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Probiotic *Bacillus subtilis* Improves Hematological, Growth, and Gut Health Performance in the Nile Tilapia (*Oreochromis niloticus*) Cultured Under Salinity Stress

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ABSTRACT

The Nile tilapia (*O. niloticus*) cultivated in waters with elevated salinity often face stress that negatively impacts their growth, immune system, and intestinal health. This study investigated the effects of adding the probiotic *B. subtilis* to the diet at various doses (10^3, 10^5, 10^7, and 10^9 CFU/mL) on the blood parameters, growth, and intestinal structure of tilapia reared at 15ppt salinity. In a 60-day feeding trial, fish fed 10^5 CFU/mL of *B. subtilis* showed significant benefits, including higher red blood cell counts, improved hematocrit levels, better growth rates and better feed conversion, as revealed by microscopes showing healthier intestinal tissue. Microscope analysis revealed healthier intestinal tissue, suggesting improved nutrient absorption and gut integrity. While white blood cell numbers showed only slight changes, the results suggested the probiotic had a positive effect on the fish's immunity. The results of this study demonstrate that the administration of dietary *B. subtilis* at an optimal dose of 10^5 CFU/mL can effectively alleviate the physiological and structural challenges caused by salinity stress, thereby supporting healthier and more productive tilapia farming without the need for antibiotic use.

INTRODUCTION

Aquaculture continues to be one of the most rapidly expanding sectors in global food production, with the Nile tilapia (*Oreochromis niloticus*) playing a vital role due to its adaptability, fast growth, and economic importance. Yet, with freshwater availability under increasing pressure, many aquaculture operations are shifting to brackish and saline waters to sustain output. This shift brings new challenges, particularly in the form of physiological stress, which compromises fish health and performance. High salinity is one of the most pressing of these challenges, disrupting osmoregulation and other physiological processes in freshwater species like the Nile tilapia. As reported by **Yamaguchi *et al.* (2018)** and **Madkour *et al.* (2025)**, when salinity exceeds 10– 15ppt, tilapia fish experience significant metabolic disturbances and greater energy requirements for ionic balance. These physiological stresses often result in reduced growth, poorer feed efficiency, and elevated stress biomarkers such as cortisol and glucose **(Dawood *et al.*, 2023; Hardi *et al.*, 2023)**. Mortality rates can increase significantly once salinity reaches 20ppt, making it a critical limit for practical aquaculture operations **(Kimera *et al.*, 2023)**.

The impact of elevated salinity goes beyond growth. Numerous studies have linked it to compromised immune responses in freshwater fish. Hyperosmotic stress has been shown to suppress immune-related gene expression and reduce the production of antibodies like immunoglobulin M (IgM) **(Fang *et al.*, 2022; Sallam *et al.*, 2024)**. This decline in immune capacity renders fish more vulnerable to disease, especially in high-density aquaculture systems already burdened by environmental stress **(Mojekwu *et al.*, 2020)**. The suppression of immune function under salinity stress threatens the long-term viability of tilapia farming, making it essential to find mitigation strategies that are both effective and sustainable **(El-Zaeem *et al.*, 2014)**.

One promising avenue is the use of probiotics in aquaculture feed, with *Bacillus subtilis* receiving particular attention for its wide-ranging benefits. Unlike antibiotics, which carry risks such as resistance and contamination, *B. subtilis* enhances fish health through natural, residue-free mechanisms. Among its most studied attributes is its capacity to secrete enzymes like amylase, protease, and lipase, which improve macronutrient digestion, feed utilization, and nutrient absorption **(Zhou *et al.*, 2010)**. These effects support better growth and more efficient feed conversion **(Panase *et al.*, 2023)**. Additionally, *B. subtilis* positively influences the intestinal microbiome, suppressing harmful bacteria and promoting a healthier gut environment.

Evidence from experimental studies supports these findings. Tilapia fed *B. subtilis*-enriched diets have shown improvements in weight gain, specific growth rate (SGR), and feed conversion ratio (FCR) **(Hamed *et al*., 2024)**. Structural benefits have also been observed in the intestinal tract, such as taller villi and increased surface area—features that are essential for optimal nutrient uptake **(Zhu *et al*., 2019)**. These physical enhancements to the gut contribute significantly to the overall growth and health of the fish.

Moreover, *B. subtilis* appears to bolster both innate and adaptive immune responses. It has been linked to elevated levels of immune markers including lysozyme, respiratory burst activity, and superoxide dismutase (SOD) **(Ridha & Azad, 2015; Addo *et al*., 2016)**. These immune responses enhance the fish’s resistance to disease and environmental stress. For example, tilapia fed *B. subtilis* were better able to withstand infections from *Aeromonas hydrophila* and *Streptococcus iniae*, two common pathogens in aquaculture **(Macias *et al*., 2024)**. Through these mechanisms, *B. subtilis* presents itself as a viable alternative to synthetic immunostimulants and antibiotics.

The drawbacks of antibiotics in aquaculture are well established. Their overuse contributes to the emergence of antibiotic-resistant bacteria, posing risks not only to fish but also to human health. Residual antibiotics in fish products can enter the food chain, leading to increased resistance in human pathogens **(Jahangiri & Esteban, 2018; Rasul *et al*., 2025)**. Furthermore, antibiotics disrupt aquatic microbial communities, diminishing biodiversity and water quality **(Wei *et al*., 2022)**. These concerns underscore the urgency for safer, more environmentally friendly alternatives. Probiotics, by contrast, are biodegradable, do not induce resistance, and support fish health through natural biological interactions **(Ridha & Azad, 2015; Rwezawula *et al*., 2025)**. Their ability to improve gut function, immune response, and nutrient utilization makes them ideal candidates for sustainable aquaculture practices **(Hasan & Banerjee, 2020; Abbas *et al*., 2025)**.

Recent studies have turned their focus specifically to the use of *B. subtilis* in saline environments. Research by **Ren *et al*. (2021)** and **Jiang *et al*. (2023)** has shown that *B. subtilis* enhances tilapia's salinity tolerance by boosting immune function and maintaining gut integrity. These effects are achieved through improved nutrient absorption, reduced inflammation, and suppression of pathogenic bacteria. In addition to supporting gut health, *B. subtilis* produces antimicrobial compounds that help keep harmful microbes at bay **(Menaga *et al*., 2019; Jiang *et al*., 2023)**.

Given these multifaceted benefits, it becomes increasingly important to identify probiotic strains like *B. subtilis* that can sustain performance under salinity stress while also promoting fish health. Nevertheless, questions remain around optimal dosing, strain-specific effects, and the long-term impact of probiotic use in saline systems. Previous research has explored different dosages of *B. subtilis* in tilapia and other fish species, indicating effective doses ranging from 10^8 to 10^{10} CFU/g feed to improve growth, immune response, and gut morphology. However, strain variations and long-term effects under salinity stress require further detailed investigation to optimize practical applications for sustainable aquaculture **(Liao *et al.*, 2023; Panase *et al.*, 2023)**.

This study was designed to address that gap by evaluating the effects of *Bacillus subtilis*-supplemented diets on immune function, growth performance, and gut histopathology in the Nile tilapia raised in high-salinity water. By combining hematological, growth, and histological analyses, this research aimed to shed light on the specific physiological mechanisms by which *B. subtilis* supports fish health in challenging aquaculture environments. The findings are expected to inform future feed strategies and contribute to building more resilient and sustainable aquaculture systems.

MATERIALS AND METHODS

1. Study site and period

The study was conducted from May to July 2025 at the Mini Hatchery Laboratory, Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar, South Sulawesi. The laboratory provided a controlled environment to simulate salinity stress commonly encountered in freshwater aquaculture. Water salinity was maintained stably at 15ppt, a level known to induce physiological stress in the Nile tilapia (*Oreochromis niloticus*) **(Yamaguchi *et al*., 2018; Madkour *et al*., 2025)**. Water temperature ranged between 27– 29°C, with dissolved oxygen levels maintained above 5mg/ L, supported by continuous aeration.

2. Preparation of fish

Juvenile Nile tilapia, averaging 7– 9cm in length, were acclimated for two weeks in brackish water at 15ppt salinity before the experiment commenced. This acclimation ensured physiological adaptation to saline conditions and uniformity of starting health status **(Kimera *et al*., 2023)**. Fish were then randomly distributed into experimental tanks.

3. Experimental design

A completely randomized design (CRD) was employed consisting of five treatment groups with three replicates each (15 tanks total). Treatments included:

- Control group (basal diet without probiotics)
- Four groups supplied with Bacillus subtilis at dosages of 10^3, 10^5, 10^7, and 10^9 CFU/mL feed, respectively.

Each tank (50L capacity) was filled with 20L aerated brackish water and stocked with 20 fish. The experiment ran for 60 days.

3. Research procedure

B. subtilis cultures were grown in nutrient broth for 24 hours, then centrifuged to concentrate cells. The bacterial pellet was resuspended in sterile saline, and a 1% suspension was evenly sprayed on the basal diet pellets, followed by air-drying at room temperature **(Addo *et al*., 2016; Panase *et al*., 2023)**. Fish were fed to apparent satiation three times daily.

4. Parameters measured

Blood profile

At the end of the trial, 3 fish per tank were randomly selected for blood sampling from the caudal vein. Erythrocyte (RBC) counts were determined using a hemocytometer after dilution with Hayem's solution. Leukocyte (WBC) counts utilized Turk's solution. Hematocrit (Hct) was measured via microcapillary tubes centrifuged at 5,000 rpm for 5 minutes **(Anderson & Siwicki, 1995)**. All procedures followed standardized protocols **(Blaxhall & Daisley, 1973)**.

Growth performance and feed utilization

Individual body weights were recorded at the beginning and end of the experiment. The following formulas were used for performance evaluation:

Weight Gain (WG) = Final Weight – Initial Weight

Specific Growth Rate (SGR) = 100 × [ln (Final Weight) – ln (Initial Weight)] / Days of Trial

Feed Conversion Ratio (FCR) = Total Feed Consumed / Total Biomass Gain

Survival Rate (SR) (%) = (Number of Surviving Fish / Initial Number) × 100

These indices offer insights into how efficiently the probiotic-enhanced diet supports physiological resilience and growth in high-salinity environments **(Addo *et al*., 2016; Niu *et al*., 2020)**.

Intestinal histopathology

At trial completion, three fish per replicate were euthanized for distal intestine sampling. Tissue samples were fixed in 10% buffered formalin for 24–48 hours, dehydrated through graded ethanol, cleared in xylene, and embedded in paraffin blocks. Sections (4– 6µm) were cut using a rotary microtome and stained with hematoxylin and eosin (H&E) for microscopic analysis of villus height, crypt depth, mucosal thickness, and inflammatory infiltration **(Moniruzzaman *et al*., 2018; Eissa *et al.*, 2022)**.

5. Data analysis

Data were analyzed via one-way ANOVA to test treatment effects on all measured parameters, followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $P< 0.05$. Statistical analyzes were performed using IBM SPSS ver. 20.

RESULTS

1. Hematological parameters

Dietary supplementation with *Bacillus subtilis* significantly influenced the hematological profiles of the Nile tilapia reared under salinity stress (15ppt). The group fed with 10^5 CFU/mL *B. subtilis* exhibited the highest erythrocyte (RBC) counts, averaging 3.3×10^6 cells/mm^3, which was statistically greater than the control and other probiotic dosage groups ($P< 0.05$). Hematocrit (Hct) values followed a similar pattern, with the 10^5 CFU/mL group showing a significantly higher mean value of 33.7% compared to all other groups ($P< 0.05$). Leukocyte (WBC) counts showed a slight, but not statistically significant, increase in probiotic-treated groups compared to the control ($P> 0.05$). Detailed hematological data are presented in Fig. (1).

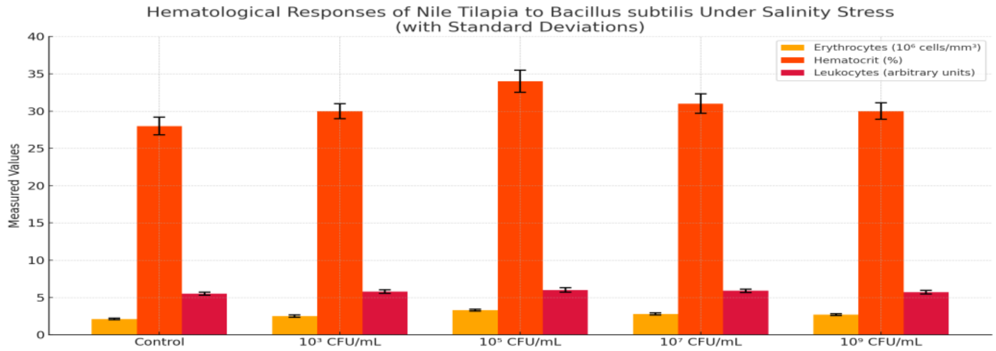

Fig. 1. Hematological responses of the Nile tilapia to *Bacillus subtilis* under salinity stress. The graph presents erythrocyte counts (10^6 cells/mm³), hematocrit (%), and leukocyte counts (arbitrary units), including standard deviations across control and probiotic-treated groups

2. Growth performance

Growth performance parameters indicated a significant improvement in fish receiving 10^5 CFU/mL *B. subtilis*. The specific growth rate (SGR) for this group was 1.32% per day, which was significantly higher than those of the control and other treatment groups ($P< 0.05$). Similarly, weight gain (WG) was the highest in the 10^5 CFU/mL group, while feed conversion ratio (FCR) was the lowest, demonstrating improved feed efficiency. Survival rates exceeded 90% across all treatments with no significant differences observed ($P> 0.05$). These growth performance metrics are summarized in Fig. (2).

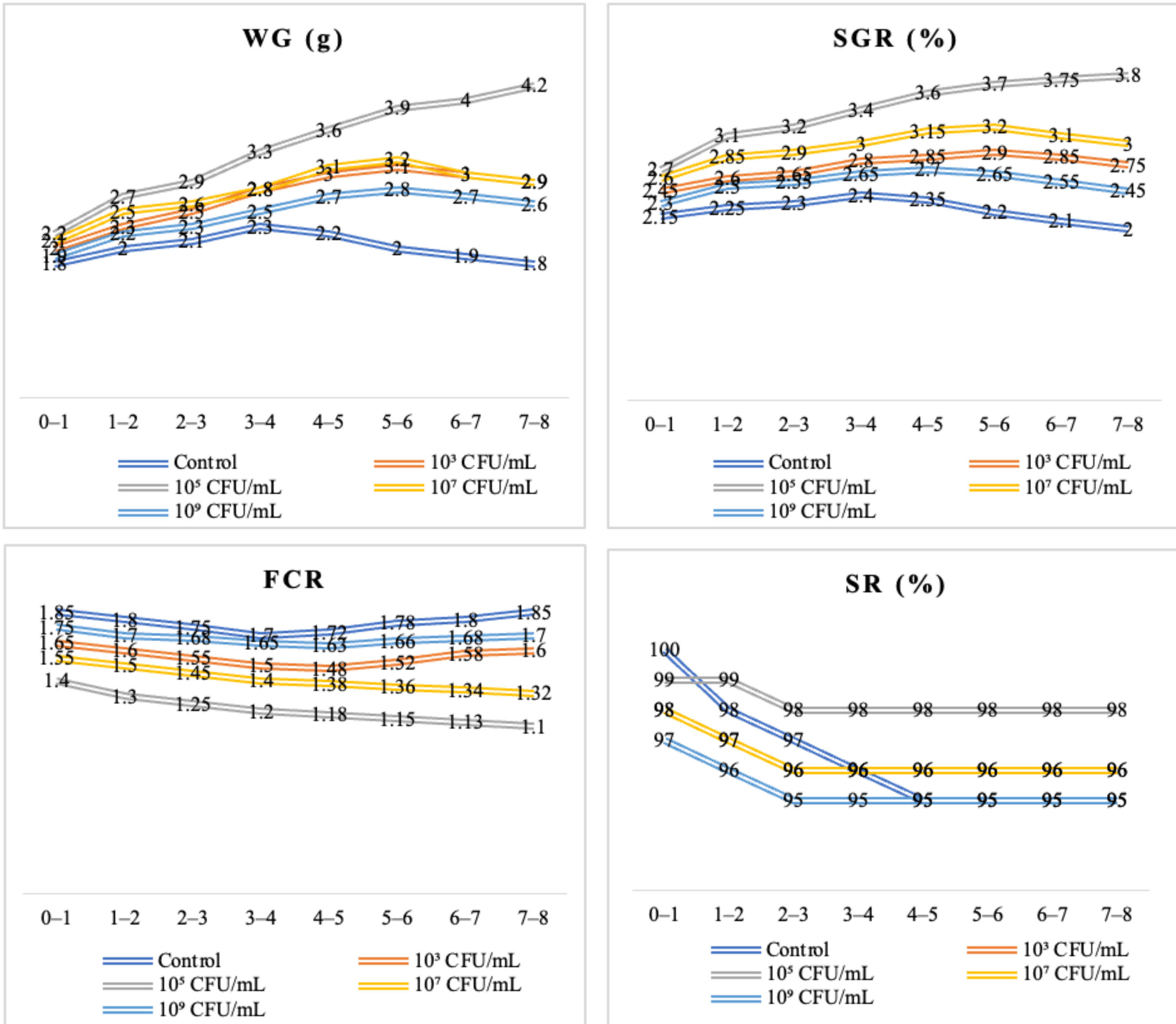

Fig. 2. Growth performance of the Nile Tilapia with *Bacillus subtilis* supplementation based on weeks of rearing. The graph displays weekly values for specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR) and survival rate (SR) across the control and various probiotic dosage treatments (10^3, 10^5, 10^7, and 10^9 CFU/mL).

3. Intestinal histology

Histological examination of distal intestinal tissues revealed significant morphological improvements in the 10^5 CFU/mL group. Fish in this group exhibited significantly increased villus height and width, deeper crypts, and thicker mucosal layers compared to the controls ($P < 0.05$). Furthermore, inflammatory infiltration in the intestinal mucosa was minimal in the probiotic 10^5 CFU/mL group versus pronounced infiltration in the control group. Representative micrographs are provided in Fig. (3). In contrast, the control fish showed signs of villus atrophy, mucosal thinning, and marked inflammatory cell presence.

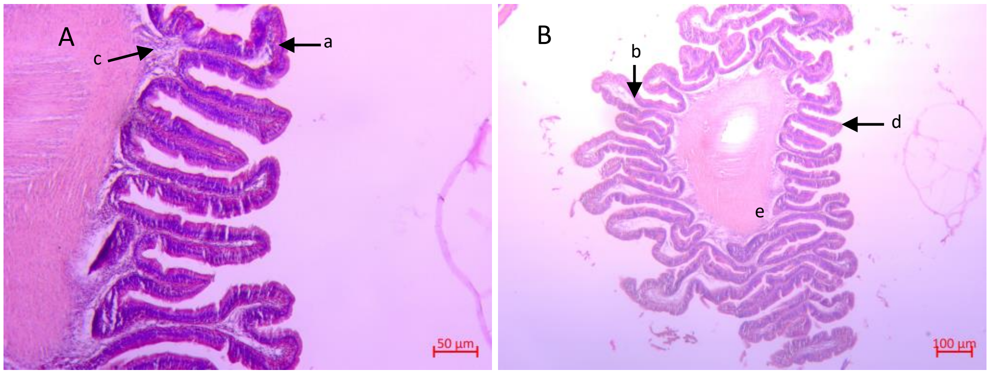

Fig. 3. Histological structure of the distal intestine in the Nile tilapia fed different diets under salinity stress (H&E staining). (A) Control group: the intestinal tissue exhibits signs of villus atrophy, reduced mucosal thickness, shortened villus height, and increased inflammatory infiltration. Crypts are shallow, and epithelial integrity appears compromised. Scale bar = 50 µm. (B) 10^5 CFU/mL B. subtilis group: a well-preserved intestinal architecture is evident, with elongated and uniformly shaped villi (a), increased villus width and height (b), deeper crypts (c), thicker mucosal layers (d), and minimal inflammatory infiltration (e). These improvements indicate better absorptive capacity and mucosal regeneration. Scale bar = 100 µm.

DISCUSSION

This study highlights the beneficial role of *Bacillus subtilis* as a dietary supplement for the Nile tilapia (*Oreochromis niloticus*) reared under salinity-induced stress. Among the measured hematological parameters, erythrocyte count emerged as the most responsive to probiotic inclusion. Fish treated with 10^5 CFU/mL of *B. subtilis* demonstrated elevated erythrocyte levels, averaging 3.3×10^6 cells/mm³. This exceeds the normal physiological range of 1.5–2.5×10^6 cells/mm³ generally observed in healthy individuals **(Cruz-García *et al.*, 2022; Panda *et al.*, 2022)**, suggesting an adaptive enhancement in oxygen transport capacity. Such a response likely reflects a physiological strategy to cope with osmotic imbalance, a mechanism also discussed by **Farrag *et al.* (2019)** and **Fonseca *et al*. (2020)**, who reported similar compensatory hematological shifts in fish exposed to elevated salinity.

A comparable trend was seen in hematocrit values. The 10^5 CFU/mL group recorded the highest mean hematocrit at 33.7%, reinforcing the notion that *B. subtilis* can support erythropoietic activity even under physiological stress. These findings are in line with those of **Abu-Elala *et al.* (2021)**, and lend further support to the concept that probiotics can serve as effective immunonutritional agents in aquaculture settings challenged by salinity fluctuations **(Farrag *et al*., 2019; Agustina & Susanto, 2024)**.

While erythrocyte and hematocrit values responded clearly to the probiotic treatments, changes in leukocyte counts were more modest. Although there was a slight increase across all *B. subtilis*-treated groups, statistical significance was not achieved. This stands in contrast to previous observations by **Kumar *et al.* (2008)** and **Docando *et al*. (2022)**, where substantial rises in neutrophil and lymphocyte populations were reported. The disparity may be due to the duration of the experiment or the absence of a pathogenic challenge, both of which could affect immune cell mobilization. Future studies incorporating stressor or pathogen exposure may offer a clearer picture of how *B. subtilis* modulates leukocyte profiles under salinity stress.

The hematological changes observed in this study likely result from a combination of mechanisms. *B. subtilis* and other probiotics have been shown to stimulate immune cell proliferation and activation, including increased lymphocyte and neutrophil numbers, as well as enhanced lysozyme activity and leukocyte responsiveness **(Won *et al*., 2020; Suphoronski *et al*., 2021; Yang *et al*., 2022)**. Additionally, probiotics have been associated with reduced plasma cortisol levels, thereby relieving stress-induced suppression of erythropoiesis **(Wu *et al*., 2020; Shukry *et al*., 2021)**. Improved intestinal health from probiotic supplementation likely contributes indirectly, through better nutrient assimilation and energy balance **(Selim & Reda, 2015; Addo *et al*., 2016)**, further supporting hematopoietic processes. Fig. (1) visually captures these hematological trends, with clearly elevated erythrocyte and hematocrit values in the 10^5 CFU/mL group. Although leukocyte counts remained stable overall, their slight upward shift indicates the potential for cumulative immune benefits.

Beyond immune-related responses, *B. subtilis* supplementation also produced significant gains in growth performance. The 10^5 CFU/mL group again stood out, achieving the highest specific growth rate (SGR) at 1.32% per day and the most substantial weight gain during the 60-day trial. This reinforces the idea that probiotic benefits are dose-dependent, with optimal effects typically achieved within a specific range—often cited as 10^6 to 10^8 CFU/g in the literature **(El-Haroun *et al*., 2006; Han *et al*., 2015)**. These findings affirm that carefully calibrated dosages are key to unlocking the full growth-enhancing potential of probiotics.

Feed conversion efficiency also followed this pattern. The lowest FCR was recorded in the 10^5 CFU/mL group, indicating better feed utilization and digestive efficiency. These outcomes align with findings from **Liu *et al*. (2021)** and **Sîrbu *et al*. (2022)**, who noted that probiotic administration improves digestive enzyme activity and supports healthier gut morphology, which in turn enhances nutrient absorption. In contrast, fish receiving either the lowest (10^3 CFU/mL) or the highest (10^9 CFU/mL) doses showed more moderate growth responses. These results suggest a saturation point beyond which additional probiotics may fail to produce further benefit—or worse, may disrupt digestion or gut homeostasis, as proposed by **Khunrang *et al.* (2021)**.

Fig. (2) presents these trends across weekly intervals, showcasing consistent increases in SGR and weight gain among fish supplemented with 10^5 CFU/mL, alongside a downward trend in FCR. Survival rates across all groups remained above 90%, with no significant differences, reinforcing the safety of the probiotic even under high salinity conditions. These results align well with earlier research by **Silva *et al*. (2017)** and **Won *et al*. (2020)**, which found similarly positive effects at higher doses ranging from 10^8 to 10^9 CFU/g.

Notably, despite using a lower probiotic concentration in this study (10^5 CFU/mL), performance outcomes were comparable to those previously reported at higher dosages. This suggests that lower dosages may be equally effective, providing economic and operational advantages for commercial aquaculture. Enhanced immune function, metabolic efficiency, and nutrient assimilation through improved gut architecture **(Addo *et al*., 2016; Kaewda *et al.*, 2024)** all contributed to these favorable growth metrics under salinity stress.

The histological findings further support the physiological and growth outcomes. Intestinal tissue from control fish showed typical signs of damage associated with osmotic stress, including atrophied villi, thinning of the mucosal layer, and notable inflammatory infiltration—features commonly reported in stressed freshwater fish **(Addo *et al*., 2016; Nawsany & Mohsen, 2019)**. Such deterioration compromises nutrient absorption and weakens intestinal barrier integrity, increasing the risk of pathogen invasion.

By contrast, tilapia in the 10^5 CFU/mL group exhibited much healthier intestinal morphology. As illustrated in Fig. (3), their intestines featured taller, more uniformly shaped villi, increased mucosal thickness, and deeper crypts, along with minimal signs of inflammation. These structural enhancements point to a more robust epithelial renewal process and improved nutrient absorption—critical under stress conditions. These outcomes mirror those of **Selim and Reda (2015)** and **Won *et al*. (2020)**, who emphasized the gut-protective properties of *B. subtilis*, particularly its ability to stimulate mucosal regeneration and maintain epithelial integrity.

Quantitative measurements confirmed the histological improvements. The 10^5 CFU/mL group showed significantly greater villus height and width, deeper crypts, and thicker mucosal layers compared to the control and higher-dose groups ($P < 0.05$). These features have been directly linked to increased digestive and absorptive capacity in studies like that of **Jian *et al.* (2022)**, who demonstrated how probiotic supplementation accelerates epithelial turnover. Interestingly, fish receiving the highest probiotic dose (10^9 CFU/mL) occasionally displayed mild epithelial disorganization, again suggesting that excessive dosing may lead to homeostatic imbalances—an issue highlighted by **Romanova *et al.* (2020)** and **Khunrang *et al*. (2021)**.

The mode of action by which *B. subtilis* exerts its histological benefits likely involves multiple pathways. The probiotic stimulates epithelial regeneration **(Sun *et al*.,**

2012), contributes to microbial homeostasis in the gut **(Zhou *et al*., 2016)**, and enhances mucus production **(Chen *et al*., 2020)**, thereby reinforcing the intestinal barrier and improving overall digestive function. These mechanisms become especially relevant in saline aquaculture, where environmental stressors often lead to early breakdowns in gut health.

Taken together, the observed improvements in gut architecture, hematological balance, and growth performance point to the value of *Bacillus subtilis* as a multi-functional additive in saline aquaculture systems. As salinity stress continues to challenge fish health and farm productivity, the use of probiotics at optimal dosages offers a practical and sustainable alternative to chemical treatments and antibiotics. These results reaffirm the findings of **Adel *et al*. (2016)**, **Kim *et al*. (2020)** and **Romanova *et al*. (2022**), who advocate for probiotics as viable tools for improving fish welfare, boosting resilience, and enhancing the overall sustainability of aquaculture.

While *Bacillus subtilis* demonstrates great promise as an environmentally friendly alternative to antibiotics, fish responses to supplementation may vary depending on rearing conditions such as water quality, environmental stress levels, and feed type. Therefore, further research is needed to evaluate the optimal dosage and effectiveness of Bacillus subtilis across diverse cultural environments. Additional studies including pathogen challenge tests and long-term trials will also be valuable to fully understand the mechanisms and broad impacts of this probiotic.

CONCLUSION

Dietary supplementation of *Bacillus subtilis* at a concentration of 10^5 CFU/mL proved highly effective in enhancing hematological health, growth performance, and intestinal integrity of the Nile tilapia (*Oreochromis niloticus*) reared under elevated salinity conditions. Fish receiving this probiotic dose consistently showed superior erythrocyte counts, hematocrit values, weight gain, specific growth rate, and feed conversion efficiency compared to both control and higher or lower dosage groups. Additionally, histological assessment revealed marked improvements in intestinal villus structure and mucosal thickness, with minimal inflammatory infiltration, indicating strengthened gut function and resilience to osmotic stress. These findings highlight the potential of Bacillus subtilis as a sustainable, antibiotic-free dietary strategy to boost fish health and productivity in saline aquaculture environments. Adoption of this optimal probiotic dose could help aquaculture producers mitigate environmental stressors while reducing reliance on chemical interventions, thereby promoting more resilient and sustainable fish farming practices in the face of increasing salinity challenges.

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