Optimizing *bacillus subtilis* dosage to boost growth and gut health in nile tilapia (*oreochromis niloticus*) in brackish water conditions

Rahmi Rahmi[1], *Nur* Insana Salam[1], *Fitri* Indah Yani[2], *Andi* Indra Jaya[3], *Akmal* Akmal[4], *Khairun* Nisaa[5], *Farhanah* Wahyu[1] dan *Bunga* Rante Tampangallo[5]

[1]Aquaculture Study Program, Faculty of Agriculture, Universitas Muhammadiyah Makassar, Makassar, Indonesia
[2]Aquaculture Study Program, Faculty of Agricultural, Animal, and Fisheries Sciences, Universitas Muhammadiyah Pare pare, Parepare, Indonesia
[3]Fisheries Research Center for Brackish Water Aquaculture and Fisheries Counseling, South Sulawesi, Indonesia
[4]Research Centre for Marine Aquaculture, National Research and Innovation Agency, Cibinong Science Center-Biology Building, Bogor, Indonesia
[5]Research Center for Veterinary, National Research and Innovation Agency, Cibinong Science Center-Biology Building, Bogor, Indonesia

Abstract. Tilapia farming is central to global food security, yet efforts to expand production into saline environments are often limited by reduced growth, metabolic strain, and increased disease susceptibility. This study examined whether synbiotic diets combining *Bacillus subtilis* with selected prebiotic substrates could improve growth, hematology, intestinal structure, immune responses, and gut microbiota in Nile tilapia exposed to salinity stress. A 12-week feeding trial, structured using a Completely Randomized Design, tested graded levels of B. *subtilis*. Growth performance, feed efficiency, and survival were measured together with hematological parameters and gut histology, including villus height and mucosal thickness. Immune gene expression and microbiota profiles were assessed through RT-PCR and Next-Generation Sequencing. Synbiotic supplementation enhanced specific growth rate, feed conversion ratio, and survival, with the optimal response at 10^5 CFU/mL. Blood indices suggested improved oxygen transport, and histology showed stronger intestinal integrity. Molecular analyses revealed greater microbial diversity and upregulated immune-related genes, offering increased resistance to *Aeromonas hydrophila*. These results demonstrate the promise of synbiotic nutrition in reducing salinity-related stress and strengthening tilapia health. Future work should include long-term trials and explore multi-strain or multi-prebiotic formulations to further refine synbiotic strategies for sustainable aquaculture.

*Corresponding author: rahmiperikanan@unismuh.ac.id

1. Introduction

Aquaculture continues to expand as a major source of animal protein worldwide, with Nile tilapia emerging as one of the most important cultured species due to its rapid growth, environmental tolerance, and affordability . Increasing global demand has placed pressure on producers to maintain high productivity while adopting environmentally responsible practices. As freshwater resources become increasingly limited, the expansion of tilapia farming into saline and brackish-water environments has become a strategic response, supported by the availability of underutilized coastal regions suitable for aquaculture [1].

The introduction of saline-tolerant strains of Nile tilapia has enabled production in coastal and arid regions where freshwater scarcity constrains conventional aquaculture. However, culture in saline conditions presents multiple biological and economic challenges. Elevated salinity often results in reduced growth, higher feed conversion ratios, and increased susceptibility to infectious diseases [3]. Feed represents up to 80% of production costs; thus, impaired feed efficiency has substantial economic consequences. Moreover, salinity stress disrupts gut microbial communities and suppresses immune function, heightening vulnerability to pathogens such as Aeromonas hydrophila and Streptococcus agalactiae [4]. These constraints highlight the need for innovative nutritional strategies capable of enhancing growth and disease resilience in tilapia under saline conditions.

Salinity acts as a major physiological and immunological stressor for Nile tilapia. While moderate salinity is tolerable, optimal performance typically occurs below 15 ppt. Exposure above 20 ppt disrupts osmoregulation, metabolism, immune defense, and increases disease risk [5]. Prolonged exposure induces shifts in endocrine signaling and metabolic pathways, lowering growth and survival and diminishing immunocompetence. Transcriptomic studies confirm that salinity stress triggers activation of osmoregulatory and stress-related genes, further illustrating the physiological burden imposed on the fish [6]. These disruptions reduce feed efficiency and increase the frequency of disease outbreaks. In response, interest has grown in synbiotic supplementation using Bacillus subtilis. Strains such as B. subtilis AAHM-BS2360 and C-3102 produce digestive enzymes, enhance nutrient absorption, stabilize gut microbiota, and strengthen mucosal defenses, making them promising candidates for improving resilience in saline aquaculture systems.

Probiotics, prebiotics, and synbiotics have therefore become widely used interventions to counteract salinity-induced stress. B. subtilis improves nutrient assimilation, modulates gut microbiota balance, and enhances immune function, while prebiotics selectively stimulate beneficial microbes and support immune regulation [7]. Numerous studies have reported that B. subtilis supplementation increases growth, reduces feed conversion ratios, enhances digestive enzyme activity, reinforces intestinal structure, and improves pathogen resistance in tilapia exposed to salinity stress [8].

Combining probiotics and prebiotics as synbiotics offers additional advantages. Synbiotic formulations have been shown to improve antioxidant capacity, disease resistance, and immune function, while also mitigating the effects of environmental contaminants such as pesticides [7]. These benefits emphasize the dual role of synbiotics in supporting fish health and reducing antibiotic dependence, both of which are essential for sustainable aquaculture.

Despite these advantages, optimizing synbiotic formulations for saline environments remains challenging. Synbiotic efficacy varies with probiotic strain, prebiotic type, and environmental conditions, and high salinity may reduce probiotic viability, limiting their biological effectiveness [9-10]. This necessitates targeted research to develop synbiotic formulations specifically suited for saline aquaculture. Advances in molecular techniques, including Next-Generation Sequencing (NGS) and Reverse Transcription PCR (RT-PCR), provide opportunities to analyze gut microbial shifts and immune gene expression with high precision [11].

NGS provides detailed insight into microbial community structure and demonstrates how synbiotic diets modify gut microbiota to enhance digestion and disease resistance [11]. RT-PCR complements these findings by quantifying changes in immune-related gene expression, clarifying the link between microbial modulation and host immune function [12]. Together, these approaches offer a robust framework for evaluating synbiotic performance.

Given the persistent challenges associated with salinity stress, synbiotic diets enriched with Bacillus subtilis and targeted prebiotics offer strong potential to improve resilience and productivity in saline tilapia farming. This study addresses key knowledge gaps by examining the effects of B. subtilis-based synbiotics on growth, hematology, gut microbiota, and immune responses in Nile tilapia exposed to salinity stress. Through combined physiological and molecular analyses, the study provides essential insights for the development of effective, sustainable functional feeds for saline aquaculture.

2. Methodology

2.1 Experimental Design

This study explored how synbiotic feeds containing Bacillus subtilis influence growth, hematology, gut microbiota, and immune responses in Nile tilapia (Oreochromis niloticus) exposed to salinity stress. A Completely Randomized Design (CRD) was applied to reduce allocation bias and account for natural variation. A total of 225 fish were used, with 15 individuals per tank and three replicates per treatment to ensure reliable comparisons. Fish received either a control diet or synbiotic diets with graded B. subtilis levels combined with prebiotics. Prior to the trial, fish underwent a two-week acclimation period while salinity was gradually raised to 15 ppt to avoid osmotic shock. The viability of B. Subtilis was confirmed using standard plate counts before and after pelletization. Sample size estimation followed power analysis guidelines to ensure adequate sensitivity for detecting treatment effects in aquaculture nutrition studies [7].

2.2 Feeding Trials

Tilapia fingerlings (10-12 cm) were acclimated in the laboratory and gradually brought to 15 ppt salinity, a level inducing physiological stress but within their tolerance limits. Fish were kept in circular fiberglass tanks with recirculating, aeration, and filtration systems, and water quality, including dissolved oxygen, pH, total dissolved solids, and ammonia was monitored daily to ensure stable conditions [6]. Diets were pelleted and enriched with graded levels of Bacillus subtilis cultured in nutrient broth and mixed with prebiotics, formulated according to tilapia nutritional requirements for protein, lipids, and carbohydrates. Fish were fed three times daily, feed intake was recorded, and growth was monitored weekly by assessing specific growth rate (SGR), feed conversion ratio (FCR), weight gain (WG), and survival rate (SR).

2.3 Hematology

At the end of the feeding trial, blood samples were collected from the caudal vein of anesthetized fish. Hematological analyses included erythrocyte and leukocyte counts, hematocrit, and hemoglobin, all established indicators of fish health [8]. RNA was extracted from intestinal tissue samples for molecular analysis, and RT-PCR was performed to assess immune-related genes such as TNF-α, IL-1β, and IL-10. These cytokines serve as markers for pro- and anti-inflammatory responses and help link synbiotic supplementation to immunological outcomes [11].

2.4 Microbiota Analysis

Gut microbiota composition was analyzed through Next-Generation Sequencing (NGS). Intestinal contents from representative fish were collected, and DNA was extracted using commercial kits. Sequencing allowed for identification of bacterial taxa and community shifts related to synbiotic intake. NGS enabled detailed profiling of microbial diversity, providing associations between microbial composition and host health [11-12]. Bioinformatic pipelines were used to assess diversity indices and community structure across treatments.

2.5 Histopathology

Intestinal samples were collected and fixed for histopathological analysis, with tissue sections stained and evaluated for villus height, crypt depth, mucosal thickness, and inflammatory infiltration, which are reliable indicators of gut health in fish fed probiotics [15]. Increased villus height and villus-to-crypt ratios signified better nutrient absorption, while lower inflammatory infiltration reflected improved intestinal resilience; all scoring was performed blindly to minimize observer bias.

3. Result and Discussion

3.1 Result

3.1.1 Immune response in Tilapia

a. Changes in erythrocyte, hematocrit, and leukocyte profiles under probiotic or synbiotic feeding

Synbiotic diets containing Bacillus subtilis improved blood parameters in Nile tilapia under salinity stress, with the 10^5 CFU/mL dose yielding the highest erythrocyte count (3.3×10^6 cells/mm^3) and hematocrit (33.7%) (Table 1). These results align with findings from [19], who reported enhanced blood indices and immune-related gene expression in probiotic-fed tilapia. Although leukocyte numbers rose slightly, the most notable improvements were in erythrocytes and hematocrit, reinforcing the value of probiotics in supporting hematological health under salinity stress .

Table 1. Hematology and Immunity Profile Changes in Tilapia During 12 Weeks of Maintenance Under Salinity (15 ppt)

Fish Maintenance (Week)	Treatment	Erythrocyte ($\times 10^6$ sel/mm³)	Hematocrit (%)	Leukocyte ($\times 10^3$ sel/mm³)	Lysozyme (U/mL)	SOD (U/mg protein)	IgM (mg/mL)
1	Control	2.6	2.6	2.6	2.6	2.6	2.6
	B. subtilis 10^5	2.9	28.8	9.0	12.6	19.5	1.20
	B. subtilis 10^9	2.7	27.4	8.7	11.8	18.6	1.10
4	Control	2.7	27.2	8.9	11.5	18.7	1.08

	B. subtilis 10^5	3.0	30.5	9.4	13.5	21.2	1.28
	B. subtilis 10^9	2.8	28.6	9.1	12.2	19.6	1.16
8	Control	2.8	28.0	9.1	11.7	19.0	1.10
	B. subtilis 10^5	3.2	32.0	9.8	14.6	22.5	1.42
	B. subtilis 10^9	2.9	29.5	9.3	12.8	20.4	1.23
12	Control	2.9	29.0	9.3	12.0	19.5	1.12
	B. subtilis 10^5	3.3	33.7	10.0	15.2	23.8	1.55
	B. subtilis 10^9	3.0	30.2	9.5	13.0	20.9	1.26

b. Influence of probiotics on innate and adaptive immune markers

Synbiotic supplementation also enhanced both innate and adaptive immune responses. Fish fed with B. Subtilis exhibited higher lysozyme activity, an essential antimicrobial peptide responsible for pathogen defense, as well as elevated superoxide dismutase (SOD) activity, which provides critical protection against oxidative stress. These findings are consistent with [21], who reported similar improvements in innate immune markers in tilapia. Adaptive immune responses were also enhanced, with probiotic-fed fish exhibiting elevated levels of immunoglobulin M (IgM), alongside increased expression of cytokines such as IL-1β and TNF-α, indicating a more robust humoral and cellular immune system [15]. Together, these results demonstrate that probiotics such as B. Subtilis provide a comprehensive enhancement of immunity by reinforcing both innate and adaptive mechanisms.

c. Dose-Response patterns in immunological outcomes of bacillus subtilis supplementation

The immune parameters in this study displayed clear dose-response patterns, with optimal outcomes observed at moderate supplementation levels. Tilapia receiving 10^5 CFU/mL B. subtilis consistently exhibited superior immune indices, including enhanced hematological profiles, lysozyme activity, and balanced leukocyte counts. These findings align with the conclusions of [13], who reported that moderate dosages produced optimal immunological benefits, whereas higher concentrations did not yield proportional improvements. At elevated levels such as 10^9 CFU/mL, diminishing returns were evident, and in some cases, immune responses were attenuated. This pattern supports the observations of3, who cautioned against supra-optimal dosing due to risks of microbiota imbalance and immune disruption. Thus, maintaining supplementation within defined thresholds is essential to maximize benefits while avoiding adverse outcomes.

3.1.2 Growth Performance in Tilapia Under Probiotic/Synbiotic Dosages

a. Effects of probiotic/synbiotic dosages on SGR, FCR, WG, and SR

Growth performance metrics revealed significant improvements among tilapia fed synbiotic diets. At the optimal supplementation of 10^5 CFU/mL, tilapia achieved a specific growth rate (SGR) of 1.32% per day and a feed conversion ratio (FCR) of 1.38, both outperforming control groups (Fig 2). Weight gain and survival rates were also higher in the treated groups, reflecting superior feed efficiency and resilience. These results are in line with earlier studies, such as those by , which showed that Bacillus strains significantly enhance growth indices. Similarly, observed improved growth and disease resistance with probiotic supplementation. These findings collectively illustrate the potential of synbiotic feeds to counteract the metabolic strain imposed by salinity, thereby enhancing productivity

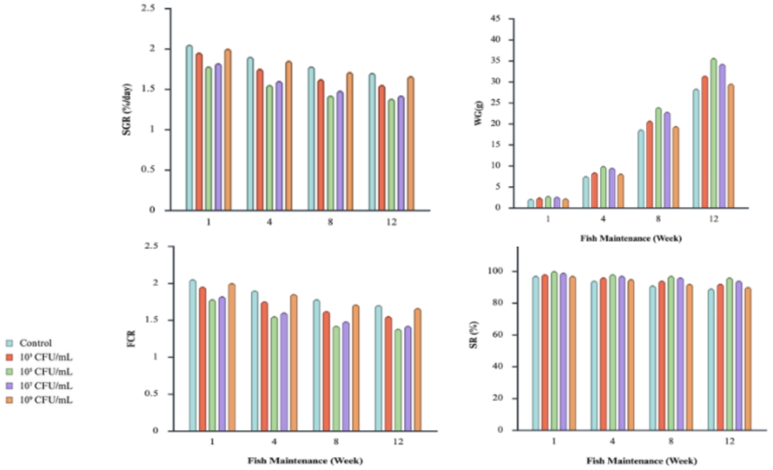

Fig 2. Growth performance (SGR, FCR, WG, SR) of Nile tilapia under different probiotic/synbiotic dosages during 12 weeks of rearing at 15 ppt salinity.

b. Cost-benefit analyses of probiotic or synbiotic supplementation

Economic considerations revealed that while feed costs increased due to probiotic inclusion, gains in biomass yield and survival substantially outweighed these expenses. Improved SGR and WG translated into higher marketable output, and reduced mortality lowered production losses. These findings echo those of [14], who reported that enhanced growth efficiency and reduced antibiotic dependency improve profitability in aquaculture. By reducing the need for costly disease treatments, synbiotic supplementation supports long-term sustainability and economic viability in commercial tilapia production.

3.1.3 Histopathological Effects of Bacillus subtilis on Intestinal Morphology in Tilapia

a. Effects on intestinal morphology

Histological evaluations revealed substantial structural improvements in the intestines of tilapia fed with synbiotic diets. Fish receiving 10^5 CFU/mL of B. *subtilis* exhibited villus heights averaging 240 µm, crypt depths of 80 µm, and mucosal thickness of approximately 65 µm, with minimal inflammatory infiltration. And with [14], who emphasized the role of probiotics in strengthening mucosal barriers. Collectively, these histological enhancements indicate that synbiotic diets improve gut resilience and functionality under salinity stress.

b. Links between gut histological improvements and nutrient absorption efficiency

The histological improvements observed were closely linked to enhanced nutrient assimilation. Higher villus-to-crypt ratios, indicative of efficient intestinal function, correlated with improved growth and feed conversion metrics. These results align with prior studies demonstrating that enhanced villus height and mucosal thickness support greater digestive enzyme activity, leading to superior nutrient absorption [9]. Such correlations confirm that structural adaptations in the gut directly mediate the performance gains achieved through probiotic supplementation.

c. Probiotic interaction with gut microbiota under salinity stress

Gut microbiota analysis demonstrated that B. *subtilis* supplementation fostered beneficial bacterial populations while suppressing opportunistic pathogens, stabilizing the intestinal environment under salinity stress. Synbiotic-fed tilapia displayed increased microbial diversity and greater representation of commensal taxa known for supporting epithelial regeneration and immune modulation. Emphasized the role of probiotics in maintaining microbial stability during environmental stress. Enhanced expression of tight junction proteins in treated groups further indicated strengthened barrier integrity, reducing pathogen translocation and supporting intestinal homeostasis. By reinforcing epithelial regeneration and sustaining microbiota balance, synbiotics provided a holistic protective effect.

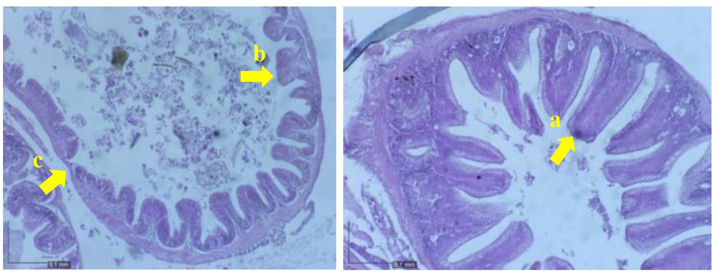

Fig 3. Representative histological sections of the intestine from control and synbiotic-fed Nile tilapia (H&E staining). Synbiotic-fed groups show increased villus height (a), thicker mucosa (b), and reduced inflammatory cell infiltration (c) compared to controls, demonstrating improved intestinal health. Scale bar = 0,1 mm

3.2 Discussion

The findings of this study indicate that supplementing Nile tilapia diets with Bacillus subtilis can markedly enhance physiological stability and resilience when the fish are exposed to salinity stress. These outcomes align closely with reports from diverse aquaculture species where probiotics have demonstrated consistent benefits for growth and immune regulation. Similar improvements have been documented in tilapia, rainbow trout, and common carp, reinforcing the notion that probiotics provide broad-spectrum support for fish experiencing environmental challenges [7].

The physiological basis for these improvements lies in the multifaceted influence of B. subtilis on gut morphology, nutrient assimilation, and immune processes. Tilapia fed synbiotic diets demonstrated more developed intestinal structures, including elongated villi, deeper crypts, and thicker mucosal layers. Such features collectively expand the absorptive surface area, allowing more efficient nutrient uptake, a critical advantage under saline conditions, where digestive function is often compromised . Alongside these structural benefits, B. subtilis plays a direct role in strengthening immune responses. Both innate and adaptive immunity were enhanced, reflected in elevated lysozyme and immunoglobulin concentrations as well as increased expression of immune-related genes [6]. These immunological improvements correspond with more robust hematological and cytokine profiles observed in this study, indicating a stronger physiological buffer against salinity-induced stress. These combined effects, enhanced digestion, improved nutrient utilization, and stronger immune responsiveness, help explain the observed increase in growth performance and the reduction in feed conversion ratios associated with B. *subtilis* supplementation .

The development of synbiotic feed plays an important role in supporting sustainable aquaculture by reducing dependence on antibiotics through improved gut health, immune resistance, and growth efficiency. However, its adoption in saltwater tilapia farming is still limited due to environmental variability that requires location-specific formulations, relatively high initial costs, and uneven farmer knowledge. Therefore, improved extension services, field trials, and clearer economic information are needed to encourage wider adoption of synbiotics [15].

Recent advancements in molecular methods have deepened understanding of how probiotics benefit the host. NGS-based studies reveal that synbiotic diets diversify the gut microbiota and promote the proliferation of beneficial bacterial taxa, improvements linked to better digestion and stronger immunity. These microbial shifts help stabilize intestinal homeostasis during salinity stress. RT-PCR analyses further demonstrate increased expression of immune markers such as lysozyme and immunoglobulin M (IgM), confirming that synbiotics influence both microbial composition and immunophysiological pathways. Together, NGS and RT-PCR provide compelling evidence that synbiotic benefits arise from integrated host-microbiota interactions [13].

Despite these advances, several research gaps remain. Identifying species-specific probiotic strains and optimized dosing strategies suited to varying environments is essential, as probiotic responses are highly strain-dependent. Longer-term effects on growth, reproduction, and ecological dynamics also require further investigation, as most studies focus on short-term performance. Future research should include multi-cycle trials, deeper exploration of host–microbiota interactions under salinity stress, and systematic evaluation of multi-strain and multi-prebiotic formulations tailored to diverse aquaculture systems.

4. Conclusion

Synbiotic feeds containing Bacillus subtilis and targeted prebiotics, especially at 10^5 CFU/mL, effectively enhance growth, feed conversion, immune resilience, blood profiles, and intestinal structure in Nile tilapia under saline conditions, as confirmed by NGS and RT-PCR markers for microbiota diversity and immune gene expression; these improvements lower disease susceptibility, offer economic and sustainability benefits by reducing antibiotic reliance, and address key aquaculture challenges, with a recommendation for further long-term trials and strain combinations to optimize sustainable applications.

References

[1] C. E. Boyd, A. A. McNevin, and R. P. Davis, “The contribution of fisheries and aquaculture to the global protein supply,” *Food Security*, vol. 14, pp. 805–827, 2022. doi: 10.1007/s12571-021-01246-9.

[2] G. Ashouri, S. H. Hoseinifar, E. El-Haroun, R. Imperatore, and M. Paolucci, “Tilapia fish for future sustainable aquaculture,” in *Novel Approaches Toward Sustainable Tilapia Aquaculture*, Springer, Cham, 2023. doi: 10.1007/978-3-031-38321-2_1.

[3] R. Rahmi et al., “Tilapia growth performance and body physical index given synbiotic feed at different salinities,” *IOP Conf. Ser. Earth Environ. Sci.*, vol. 1137, p. 012029, 2023. doi: 10.1088/1755-1315/1137/1/012029.

[4] Q. A. Le and D. Pham, "Detection of *Streptococcus agalactiae* causing hemorrhage and eye edema in Nile tilapia by nested PCR," *IOP Conf. Ser. Earth Environ. Sci.*, vol. 1399, p. 012004, 2024. doi: 10.1088/1755-1315/1399/1/012004.

[5] A. H. A. Almaaty, M. A. H. El-Kady, and H. A. A. Mansour, "Differential expression of aromatase genes as a stress response to salinity in Nile tilapia," *Egypt. J. Aquat. Biol. Fish.*, vol. 23, pp. 205–215, 2019. doi: 10.21608/ejabf.2019.27295.

[6] R. V. Azevedo et al., "Responses of Nile tilapia to different levels of water salinity," *Lat. Am. J. Aquat. Res.*, vol. 43, pp. 828–835, 2017. doi: 10.3856/vol43-issue5-fulltext-3.

[7] L.-G. Wang et al., "Effects of different water quality regulators on growth performance, immunologic function, and domestic water quality of GIFT tilapia," *PLoS One*, vol. 18, p. e0290854, 2023. doi: 10.1371/journal.pone.0290854.

[8] R. Rahmi et al., "Growth analysis and innate immune response of tilapia fed with synbiotic feeds in brackish water," *Int. J. Vet. Sci.*, vol. 23, p. 278, 2024. doi: 10.47278/journal.ijvs/2023.100.

[9] P. Puri, J. G. Sharma, and R. Singh, "Biotherapeutic microbial supplementation for ameliorating fish health," *Anim. Health Res. Rev.*, vol. 23, pp. 113–135, 2022. doi: 10.1017/S1466252321000165.

[10] P. J. Say et al., "Long-term application of a synbiotic chitosan and *Acinetobacter* mixture on hybrid catfish," *Microorganisms*, vol. 11, p. 1807, 2023. doi: 10.3390/microorganisms11071807.

[11] M. Aftabgard et al., "Combined efficiency of isomaltooligosaccharides and *Bacillus* spp. on Caspian brown trout," *Probiotics Antimicrob. Proteins*, vol. 11, pp. 198–206, 2019. doi: 10.1007/s12602-017-9361-z.

[12] M. Aftabgard et al., "Functional, gut digestive, and serum antioxidant factors in *Salmo trutta caspius* fingerlings with synbiotic diet," *Arch. Pol. Fish.*, vol. 26, pp. 31–38, 2018. doi: 10.2478/aopf-2018-0004.

[13] M. H. Islam et al., "Boosting growth, muscle development, and intestinal morphology in *Mystus cavasius* with dietary synbiotics," *Aquac. Res.*, 2025. doi: 10.1155/are/3638368.

[14] P. Puri, R. Singh, and J. G. Sharma, "Encapsulations and co-treatments of microbial feed supplements in finfish aquaculture," *Benef. Microbes*, vol. 14, pp. 281–302, 2023. doi: 10.3920/BM2022.0087.

[15] Q. Luo et al., "Dietary α-ketoglutarate alleviates glycinin-induced intestinal damage in mirror carp," *Front. Immunol.*, vol. 14, p. 1140012, 2023. doi: 10.3389/fimmu.2023.1140012.