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Original Research Article

Effects of dietary supplementation of *Bacillus subtilis* DSM 32315 on growth, immune response and acute ammonia stress tolerance of Nile tilapia (*Oreochromis niloticus*) fed with high or low protein diets

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ABSTRACT

Aquatic animals have benefited from *Bacillus subtilis*-based probiotics over the past few decades. This study evaluated the effects of *B. subtilis* DSM 32315 probiotics as a feed additive on growth, immune response and resistance to acute ammonia challenge in Nile tilapia. Specifically, four supplemental levels (0%, 0.1%, 0.2%, and 0.3%) of *B. subtilis* probiotics were tested under two dietary protein levels (32% and 28%). Five replicate tanks were randomly allotted to each dietary treatment, with each tank containing 30 Nile tilapia. After 8 weeks of feeding, Nile tilapia in each tank were exposed to 43.61 mg/L of total ammonia nitrogen for 48 h. The results revealed that reducing protein levels from 32% to 28% did not affect growth performance or antioxidant capacity. However, the low protein diet tended to induce an inflammatory effect shown by increased expressions of *TGF-β* and *IFN-γ* genes ($P < 0.05$) in the liver. The impact was alleviated by the probiotic supplementation. Compared with the non-supplemented group, 0.1% probiotic supplementation remarkably increased plasma lysozyme activity, total antioxidant capacity and complement C3 and interleukin-10 mRNA levels ($P < 0.05$) in the 28% protein diet, while higher supplementation of probiotics (0.3%) was shown to be beneficial for the high protein diet (32%). In both the dietary protein levels, 0.1% supplementation of probiotics promoted the antioxidant capacity of Nile tilapia before exposure to ammonia stress but higher probiotic supplementation (0.3%) proved to be necessary under ammonia stress as evidenced by higher fish survival rate. Results exhibited that supplementation with *B. subtilis* probiotics had a better effect on the intestinal morphology (villi height and width) regardless of protein levels. In conclusion, dietary supplementation of *B. subtilis* DSM 32315 probiotics at 0.1% in the low protein diet and up to 0.3% in the high protein diet showed beneficial effects on the growth, immunity, and antioxidant capacity of Nile tilapia. Under ammonia stress conditions, the higher supplementation of *B. subtilis* DSM 32315 probiotics at 0.3% improves stress tolerance of Nile tilapia despite the two dietary protein levels (32%; 28%).

1. Introduction

The Nile tilapia (*Oreochromis niloticus*) is a commercially important, worldwide warm-water fish species (Guimarães et al., 2014). Intensive farming of Nile tilapia has rapidly expanded, making it the world's second most widely farmed fish, after carp (FAO, 2018). However, ammonia concentrations may reach high levels in intensive aquaculture systems, particularly in recirculating

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ones. Ammonia exists in two chemical forms in water: ionized and un-ionized. The former may cause cell death by destroying cell functions, whereas the latter is more toxic (Frías-Espericueta et al., 1999). There is considerable evidence showing that high ammonia levels induce poor growth performance, oxidative stress, metabolic disorders, and immunosuppression in aquatic animals (Cheng et al., 2015; Ren et al., 2016; Shi et al., 2015; Sun et al., 2012). In this regard, attempts to improve the resistance of Nile tilapia to ammonia stress have emerged as an important topic in Nile tilapia farming.

Functional feed additives have been demonstrated to alleviate the adverse effects of excessive ammonia on fish and shrimp. For instance, dietary yeast hydrolysate and brewer's yeast supplementation could enhance the resistance of ammonia nitrogen stress in Pacific white shrimp (*Litopenaeus vannamei*) (Jin et al., 2018). *Bacillus subtilis*, a Gram-positive bacterium, is well-known for its inherent property to produce spores and display resistance to various environmental stresses (Hong et al., 2005). Previous studies suggested dietary *B. subtilis* HAINUP40 supplementation enhances Nile tilapia growth, digestive enzyme activities, innate immune responses and disease resistance (Liu et al., 2017). Similarly, Telli et al. (2014) discovered that *B. subtilis* improved the nonspecific immune response of Nile tilapia at high stocking density. Other similar studies have also confirmed that *B. subtilis* enhances growth performance and immune status in fish and shrimp (Olmos et al., 2020; Touraki et al., 2012). However, whether *B. subtilis* positively improves resistance to ammonia nitrogen stress must be verified further.

Dietary protein is one of the most costly nutrient components in feed, playing a major role in determining feed quality and fish performance. Dietary protein quality is assessed by its amino acid profile than the crude protein content itself. Optimal dietary protein levels of Nile tilapia vary from 30% to 42% and may be affected by size, dietary protein source and level, feeding strategy, management method, and experimental conditions (Galagarza et al., 2018; Liu et al., 2017; Van et al., 2021; Xia et al., 2018). With the development of commodity feed in Nile tilapia, plant-based protein ingredients have received considerable attention due to their nutritional value and contribution to sustainability. Plant-based soy, corn, or wheat are good candidates as they are produced with high protein concentrations with few antinutritional factors (Bratosin et al., 2021). However, excessive plant protein in the diet harms fish and shrimp (Kuebutornye et al., 2019). A study by Teodósio (2020) in Nile tilapia showed a possibility of reducing dietary protein levels from 36% to 30% when the diet is balanced for amino acids, producing similar growth performance with improved dietary protein utilization and reduced environmental impact. However, further reducing the dietary protein level to 28% impaired fish performance. However, *B. subtilis* can promote host digestion and absorption of nutrients by degrading numerous natural substrates (Latorre et al., 2016). Nevertheless, whether dietary *B. subtilis* supplementation can eliminate the side effects of plant-based protein in Nile tilapia feed is unknown.

B. subtilis strain DSM 32315 was isolated by screening more than 500 naturally occurring *Bacillus* strains. *B. subtilis* DSM 32315 exhibited beneficial properties, such as inhibiting harmful bacteria (*Escherichia coli* and *Clostridium perfringens*) and resistance to natural bile salts. Furthermore, *B. subtilis* DSM 32315 can be prepared rapidly and continuously, making it easy to scale up production while avoiding antibiotic resistance. This study applies a feed formula of plant-based protein sources and a new probiotic product containing *B. subtilis* DSM 32315 at a concentration of 2×10^9 CFU/g (with calcium carbonate as the carrier). The present research aims to evaluate the effect of dietary *B. subtilis* DSM 32315 supplementation at two dietary protein levels on Nile tilapia growth, immune response, and resistance to acute ammonia exposure.

2. Materials and methods

2.1. Animal ethics statement

All the experimental procedures were strictly performed according to the official recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The study protocol was approved by the Experimental Animal Ethics Committee of Sun Yat-sen University. The protocol number is Evonik Trial No. 37.87.19004.

2.2. Diet preparation

The formulation and nutritional composition of 8 diets are presented in Table 1. *B. subtilis* DSM 32315 probiotic product was provided by Evonik (China) Co., Ltd., containing *B. subtilis* DSM 32315 at a concentration of 2×10^9 CFU/g (with calcium carbonate as the carrier). The primary protein sources were soybean meal, rapeseed meal, concentrated cottonseed and defatted rice bran. Primary lipid sources were soybean oil and soybean lecithin. The carbohydrate source was wheat flour. Four *B. subtilis* probiotic supplementation levels (0%, 0.1%, 0.2%, and 0.3%) were added under two protein diets (32% and 28%). Accordingly, each diet was labeled as P32-0, P32-0.1, P32-0.2, P32-0.3, P28-0, P28-0.1, P28-0.2, and P28-0.3, respectively. Diets were prepared using a modified method of Niu et al. (2010) as follows: all dry ingredients of the experimental diets were accurately weighed, combined and thoroughly mixed for 5 min, then oil was added and further stirred by hand in a basin. Hand-mixed ingredients were then transferred to a Hobart mixer (A-200T Mixer Bench Model unit, Resell Food Equipment Ltd., Ottawa, Canada) and mixed for 15 min to ensure the homogeneity of the ingredients. Afterward, deionized water (30%, vol/wt) was added and mixed for a further 10 min. The wet mixture was placed into a mono-screw extruder and the mold nozzle diameter ranged from 1.5 to 2.5 mm (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China). All diets were dried at 25 °C for 24 h until the moisture was reduced to less than 100 g/kg. All dry diets were stored at −20 °C to preserve the diet quality.

2.3. Animals and environmental conditions

Healthy juvenile mixed Nile tilapia (length: 3 to 5 cm) were acquired from Guangdong Provincial Fishery Germplasm Conservation Center (Guangzhou, China) and checked by a veterinarian. Nile tilapia were acclimated to the experimental conditions and fed with the P32-0 diet for 1 week before the experiment. A total of 1,200 Nile tilapia with an initial body weight (IBW) of approximately 3.03 ± 0.02 g were distributed randomly into 40 tanks (240 L, 0.8 m^2 bottom, 5 tanks per diet, 30 Nile tilapia per tank). For the feeding trial, water quality was consistently measured throughout the experiment using portable digital instruments to estimate the water temperature, dissolved oxygen, and pH levels. The temperature was maintained at 25 ± 1 °C using thermostats. Approximately 80% of the water was exchanged once weekly to retain water quality and freshly sterilized water was added to reach 80% of the final volume. Ideal water quality was maintained across the experimental tanks by keeping dissolved oxygen >5 mg/L, ammonia < 0.01 mg/L, H_2S < 0.05 mg/L and pH at 6.8 to 7.5. The Nile tilapia were raised in a constant flow water system.

During the 8-week experiment, Nile tilapia were fed by hand twice daily (at 09:00 and 17:00) at 5% of the IBW. During each feeding, uneaten feed was collected from the bottom of the tanks 2 h after feeding. If the feed was eaten completely, the next meal was increased by 20% of the previous meal; if there was feed waste,

Table 1
Ingredients and nutrient composition of eight experimental diets (%, dry matter basis).

Item	P32-0	P32-0.1	P32-0.2	P32-0.3	P28-0	P28-0.1	P28-0.2	P28-0.3
Ingredients								
Soybean meal	24.00	24.00	24.00	24.00	24.00	24.00	24.00	24.00
Rapeseed meal	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00
Concentrated cottonseed protein	13.00	13.00	13.00	13.00	4.00	4.00	4.00	4.00
Wheat flour	22.23	22.13	22.03	21.93	22.71	22.61	22.51	22.41
Defatted rice bran	14.00	14.00	14.00	14.00	22.00	22.00	22.00	22.00
Probiotics[1]	0.00	0.10	0.20	0.30	0.00	0.10	0.20	0.30
Soybean oil	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20
Soybean lecithin	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[3]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin C	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Choline chloride (50%)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
DL-Met (99%)	0.34	0.34	0.34	0.34	0.36	0.36	0.36	0.36
L-Lys HCl (79%)	0.60	0.60	0.60	0.60	0.78	0.78	0.78	0.78
L-Thr	0.13	0.13	0.13	0.13	0.22	0.22	0.22	0.22
L-Trp	–	–	–	–	0.06	0.06	0.06	0.06
L-Ile	–	–	–	–	0.06	0.06	0.06	0.06
L-Val	–	–	–	–	0.03	0.03	0.03	0.03
L-His	–	–	–	–	0.08	0.08	0.08	0.08
Sum	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient composition[4]								
Dry matter	90.19	90.10	90.29	90.07	89.72	89.90	90.02	89.85
Crude protein	32.21	32.08	32.12	32.18	28.16	28.04	28.14	28.23
Crude lipid	5.40	5.37	5.44	5.38	5.42	5.46	5.50	5.36
Ash	5.26	5.24	5.23	5.26	5.34	5.30	5.28	5.36
Lys	1.96	1.92	1.90	1.94	1.91	1.90	1.89	1.87
Met	0.81	0.80	0.78	0.80	0.79	0.77	0.76	0.78
Cys	0.57	0.55	0.53	0.55	0.51	0.50	0.49	0.48
Met + Cys	1.39	1.37	1.36	1.36	1.31	1.30	1.29	1.28
Thr	1.29	1.27	1.28	1.27	1.25	1.24	1.23	1.23
Trp	0.40	0.38	0.39	0.39	0.41	0.40	0.40	0.39
Arg	2.49	2.47	2.46	2.45	1.92	1.90	1.89	1.90
Ile	1.23	1.20	1.21	1.22	1.17	1.15	1.15	1.13
Leu	2.17	2.14	2.15	2.15	1.96	1.95	1.93	1.92
Val	1.46	1.42	1.43	1.42	1.33	1.32	1.30	1.31
His	0.83	0.82	0.80	0.81	0.79	0.78	0.77	0.77
Phe	1.54	1.53	1.52	1.51	1.31	1.30	1.29	1.28
Bacillus subtilis	0	0.096	0.194	0.291	0	0.097	0.192	0.288

[1] *Bacillus subtilis* DSM32315 (2×10^9 CFU/g) was provided by Evonik (China) Co., Ltd., Guangzhou, China.
[2] Mineral premix provides the following per kilogram of mineral premix: P 120 g, Ca 120 g, Mg 15 g, Fe 1.5 g, Zn 4.2 g, Cu 2.1 g, K 75 g, Co 0.11 g, Mn 1.6 g, Se 0.01 g, Mo 0.005 g, Al 0.025 g, I 0.4 g, cellulose was used as a carrier.
[3] Vitamin premix provides the following per kilogram of vitamin premix: vitamin D_3 0.6 MIU, vitamin B_1 3.6 g, vitamin B_2 7.2 g, vitamin B_6 6.6 g, vitamin B_{12} 0.02 g, vitamin E 16.5 g, vitamin K_3 2.4 g, vitamin B_3 14.4 g, vitamin B_5 4 g, biotin 0.02 g, folic acid 1.2 g, inositol 30 g, vitamin C 100 g, cellulose was used as a carrier.
[4] Measured value (dry matter basis).

the next meal was reduced by 20% of the previous meal. In this way overfeeding was minimized and Nile tilapia were fed close to satiation.

2.4. Growth performance measuring and sampling

At the beginning of the feeding trial, Nile tilapia were bulk weighed, keeping a coefficient of variation below 15% within tank and below 10% between treatments. At the end of the feeding trial, Nile tilapia from each tank were weighed and counted. Daily feed data and survival were recorded. From the recorded parameters, IBW, final body weight (FBW), weight gain (WG), survival rate, feed conversion ratio (FCR) and specific growth rate (SGR), were calculated as previously described (Van et al., 2021). At the end of the trial, 4 fish selected at random from each tank were anaesthetized by an overdose of MS-222 (200 ± 10 mg/L), their weights were measured, and livers and blood samples were collected. Livers were immediately put into RNAlater (cat. no. AM7022, Thermo Fisher Scientific, Waltham, MA, USA) and frozen at −80 °C for further determination of enzyme activity and gene expression. Blood samples were withdrawn by puncturing the caudal vessels using a heparin–lithium syringe. Plasma samples were obtained by centrifugation at 14,000 × *g* for 10 min at 4 °C, then stored at −20 °C until lysozyme (LZM) analysis was conducted. For intestinal morphology measurements, 3 fish from each tank were randomly selected, and their midgut (a length of about 1 cm from the middle of the first to the last bend of the intestine) was excised and used for the measurements.

2.5. Feed and body chemical composition

At the end of the feeding trial, Nile tilapia were fasted for 24 h and 5 Nile tilapia from each tank were randomly selected for proximate body chemical composition. The samples (whole body) and feeds were oven-dried at 105 °C for 36 h. The samples and feeds were pulverized, crude protein (nitrogen × 6.25) was determined by the Kjeldahl method (Tecator 1030 automatic analyzer, Tecator, Helsingborg, Sweden), crude lipid was analyzed by the ether-extraction method using a Soxtec System HT (Soxtec System HT6, Tecator, Hoganas, Sweden), and ash content was determined by incineration in a muffle furnace (M110, Thermo Fisher Scientific, Waltham, MA, USA) at 550 °C for 5 h.

2.6. Challenge with acute ammonia exposure

Following the 56-d feeding trial, an acute ammonia challenge experiment was conducted by exposing tilapia to median lethal dose of total ammonia nitrogen at concentration of 43.61 mg/L for 48 h. Eight fish randomly selected from each tank (240 L, 0.8 m^2 bottom; the water volume was 180 L) were used for this experiment. Water temperature was controlled at 25 ± 1 °C and pH at 7.5 ± 0.3 during the acute ammonia challenge test. Nile tilapia mortality was recorded every 6 h during 48-h stress test, and dead Nile tilapia were removed. At the end of 48 h, livers of surviving fish from each tank (n = 4 fish per tank) were collected and frozen at −80 °C until further analysis.

2.7. Enzyme activity assays

Liver samples of Nile tilapia (n = 4 fish per tank) from the feeding trial and the challenge test were homogenized to estimate antioxidants, including total antioxidant capacity (T-AOC), and the malondialdehyde (MDA) content via commercial kits (cat. no. A015 and A003, respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Plasma LZM activity was measured using commercial kits (cat. no. A050, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by following the protocol provided by the manufacturer. The levels of these indices were measured by spectrophotometry based on the absorbance of light at a specified wavelength: 405 nm for T-AOC activity, 532 nm for MDA content and 530 nm for plasma LZM activity. Each experiment was repeated 3 times.

2.8. Gene quantification in liver

Total RNA was extracted from the liver samples of individual Nile tilapia using the RNeasy animal RNA extraction kit (cat. no. R0024, Beyotime, Shanghai, China) according to the manufacturer's protocol, and the first-strand cDNA was synthesized by a PrimeScript RT Reagent kit (cat. no. RR037A, Takara Bio Inc., Beijing, China). Based on the sequences from GenBank, the primers of 10 genes, including heat shock protein 70 (*HSP70*), interleukin-10 (*IL-10*), interferon gamma (*IFN-γ*), elongation factor-1 alpha (*EF-1α*), complement C3, transforming growth factor-β (*TGF-β*), glutathione reductase (*GSR*), nuclear factor erythroid 2-related factor 2 (*Nrf2*), Kelch-like ECH-associated protein 1 (*Keap1*) and superoxide dismutase (*SOD*) shown in Table 2 were designed by SnapGene software. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed in a 384-well plate using Applied Biosystems SYBR Green (cat. no. A25780, Invitrogen, California, USA), and the $2^{-\Delta\Delta Ct}$ method was used for the relative quantification of the above genes.

2.9. Intestinal microvilli morphology

The midgut samples midguts (n = 3 per tank) were fixed in 4% formaldehyde-buffered solution, then embedded in a paraffin block, and sectioned into 4 μm slices, and finally stained with hematoxylin and eosin (H&E) for histological examination under a light microscope (NikonNi-U, Nikon Corporation, Tokyo, Japan). The villi were chosen to be as evenly spaced around the midgut sample as possible. Villi height and width were measured randomly 5 times per slice by a computer-operated image picture analysis system and expressed in micron (μm) at 4× magnification.

2.10. Statistical analysis

The experiment was repeated 3 times (n = 5 Nile tilapia per treatment). The mean and standard deviation of the groups were estimated and checked for normality and homogeneity. Related data on growth performance, antioxidant enzyme activity, and gene expression were analyzed using GraphPad Prism 8.0.1 software. Data were subjected to two-way analysis of variance (ANOVA) to determine the interactions between dietary protein and probiotic supplementation levels. If the effect of a factor was significant ($P < 0.05$), Duncan's comparison test was performed on the average value of a single factor. If the interaction between two factors was significant ($P < 0.05$), Duncan's comparison test was performed on the average value of each treatment.

Table 2
Sequences of primers used in this study.

Genes	Primers (5′ to 3′)	GenBank no.
HSP70	F: AGCTGCCATTGCTTATGGGT	XM_003448890.5
	R: TCCTCCCCACCAAGATGAGT	XM_003448890.5
IL-10	F: GCTGCTAGATCAGTCCGTCG	XM_013269189.3
	R: ATCTCTTCAGTCACTCCGGC	XM_013269189.3
IFN-γ	F: TGGGTGGTGTTTTGGAGTCG	NM_001287402.1
	R: TAGCGAGCCTGAGTTGTTGG	NM_001287402.1
EF-1α	F: GACCTTCATTCTCGCTACTCC	NM_001279647.1
	R: TTCCATTCACATCCACCTTCTC	NM_001279647.1
Complement C3	F: CCTTGCCCTAGAAACACACAC	XM_019347500.2
	R: TCCACGGACAGCAGAATAAGG	XM_019347500.2
TGF-β	F: AACTACTGCATGGGGTCCTG	XM_025897821.1
	R: GTGTTGCCTCCCCACATAGT	XM_025897821.1
GSR	F: AGAAGTTCCAGGGGCAAGTC	XM_005467348.4
	R: TGAGGGATGTTTTGGACCCC	XM_005467348.4
Nrf2	F: CTGCCGTAAACGCAAGATGG	XM_003447296.5
	R: TACAAGCTGTTGAGCTGCCA	XM_003447296.5
Keap1	F: TTCAACTTGTCCCACTGCCA	XM_003447926.4
	R: ACAGCCTGGAGTAAGGCTTG	XM_003447926.4
SOD	F: CGGTTTGCGTTTTGAAGGGA	XM_003446807.5
	R: ATGCACCCGTTTGTGTTGTC	XM_003446807.5

HSP70 = heat shock protein 70; *IL-10* = interleukin-10; *IFN-γ* = interferon gamma; *EF-1α* = elongation factor-1 alpha; *TGF-β* = transforming growth factor-β; *GSR* = glutathione reductase; *Nrf2* = nuclear factor erythroid 2-related factor 2; *Keap1* = Kelch-like ECH-associated protein 1; *SOD* = superoxide dismutase.

3. Results

3.1. Growth performance

No significant differences were detected between the 32% and 28% protein diets in WG and FCR, however, these two diets responded differently to the *B. subtilis* probiotic supplementation (Table 3). Highly significant interactions between dietary protein and *B. subtilis* probiotic levels were observed for FBW, WG and SGR (Table 3; $P < 0.001$). The maximum FBW, WG and SGR were observed in the groups fed the P32-0.3 and P28-0.1 diets, while the lowest performance was observed in fish fed the P28-0.3 diet. Concerning survival rate and FCR, there was no interaction between dietary protein and *B. subtilis* probiotic level (P = 0.076 and P = 0.208, respectively).

3.2. Whole body composition

Table 4 displays the whole body composition (%, wet weight) of Nile tilapia fed with the eight experimental diets. According to two-way ANOVA, dietary protein and *B. subtilis* probiotic levels did not interact significantly in moisture, ash, crude lipid, and crude protein ($P > 0.05$). Moisture and crude protein differ between protein levels ($P < 0.05$) but are unaffected by dietary *B. subtilis* probiotic supplementation level ($P > 0.05$). Regardless of *B. subtilis* supplementation, Nile tilapia fed the 32% protein diet had higher contents of moisture and crude protein than those fed the 28% protein diet ($P < 0.05$).

Table 3
The effect of eight different experimental diets on growth performance of Nile tilapia.

Item	*B. subtilis* probiotics, %	Protein, %	IBW, g	FBW, g	WG, %	SGR, %/day	Survival, %	FCR
P32-0	0	32	3.02 ± 0.01	46.92 ± 2.34^{b}	1,453 ± 76.85^{b}	4.89 ± 0.09^{b}	90.00 ± 3.54	1.35 ± 0.03
P32-0.1	0.1	32	3.02 ± 0.01	49.24 ± 1.79^{bc}	1,529 ± 61.84^{bc}	4.98 ± 0.07^{bc}	96.00 ± 1.00	1.34 ± 0.07
P32-0.2	0.2	32	3.02 ± 0.01	50.07 ± 0.57^{bc}	1,559 ± 18.44^{bc}	5.01 ± 0.02^{bc}	91.00 ± 1.87	1.33 ± 0.04
P32-0.3	0.3	32	3.04 ± 0.01	54.16 ± 1.75^{c}	1,683 ± 57.15^{c}	5.14 ± 0.06^{c}	87.00 ± 2.55	1.33 ± 0.05
P28-0	0	28	3.03 ± 0.01	49.69 ± 1.12^{bc}	1,539 ± 39.04^{bc}	4.99 ± 0.04^{bc}	91.00 ± 1.00	1.31 ± 0.03
P28-0.1	0.1	28	3.03 ± 0.01	53.83 ± 3.08^{c}	1,677 ± 100.4^{c}	5.13 ± 0.11^{c}	88.00 ± 1.22	1.26 ± 0.02
P28-0.2	0.2	28	3.03 ± 0.01	45.59 ± 2.08^{b}	1,403 ± 69.78^{ab}	4.83 ± 0.08^{ab}	85.00 ± 4.18	1.50 ± 0.09
P28-0.3	0.3	28	3.03 ± 0.01	40.03 ± 1.55^{a}	1,223 ± 49.81^{a}	4.61 ± 0.07^{a}	91.00 ± 2.92	1.33 ± 0.05
Means of main effect								
B. subtilis probiotics, %								
0			3.03 ± 0.01	48.31 ± 1.27^{A}	1,496 ± 50.45^{A}	4.94 ± 0.03^{A}	90.50 ± 1.45	1.33 ± 0.03^{A}
0.1			3.03 ± 0.01	51.54 ± 1.90^{B}	1,603 ± 79.54^{B}	5.05 ± 0.08^{B}	92.00 ± 1.00	1.30 ± 0.04^{A}
0.2			3.03 ± 0.01	47.83 ± 1.32^{A}	1,481 ± 50.45^{A}	4.92 ± 0.02^{A}	88.00 ± 2.75	1.41 ± 0.03^{B}
0.3			3.04 ± 0.01	47.05 ± 1.60^{A}	1,453 ± 46.89^{A}	4.88 ± 0.05^{A}	89.00 ± 2.08	1.33 ± 0.05^{A}
Protein, %								
32			3.02 ± 0.01	50.00 ± 1.61^{y}	1,556 ± 34.68	5.03 ± 0.02^{y}	91.00 ± 1.79	1.34 ± 0.04
28			3.03 ± 0.01	47.28 ± 1.14^{x}	1,460 ± 50.47	4.89 ± 0.06^{x}	88.75 ± 2.49	1.35 ± 0.05
Two-way ANOVA (*P*-value)								
B. subtilis probiotics			0.866	<0.01	<0.01	<0.01	0.432	<0.05
Protein			0.084	<0.05	0.267	<0.001	0.280	0.156
Interaction			0.626	<0.001	<0.001	<0.001	0.076	0.208

B. subtilis = *Bacillus subtilis*; IBW = initial body weight; FBW = final body weight; WG = weight gain; SGR = specific growth rate; FCR = feed conversion ratio.
Values are mean ± SEM of 5 replicates.
[a–c]Different lowercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments by Duncan's comparison test.
[A, B]Different uppercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments with graded *B. subtilis* probiotics by Duncan's comparison test.
[x, y]Different superscript letters within a column denote significant differences ($P < 0.05$) between 32% and 28% protein levels by Duncan's comparison test.

Table 4
Whole fish compositions (%, wet weight) of Nile tilapia fed eight different experimental diets.

Item	*B. subtilis* probiotics, %	Protein, %	Moisture	Crude lipid	Crude protein	Ash
P32-0	0	32	73.02 ± 1.01	7.85 ± 0.98	14.43 ± 1.59	0.37 ± 0.03
P32-0.1	0.1	32	73.79 ± 0.47	8.05 ± 1.07	15.89 ± 0.60	0.39 ± 0.05
P32-0.2	0.2	32	72.02 ± 0.58	9.09 ± 1.49	15.76 ± 0.12	0.36 ± 0.04
P32-0.3	0.3	32	72.48 ± 0.58	8.51 ± 0.52	16.03 ± 1.11	0.38 ± 0.02
P28-0	0	28	70.87 ± 0.48	9.55 ± 1.19	15.15 ± 0.60	0.36 ± 0.02
P28-0.1	0.1	28	70.40 ± 0.75	9.27 ± 0.76	15.08 ± 1.01	0.36 ± 0.04
P28-0.2	0.2	28	70.79 ± 0.34	9.37 ± 0.21	14.94 ± 0.26	0.38 ± 0.03
P28-0.3	0.3	28	70.67 ± 1.16	9.10 ± 2.67	15.48 ± 1.45	0.37 ± 0.04
Means of main effect						
B. subtilis probiotics, %						
0			71.95 ± 0.23	8.70 ± 0.53	14.79 ± 0.75	0.37 ± 0.02
0.1			72.10 ± 0.56	8.66 ± 0.84	15.48 ± 0.31	0.38 ± 0.03
0.2			71.40 ± 0.34	9.23 ± 1.06	15.35 ± 0.50	0.37 ± 0.03
0.3			71.56 ± 0.34	8.81 ± 0.37	15.75 ± 0.74	0.38 ± 0.04
Protein, %						
32			72.83 ± 0.35^{y}	8.38 ± 0.77	15.52 ± 0.56^{y}	0.38 ± 0.03
28			70.69 ± 0.37^{x}	9.32 ± 1.58	15.16 ± 0.68^{x}	0.37 ± 0.02
Two-way ANOVA (*P*-value)						
B. subtilis probiotics			0.760	0.416	0.885	0.057
Protein			<0.05	0.267	<0.05	0.160
Interaction			0.504	0.530	0.088	0.163

B. subtilis = *Bacillus subtilis*.
Values are mean ± SEM of 5 replicates.
[x, y]Different superscript letters within a column denote significant differences ($P < 0.05$) between 32% and 28% protein levels by Duncan's comparison test.

3.3. Immune biochemical parameters

As shown in Table 5, the liver LZM activity in Nile tilapia was significantly affected by dietary *B. subtilis* probiotics ($P < 0.001$), but not by protein level ($P = 0.291$). The two factors did not interact significantly ($P = 0.642$). The LZM activity of Nile tilapia fed *B. subtilis* supplemented diets showed no clear trend with the highest appearing in the P28-0.1 diet. The immune-related genes (*HSP70*, *TGF-β*, *IFN-γ*, and *IL-10*) exhibited significant interactions between the two factors ($P < 0.001$), except for complement C3 ($P = 0.110$) in the liver samples of Nile tilapia. The expression of stress-indicating gene *HSP70* showed the opposite trend to fish growth performance with increasing levels of *B. subtilis* supplementation for the two protein levels. At a high protein level, *HSP70* tended to decrease, while the opposite trend for the low protein diet was observed with increasing levels of *B. subtilis* supplementation. Known for its potent anti-inflammatory effect, *IL-10* had a higher expression level in the fish fed P32-0.3 and P28-0.1 than those fed the other levels of *B. subtilis* probiotics in the two protein levels, except for the P32-0.1 diet in 32% protein level. Among the other immune indicators, *TGF-β* showed elevated levels in the non-supplemented low protein diet, and other low protein diets with

Table 5
Relative immune biochemical parameters of Nile tilapia fed eight different experimental diets.

Item	*B. subtilis* probiotics, %	Protein, %	LZM, U/mL	*HSP70* (fold change)	Complement C3 (fold change)	*TGF-β* (fold change)	*IFN-γ* (fold change)	*IL-10* (fold change)
P32-0	0	32	154.23 ± 5.68	0.98 ± 0.26^{b}	1.00 ± 0.23	1.00 ± 0.22^{a}	0.93 ± 0.31^{a}	1.00 ± 0.56^{a}
P32-0.1	0.1	32	215.45 ± 9.14	0.52 ± 0.40^{a}	1.33 ± 0.59	1.69 ± 0.55^{ab}	1.17 ± 0.13^{a}	2.15 ± 0.30^{ab}
P32-0.2	0.2	32	151.02 ± 4.43	0.53 ± 0.31^{a}	1.45 ± 0.49	1.11 ± 0.42^{a}	1.36 ± 0.36^{a}	1.23 ± 0.95^{a}
P32-0.3	0.3	32	182.80 ± 10.0	0.43 ± 0.25^{a}	1.81 ± 0.13	1.52 ± 0.07^{ab}	0.80 ± 0.25^{a}	3.20 ± 0.83^{b}
P28-0	0	28	124.67 ± 2.60	0.89 ± 0.17^{ab}	1.06 ± 0.06	2.41 ± 0.62^{b}	4.88 ± 0.47^{b}	1.52 ± 0.02^{a}
P28-0.1	0.1	28	245.35 ± 41.30	0.66 ± 0.07^{a}	2.41 ± 0.71	1.04 ± 0.22^{a}	1.78 ± 0.63^{a}	4.54 ± 0.30^{c}
P28-0.2	0.2	28	147.46 ± 6.46	1.29 ± 0.31^{b}	2.05 ± 0.73	1.14 ± 0.02^{a}	1.67 ± 0.53^{a}	1.89 ± 0.29^{ab}
P28-0.3	0.3	28	212.50 ± 17.96	1.19 ± 0.51^{b}	1.72 ± 0.21	1.14 ± 0.08^{a}	4.15 ± 0.89^{b}	1.75 ± 0.07^{a}
Means of main effect								
B. subtilis probiotics, %								
0			139.45 ± 3.10^{A}	0.94 ± 0.15^{B}	1.03 ± 0.02^{A}	1.71 ± 0.31	2.91 ± 0.37^{B}	1.26 ± 0.07^{A}
0.1			230.40 ± 23.71^{B}	0.59 ± 0.27^{A}	1.87 ± 0.35^{B}	1.37 ± 0.15	1.48 ± 0.23^{A}	3.34 ± 0.17^{B}
0.2			149.24 ± 2.75^{A}	0.91 ± 0.40^{B}	1.75 ± 0.21^{B}	1.13 ± 0.04	1.52 ± 0.45^{A}	1.56 ± 0.25^{A}
0.3			197.65 ± 8.76^{B}	0.81 ± 0.08^{B}	1.77 ± 0.11^{B}	1.33 ± 0.02	2.48 ± 0.53^{B}	2.48 ± 0.08^{B}
Protein, %								
32			175.70 ± 6.73	0.62 ± 0.35^{x}	1.40 ± 0.41^{x}	1.33 ± 0.25^{x}	1.07 ± 0.15^{x}	1.90 ± 0.27^{x}
28			182.25 ± 15.79	1.01 ± 0.07^{y}	1.81 ± 0.07^{y}	1.43 ± 0.05^{y}	3.12 ± 0.27^{y}	2.43 ± 0.12^{y}
Two-way ANOVA (*P*-value)								
B. subtilis probiotics			<0.001	<0.05	<0.05	0.096	<0.01	<0.001
Protein			0.291	<0.001	<0.05	<0.05	<0.001	<0.01
Interaction			0.642	<0.001	0.110	<0.001	<0.001	<0.001

B. subtilis = *Bacillus subtilis*; LZM = lysozyme; *HSP70* = heat shock protein 70; *TGF-β* = transforming growth factor-β; *IFN-γ* = interferon gamma; *IL-10* = interleukin-10.
Values are mean ± SEM of 5 replicates.
[a–c]Different lowercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments by Duncan's comparison test.
[A, B]Different uppercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments with graded *B. subtilis* probiotics by Duncan's comparison test.
[x, y]Different superscript letters within a column denote significant differences ($P < 0.05$) between 32% and 28% protein levels by Duncan's comparison test.

0.1% to 0.3% *B. subtilis* supplementation showed generally lower levels. A similar trend was also observed for *IFN-γ* expression, although higher expression was also observed in the low protein diet at 0.3% *B. subtilis* supplementation. As main effects, complement C3 showed lower expression levels in the non-supplemented diet versus the supplemented diets, and for the high protein diet versus the low protein diet. Regardless of *B. subtilis* supplementation, Nile tilapia fed 28% protein diet had higher *HSP70*, complement C3, *TGF-β*, *IFN-γ*, and *IL-10* gene expression.

3.4. Acute ammonia challenge test

Table 6 presents the mortality of Nile tilapia under acute ammonia resistance for the eight different diet treatments. The two factors interacted significantly with mortality. The mortality of Nile tilapia showed an interaction effect between dietary *B. subtilis* probiotics and dietary protein levels ($P < 0.001$). The mortality rate of Nile tilapia fed P32-0.3, P28-0.1 and P28-0.3 diets was significantly lower than with other diets ($P < 0.001$).

3.5. Antioxidant enzyme activity before and after acute ammonia challenge

We tested T-AOC and MDA content in liver before and after acute ammonia challenge in the eight different diets (Table 7). The *B. subtilis* probiotics significantly affected the T-AOC and the MDA contents from the feeding trial and challenge test. Before the challenge, the interaction between dietary protein and *B. subtilis* probiotic level significantly affected T-AOC ($P < 0.01$). As observed for growth performance, in the low protein group, T-AOC peaked in Nile tilapia fed with the P28-0.1 diet, while that in the high protein group generally increased with increasing *B. subtilis* supplementation. On the other hand, the content of MDA significantly decreased with supplementation of *B. subtilis* probiotics ($P < 0.01$), but was not affected by dietary protein level ($P = 0.368$), nor did it show any interaction effect between the two factors ($P = 0.111$). After acute ammonia challenge, two-way ANOVA showed no significant interactions between T-AOC and MDA content ($P > 0.05$). The T-AOC of Nile tilapia was significantly affected only by dietary *B. subtilis* supplementation with fish fed 0.3% showing significantly higher levels than others ($P < 0.05$). Both *B. subtilis* probiotics and protein treatments had main effects on the content of MDA after the ammonia challenge. Nile tilapia fed with 0.1% to 0.3% *B. subtilis*

Table 6
Mortality of Nile tilapia fed eight different experimental diets under acute ammonia challenge.

Item	*B. subtilis* probiotics, %	Protein, %	Mortality, %
P32-0	0	32	22.50 ± 6.12^{b}
P32-0.1	0.1	32	27.50 ± 6.12^{b}
P32-0.2	0.2	32	27.50 ± 6.12^{b}
P32-0.3	0.3	32	7.50 ± 5.00^{a}
P28-0	0	28	27.50 ± 2.50^{b}
P28-0.1	0.1	28	7.50 ± 7.50^{a}
P28-0.2	0.2	28	30.00 ± 6.37^{b}
P28-0.3	0.3	28	10.00 ± 4.68^{a}
Means of main effect			
B. subtilis probiotics, %			
0			25.00 ± 6.12^{B}
0.1			17.50 ± 7.50^{A}
0.2			28.75 ± 2.50^{B}
0.3			8.75 ± 5.00^{A}
Protein, %			
32			21.25 ± 7.50
28			18.75 ± 5.00
Two-way ANOVA (*P*-value)			
B. subtilis probiotics			<0.001
Protein			0.118
Interaction			<0.001

B. subtilis = *Bacillus subtilis*.
Values are mean ± SEM of 5 replicates.
[a, b]Different lowercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments by Duncan's comparison test.
[A, B]Different uppercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments with graded *B. subtilis* probiotics by Duncan's comparison test.

Table 7
Antioxidant indicators in the livers of Nile tilapia fed eight different experimental diets before and after acute ammonia stress.

Item	*B. subtilis* probiotics, %	Protein, %	Before acute ammonia stress		After acute ammonia stress	
			T-AOC, U/mgprot	MDA, nmol/mgprot	T-AOC, U/mgprot	MDA, nmol/mgprot
P32-0	0	32	0.96 ± 0.13[a]	1.80 ± 0.08	0.62 ± 0.08	1.24 ± 0.07
P32-0.1	0.1	32	1.34 ± 0.13[a]	1.09 ± 0.12	0.13 ± 0.01	0.52 ± 0.12
P32-0.2	0.2	32	1.51 ± 0.15[ab]	1.16 ± 0.08	0.40 ± 0.03	1.11 ± 0.19
P32-0.3	0.3	32	2.21 ± 0.61[b]	0.70 ± 0.08	0.57 ± 0.02	0.63 ± 0.06
P28-0	0	28	1.17 ± 0.06[a]	1.77 ± 0.35	0.15 ± 0.02	1.68 ± 0.26
P28-0.1	0.1	28	3.43 ± 0.20[c]	0.66 ± 0.13	0.75 ± 0.11	1.03 ± 0.02
P28-0.2	0.2	28	1.28 ± 0.01[a]	0.92 ± 0.13	0.46 ± 0.05	0.99 ± 0.24
P28-0.3	0.3	28	1.30 ± 0.31[a]	2.05 ± 0.29	0.50 ± 0.06	1.32 ± 0.15
Means of main effect						
B. subtilis probiotics, %						
0			1.07 ± 0.07[A]	1.79 ± 0.05[B]	0.39 ± 0.05[A]	1.46 ± 0.12[B]
0.1			2.39 ± 0.10[B]	0.88 ± 0.12[A]	0.44 ± 0.08[A]	0.78 ± 0.05[A]
0.2			1.40 ± 0.23[A]	1.04 ± 0.07[A]	0.43 ± 0.09[A]	1.05 ± 0.06[A]
0.3			1.76 ± 0.40[B]	1.38 ± 0.02[A]	0.54 ± 0.02[B]	0.98 ± 0.05[A]
Protein, %						
32			1.51 ± 0.21	1.19 ± 0.13	0.43 ± 0.03	0.88 ± 0.03[x]
28			1.79 ± 0.08	1.35 ± 0.01	0.48 ± 0.06	1.26 ± 0.10[y]
Two-way ANOVA (*P*-value)						
B. subtilis probiotics			<0.01	<0.01	<0.05	<0.05
Protein			0.644	0.368	0.285	<0.001
Interaction			<0.01	0.111	0.096	0.311

B. subtilis = *Bacillus subtilis*; T-AOC = total antioxidant capacity; MDA = malondialdehyde.
Values are mean ± SEM of 5 replicates.
[a–c]Different lowercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments by Duncan's comparison test.
[A, B]Different uppercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments with graded *B. subtilis* probiotics by Duncan's comparison test.
[x, y]Different superscript letters within a column denote significant differences ($P < 0.05$) between 32% and 28% protein levels by Duncan's comparison test.

supplementation had significantly lower content of MDA than without *B. subtilis* supplementation and the MDA content was significantly increased with the reduction of protein level ($P < 0.05$).

3.6. Antioxidant related gene expression

Table 8 displays the relative mRNA expression levels of antioxidant-related genes after the acute ammonia challenge. *Nrf2*, *Keap1*, and *SOD* gene expression ($P < 0.01$) exhibited significant interaction between the two factors, except for *GSR* ($P = 0.619$). The expression of *Nrf2* in fish fed the P28-0 and P28-0.3 diets were higher than P28-0.1 and P28-0.2 diets ($P < 0.05$). However, the trend was not consistent for the *Keap1* and *SOD* genes. *Keap1* showed a higher level of expression both in the high protein diets and in the low protein diet with no *B. subtilis* supplementation diet compared with the low protein *B. subtilis* supplemented diets. Besides, as main effect, Nile tilapia fed 28% protein diet tended to have higher *Nrf2* and *SOD* and lower *Keap1* gene expression than those fed 32% protein diet ($P < 0.01$).

3.7. Intestinal histology

Fig. 1 and Table 9 depict the morphology and parameters of the intestines after ammonia stress. We observed significant interactions between dietary protein and *B. subtilis* probiotic levels on intestinal villi height ($P < 0.001$) but not on their width ($P = 0.867$) in Nile tilapia. *B. subtilis* probiotic supplementation could significantly increase the intestinal villi height and width, especially in the low protein diets, but the trend in villi height was the opposite in the high protein diet with a peak observed in the 0.1% probiotic supplementation diet. Increasing dietary protein level from 28% to 32% significantly increased the intestinal villi height ($P < 0.001$) while the opposite trend was observed for intestinal villi width for the two dietary protein levels ($P = 0.052$). For both the dietary protein levels, probiotic supplementation produced increased villi width in Nile tilapia.

4. Discussion

In the present study, we reduced the dietary protein level by reducing cottonseed protein concentrate and increasing rice bran. Rice bran is known to be rich in carbohydrates from starch and dietary fiber (mainly insoluble fractions, non-starch polysaccharides) (Gul et al., 2015). Cottonseed protein concentrate typically contains a high level of protein (>65%) and low levels of anti-nutrients (e.g., 7.9 mg/kg gossypol) (Shen et al., 2020). A study in tilapia showed reduced performance with increasing levels of cottonseed protein concentrate (Li et al., 2021). The study showed that higher gossypol content affected gut morphology and reduced fish performance. Additionally, the study did not balance the diets for amino acids and contained significantly lower levels of several amino acids including Met, Lys and Thr which could have been an additional factor affecting fish performance. In our study, we used a moderate amount of cottonseed concentrate in the high protein (13%) diet and balanced the diet for amino acid profile. At non-supplementation of *B. subtilis* probiotics, the low protein diet showed no difference in the growth performance compared with the high protein diet. In fact, the low protein diet produced numerically better performance which is speculated to be due to the reduced level of gossypol, but its content was not measured in our study. The increase in the growth performance of tilapia on the high protein diet supplemented with *B. subtilis* could indicate a positive effect of probiotic supplementation. However, in the low protein diet, the performance peaked at 0.1% *B. subtilis* probiotic supplementation but then decreased at higher supplementation levels. Surprisingly, only in the low protein diet (28%) did the high supplementation of *B. subtilis* probiotics (0.3%) reduce performance, whereas the opposite trend was recorded in the high protein diet. Nevertheless, the same diet (28% protein, 0.3% probiotics) showed significantly lower mortality compared with the non-

Table 8
Antioxidant-related gene expression from the livers of Nile tilapia fed eight different experimental diets after acute ammonia challenge.

Item	*B. subtilis* probiotics, %	Protein, %	*Nrf2* (fold change)	*Keap1* (fold change)	*GSR* (fold change)	*SOD* (fold change)
P32-0	0	32	1.00 ± 0.37^{a}	1.00 ± 0.21^{b}	0.97 ± 0.22	1.00 ± 0.12^{a}
P32-0.1	0.1	32	0.99 ± 0.49^{a}	1.15 ± 0.05^{b}	0.93 ± 0.12	1.20 ± 0.19^{a}
P32-0.2	0.2	32	0.87 ± 0.20^{a}	0.93 ± 0.12^{b}	1.11 ± 0.39	1.15 ± 0.24^{a}
P32-0.3	0.3	32	0.94 ± 0.33^{a}	0.99 ± 0.45^{b}	1.03 ± 0.11	1.06 ± 0.04^{a}
P28-0	0	28	1.77 ± 0.27^{b}	1.16 ± 0.04^{b}	1.16 ± 0.03	1.37 ± 0.10^{a}
P28-0.1	0.1	28	0.71 ± 0.10^{a}	0.45 ± 0.32^{a}	1.07 ± 0.31	2.38 ± 0.10^{b}
P28-0.2	0.2	28	0.96 ± 0.49^{a}	0.39 ± 0.10^{a}	0.96 ± 0.51	1.35 ± 0.26^{a}
P28-0.3	0.3	28	1.88 ± 0.04^{b}	0.63 ± 0.15^{a}	1.24 ± 0.22	2.44 ± 0.09^{b}
Means of main effect						
B. subtilis probiotics, %						
0			1.39 ± 0.24^{B}	1.08 ± 0.13	1.07 ± 0.10	1.19 ± 0.08^{A}
0.1			0.85 ± 0.10^{A}	0.80 ± 0.07	1.00 ± 0.22	1.79 ± 0.17^{B}
0.2			0.92 ± 0.35^{A}	0.66 ± 0.12	1.04 ± 0.12	1.25 ± 0.12^{A}
0.3			1.41 ± 0.09^{B}	0.81 ± 0.05	1.13 ± 0.15	1.75 ± 0.07^{B}
Protein, %						
32			0.95 ± 0.25^{x}	1.03 ± 0.17^{y}	1.01 ± 0.27	1.10 ± 0.02^{x}
28			1.33 ± 0.18^{y}	0.66 ± 0.21^{x}	1.10 ± 0.07	1.85 ± 0.12^{y}
Two-way ANOVA (*P*-value)						
B. subtilis probiotics			<0.05	0.083	0.845	<0.001
Protein			<0.05	<0.001	0.419	<0.001
Interaction			<0.01	<0.01	0.619	<0.001

B. subtilis = *Bacillus subtilis*; *Nrf2* = nuclear factor erythroid 2-related factor 2; *Keap1* = Kelch-like ECH-associated protein 1; *GSR* = glutathione reductase; *SOD* = superoxide dismutase.
Values are mean ± SEM of 5 replicates.
[a, b]Different lowercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments by Duncan's comparison test.
[A, B]Different uppercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments with graded *B. subtilis* probiotics by Duncan's comparison test.
[x, y]Different superscript letters within a column denote significant differences ($P < 0.05$) between 32% and 28% protein levels by Duncan's comparison test.

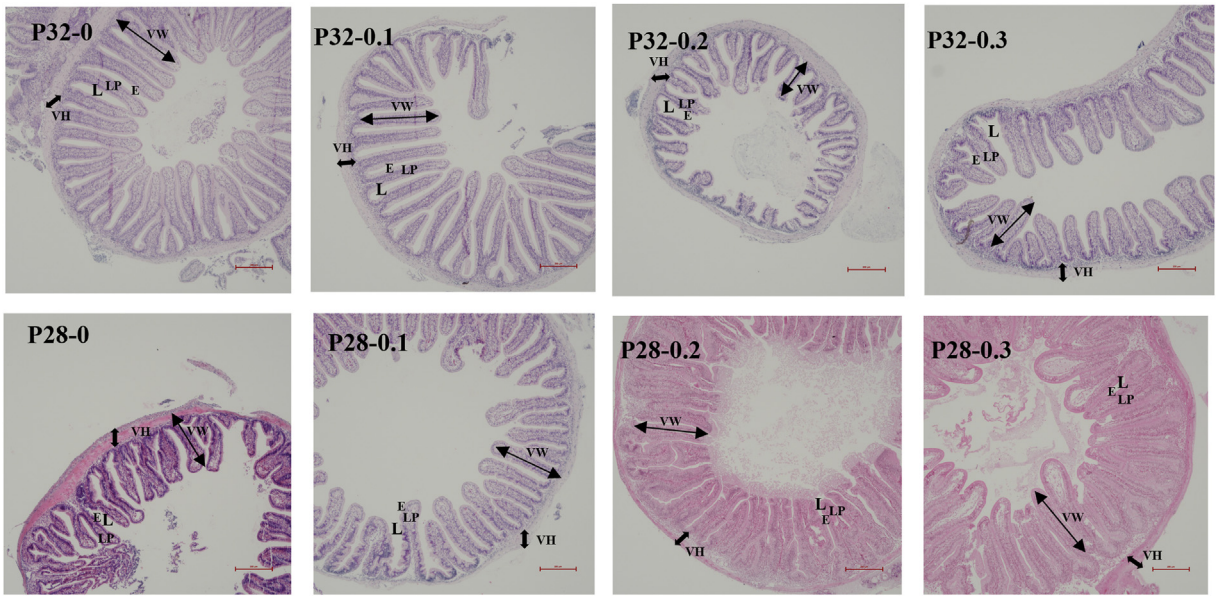

Fig. 1. The effect of eight different diets on intestinal morphology of Nile tilapia after acute ammonia challenge for 48 h. L = lumen; E = enterocytes; LP = lamina propria; VH = villi height; VW = villi width. Scale bar, 200 μm; original magnification, 4×.

supplemented diet with low protein after the ammonia challenge. This indicates that there is a positive effect of *B. subtilis* supplementation under stressful conditions in both high and low protein diets. To our knowledge, while there are several studies investigating the effects of probiotics in diets replacing fish meal or soybean meal with alternative protein sources (Ringø et al., 2016), no study has been conducted to understand how the dietary protein level interacts with probiotics in the fish gut and how this affects fish performance.

Dietary *B. subtilis* supplementation generally improves growth performance and immune capacity of Nile tilapia (Netea et al., 2010; Srisapoome and Areechon, 2017). Laraflores et al. (2010) reported that dietary probiotic supplementation significantly improved WG and FCR when Nile tilapia diets contained 27% protein. Similarly, the present study revealed that the feed formulation with P32-0.3 and P28-0.1 diets could improve Nile tilapia growth performance. However, Telli et al. (2014) reported that dietary *B. subtilis* did not improve Nile tilapia growth performance. The

Table 9
Effects of diets on intestinal health indicators of Nile tilapia fed eight different experimental diets after acute ammonia challenge.

Item	*B. subtilis* probiotics, %	Protein, %	Intestinal villi height, μm	Intestinal villi width, μm
P32	0	32	465.30 ± 3.87^{bc}	79.41 ± 2.55
P32-0.1	0.1	32	491.26 ± 10.94^{c}	78.81 ± 1.30
P32-0.2	0.2	32	408.25 ± 44.30^{ab}	99.66 ± 4.51
P32-0.3	0.3	32	350.15 ± 13.17^{a}	96.34 ± 3.90
P28	0	28	354.00 ± 15.19^{a}	89.32 ± 6.11
P28-0.1	0.1	28	389.59 ± 10.45^{a}	85.16 ± 2.64
P28-0.2	0.2	28	453.06 ± 12.79^{bc}	102.48 ± 3.66
P28-0.3	0.3	28	497.99 ± 13.71^{c}	100.19 ± 4.27
Means of main effect				
B. subtilis probiotics, %				
0			409.65 ± 7.56^{A}	84.37 ± 3.74^{A}
0.1			440.43 ± 10.75^{B}	81.99 ± 1.43^{A}
0.2			430.66 ± 20.35^{B}	101.07 ± 3.75^{B}
0.3			424.07 ± 5.43^{B}	98.27 ± 2.39^{B}
Protein, %				
32			428.73 ± 10.39^{y}	88.57 ± 2.96
28			423.66 ± 11.33^{x}	94.29 ± 3.42
Two-way ANOVA (*P*-value)				
B. subtilis probiotics			<0.001	<0.001
Protein			<0.001	0.052
Interaction			<0.001	0.867

B. subtilis = *Bacillus subtilis*.
Values are mean ± SEM of 5 replicates.
[a–c]Different lowercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments by Duncan's comparison test.
[A, B]Different uppercase superscript letters within a column denote significant differences ($P < 0.05$) among the treatments with graded *B. subtilis* probiotics by Duncan's comparison test.
[x, y]Different superscript letters within a column denote significant differences ($P < 0.05$) between 32% and 28% protein levels by Duncan's comparison test.

potential ability of *B. subtilis* to enhance growth performance might be due to beneficial digestive enzyme secretion; thus it is a pity that examining the impact of digestive enzymes was excluded in the current study. Further investigations are required to elucidate the mechanisms related to *B. subtilis*. In our study, measured parameters on immune response, antioxidant capacity, intestinal morphology provided evidence on the mode of action supporting some of the benefits of *B. subtilis* probiotics on growth performance and stress resistance of tilapia.

LZM, the first line of defense in plasma, is abundant in mucus, lymphoid tissue, plasma, and other fluid components of fish (Magnadóttir, 2006). This study's findings exhibited that probiotic addition increased LZM content in plasma, which is in agreement with the results of others (Addo et al., 2017; Galagarza et al., 2018; Selim and Reda, 2015) who reported that *B. subtilis* probiotics could improve the LZM content in aquaculture animals. A high LZM content in Nile tilapia may be due to the up-regulation of *LZM* mRNA levels caused by challenge to the immune system. LZM expression activates the complement and the phagocyte systems (Jollès and Jollès, 1984). The present study indicated that *B. subtilis* probiotic addition increased complement C3 gene expression. Complement C3 is the core of the complement system, a mediator of innate immunity and a nonspecific defense mechanism against pathogens (Muller-Eberhard, 1988). This suggests that *B. subtilis* probiotics may enhance non-specific immune responses. A similar result was observed in Hulong grouper fed with *B. subtilis*-supplemented diets at 10^6 to 10^{10} CFU/g (Zhou et al., 2020).

Previous studies have suggested that probiotic-supplemented diets resulted in significantly higher *TGF-β* and *IFN-γ* expressions than a basal diet (Won et al., 2020; Xia et al., 2018). Our study results showed that 28% protein diets tended to promote the expression of pro-inflammatory genes such as *TGF-β* and *IFN-γ* but the supplementation of *B. subtilis* probiotics alleviates the impact, as shown by reduction in the expression of these genes. The expression of anti-inflammatory genes (*IL-10*) showed an inconsistent response to probiotic supplementation between the two dietary protein levels.

The frequent occurrence of ammonia stress is considered a significant constraint in the aquaculture industry. *B. subtilis* administration enhanced the resistance of Nile tilapia against various diseases (Abarike et al., 2018; Han et al., 2015), which also improved the survival capacity of Nile tilapia (Abraham et al., 2007). This study tested the potential resistance of *B. subtilis* against acute ammonia stress. The results suggested that adding *B. subtilis* probiotics could improve the survival and antioxidant ability against ammonia stress, whereas dietary protein reduction did not make a difference to ammonia stress capacity of Nile tilapia. Unlike for growth performance, across both the dietary protein levels, higher levels of *B. subtilis* probiotic supplementation (0.3%) improved the survival rate of Nile tilapia. This suggested that the *B. subtilis* DSM 32315 strain-based probiotics is a good candidate to enhance the anti-stress ability of Nile tilapia.

Reactive oxygen species (ROS) maintain a dynamic balance between constantly being generated and removed under normal physiological conditions (Zhang et al., 2013). The present study discovered that *B. subtilis* probiotic supplementation drastically increased the T-AOC regardless of the presence or absence of acute ammonia challenge, while, the T-AOC in the liver decreased significantly in all diets following the acute ammonia challenge test. This reduction indicated that ammonia stress led to imbalance in the intracellular buffer systems and oxidative damage. Qiang et al. (2011) suggested that the increased ammonia concentrations in the rearing environment promoted the MDA content in the livers of Nile tilapia. MDA content, a bioindicator of oxidative stress, was measured in the liver. MDA markedly declined in Nile tilapia fed the diets with *B. subtilis* supplementation, regardless of the presence or absence of acute ammonia challenge. These findings indicated that *B. subtilis* probiotic supplementation reduced lipid peroxidation injury in Nile tilapia. Similarly, *B. subtilis* probiotics enhance the antioxidant enzyme activity in juvenile *L. vannamei* (Tao et al., 2022). These findings reveal that dietary supplementation of 0.1% and 0.3% *B. subtilis* probiotics reduced reactive oxygen species produced by ammonia stress and maintained the host's health. In response to stressors, *Nrf2* and *Keap1* can dissociate from the cytoplasm, allowing *Nrf2* to translocate to the nucleus, and activating the transcription of downstream antioxidant enzyme genes (Lee and Johnson, 2004). This study observed *Nrf2* down-regulation and *SOD* up-regulation in the 0.1% *B. subtilis* probiotics diet, implying that 0.1% *B. subtilis* probiotics could inhibit the Nrf2-Keap1 signal pathway and increase *SOD* gene expression with acute ammonia challenge.

The villi height, width, and muscular layer thickness are good indicators of intestinal health (Khojasteh, 2012). *Bacillus amyloliquefaciens* supplementation increased villi length, improving intestinal morphology (Reda and Selim, 2015). These changes in intestinal morphology likely result from the complete utilization of carbohydrates and short-chain fatty acid production. The findings indicated that *B. subtilis* probiotic supplementation improved intestinal morphology regardless of protein level, and the benefit was remarkable in low-protein treatments. Furthermore, Nile tilapia fed with the P32-0.3 diet reduced villi height, indicating that at a low protein level, higher supplementation positively impacts the gut morphology. It is possible that dietary protein levels, due to differences in ingredient composition, can differentially alter intestinal microflora and affect the intestinal morphology. Further investigation is needed to elucidate these differences completely.

5. Conclusions

In summary, a low protein diet (28%) did not result in differences in the performance of tilapia compared with a high protein diet (32%). However, supplementation of *B. subtilis* probiotics produced different performances between the high and low protein diets. Dietary supplementation of *B. subtilis* DSM 32315 probiotics at 0.1% in the low protein diet and up to 0.3% in the high protein diet showed beneficial effects on the growth, immunity, and antioxidant capacity of Nile tilapia. Studying ammonia stress showed the need for higher supplementation (0.3% in the diet) of *B. subtilis* DSM 32315 probiotics to improve the stress tolerance and reduce mortality of Nile tilapia, despite the two dietary protein levels (32%; 28%).

Author contributions

Author contributions were as follows. **Jin Niu** and **Juyun He:** Designed the study. **Xuanshu He** and **Hanlin Wei:** Carried out the rearing trial. **Ziqiao Wang**, **Zhihong Liao**, **Wei Zhao**, **Yantao Liu**, **Zhenxiao Zhuang** and **Karthik Masagounder:** Analyzed parts of results. **Zhihong Liao:** Wrote this paper with suggestions from **Jin Niu**, **Juyun He** and **Karthik Masagounder**.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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