analysis was conducted utilizing a one-way ANOVA through SPSS version 27. In the case of a significant difference ($P< 0.05$), Duncan's test was used to assess the differences between the treatments.

RESULTS

Immune response

Dietary enrichment influenced significantly the key immune response parameters in *Anguilla bicolor* glass eels. The study of phagocytic activity reveals a significant increase in phagocytic activity of *Anguilla bicolor* seeds ($P< 0.05$) across all enrichment treatments compared to the non-enrichment treatment (P0). The prebiotic treatment (P2) demonstrated the highest phagocytic activity at 51.93% ± 0.052%, followed by the synbiotic treatment (P3) at 44.22% ± 0.157%, and the probiotic treatment (P1) at 40.0% ± 0.0%. The non-enrichment treatment (P0) exhibited the lowest value at 32.10% ± 0.072%. Duncan's post hoc test results revealed significant differences ($P< 0.05$) between all enrichment treatments and the control, as well as among the treatments, indicating that the type of enrichment material influences significantly the phagocytic activity (Fig. 1).

The total erythrocyte count in *Anguilla bicolor* (glass eel) larvae showed significant differences among the treatments ($P< 0.05$), as demonstrated by the analysis of variance. The prebiotic treatment (P2) resulted in the highest erythrocyte count at $24 \times 10^4 \pm 0 \times 10^4$ cells/mL, while the synbiotic treatment (P3) recorded a count of $20 \times 10^4 \pm 1.41 \times 10^4$ cells/mL. The non-enrichment treatment (P0) yielded a total erythrocyte count of $6\times10^4 \pm 1.41\times10^4$ cells/mL, whereas the probiotic treatment (P1) exhibited the lowest count of $4\times10^4 \pm 1.41\times10^4$ cells/mL. Duncan's post hoc test revealed significant differences between the prebiotic and synbiotic treatments compared to the probiotic and control treatments (Fig. 1).

The differential leukocyte analysis of *Anguilla bicolor* larvae reveals that the composition of leukocyte types is significantly affected by enrichment with live feed (*Artemia*). The differences in the proportions of neutrophils, lymphocytes, and monocytes indicate particular physiological and immunological conditions associated with the treatments. Monocytes were exclusively identified in the non-enrichment treatment (P0) and probiotic treatment (P1), with frequencies of 6.25% and 6.6%, respectively. Monocytes were absent in both the prebiotic treatment (P2) and the synbiotic treatment (P3). Neutrophils demonstrated a notable increase in the probiotic treatment (P1) at 33.3%, while synbiotics (P3) showed a rise of 31.58%, and the prebiotic treatment (P2) indicated an increase of 22.22%. The non-enriched treatment (P0) demonstrated a modest increase of 18.75%. The prebiotic treatment (P2) exhibited the highest lymphocyte percentage at 77.78%, followed by the control (P0) at 71.88%, the synbiotic (P3) at 68.42%, and the probiotic (P1) at 60.0% (Fig. 1).

Intestinal histology

Intestinal histological observations indicated notable changes across all treatments. The unenriched treatment (P0) displayed short and irregular villi, accompanied by a significant reduction in goblet cells. Probiotic treatment (P1) enhanced villi length and density while increasing the number of goblet cells. The prebiotic treatment (P2) improved significantly the villi architecture and goblet cell activity, indicating a more developed intestinal structure compared to both P0 and P1 treatments. The synbiotic treatment (P3) exhibited the most favorable histological characteristics, characterized by elongated and regular villi, along with a significant increase in goblet cells (Fig. 2). This treatment led to increased mucus production and the activation of gut-associated lymphoid tissue (GALT), suggesting an enhanced mucosal barrier and improved absorption capacity. The findings are derived from qualitative comparisons, as histological observations were representative rather than subjected to statistical analysis.

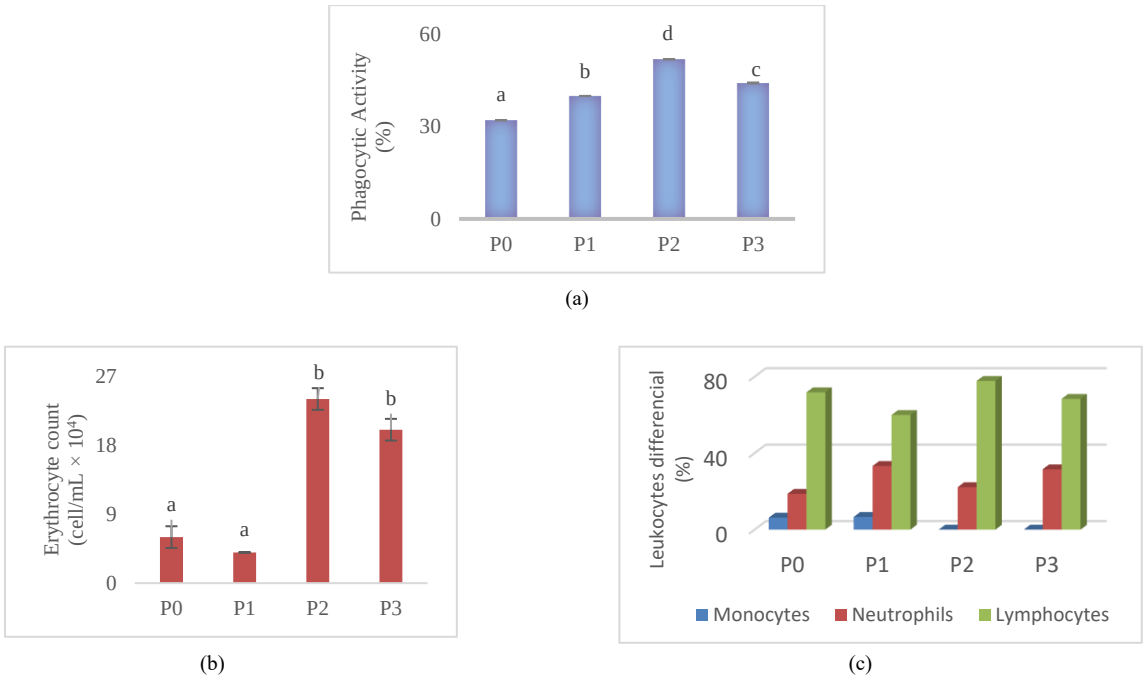


Fig. 1. Immune response. Different letters on the bar chart signify statistically significant differences (P<0.05, Duncan post-hoc test). (a) Assessment of phagocytic activity in glass eel blood. (b) Erythrocyte count. (c) Differential leukocytes

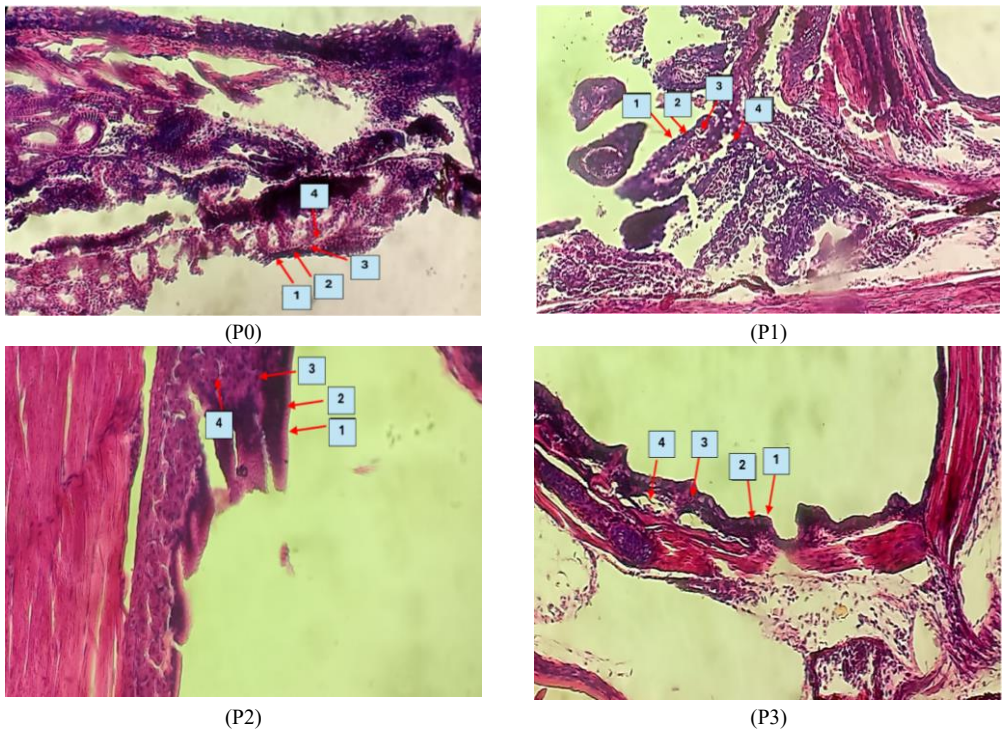


Fig. 1. Histology of glass eel intestines. (P0) unenriched treatment, (P1) probiotic enrichment, (P2) prebiotic enrichment, (P3) synbiotic enrichment. Description: 1. Epithelial cells; 2. Villi; 3. Goblet cells; 4. Connective tissue

Intestinal microbiota

At the conclusion of the trial, the overall bacterial count was low, $4.95 \times 10^5 \pm 0.49 \times 10^5$ CFU/g in the intestines of glass eels in the unenriched treatment (P0). The probiotic treatment (P1) reduced overall bacterial count to $1.75 \times 10^5 \pm 0.35 \times 10^5$ CFU/g compared to the unenriched treatment (P0). This suggests *Lactobacillus* sp. may inhibit other microorganisms. In contrast, prebiotic (P2) and synbiotic (P3) treatments increased significantly the total bacterial counts in glass eels, reaching $9.85 \times 10^5 \pm 0.07 \times 10^5$ CFU/g and $10.25 \times 10^5 \pm 0.35 \times 10^5$ CFU/g, respectively, indicating enhancement of the intestinal microbiota. Variance analysis revealed that adding probiotics, prebiotics, and synbiotics to *Artemia* impacted significantly the outcomes ($P< 0.05$). Duncan's post hoc test showed significant differences between probiotic treatment (P1) and unenriched treatment (P0). Comparing probiotic treatment (P1) to prebiotic (P2) and synbiotic (P3) showed substantial differences. Although the enrichment treatment differed significantly from the unenrichment (P0), there was no significant difference in the total bacterial count between the prebiotic (P2) and synbiotic (P3) treatments. In glass eel intestines, synbiotic treatment (P3) showed the highest number of lactic acid bacteria (LAB) at $19.76 \times 10^5 \pm 1.24 \times 10^5$ CFU/g. Next, the unenriched treatment (P0) had $16.3 \times 10^5 \pm 5.52 \times 10^5$ CFU/g, the prebiotic treatment (P2) had $16.1 \times 10^5 \pm 0.9 \times 10^5$ CFU/g, and the probiotic treatment (P1) had the lowest concentration at $13.45 \times 10^5 \pm 5.5 \times 10^5$ CFU. Variance analysis revealed a substantial impact of probiotics, prebiotics, and synbiotics on *Artemia* enrichment treatment ($P< 0.05$). Duncan's post hoc test showed no significant variations in glass eel gut lactic acid bacteria counts between unenriched (P0), probiotic (P1), prebiotic (P2), and synbiotic (P3) treatments at the study's end (Fig. 3).

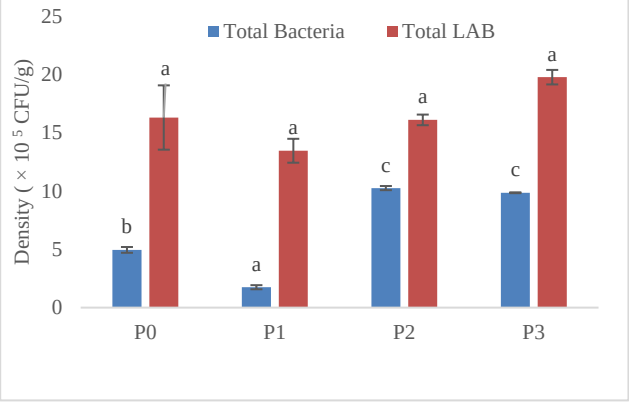
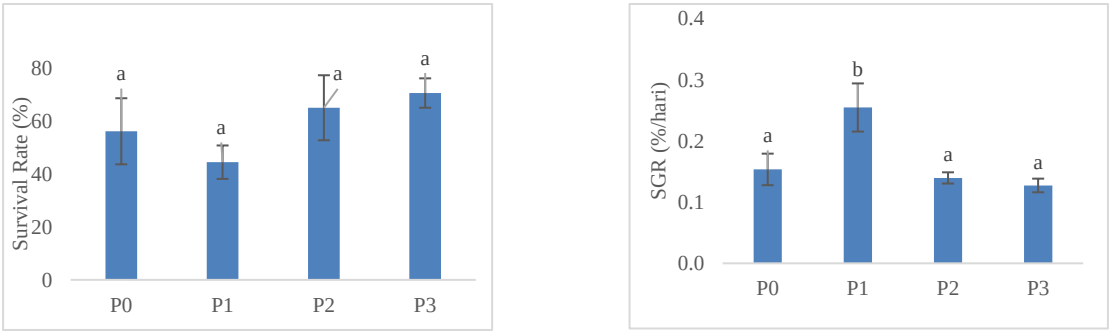


Fig. 2. Total bacterial count and total lactic acid bacterial count (LAB). Different letters denote statistically significant differences ($P<0.05$, Duncan post-hoc test)

Survival rate and specific growth rate

The survival rate (SR) serves as a vital metric in aquaculture, indicating the efficacy of management practices and the adaptability of seed to environmental conditions and dietary modifications. This study found that the synbiotic treatment (P3) achieved the highest survival rate at 70.6 ± 5.6%, followed by the prebiotic treatment (P2) at 65.0 ± 12.3%. The unenriched treatment (P0) had a survival rate of 56.1 ± 12.48%, while the probiotic treatment (P1) recorded the lowest survival rate at 44.4 ± 6.3%. Variance

analysis indicated significant differences among treatments (P<0.05); however, Duncan post hoc testing showed no significant differences in SR at the conclusion of the study. The probiotic treatment (P1) exhibited the highest specific growth rate (SGR) of 0.255 ± 0.072%/day, which was statistically significant (P< 0.05) compared to the other treatments. The SGR for the unenriched treatment (P0), prebiotics treatment (P2), and synbiotic treatment (P3) were 0.154 ± 0.038%/day, 0.140 ± 0.002%/day, and 0.127 ± 0.049%/day, respectively. However, Duncan's post-hoc test indicated no significant differences among these treatments (Fig. 4). The findings indicate that certain probiotics effectively enhance the daily growth of glass eel *Anguilla bicolor*.



(a) (b)

Fig. 3. (a) Survival rate of glass eels (*Anguilla bicolor*). (b) Specific growth rate of glass eels. Different letters denote statistically significant differences (P<0.05, Duncan post-hoc test)

DISCUSSION

The present study demonstrates that synbiotic-enriched *Artemia*, which combines *Lactobacillus* sp. and inulin, enhances significantly the immune response, gut health, and survival in *Anguilla bicolor* glass eels. The findings support previous studies indicating the positive impact of synbiotics on fish health, microbiota stability, and pathogen resilience **(Mugwanya *et al.*, 2022; Abd El-Hamid *et al.*, 2024; Linda *et al.*, 2025)**. The increase in phagocytic activity and immune cell counts (neutrophils and lymphocytes) in the synbiotic and prebiotic groups suggests stimulation of both innate and adaptive immunity. Comparable findings have been reported in tilapia and catfish, indicating that synbiotic supplementation improved non-specific immune parameters and increased resistance to infection **(Chouayekh *et al.*, 2023; Abd El-Hamid *et al.*, 2024; Wang *et al.*, 2024)**. The immune-enhancing effect is closely associated with the fermentation of inulin, resulting in the production of short-chain fatty acids (SCFAs) that serve as signalling molecules for immune cells and facilitate anti-inflammatory processes **(Corrêa *et al.*, 2023; Sheng *et al.*, 2023)**. In this context, inulin enhanced phagocytic activity via SCFA-mediated immune modulation, whereas synbiotics provided a more balanced effect by promoting microbial stability and host resilience.

Histological observations corroborate these immunological findings, indicating that synbiotic supplementation led to elongated villi, increased goblet cell density, and improved mucosal integrity. The modifications enhanced nutrient absorption and

reinforced mucosal defences against pathogen invasion **(De Marco *et al*., 2023; Linda *et al*., 2025)**. The activation of gut-associated lymphoid tissue (GALT) in the synbiotic group indicates a reinforced mucosal immune barrier, a critical feature during early larval stages **(Simón *et al*., 2021)**. The improvements noted in villi length and goblet cell counts correlate with enhanced nutrient absorption and increased secretion of mucus containing antimicrobial compounds, thereby reinforcing the gut's protective function **(Chouayekh *et al*., 2023; De Marco *et al*., 2023)**.

The synbiotic group demonstrated significantly elevated counts of lactic acid bacteria (LAB), suggesting that supplementation facilitated the colonisation of beneficial bacteria. LABs are recognized for their role in competitive exclusion of pathogens, modulation of immune responses, and enhancement of intestinal homeostasis **(Ringø *et al*., 2018; Wang *et al*., 2021; Linda *et al*., 2025)**. The improvements in gut morphology, mucus production, and LAB colonization demonstrate the role of synbiotics in enhancing gut health and mucosal immunity, resulting in improved survival outcomes.

A significant trade-off was identified in the probiotic-only group, which attained the highest specific growth rate (SGR) while exhibiting the lowest survival rate. *Lactobacillus* sp. has been shown to enhance nutrient digestion and growth **(Hoseinifar *et al*., 2017)**. However, excessive colonisation by a single strain may disrupt microbial balance, leading to reduced colonization resistance and increased susceptibility to opportunistic infections **(Liu *et al*., 2022; Mugwanya *et al*., 2022)**. This outcome indicates that probiotics alone may enhance growth while compromising health, whereas synbiotics offer a more sustainable approach by preserving microbial balance and host resilience **(Politis *et al*., 2023)**.

The findings of this study demonstrate the diverse advantages of synbiotic-enriched *Artemia*, which markedly improved immune responses, gut health, and survival rates in glass eels. Inulin has been identified as a significant enhancer of phagocytic activity through SCFA-mediated pathways. Concurrently, structural enhancements in the gut, including the elongation of intestinal villi, an increase in goblet cell numbers, and the activation of GALT, collectively strengthen digestive function and defence mechanisms. This study highlights the necessity of achieving a careful balance in synbiotic formulations; while probiotics alone effectively promoted growth, their application was linked to decreased survival rates. The results affirm the potential of synbiotic live feeds for eel hatchery operations and suggest broader applications across various tropical aquaculture species. Further research is necessary to enhance probiotic-prebiotic combinations, establish optimal dosages, and confirm the efficacy of these formulations at larger production scales **(Chouayekh *et al*., 2023; Politis *et al*., 2023; Wang *et al*., 2024; Linda *et al*., 2025)**.

CONCLUSION

This study shows that synbiotic-enriched *Artemia*, which contains *Lactobacillus* sp. and inulin, improves stomach shape, immunological responses, and survival rates in *Anguilla bicolor* glass eels. Villi elongation, goblet cell density, and gut-associated

lymphoid tissue activation indicate that SCFA synthesis and mucosal barriers stimulated the immune system. Lactic acid bacteria colonization improved gut health and caused pathogen exclusion. High doses of single-strain probiotics promoted growth but decreased survival, suggesting that they may upset gut microbial equilibrium. Synbiotics improved microbial stability, host resilience, and growth support. Synbiotic-enriched live feeds may be a sustainable option for eel hatcheries, improving seed quality and larval survival. Other high-value tropical aquaculture species besides eels may use this strategy. Commercial aquaculture functional feeds can be improved by optimizing probiotic-prebiotic combinations and dosage and confirming them in large hatcheries.

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