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Effects of dietary supplementation with garlic and *Bacillus subtilis* on growth performance and stress of *Anabas testudineus*

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Abstract The effects of dietary supplementation with garlic and *B. subtilis* on the growth performance and stress tolerance of *A. testudineus* were investigated to utilize a total of 12 tanks with sex-reversed fish averaging 1.86±0.559 g, the experiment was conducted over 8 weeks with four dietary treatments: control, garlic, *B. subtilis* at 1 x 10^5 CFU/kg, 1 x 10^7 CFU/kg, and 1 x 10^9 CFU/kg. Growth parameters including final weight, mean length gain, weight gain, percentage weight gain, average daily growth, and specific growth rate showed significant improvements ($p<0.05$) in fish fed the diet supplemented with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, compared to other treatments. This diet also resulted in the highest survival rate (100%) compared to the control (87.50%). Feed efficiency metrics such as average daily feed intake, feed conversion ratio, and feed efficiency were significantly better in the 1 x 10^9 CFU/kg treatment group. Water quality analysis indicated no significant differences in temperature ($p>0.05$) but showed better pH and dissolved oxygen levels in the same treatment group. Carcass composition revealed a higher fillet percentage and lower skeletal proportion in fish fed with the garlic and *B. subtilis* diet at 1 x 10^9 CFU/kg. Proximate analysis of the fish flesh demonstrated higher crude protein and ether extract content for the same diet. Additionally, stress tolerance experiments under temperature fluctuations revealed that fish receiving the garlic and *B. subtilis* diet at 1 x 10^9 CFU/kg exhibited significantly lower mortality rates under stress conditions at 15°C and 35°C. Overall, dietary supplementation with garlic and *B. subtilis*, particularly at 1 x 10^9 CFU/kg, enhanced growth performance, feed efficiency, and stress resilience in *A. testudineus.*

Keywords: *Anabas testudineus*, *Bacillus subtilis*, Dietary supplementation, Garlic, Growth performance

Introduction

Aquaculture, the cultivation of aquatic organisms such as fish, shellfish, and aquatic plants, has become a crucial component of global food production. As the world's population increases and the demand for protein rises—particularly in developing countries where fish is a primary dietary protein source—

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aquaculture presents a vital solution (FAO, 2022). This industry not only supports food security but also contributes significantly to the global economy by providing livelihoods for millions and easing pressure on overexploited wild fish stocks (FAO, 2022). The growth of aquaculture is driven by its potential for sustainable seafood production, addressing the decline in wild fish populations caused by overfishing and environmental degradation. Despite its benefits, the sector faces challenges such as disease management, water quality control, and environmental stress mitigation, all of which are critical for improving efficiency and sustainability. The climbing perch *(Anabas testudineus)*, a freshwater fish native to Southeast Asia, is a promising species for aquaculture due to its adaptability to various aquatic environments, including freshwater and brackish water (Debroy *et al*., 2022). Known for its ability to breathe air and migrate over land to find suitable habitats, *A. testudineus* demonstrates significant resilience (Gonzalez *et al*., 2019). However, it faces challenges related to environmental stressors, with fluctuations in water quality parameters such as salinity and pH adversely affecting growth, health, and survival (Kohinoor *et al*., 2016). Environmental stressors can lead to decreased growth rates, increased disease susceptibility, and higher mortality, impacting the economic viability of aquaculture ventures. Managing environmental stressors is essential for maintaining fish health and optimizing growth. Variations in temperature, salinity, pH, and oxygen levels can cause physiological and biochemical changes that negatively affect growth, feed utilization, and immune function (Barton, 2002). Many fish species, including *A. testudineus*, are sensitive to salinity fluctuations, which can disrupt osmoregulation, impair growth, and weaken immune responses (Kohinoor *et al*., 2016). Similarly, extreme pH levels can disrupt physiological processes such as respiration and enzyme activity, making stable pH levels crucial for fish health (Martinez *et al*., 2010). Temperature extremes can stress fish, affecting their metabolism and immune responses (Pankhurst and Porter, 2001). Maintaining optimal temperatures is crucial for the health and productivity of fish populations. To mitigate the effects of environmental stressors and improve fish health, dietary supplements such as garlic *(Allium sativum)* and probiotics like *Bacillus subtilis* have been explored. Garlic is known for its medicinal properties, including antioxidant, antimicrobial, and immunostimulatory effects (Haris, 2001; Allison *et al*., 2006). In aquaculture, garlic is used to enhance fish health by boosting immune responses and improving disease resistance. The active compound allicin in garlic has antimicrobial properties that may reduce infection rates (Yang *et al*., 2010). Probiotics like *B. subtilis* are live microorganisms that offer health benefits when administered in adequate amounts (FAO and WHO, 2001). *B. subtilis* has been studied for its positive effects on gut health, immune function, and stress resilience in fish (Balcázar *et al*., 2008; Pandiyan *et al*., 2013). Probiotics can improve nutrient utilization, gut flora balance, and the fish's ability to manage environmental stressors. Research has shown promising results for dietary supplements in improving fish health and performance. Garlic supplementation has been found to enhance growth and immune responses in various fish species, including tilapia and catfish (Gafar *et al*., 2012; Huang *et al*., 2001). Similarly, *B. subtilis* has been shown to improve growth rates, feed conversion ratios, and stress tolerance in species like trout and shrimp (Balcázar *et al*., 2008; Saleh *et al*., 2018). Attia *et al*. (2020) highlighted the benefits of probiotics in fish feed, noting improvements in growth performance and health. Panigrahi *et al*. (2005) found that probiotics could enhance disease resistance and stress tolerance. However, more targeted research is needed to understand the effects of garlic and *B. subtilis* specifically for *A. testudineus.* Investigating how these supplements influence growth performance and stress resilience under various environmental conditions is essential for optimizing aquaculture practices.

The objective was to evaluate the impact of dietary supplementation with garlic powder and *Bacillus subtilis* on the growth performance and stress resilience of *Anabas testudineus*. Specifically, it aimed to assess key growth parameters such as final weight, weight gain, length gain, and specific growth rate. Additionally, the study focused on the fish's ability to withstand environmental stressors by measuring survival rates, behavioral responses, and physiological stress indicators.

Materials and methods

Preparation of experimental units

Cement tanks (100 cm in diameter, 50 cm in height, with a water depth of 30 cm) equipped with continuous aeration were used. Thirty-day-old sex-reversed *A. testudineus* were obtained from a commercial farm and stocked in each tank at a density of 40 fish per tank (equivalent to 50.63 fish/m²). A total of twelve tanks were divided into four experimental groups, with three replicates per group. The study investigated the effects of dietary garlic powder and *B. subtilis* on the growth and stress response of *A. testudineus* over 8 weeks.

Preparation of experimental diet

Diets were formulated with varying levels of garlic and *B. subtilis*. Garlic was sourced from Sisaket Province, cleaned, peeled, sliced, and dried at 50°C for

72 hours before being ground and stored at 4°C. *B. subtilis* was incorporated into the diets at different concentrations, as detailed in Table 1.

Table 1. Dietary formulation and proximate composition analysis of experimental diets containing different levels of garlic and *B. subtilis*

Ingredient (g dry weight)	**Dietary garlic + *B. subtilis* (CFU/kg)**			
	Control	$1x10^5$	$1x10^7$	$1x10^9$
Fish meal (CP 60%)	320	320	320	320
Soybean meal	160	160	160	160
Tapioca residue	160	160	160	160
Tapioca Chip	140	140	140	140
Rice bran	85	85	85	85
Palm kernel meal	80	80	80	80
Thickener	25	25	25	25
Vit–min premixa	10	10	10	10
A. sativum	20	20	20	20
B. subtilis	-	$1x10^5$	$1x10^7$	$1x10^9$
Proximate composition				
Dry matter	89.80	90.00	90.01	90.04
Organic matter	30.0	30.2	30.3	30.6
Crude protein	27.7	27.7	27.7	27.7
Ash	14.7	14.7	14.8	14.8
Ether extract	3.3	3.3	3.3	3.3
Crude fiber	9.6	9.6	9.7	9.7

Feeding and water management

Fish were fed the experimental diets to satiation twice daily (0900H and 1700H). Before the experiment, they were acclimated and fed a commercial diet at 5% of their body weight per day. Weekly, 80% of the water in the tanks was replaced, and water quality was maintained by removing sediments and adding fresh water.

Experimental plan

This experiment employed a completely randomized design with 4 treatment groups and 3 replicates per group to assess the impact of garlic and *B. subtilis* supplementation on the growth of *A. testudineus* over 8 weeks. The treatments were as follows: experimental set 1: *B. subtilis* 0 CFU/kg of diet (control), experimental set 2: *B. subtilis* 1 x 10^5 CFU/kg of diet, experimental set 3: *B. subtilis* 1 x 10^7 CFU/kg of diet and experimental set 4: *B. subtilis* 1 x 10^9 CFU/kg of diet. Each treatment was supplemented with 20 grams of garlic per kilogram of diet. The diets were mixed, pelletized to a size of 2 mm, dried, and stored in plastic bags at 4°C until further use. The dried diet was packaged into plastic bag and stored frozen at -20°C until use. The pelleted diets were mechanically dried and stored in plastic bags at 4°C until further use.

Data collection

The growth rate of fish was randomly sampled from 30% of the total fish population, with each sample taken in duplicate. Data were recorded biweekly over an 8-week period. The percentage weight gain (PWG) was calculated using the method described by De Silva and Anderson (1995), as shown in Equation 1.

$$\text{PWG (\%)} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \times 100 \quad \text{.................................(1)}$$

The mean length gain (LG) was calculated using the method described by De Silva and Anderson (1995), as shown in Equation 2.

$$\text{LG (cm)} = \text{Final length} - \text{Initial length} \quad \text{.......................................(2)}$$

The specific growth rate (SGR) was calculated using the method described by Steffens (1989), as presented in Equation 3.

$$\text{SGR (\%/day)} = \frac{(\ln \text{final weight} - \ln \text{Initial weight})}{\text{number of days}} \times 100 \quad \text{..........................(3)}$$

The average daily growth (ADG) was calculated using the method described by De Silva and Anderson (1995), as presented in Equation 4.

$$\text{ADG (g/fish/day)} = \frac{\text{body weight gain (g)}}{\text{number of days}} \quad \text{..................................(4)}$$

The survival rate (SR) was calculated using the method described by Steffens (1989), as shown in Equation 5.

$$\text{Survival rate (\%)} = \frac{\text{number of surviving at the end of the production cycle}}{\text{total number at the beginning of the cycle}} \times 100 \quad \text{....(5)}$$

The efficiency of the diet used for the fish was recorded at the end of the 8-week experiment. The average daily feed intake (ADFI) was calculated using the method described by De Silva and Anderson (1995), as presented in Equation 6.

$$\text{ADFI (g/day)} = \frac{\text{amount of diet eaten (g)}}{\text{number of days}} \quad \text{......................................(6)}$$

The feed conversion ratio (FCR) was calculated using the method described by Steffens (1989), as presented in Equation 7.

$$\text{FCR} = \frac{\text{Total weight of diet (dry) eaten by fish}}{\text{increased fish weight}} \quad \text{.................................(7)}$$

The feed efficiency (FE) was calculated using the method described by De Silva and Anderson (1995), as shown in Equation 8.

FE (%) = 1 / Feed conversion ratio x 100 …………………...………………(8)

Water quality in fish ponds was monitored weekly at a depth of 15 cm at 1100H before water changes over an 8-week period. The recorded parameters included water temperature (°C), pH value, and dissolved oxygen (DO).

Carcass composition: At the end of the experiment, five fish samples were collected. The samples were processed by cutting open the abdomen and dividing the carcasses into three parts: fillet, skeleton, and viscera. Each part was then weighed, and the proportions were calculated as shown in Equations 9 and 10.

$$\text{Fillet (\%)} = \frac{\text{Meat weight}}{\text{weight of fish}} \times 100 \quad (9)$$

$$\text{Skeleton (\%)} = \frac{\text{Skeleton weight}}{\text{weight of fish}} \times 100 \quad (10)$$

The hepatosomatic index (HSI) was calculated using the method described by Anwar and Jafri (1995), as presented in Equation 11.

$$\text{HSI (\%)} = \frac{\text{liver weight (g)}}{\text{weight of fish (g)}} \times 100 \quad (11)$$

The viscerosomatic index (VSI) was described by Anwar and Jafri (1995) and was calculated as shown in Equation 12.

$$\text{VSI (\%)} = \frac{\text{internal organ weight (g)}}{\text{weight of fish (g)}} \times 100 \quad (12)$$

Proximate Composition: The proximate composition of fish flesh, including moisture, crude protein, crude fat, and ash, was analyzed using the methods of AOAC (2016).

Stress challenge

The dietary treatments consisted of garlic supplementation at 20 g per kg of feed and *B. subtilis* at a concentration of 1 x 10^9 CFU per kg of feed, applied as follows: Control Group: Standard diet with 27.7% protein for 45 days. Garlic and *B. subtilis* Supplementation Groups: Diets supplemented with garlic and *B. subtilis* for 15, 30, and 45 days. Post-supplementation Group: Supplemented diet for 45 days, then standard diet for 15 days (45 + 15 Days).

Testing for stress responses

Temperature stress experiments were conducted in aquaria (45 cm x 90 cm x 46 cm, water depth 30 cm) using ice and cooling systems to reach 15°C and heating systems to reach 35°C. For salinity stress, fish were kept in aquaria with the same dimensions, with salinity levels adjusted to 25 ppt and 40 ppt. pH stress was tested in similar aquaria, with pH levels set at 4 and 10. Ten fish per replicate were exposed to each condition, and mortality and behavioral changes were monitored at 5, 15, 30, 60, 90, 120, 180, and 360 minutes. Mortality rates were recorded daily for each group.

Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA). Differences between means were assessed using Duncan's New Multiple Range Test at a 95% confidence level.

Results

Growth performance rate of A. testudineus

The growth performance of *A. testudineus* was evaluated in response to dietary supplementation with garlic and different concentrations of *B. subtilis*. The results are summarized in Table 2. Final Weight: Fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg had the highest final weight of 32.63±0.480 g, significantly different from other treatments. The final weights for the treatments with 1 x 10^7, 1 x 10^5 CFU/kg, and the control were 31.96±0.482 g, 30.81±0.431 g, and 30.56±0.855 g, respectively. Mean Length Gain: The highest mean length gain of 6.62±0.167 cm was observed in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, which was significantly higher than other treatments. The mean length gains for the other treatments were 6.41±0.155 cm, 6.03±0.136 cm, and 5.98±0.311 cm, respectively. Weight Gain: Fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg showed the greatest weight gain of 30.77±0.480 g, significantly different from other treatments. The gains for other treatments were 30.10±0.482 g, 28.95±0.431 g, and 28.70±0.855 g, respectively. Percentage Weight Gain: The highest percentage weight gain of 1,654.39±25.807% was recorded in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments. The percentages for other treatments were 1,618.50±25.928%, 1,556.23±23.162%, and 1,542.98±45.990%, respectively. Average Daily Growth: The average daily growth was highest in the garlic and *B. subtilis* at 1 x 10^9 CFU/kg with 0.55±0.010 g/fish/day, significantly higher than other treatments. The average daily growth for other treatments were 0.54±0.010 g/fish/day, 0.52±0.008 g/fish/day, and 0.51±0.016 g/fish/day, respectively. Specific Growth Rate: The highest specific growth rate of 6.12±0.028%/day was observed in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments. The specific growth rates for other treatments were 6.08±0.029%/day, 6.01±0.027%/day, and 5.99±0.052%/day, respectively. Survival Rate: The highest survival rate of 100.00±0.000% was found in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly higher than other treatments. The survival rates for other treatments were 98.33±1.443%, 95.00±2.500%, and 87.50±2.500%, respectively (Table 2).

Feed efficiency

Average Daily Feed Intake (ADFI): The highest ADFI of 0.79±0.012%/day was observed in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments ($p<0.05$). The ADFI for other treatments were 0.85±0.015%/day, 0.94±0.031%/day, and 1.00±0.010%/day, respectively. Feed Conversion Ratio (FCR): The best feed conversion ratio of 1.52±0.064 was achieved by fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments ($p<0.05$). The FCR for other treatments were 1.69±0.012, 1.80±0.085, and 1.99±0.023, respectively. Feed Efficiency (FE): The highest feed efficiency of 65.76±0.630% was observed in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments ($p<0.05$). The feed efficiency for other treatments were 58.96±0.390%, 55.47±0.601%, and 50.32±0.611%, respectively (Table 2).

Table 2. Growth performance and feed efficiency of fish fed diets supplemented with different levels of garlic for 8 weeks

Parameters	Garlic 20 g/kg diet and *B. subtilis* (CUF/kg diet)			
	0	**1 x 10^5**	**1 x 10^7**	**1 x 10^9**
Initial weight (g)	1.86±0.559	1.86±0.559	1.86±0.559	1.86±0.559
Final weight (g)	30.56±0.855^a	30.81±0.431^a	31.96±0.482^b	32.63±0.480^c
LG (cm)	5.98±0.311^a	6.03±0.136^a	6.41±0.155^b	6.62±0.167^c
WG (g)	28.7±0.855^a	28.95±0.431^a	30.10±0.482^b	30.77±0.480^c
PWG (%)	1542.98±45.990^a	1556.23±23.162^a	1618.50±25.928^b	1654.39±25.807^c
ADG (g/fish/day)	0.51±0.016^a	0.52±0.008^a	0.54±0.010^b	0.55±0.010^c
SGR (%/day)	5.99±0.052^a	6.01±0.027^a	6.08±0.029^b	6.12±0.028^c
SR (%)	87.50±2.500^a	95.00±2.500^b	98.33±1.443^{bc}	100.00±0.000^c
ADFI (g/day)	1.00±0.010^a	0.94±0.031^b	0.85±0.015^c	0.79±0.012^d
FCR	1.99±0.023^a	1.80±0.085^b	1.69±0.012^b	1.52±0.064^c
FE (%)	50.32±0.611^a	55.47±0.601^b	58.96±0.390^b	65.76±0.630^c

Each value indicates the average ± standard deviation. Means in each row with different superscripts are significantly different ($p < 0.05$). Abbreviations: LG = Mean length gain; WG = Weight Gain; PWG = Percentage Weight Gain; ADG = Average diary growth; SGR = Specific growth rate; SR = survival rate; ADFT = Average Daily Feed Intake; FCR = Feed conversion ratio; FE = Feed efficiency

Water quality

Temperature: No significant differences in water temperature were observed among different treatments. The average temperatures ranged from 0.79±0.012°C to 1.00±0.010°C. pH: Significant differences in pH were noted, with the highest pH of 7.17±0.058 observed in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg. Other treatments recorded pH values of 6.93±0.058, 6.67±0.058, and 6.57±0.058. Dissolved Oxygen (DO): The highest DO of 6.47±0.058 mg/L was found in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments. The DO for other treatments were 6.27±0.058 mg/L, 6.07±0.015 mg/L, and 6.00±0.058 mg/L (Table 3).

Carcass composition

Fillet Proportion: The highest fillet proportion of 36.40±0.365% was observed in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments. The proportions for other treatments were 35.10±0.820%, 34.14±0.672%, and 33.57±0.480%. Skeleton Proportion: The lowest skeleton proportion of 53.61±0.404% was found in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments. The proportions for other treatments were 55.09±0.658%, 56.52±0.620%, and 57.29±0.331%. Hepatosomatic Index (HSI): The highest HSI of 2.79±0.090 was recorded in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments. The HSIs for other treatments were 2.53±0.108, 2.26±0.090, and 1.95±0.050. Viscerosomatic Index (VSI): The highest VSI of 9.99±0.173% was observed in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments. The VSIs for other treatments were 9.81±0.281%, 9.34±0.320%, and 9.14±0.321% (Table 3).

Proximate composition

Dry Matter and Ash: No significant differences in dry matter and ash percentages were observed ($p>0.05$), as presented in Table 3. Crude Protein: The highest crude protein content of 77.74±1.385% was found in fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, significantly different from other treatments ($p<0.05$). The crude protein contents for other treatments were 74.58±0.398%, 72.65±1.260%, and 71.79±0.676%. Ether Extract: The highest ether extract content of 2.45±0.049% was found in fish fed with garlic and *B. subtilis* at 1 x 10^5 CFU/kg, significantly different from other treatments. The ether extracts for other treatments were 2.06±0.116%, 1.20±0.087%, and 1.13±0.119% (Table 3).

Effect of dietary supplementation with garlic and B. subtilis on temperature-induced stress

The effects of garlic and *B. subtilis* supplementation on temperature-induced stress in *A. testudineus* were evaluated at two temperatures: 15°C and 35°C. At 15°C: 5 minutes**:** The control group had a significantly higher cumulative mortality rate (56.67%) compared to the garlic and *B. subtilis* supplementation groups (Table 4). The mortality rates for these groups were 33.33% for the 15-day supplement, 20.00% for the 30-day supplement, 10.00% for the 45-day supplement, and 6.67% for the 45+15-day supplement. At 15 minutes: The control group showed the highest mortality rate (100.00%). The rates for the garlic and *B. subtilis* groups were 80.00% (15-day), 63.33% (30-day), 43.33% (45-day), and 36.67% (45+15-day) at 30 minutes.

Table 3. Water quality, carcass composition, and proximate analysis of fish-fed diets supplemented with different levels of garlic for 8 weeks

Parameters	Garlic 20 g/kg diet and *B. subtilis* (CUF/kg diet)			
	0	1 x 10^5	1 x 10^7	1 x 10^9
Water quality				
Temperature (°C)	28.40±0.100	28.47±0.058	28.30±0.100	28.39±0.116
pH	6.57±0.058^a	6.67±0.058^a	6.93±0.058^b	7.17±0.058^c
DO (mg/L)	6.00±0.058^a	6.07±0.015^a	6.27±0.058^b	6.47±0.058^c
Carcass composition				
Fillet (%)	33.57±0.480^a	34.14±0.672^a	35.10±0.820^b	36.40±0.365^c
Skeleton (%)	57.29±0.331^d	56.52±0.620^c	55.09±0.658^b	53.61±0.404^a
HIS (%)	1.95±0.050^a	2.26±0.090^a	2.53±0.108^b	2.79±0.090^b
VSI (%)	9.14±0.321^a	9.34±0.320^a	9.81±0.281^b	9.99±0.173^b
Proximate analysis				
Dry matter (%)	23.61±0.639	23.19±0.7193	23.56±0.564	23.91±0.5198
Crude protein (%)	71.79±0.676^a	72.65±1.260^a	74.58±0.398^b	77.74±1.385^c
Ether extract (%)	2.06±0.116^b	2.45±0.049^c	1.13±0.119^a	1.20±0.087^a
Ash (%)	18.58±0.785	18.35±1.091	18.06±0.675	17.65±1.179

Each value indicates the average ± standard deviation. Means in each row with different superscripts are significantly different ($p < 0.05$). Abbreviations: DO = Dissolved Oxygen; HIS = Hepatosomatic index; VSI = Viscerosomatic index

The control group also exhibited the highest mortality rate (100.00%), as presented in Table 4. The garlic and *B. subtilis* groups had mortality rates of 93.33% (15-day), 83.33% (30-day), 63.33% (45-day), and 50.00% (45+15-day). At 60 minutes: The control and the 15-day and 30-day supplementation groups had a cumulative mortality rate of 100.00%. The 45-day and 45+15-day supplementation groups had lower rates of 76.67% and 60.00%, respectively. At

90 minutes: The control and the 15, 30, and 45-day supplementation groups all reached 100.00% mortality. The 45+15-day group had a rate of 70.00%. At 120 minutes:- all groups reached 100.00% mortality ($p>0.05$). At 180 minutes: All groups reached 100.00% mortality. At 35°C: 5 minutes: No significant differences were observed among groups, with all having 100% survival. At 15 minutes: The control group had a higher cumulative mortality rate (56.67%) compared to the garlic and *B. subtilis* group, which had a rate of 6.67%. At 30 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67%. At 60 minutes: The control group had a mortality rate of 13.33%, and the 15-day supplementation group had 6.67% ($p<0.05$). At 90 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67%. At 120 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67%. At 180 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67%. At 360 minutes: The control group had a mortality rate of 13.33%, and the 15-day supplementation group had 6.67% (Table 4).

Table 4. Cumulative mortality rate (%) challenge of *A. testudineus* as affected by incorporation of garlic (20 g/kg diet) and *B. subtilis* (1×10^9 CUF/kg diet) in the diets

Challenge		Cumulative mortality rate (%)				
		0 Day	15 Days	30 Days	45 Days	45+15 Days
5 min	Temperature 15°C	56.67 ± 5.773^{d}	33.33 ± 5.773^{d}	20.00 ± 0.000^{c}	10.00 ± 0.000^{b}	6.67 ± 5.773^{a}
	Temperature 35°C	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
	Salinity 25 ppt	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
	Salinity 40 ppt	6.67 ± 5.773^{b}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}
	pH 4	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
	pH 10	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
15 min	Temperature 15°C	100.00 ± 0.000^{c}	86.67 ± 15.275^{c}	63.33 ± 5.774^{b}	43.33 ± 5.774^{a}	36.67 ± 5.774^{a}
	Temperature 35°C	13.33 ± 5.774^{b}	6.67 ± 5.774^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}
	Salinity 25 ppt	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
	Salinity 40 ppt	36.67 ± 11.547^{b}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}
	pH 4	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
	pH 10	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
30 min	Temperature 15°C	100.00 ± 0.000^{d}	$93.33^{c}\pm5.774$	$83.33^{b}\pm5.774$	$63.33^{a}\pm5.774$	$50.00^{a}\pm10.000$
	Temperature 35°C	13.33 ± 5.774^{b}	6.67 ± 5.774^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}
	Salinity 25 ppt	3.33±5.774	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
	Salinity 40 ppt	80.00 ± 10.000^{c}	23.33 ± 5.774^{b}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}	0.00 ± 0.000^{a}

Challenge		Cumulative mortality rate (%)				
		0 Day	15 Days	30 Days	45 Days	45+15 Days
	pH 4	23.33±5.774^{b}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	pH 10	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
60 min	Temperature 15°C	100.00±0.000^{c}	100.00±0.000^{b}	100.00±0.000^{a}	76.67±5.774^{a}	60.00±10.000^{a}
	Temperature 35°C	13.33±5.774^{b}	6.67±5.774^{b}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 25 ppt	10.00±0.000^{b}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 40 ppt	100.00±0.000^{d}	66.67±5.774^{c}	26.67±5.774^{b}	16.67±5.774^{a}	13.33±5.774^{a}
	pH 4	50.00±17.321^{b}	13.33±5.774^{a}	10.00±0.000^{a}	3.33±5.774^{a}	3.33±5.774^{a}
	pH 10	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
90 min	Temperature 15°C	100.00±0.000^{b}	100.00±0.000^{b}	100.00±0.000^{b}	100.00±0.000^{b}	70.00±10.000^{a}
	Temperature 35°C	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 25 ppt	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 40 ppt	100.00±0.000^{c}	100.00±0.000^{c}	60.00±10.000^{b}	43.33±11.547^{a}	36.67±5.774^{a}
	pH 4	70.00±10.000^{c}	36.67±5.774^{b}	30.00±10.000^{b}	10.00±10.000^{a}	10.00±10.000^{a}
	pH 10	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
120 min	Temperature 15°C	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	Temperature 35°C	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 25 ppt	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 40 ppt	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	pH 4	100.00±0.000^{c}	100.00±0.000^{c}	100.00±0.000^{c}	56.67±11.547^{b}	33.33±11.547^{a}
	pH 10	66.67±5.774^{d}	56.67±5.774^{cd}	46.67±5.774^{bc}	43.33±5.774^{ab}	33.33±5.774^{a}
180 min	Temperature 15°C	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	Temperature 35°C	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 25 ppt	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 40 ppt	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	pH 4	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	pH 10	100.00±0.000^{b}	100.00±0.000^{b}	100.00±0.000^{b}	90.00±10.000^{a}	83.33±5.774^{a}
360 min	Temperature 15°C	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	Temperature 35°C	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 25 ppt	13.33±5.774^{b}	6.67±5.774^{a}	0.00±0.000^{a}	0.00±0.000^{a}	0.00±0.000^{a}
	Salinity 40 ppt	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	pH 4	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000
	pH 10	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000	100.00±0.000

Each value indicates the average ± standard deviation. Each value indicates the average ± standard deviation. Means in each row with different superscripts are significantly different ($p < 0.05$)

Effect of dietary supplementation with garlic and B. subtilis on salinity-induced stress

The study evaluated the stress response to salinity changes at levels of 25 ppt and 40 ppt. At 25 ppt, 5 minutes: No significant differences among groups, with all showing 100% survival. At 15 minutes: No significant differences, with all groups showing 100% survival ($p>0.05$). At 30 minutes: The control group showed a mortality rate of 3.33%, while other groups had no significant differences. At 60 minutes: The control group had a mortality rate of 10.00%, with no significant differences among other groups. At 90 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67% ($p<0.05$). At 120 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67%. At 180 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67%. At 360 minutes: The control group had the highest mortality rate (13.33%), while the 15-day supplementation group had 6.67%. At 40 ppt, 5 minutes: The control group had a mortality rate of 6.67%, while other groups had no significant differences. At 15 minutes: The control group had a mortality rate of 36.67%, while other groups had no significant differences. At 30 minutes: The control group had the highest mortality rate (80.00%), while the 15-day supplementation group had 23.33%. At 60 minutes: The control group had the highest mortality rate (50.00%), while the 15, 30, 45, and 45+15-day groups had rates of 66.67%, 26.67%, 16.67%, and 13.33%. At 90 minutes: The control and 15-day supplementation groups had 100% mortality, while the 30, 45, and 45+15-day groups had rates of 60.00%, 43.33%, and 36.67%. At 120 minutes: The control group had the highest mortality rate (100.00%), while the 15-day supplementation group had 6.67% ($p<0.05$). At 180 minutes: The control group had the highest mortality rate (100.00%), while the 15-day supplementation group had 6.67% ($p<0.05$). At 360 minutes: All groups had 100% mortality (Table 4).

Effect of dietary supplementation with garlic and B. subtilis on pH-induced stress

The effect of garlic and *B. subtilis* supplementation on pH-induced stress was tested at pH levels of 4 and 10. At pH 4, 15 minutes: No significant differences, with all groups showing 100% survival. At 30 minutes: The control group had a mortality rate of 23.33%, while the garlic and *B. subtilis* groups had no significant differences. At 60 minutes: The control group had the highest mortality rate (50.00%), while the 15, 30, 45, and 45+15-day groups had rates of 13.33%, 10.00%, 3.33%, and 3.33%, respectively. At 90 minutes: The control group had a mortality rate of 70.00%, while the 15, 30, 45, and 45+15-day groups had rates of 36.67%, 30.00%, 10.00%, and 10.00%, respectively. At 120 minutes: All groups reached 100% mortality. At 180 minutes: All groups reached 100% mortality. At pH 10, 15 minutes: No significant differences, with all groups showing 100% survival. At 30 minutes: No significant differences, with all groups showing 100% survival. At 60 minutes: The control group had a mortality rate of 100.00%, while the 15, 30, 45, and 45+15-day groups had rates of 56.67%, 46.67%, 43.33%, and 33.33%, respectively. At 90 minutes: The control group had a mortality rate of 100.00%, while the 15, 30, 45, and 45+15-day groups had rates of 56.67%, 46.67%, 43.33%, and 33.33%, respectively. 120 minutes: The control group had 100% mortality, while the 15-day supplementation group had 70.00%. 180 minutes: The control group had 100% mortality, while the 15-day supplementation group had 30.00% (Table 4).

Discussion

Growth performance rate

The fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg achieved the highest final weight (32.63±0.480 g), mean length gain (6.62±0.167 cm), weight gain (30.77±0.480 g), percentage weight gain (1,654.39±25.807%), average daily growth (0.55±0.010 g/fish/day), and specific growth rate (6.12±0.028%/day). These results suggest that this treatment significantly improved growth metrics compared to lower concentrations of *B. subtilis* and the control group. The enhanced growth performance observed can be attributed to the combined effects of garlic and high concentrations of *B. subtilis*, which may positively influence digestion, nutrient absorption, and overall health (Güroy *et al*., 2024; Zare *et al*., 2021). The survival rate was also highest (100.00±0.000%) in the fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg, which indicates improved health and resilience against environmental stress. This aligns with findings from other studies showing that garlic and probiotics can enhance fish health and survival rates by modulating the gut microbiota and improving immune responses (Adorian *et al*., 2018; Liao *et al*., 2022).

Feed efficiency

In terms of feed efficiency, the highest average daily feed intake (0.79±0.012%/day) and feed efficiency (65.76±0.630%) were observed in the same treatment group. Conversely, the feed conversion ratio (FCR) was the lowest (1.52±0.064) for this group, indicating more efficient feed utilization. This is consistent with research indicating that probiotics can enhance feed utilization by promoting better digestion and nutrient absorption (Yılmaz and Tan, 2022). The improved feed efficiency and lower FCR observed in the garlic and *B. subtilis* treatment group suggest that the combination of these additives has a synergistic effect on feed utilization. This finding is supported by studies on the effects of garlic and probiotics on growth performance and feed efficiency in various fish species (Ibrahim Saad *et al*., 2021; Petruța *et al*., 2023).

Water quality

This study highlights key aspects of water quality related to different treatments for *A. testudineus*. Water temperature remained stable across treatments, with no significant differences ($p>0.05$), ensuring that temperature did not affect the treatment outcomes. The temperature range of 0.79±0.012°C to 1.00±0.010°C was appropriate for the species. Significant differences in pH were observed, with the highest pH of 7.17±0.058 in fish fed garlic and *B. subtilis* at 1 x 10^9 CFU/kg. This increase in pH is likely due to the buffering capacity of these additives, which may improve water stability and fish health. Other treatments had pH values between 6.57±0.058 and 6.93±0.058. Dissolved Oxygen (DO) levels were also significantly higher in the garlic and *B. subtilis* group (6.47±0.058 mg/L). Elevated DO levels benefit fish respiration and health, suggesting that the garlic and probiotics enhance both water quality and fish vitality. These findings are consistent with previous research showing that dietary supplements like garlic and probiotics can positively influence water quality and fish health (Güroy *et al*., 2024; Zare *et al*., 2021).

Carcass composition

The carcass composition results reflect significant improvements in fillet proportion, skeleton proportion, hepatosomatic index (HSI), and viscerosomatic index (VSI) for fish receiving garlic and *B. subtilis* at 1 x 10^9 CFU/kg. The higher fillet proportion and lower skeleton proportion indicate better growth performance and more efficient resource allocation towards muscle development rather than skeletal growth. The increase in HSI and VSI further supports the beneficial effects of this supplementation, suggesting improved overall body condition and internal organ health. These findings align with previous studies showing that dietary additives like garlic and probiotics can positively impact fish growth and body composition (Zare *et al*., 2021; Liao *et al*., 2022).

Proximate composition

The proximate composition analysis revealed significant differences in crude protein and ether extract contents. Fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg exhibited the highest crude protein content, suggesting that this supplementation enhances protein utilization. The increase in ether extract content in fish fed with garlic and *B. subtilis* at 1 x 10^5 CFU/kg points to improved fat deposition. These results are consistent with findings from previous studies that dietary supplements can influence nutrient digestibility and composition in fish (Güroy *et al*., 2024; Ibrahim Saad *et al*., 2021).

Stress challenge

The results of this study demonstrated that dietary supplementation with garlic and *B. subtilis* can significantly mitigate temperature-induced stress in *A. testudineus*. At 15°C, a lower cumulative mortality rate was observed in fish receiving garlic and *B. subtilis* supplementation compared to the control group. Specifically, the 45-day and 45+15-day supplementation groups showed the most significant reductions in mortality rates. These findings are consistent with previous studies that highlight the benefits of garlic and probiotics in enhancing fish health and stress resilience. For instance, garlic has been shown to improve antioxidant defense mechanisms and reduce oxidative stress in fish (Zare *et al*., 2021). Similarly, *B. subtilis* has been reported to enhance the immune response and growth performance in various fish species (Juliana *et al*., 2018). When subjected to a higher temperature of 35°C, the control group exhibited significantly higher mortality rates compared to the garlic and *B. subtilis* supplemented groups, particularly in the 15-day supplementation group. This suggests that garlic and *B. subtilis* may help in stabilizing physiological processes under thermal stress, corroborating findings by Liao *et al*. (2022), who reported that natural feed supplements improve stress resistance in fish. In response to salinity-induced stress, both garlic and *B. subtilis* supplementation again demonstrated beneficial effects. At 25 ppt, significant differences in mortality rates were observed, with the control group showing higher rates compared to the supplemented groups. The findings align with earlier research indicating that garlic and probiotics can enhance stress tolerance and overall health in fish (Abd El-Ghany, 2024). At higher salinity levels (40 ppt), the supplementation groups showed markedly lower mortality rates compared to controls, particularly in the 15-day and 30-day supplementation groups. This is consistent with results from studies on the effectiveness of garlic and probiotics in improving fish survival under saline conditions (Ghafarifarsani *et al*., 2021). The pH-induced stress experiments revealed that garlic and *B. subtilis* supplementation could significantly reduce mortality rates at pH 4, with the most pronounced effects observed in the 15, 30, and 45-day supplementation groups. At pH 10, the control group had higher mortality rates compared to the supplemented groups, particularly in the 15-day supplementation group. These results support previous findings that dietary supplements can ameliorate stress responses related to pH changes (Ibrahim Saad *et al*., 2021). Overall, this study underscores the potential of garlic and *B. subtilis* as effective dietary supplements to enhance stress resilience and overall health in *A. testudineus* under various environmental stressors. These findings are supported by a body of literature demonstrating the positive effects of similar dietary interventions in aquaculture (Vijayaram *et al*., 2024; Yılmaz and Tan, 2022).

This study demonstrated that dietary supplementation with garlic and *B. subtilis* significantly improved the growth performance, feed efficiency, and stress resilience of *A. testudineus*. Fish fed with garlic and *B. subtilis* at 1 x 10^9 CFU/kg showed the highest gains in weight, feed utilization, and survival rates. The supplements positively influenced water quality, carcass composition, and protein content, while mitigating stress effects from temperature, salinity, and pH changes. These findings suggest that garlic and *B. subtilis* have synergistic benefits in enhancing fish health and resilience, supporting their use in aquaculture.

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