

Article

Sand Substrate Thickness Regulates Growth Performance, Intestinal Antioxidant Defense, and Gut Microbiota in an Experimental Culture of *Marsupenaeus japonicus*

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Simple Summary

Shrimp farming often uses artificial ponds, but it is unclear how much sand should be placed at the bottom to best support shrimp health and growth. This study tested four different sand thicknesses in a 120-day farming experiment with kuruma shrimp to determine which condition was most suitable. We found that shrimp raised without sand grew more slowly and had much lower survival rates. In contrast, shrimp raised on a sand layer 10 cm thick grew faster, survived better, and showed healthier intestines. These shrimps also experienced less damage caused by internal stress and had fewer damaged intestinal cells. In addition, the community of helpful microorganisms living in the shrimp intestine was richer and more balanced when a 10 cm sand layer was used, while the absence of sand reduced this diversity. Very thick sand layers did not provide additional benefits and may increase health risks. Overall, this study shows that using an appropriate sand thickness, especially 10 cm, can improve shrimp growth, intestinal health, and long-term survival. These findings provide practical guidance for shrimp farmers and can help make shrimp production healthier, more efficient, and more sustainable.

Abstract

Kuruma shrimp (*Marsupenaeus japonicus*) exhibit natural sand-burrowing behavior, but the optimal sand substrate thickness for industrial farming remains unclear. This study evaluated the effects of different sand layer thicknesses on growth performance, intestinal health, oxidative status, and gut microbiota in *Marsupenaeus japonicus*. A 120-day controlled farming experiment was conducted using four sand substrate treatments: 0 cm (no sand), 5 cm, 10 cm, and 20 cm, with three replicate ponds per treatment. Growth indices, survival rate, intestinal histology, antioxidant enzyme activity, gene expression, and gut microbial composition were analyzed. Shrimp reared without sand showed markedly reduced growth and survival, increased intestinal damage, and higher oxidative stress. In contrast, shrimp cultured with a 10 cm sand layer exhibited improved growth and survival, lower intestinal oxidative damage and cell apoptosis, and healthier intestinal structure. This condition also supported a more diverse and stable intestinal microbial community and a lower abundance of opportunistic pathogenic bacteria compared with thinner or thicker sand layers. Overall, these results indicate that a sand substrate thickness of 10 cm provides



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the most favorable balance between growth, intestinal health, and microbial stability, offering practical guidance for optimizing kuruma shrimp aquaculture.

Keywords: *Marsupenaeus japonicus*; sand substrate; growth; oxidative stress; apoptosis; gut microbiota; 16S rRNA

1. Introduction

Marsupenaeus japonicus (Decapoda: Penaeidae), commonly known as kuruma shrimp, is an economically important crustacean widely distributed throughout the Indo-West Pacific [1]. It is currently farmed in multiple Asian and European countries and accounts for more than 5% of global shrimp production [2]. China is the leading producer, with an annual output of 45,968 tons in 2024 [3]. Owing to its natural burrowing behavior, *M. japonicus* is traditionally cultivated using ecological farming approaches, and recent industrial systems increasingly incorporate sand substrates to improve production efficiency. Substrate quality is therefore a critical determinant of successful industrial farming of *M. japonicus* [4].

This issue is particularly relevant as high-density polyethylene (HDPE)-lined systems are increasingly replacing traditional earthen ponds, owing to their superior control of seepage, dike erosion, sediment intrusion, disease transmission, and harvesting efficiency. Given the inherently slow growth rate of *M. japonicus*, the integration of nursery and grow-out phases is commonly used to promote compensatory growth and shorten the culture period. In addition to substrate composition, sediment depth plays a crucial role in influencing shrimp survival and growth, as optimal conditions help maintain a stable microbial–shrimp equilibrium [5]. Burrowing behavior and sediment distribution have been well documented in earthworms [6], and soil depth preferences are known to vary among species in relation to organic matter distribution [7]. Considering the depth-dependent effects of sediment on feeding and burrowing behavior [8], the influence of sediment thickness on kuruma shrimp warrants systematic investigation.

Optimizing shrimp cultivation requires a thorough understanding of both physiological and behavioral responses. Kuruma shrimp exhibit inherent burrowing behavior and nocturnal feeding habits [4], which necessitate the provision of sandy substrates during the grow-out phase and the implementation of nighttime feeding protocols. Although substrate characteristics such as composition and particle size have been investigated [9], the sand thickness preferences of juvenile *M. japonicus* remain poorly characterized. We hypothesized that sediment depth influences survival and growth performance throughout the ontogeny of *M. japonicus*. To test this hypothesis, early juveniles were cultured under three sediment depths in a controlled grow-out experiment. This study elucidates the adaptive responses of kuruma shrimp to varying sand substrates and provides a scientific basis for optimizing healthy and efficient shrimp cultivation.

2. Material and Methods

2.1. Ethical Considerations

All animal procedures were conducted in strict accordance with the Guidelines for the Care and Use of Laboratory Animals of China. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Yellow Sea Fisheries Research Institute (Qingdao, China; Approval No. YSFRI2022026).

2.2. Animals Acquisition and Rearing Conditions

The experiment was carried out at the factory-based shrimp farming facilities of Changyi Haifeng Aquatic Breeding Co., Ltd., Weifang, China. Postlarvae of kuruma shrimp were obtained from wild broodstock originating from Penghu, Taiwan. The post-larvae had an initial average body length of 0.9 ± 0.1 cm and were nursery-reared for 28 days until reaching an average body length of 2.18 ± 0.12 cm and a body weight of 0.093 ± 0.01 g, after which they were transferred to the experimental culture system. A total of 12 culture ponds were used in this study. All ponds were rectangular cement tanks with an area of 8 m² and a water depth of 1 m, each equipped with a drainage outlet located at the inner bottom side of the pond.

2.3. Culture System and Experimental Design

Four sand substrate treatments were established according to substrate thickness: 0 cm (non-sand control), 5 cm (low-sand group, LG), 10 cm (medium-sand group, MG), and 20 cm (thick-sand group, TG). Each treatment was conducted in triplicate, with 1400 shrimp stocked per pond, corresponding to an intensive culture density of 175 shrimp per square meter. All other environmental and management conditions were maintained consistently across treatments. The substrate consisted of a sandy bed composed of 70% sand (particle size 500–700 µm) and 30% soil (particle size 100–250 µm). Sundried beach sand was sieved through a 700 µm mesh and retained on a 500 µm mesh, while sun-dried pond soil was sieved through 250 µm and 100 µm meshes. The processed substrates were disinfected with bleaching powder at 60 ppm (available chlorine 20 ppm), thoroughly rinsed with clean water, and air-dried prior to use.

The experiment lasted for 120 days. When shrimp body length was less than 4.5 cm, they were fed four times daily at 06:00, 12:00, 18:00, and 24:00. Once body length exceeded 4.5 cm, feeding frequency was reduced to three times daily at 09:00, 18:00, and 24:00. During the first two months, shrimp were primarily fed frozen brine shrimp, whereas a formulated pellet diet containing 45% crude protein was used during the latter two months. Routine management procedures were performed daily at 08:00, including waste removal, partial water exchange, and the removal of molted shells. The daily water exchange volume was approximately 30% of the total culture water volume.

2.4. Collection of Samples

After the initiation of the experiment, intestinal tissue samples of *M. japonicus* were collected on days 60, 90, and 120. On day 120, intestinal segments from six shrimp per replicate ($n = 3$ replicates per treatment) were collected and immediately fixed in 4% paraformaldehyde (Sangon Biotech, Shanghai, China). Of these, three biological replicates (one shrimp per replicate) were processed for histological analysis. To minimize the physiological variability associated with the molting cycle, shrimp were sampled during the intermolt stage (Stage C) [10]. For biochemical and molecular analyses, intestinal tissue samples weighing approximately 80–100 mg were excised, rapidly frozen, and stored at -80 °C until further use.

2.5. Basal Growth Index

At the end of the experimental period, the final body weight and body length of all shrimp were measured. Survival rate (SR), body length growth rate (BLGR), weight gain rate (WGR), and specific growth rate (SGR) were calculated using the following formulas:

$$\text{SR (\%)} = (\text{total shrimp} - \text{dead shrimp}) / \text{total shrimp} \times 100$$

$$\text{BLGR (\%)} = (\text{final body length} - \text{initial body length}) / \text{initial body length} \times 100$$

$$\text{WGR (\%)} = (\text{final weight} - \text{initial weight}) / \text{initial weight} \times 100$$

$$\text{SGR (\%/day)} = (\ln \text{ final weight} - \ln \text{ initial weight}) / \text{culture duration days} \times 100$$

2.6. Observations of Intestine Structure and Apoptosis

Intestinal histomorphological analysis was performed using established methods [11]. Briefly, intestinal samples were fixed in 4% paraformaldehyde for 24 h at 4 °C and then rinsed under running water for 8 h. Samples were dehydrated through a graded ethanol series (70%, 80%, 90%, and 100%), cleared in xylene, embedded in paraffin, and sectioned at a thickness of 4 µm using a Leica RM2016 microtome (Leica Microsystems, Wetzlar, Germany). Sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus, Tokyo, Japan). Histomorphometric parameters were quantified using the CaseViewer software (V.2.4, Budapest, Hungary). Unless otherwise stated, all chemical reagents were of analytical grade and supplied by Sinopharm Group Co., Ltd. (Shanghai, China).

2.7. Analysis of Antioxidant Enzyme Activity

Intestinal tissues stored at −80 °C were thawed on ice and homogenized (1:9, *w/v*) in phosphate-buffered saline (PBS; pH 7.2). The homogenates were centrifuged at 9300× *g* for 10 min, and the supernatants were collected for subsequent analyses. Total antioxidant capacity (T-AOC; Cat. No. A015-1-1) and glutathione peroxidase (GSH-PX; Cat. No. A005-1-2) activities were measured by colorimetric methods at 520 nm and 412 nm, respectively. Superoxide dismutase (SOD; Cat. No. A001-3-1) activity was determined using the WST-1 method at 450 nm. Catalase (CAT) activity was measured using a catalase assay kit (Cat. No. A007-1-1; ammonium molybdate method) at 405 nm. Malondialdehyde (MDA; Cat. No. A003-1-1) content was quantified using the thiobarbituric acid (TBA) method at 532 nm. All assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Protein concentrations were determined using the Bradford assay with bovine serum albumin as the standard [12]. All measurements were performed in triplicate using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA).

2.8. Gene Expression Analysis of Antioxidant Capacity and Energy (Carbohydrate, Lipid and Protein) Metabolism by Real-Time Quantitative PCR (qPCR)

Approximately 80–100 mg of intestinal tissue was weighed, and total RNA was extracted using RNA Isolater Total RNA Extraction Reagent (Vazyme Biotech Co., Ltd., Nanjing, China). RNA concentration and purity were assessed using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse transcribed into complementary DNA (cDNA) using the PrimerScript™ One Step RT-PCR Kit (Vazyme Biotech Co., Ltd., Nanjing, China), following the manufacturer's instructions. The transcription levels of genes related to antioxidant capacity (*SOD*, *CAT*, and heat shock protein 70, *HSP70*) and cellular immune defense (lysozyme, *Lzm*, and *caspase-3*) were quantified, with β-actin used as the internal reference gene. Primer sequences for all target genes are listed in Table 1. Amplification efficiencies were determined using a serial dilution of cDNA, and all primer pairs showed efficiencies ranging from 90% to 110% (Table 1).

Quantitative PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq II (Vazyme Biotech Co., Ltd., Nanjing, China). Each reaction was carried out in a 20 µL volume containing 10 µL of 2× SYBR Green I Master Mix, 1.0 µL of each gene-specific primer (10 µM), 6 µL of DEPC-treated water, and 2 µL of cDNA template [13]. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s; 45 cycles of denaturation at 95 °C for 5 s

and annealing at 58 °C for 30 s; followed by melt curve analysis at 95 °C for 5 s, 60 °C for 60 s, heating to 95 °C, and cooling to 50 °C for 30 s. Relative gene expression levels were calculated using the 2^{−ΔΔCT} method, as described by Livak and Schmittgen [14].

Table 1. Primers used for real-time quantitative PCR.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplification Efficiencies (%)
SOD	TCAATCCTCTCCCACACA	CACAGACAGGCAGAGCAGT	96
CAT	GCCTCAGGAGAACGTTGTTG	TGAGCTTGTCTGAGAGTGGG	92
Hsp70	ACAAGTCCATCAACCCCGAT	GGTGAAGGTCTGAGTCTGCT	90
Lzm	CAGTAGTGGCTGTGTCATGC	AGGTATGCACGGACAGTCTC	110
Caspase-3	CTCTCACGACGCCTACAT	TCCCTGTTGTTTCCTGTTT	97
β-actin	TCCACGAGACCACATACAAC	CACTTCCTGAACGATTGA	100

2.9. Gut Microbiota Profiling

Total genomic DNA of the hindgut microbiota from the control, LG, MG, and TG groups was extracted, and DNA quality and concentration were assessed prior to downstream analyses. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Amplified products were verified by 2% agarose gel electrophoresis, purified using a commercial gel extraction kit (Axygen Biosciences, Union City, CA, USA), and quantified with a Quantus™ Fluorometer (Promega, Madison, WI, USA). Sequencing libraries were constructed using the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific Corp., Austin, TX, USA) and paired-end sequencing (2 × 300 bp) was performed on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) according to standard protocols provided by Majorbio Bio-Pharm Technology (Shanghai, China). Raw sequencing data were processed, and operational taxonomic units (OTUs) were clustered at a 97% sequence similarity threshold using UPARSE (<http://www.drive5.com/uparse/>, accessed 15 January 2025). Alpha diversity indices and rarefaction curves were calculated using Mothur (<http://www.mothur.org/>, accessed 15 January 2025), while beta diversity analyses were conducted using QIIME (v2020.2.0). Data visualization and statistical plotting were performed using R software (v3.3.1).

2.10. Statistical Analysis

All statistical analyses were performed using IBM® SPSS Statistics Version 26.0 (IBM Corp., Armonk, NY, USA), with statistical significance set at *p* < 0.05. Quantitative data are presented as the mean ± standard error (SE) based on three biological replicates (*n* = 3). Prior to analysis, data were assessed for normality and homogeneity of variance. When these assumptions were satisfied, differences among groups were evaluated using one-way analysis of variance (ANOVA), followed by Duncan’s multiple range test for post hoc comparisons. When the assumptions of normality or homoscedasticity were not met, the nonparametric Kruskal–Wallis test was applied. Principal component analysis (PCA) was conducted using OriginPro 2021 software (OriginLab Corporation, Northampton, MA, USA) to explore relationships among variables across experimental groups.

3. Results

3.1. Growth Performance

As shown in Table 2, the growth rate, weight gain rate, and SGR of *M. japonicus* in the control group were consistently the lowest across all sampling periods compared with those in the sand substrate groups, with significant differences observed (*p* < 0.05).

Compared with the MG and TG groups, shrimp in the LG group exhibited inferior growth performance. By day 120, the growth rate, weight gain rate, and SGR in the LG group were significantly lower than those in the MG and TG groups. Throughout the experimental period, shrimp cultured in the MG and TG groups maintained relatively high growth levels, with no significant differences in growth performance between these two groups. Survival rates in the control, LG, MG, and TG groups were 20.5%, 78.5%, 85.7%, and 87.3%, respectively. The control group showed the lowest survival rate, which was significantly lower than that of all sand substrate groups ($p < 0.05$). Survival in the LG group was significantly lower than that in the MG and TG groups, whereas the TG group exhibited the highest survival rate, although no significant difference was detected between the TG and MG groups.

Table 2. Growth performance of cultured *M. japonicus* under varying sand substrate thicknesses.

Growth Indicators (%)	Control	LG	MG	TG
30 d Length growth rate	61.11 ± 0.82 ^a	86.90 ± 1.74 ^b	95.69 ± 2.42 ^c	91.29 ± 2.27 ^{bc}
60 d Length growth rate	128.94 ± 2.95 ^a	155.08 ± 4.31 ^b	155.16 ± 3.10 ^b	160.18 ± 0.76 ^b
90 d Length growth rate	184.98 ± 1.15 ^a	197.02 ± 1.25 ^b	204.98 ± 1.18 ^c	203.17 ± 0.88 ^c
120 d Length growth rate	216.05 ± 2.54 ^a	227.17 ± 0.96 ^b	237.31 ± 0.56 ^c	237.48 ± 3.34 ^c
30 d Weight gain rate	381.36 ± 14.70 ^a	633.51 ± 47.90 ^b	802.69 ± 14.95 ^c	718.64 ± 19.77 ^d
60 d Weight gain rate	1308.96 ± 13.69 ^a	1836.92 ± 108.47 ^b	1910.57 ± 67.67 ^{bc}	2030.82 ± 33.45 ^c
90 d Weight gain rate	2205.56 ± 52.46 ^a	3448.03 ± 191.60 ^b	3832.80 ± 85.41 ^c	3607.89 ± 124.69 ^{bc}
120 d Weight gain rate	4534.59 ± 60.57 ^a	4772.76 ± 59.81 ^b	5148.21 ± 18.50 ^c	5318.46 ± 114.30 ^c
30 d Specific growth rate	5.31 ± 0.01 ^a	6.64 ± 0.22 ^b	7.33 ± 0.06 ^c	7.05 ± 0.008 ^c
60 d Specific growth rate	4.41 ± 0.02 ^a	4.94 ± 0.09 ^b	5.00 ± 0.06 ^b	5.10 ± 0.03 ^b
90 d Specific growth rate	3.49 ± 0.03 ^a	3.96 ± 0.06 ^b	4.08 ± 0.02 ^c	4.01 ± 0.04 ^{bc}
120 d Specific growth rate	3.20 ± 0.01 ^a	3.24 ± 0.01 ^b	3.30 ± 0.003 ^c	3.33 ± 0.02 ^c
Survival rate	20.57 ± 2.63 ^a	78.54 ± 1.26 ^b	85.71 ± 0.91 ^c	87.32 ± 0.56 ^c

^{a–d} The values in the same row sharing different superscript letters are significantly different.

3.2. Analysis of Oxidative Stress

As shown in Table 3, T-AOC levels in *M. japonicus* varied significantly among sediment treatments. At day 90, T-AOC declined markedly in all groups, with the most pronounced decrease observed in the control group, resulting in significantly lower levels than those in the sand substrate groups. By day 120, T-AOC levels continued to decrease across all treatments; however, values in the MG and TG groups remained significantly higher than those in the control group. SOD activity increased by day 90 and subsequently decreased by day 120 in all groups. SOD activity was consistently highest in the control group throughout the experimental period. At day 120, SOD activity in the MG group was significantly lower than that in the control group but did not differ significantly from that in the TG group. CAT activity differed among sediment treatments. At day 60, CAT activity was significantly lower in all sand substrate groups than in the control group ($p < 0.05$). CAT activity increased by day 90 and declined by day 120 in the sand substrate groups, whereas it increased progressively in the control group throughout the culture period. GSH content decreased by day 90 and increased by day 120 in the control, LG, and TG groups. In contrast, GSH levels increased continuously in the MG group during the experiment, resulting in significantly higher values than those in the control group. At day 90, GSH-PX activity was significantly lower in the control group and significantly higher in the TG group compared with the other groups, while activities in the LG and MG groups decreased but without significant differences. By day 120, GSH-PX activity declined significantly in all groups, with the most pronounced decrease observed in the MG group, which differed significantly from the other treatments. MDA content, an indicator of intestinal oxidative damage, exhibited distinct temporal patterns among groups. At

day 90, MDA content decreased significantly in the control group but increased slightly by day 120. In contrast, MDA content decreased progressively in all sand substrate groups throughout the experimental period. At day 60, MDA content was significantly lower in the TG group than in the other groups; however, this difference gradually diminished over time. By day 120, the lowest MDA content was observed in the MG group, which was significantly lower than that in all other groups.

Table 3. Intestinal immunity and antioxidant parameters in *M. japonicus* under sand thickness gradients.

Parameters (%)	Control	LG	MG	TG
60 d T-AOC activity	1.71 ± 0.02 ^a	1.71 ± 0.03 ^a	1.56 ± 0.04 ^b	1.28 ± 0.03 ^c
90 d T-AOC activity	0.42 ± 0.03 ^a	0.67 ± 0.02 ^b	0.53 ± 0.03 ^c	0.68 ± 0.02 ^b
120 d T-AOC activity	0.41 ± 0.01 ^a	0.38 ± 0.02 ^b	0.45 ± 0.008 ^c	0.46 ± 0.008 ^c
60 d SOD activity	29.52 ± 0.48 ^a	25.04 ± 0.35 ^b	23.31 ± 0.68 ^c	27.05 ± 3.38 ^d
90 d SOD activity	43.44 ± 0.44 ^a	32.37 ± 0.40 ^b	34.42 ± 0.49 ^c	33.48 ± 0.60 ^d
120 d SOD activity	30.24 ± 0.48 ^a	29.55 ± 0.98 ^{ab}	28.11 ± 0.39 ^c	28.61 ± 0.25 ^{bc}
60 d CAT activity	0.22 ± 0.007 ^a	0.09 ± 0.007 ^b	0.16 ± 0.006 ^c	0.17 ± 0.004 ^c
90 d CAT activity	0.25 ± 0.007 ^a	0.21 ± 0.005 ^b	0.29 ± 0.004 ^c	0.32 ± 0.005 ^d
120 d CAT activity	0.27 ± 0.007 ^a	0.15 ± 0.002 ^b	0.26 ± 0.009 ^a	0.31 ± 0.004 ^c
60 d GSH content	29.47 ± 0.73 ^a	31.42 ± 0.57 ^b	25.76 ± 1.15 ^c	27.88 ± 0.83 ^a
90 d GSH content	16.62 ± 0.72 ^a	21.46 ± 0.97 ^b	31.46 ± 0.67 ^c	22.15 ± 0.97 ^b
120 d GSH content	24.37 ± 0.94 ^a	22.01 ± 1.09 ^b	41.22 ± 0.75 ^c	52.36 ± 0.90 ^d
60 d GSH-PX activity	34.92 ± 0.58 ^a	37.17 ± 0.71 ^b	38.11 ± 1.01 ^b	35.62 ± 0.41 ^a
90 d GSH-PX activity	18.12 ± 0.95 ^a	34.99 ± 0.87 ^b	33.92 ± 0.71 ^b	45.93 ± 0.34 ^c
120 d GSH-PX activity	13.21 ± 0.73 ^a	19.42 ± 0.62 ^b	8.51 ± 0.66 ^c	14.61 ± 1.03 ^a
60 d MDA content	1.75 ± 0.03 ^a	1.76 ± 0.03 ^a	1.70 ± 0.04 ^b	1.39 ± 0.02 ^c
90 d MDA content	0.39 ± 0.05 ^a	0.50 ± 0.03 ^b	0.79 ± 0.03 ^c	0.57 ± 0.04 ^d
120 d MDA content	0.46 ± 0.02 ^a	0.41 ± 0.02 ^b	0.32 ± 0.02 ^c	0.37 ± 0.03 ^b

^{a–d} The values in the same row sharing different superscript letters are significantly different.

As shown in Figure 1A, SOD expression was significantly higher in the TG group than in the other groups at days 60 and 90, whereas the highest expression level at day 120 was observed in the control group. Figure 1B shows that CAT expression was highest in the control group at days 60 and 90; at day 120, CAT expression peaked in the MG group but did not differ significantly from that in the TG group. Figure 1C demonstrates that Hsp70 expression remained relatively stable in the control group throughout the experiment, whereas sand substrate groups exhibited a biphasic pattern characterized by an initial decrease followed by an increase. Figure 1D shows consistently low Po expression in the control group, with peak expression observed in the TG group at days 60 and 90 and shifting to the LG group by day 120. As shown in Figure 1E,F, *Lzm* and *caspase-3* expression in the sand substrate groups followed a U-shaped pattern, decreasing initially and increasing thereafter; at day 120, the highest expression levels were detected in the TG group, while the control group exhibited the lowest levels.

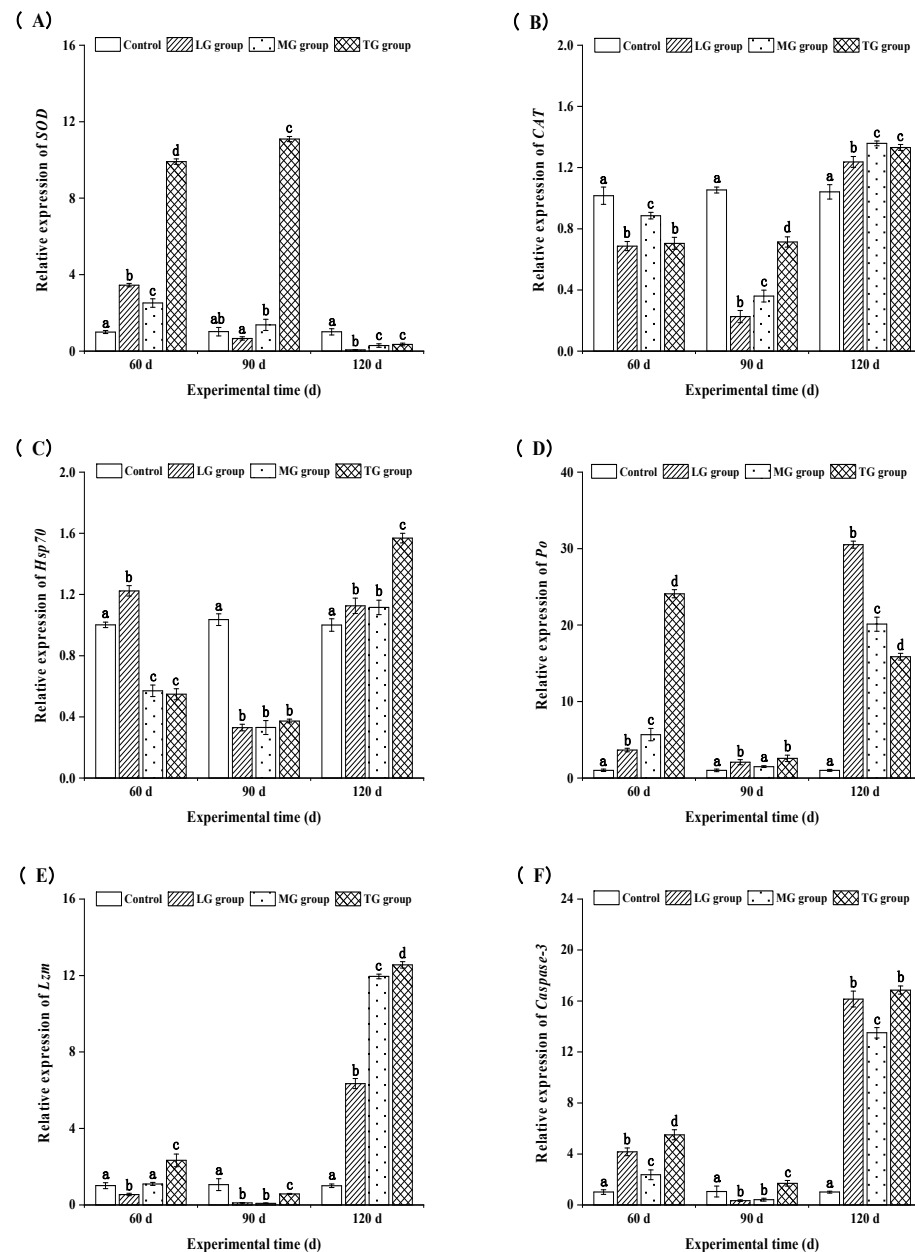


Figure 1. Immunity and antioxidant gene expression in *M. japonicus* intestine under sand thickness variations. (A) *SOD* gene, (B) *CAT* gene, (C) *Hsp70* gene, (D) *Po* gene, (E) *Lzm* gene, (F) *Caspase-3* gene. ^{a–d} The values in the same row sharing different superscript letters are significantly different.

3.3. Changes of the Intestine Histological Structure of *M. japonicus*

After 120 days of culture, H&E staining revealed mild swelling and congestion of the intestinal wall in the control group (Figure 2). In contrast, the LG group exhibited pronounced intestinal wall thickening, which was primarily attributable to severe congestion and inflammatory infiltration. No obvious congestion or inflammation was observed in the MG or TG groups, and intestinal wall thickness in these groups was significantly reduced compared with that in the control and LG groups. Apoptosis in intestinal tissues was assessed using the TUNEL assay (apoptotic cells shown in green; Figure 3). The basal lamina exhibited swelling and fragmentation in response to differences in substrate thickness. Quantitative analysis showed that apoptotic cell numbers were lowest in the control group and highest in the LG group. With increasing substrate thickness, the number of apoptotic cells decreased significantly in the MG and TG groups.

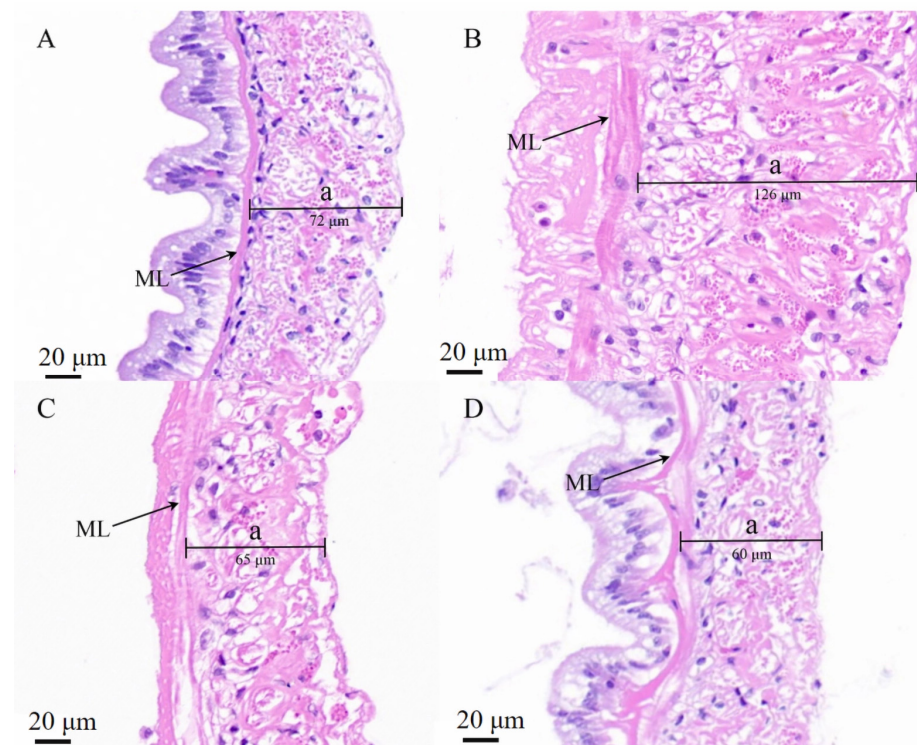


Figure 2. Intestinal histopathology. (A) control group, (B) LG group, (C) MG group, (D) TG group. Abbreviations: ML, muscular layer; a, intestinal wall thickness.

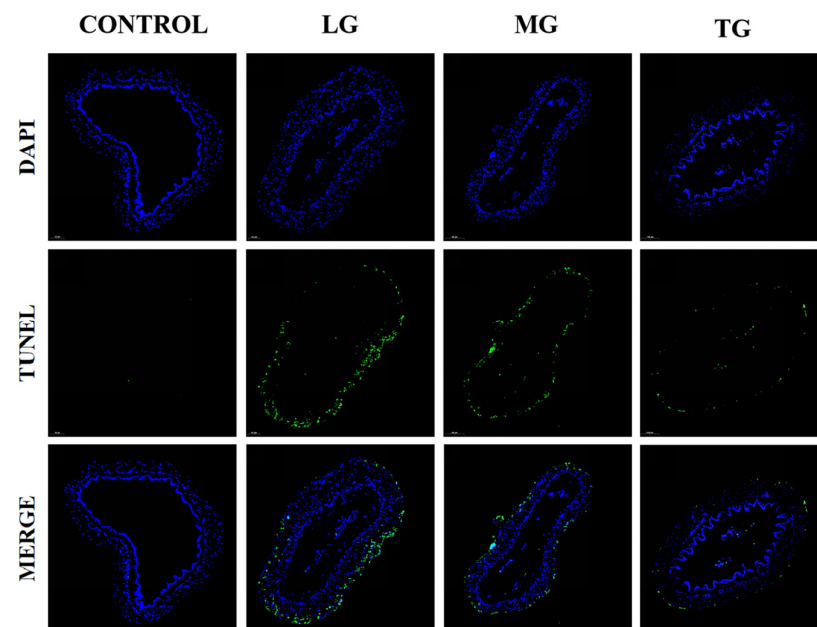


Figure 3. Gut epithelial apoptosis in *M. japonicus*.

3.4. Gut Microbiota

3.4.1. Diversity

The Ace and Chao indices were used to assess microbial abundance, whereas the Shannon and Simpson indices were used to evaluate microbial diversity. As shown in Table 4, the Ace and Chao indices in the control group were significantly lower than those in the sand substrate groups, indicating that the absence of a sand substrate reduced intestinal microbial abundance in *M. japonicus*. The Simpson diversity index was significantly higher

in the MG group than in the LG and TG groups, suggesting that a sand substrate thickness of 10 cm was more conducive to enhancing intestinal microbial diversity in *M. japonicus*.

Table 4. Intestinal microbiota alpha diversity in *M. japonicus* across sand thicknesses.

Groups	Shannon Index	Simpson Index	ACE Index	Chao Index
Control	4.05 ± 0.16 ^a	0.07 ± 0.002 ^a	417.18 ± 13.30 ^a	419.68 ± 14.99 ^a
LG	4.38 ± 0.18 ^b	0.05 ± 0.002 ^b	546.56 ± 14.99 ^b	546.81 ± 12.66 ^b
MG	3.99 ± 0.15 ^a	0.09 ± 0.003 ^c	441.81 ± 18.32 ^c	442.39 ± 17.46 ^c
TG	4.37 ± 0.12 ^b	0.04 ± 0.003 ^d	512.59 ± 18.14 ^d	513.96 ± 17.15 ^d

^{a–d} The values in the same row sharing different superscript letters are significantly different.

As illustrated in Figure 4, NMDS and PCoA analyses demonstrated a clear separation between the intestinal bacterial community of the control group and those of the sand substrate groups, whereas the sand substrate groups exhibited partial overlap. In terms of sample distribution, samples from the TG group were more tightly clustered, while samples from the other groups showed greater dispersion.

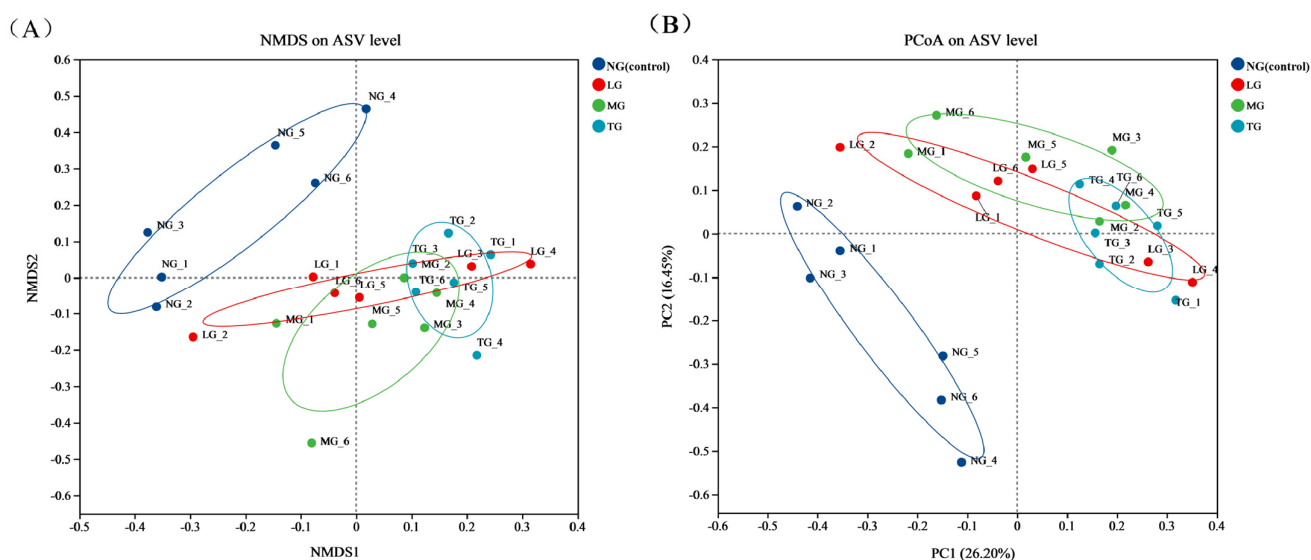


Figure 4. Beta diversity analysis of intestinal microbiota in *M. japonicus*. (A) Nonmetric multidimensional scaling (NMDS); (B) Principal coordinates analysis (PCoA).

3.4.2. Key Species Responsible for Differences in Gut Microbiota Across Sand Thicknesses

At the phylum level, the predominant intestinal microbiota (relative abundance $\geq 1\%$) across all experimental groups of *M. japonicus* consisted mainly of *Proteobacteria*, *Bacteroidetes*, *Actinobacteriota*, and *Firmicutes* (Figure S1). The relative abundances of *Proteobacteria* and *Firmicutes* were lower in the sand substrate groups than in the control group, whereas *Actinobacteriota* were more abundant in the sand substrate groups. At the genus level, dominant taxa (relative abundance $\geq 1\%$) included *Vibrio*, *Photobacterium*, *Ruegeria*, and members of the families *Rhodobacteraceae*, *Thiotrichaceae*, and *Flavobacteriaceae* (Figure S2). Compared with the control group, the sand substrate groups exhibited higher proportions of *Photobacterium* and *Actinomarinales*, along with lower proportions of *Rhodobacteraceae*, *Ruegeria*, *Thiotrichaceae*, *Enterococcus*, and *Motilimonas*. The dominant taxa in each group were identified as *Thiotrichaceae* in the control group, *Rhodobacteraceae* in the LG group, *Photobacterium* in the MG group, and *Vibrio* in the TG group. Notably, *Photobacterium* in the LG group and *Vibrio* in the TG group each reached relative abundances of approximately 20%, which were significantly higher than those observed in the other groups.

A total of 219 amplicon sequence variants (ASVs) were shared among all four groups, with 283 ASVs shared between the control and LG groups, 277 between the control and MG groups, and 262 between the control and TG groups (Figure S3). The control group contained the highest number of unique ASVs (81), whereas the MG group had the lowest number (59). Pie chart analysis showed that the three most abundant taxa in the control and LG groups were *Thiotrichaceae*, *Rhodobacteraceae*, and *Ruegeria*. In the control and MG groups, the dominant taxa were *Photobacterium*, *Rhodobacteraceae*, and *Thiotrichaceae*, while the control and TG groups were dominated by *Vibrio*, *Rhodobacteraceae*, and *Ruegeria*. Across all groups, the three most prevalent taxa were *Vibrio*, *Photobacterium*, and *Rhodobacteraceae*.

The Kruskal–Wallis rank sum test results indicated that the relative abundance of *Proteobacteria* was significantly higher in the control group than in the sand substrate groups, whereas the abundances of *Actinobacteriota*, *Chloroflexi*, and *Patescibacteria* were significantly lower in the control group (Figures S4 and S5). Among the sand substrate groups, *Proteobacteria* abundance was highest in the MG group and lowest in the LG group. *Actinobacteriota* accounted for a substantial proportion of the microbiota in the sand substrate groups, indicating marked dominance, while *Chloroflexi* abundance was significantly higher in the LG group than in the other groups.

As shown in Figure S6, LEfSe analysis revealed that *Pseudoalteromonadaceae* were significantly enriched in the control group compared with the LG group, whereas *Actinobacteriota* were significantly enriched in the LG group. In comparisons between the control and MG groups, *Alteromonadales* were more enriched in the control group, while *Photobacterium* and *Actinobacteriota* were significantly enriched in the MG group. Similarly, comparisons between the control and TG groups showed significant enrichment of *Pseudoalteromonadaceae* in the control group and *Actinobacteriota* in the TG group.

3.4.3. Effect of Sand Thickness on the Function of Gut Microbiota

As shown in Figure 5, the predicted functions of the intestinal microbiota were predominantly associated with primary metabolic pathways across all experimental groups. Functions related to Genetic Information Processing, Environmental Information Processing, and Cellular Processes were present at lower relative abundances. Secondary pathway analysis further revealed that gene functions were mainly enriched in carbohydrate metabolism and amino acid metabolism within the Metabolic pathways category; replication and repair within Genetic Information Processing; membrane transport within Environmental Information Processing; and cellular community–prokaryotes within Cellular Processes. No significant differences in the relative abundance of predicted functional pathways were observed among the experimental groups, indicating a high degree of functional stability in the intestinal microbiota of *M. japonicus* and a minimal impact of sand substrate thickness on overall microbial functional potential.

Sediment plays a vital role in aquaculture by providing nutrients, regulating water quality, and serving as an ecological matrix that supports the survival and reproduction of diverse beneficial and benthic organisms. The behavior and growth of crustaceans are influenced by multiple sediment-related factors, including substrate presence, type, and thickness [15,16]. The present study demonstrates that the survival and growth of *M. japonicus* from the early juvenile to adult stages are strongly dependent on sand substrate thickness. In particular, shrimp reared under non-sand and low-sand conditions exhibited markedly reduced survival and growth performance. In natural aquatic systems, the sediment–water interface represents a zone of intense physical, chemical, and biological interactions, which directly influence the feeding and burrowing behaviors of benthic macroinvertebrates.

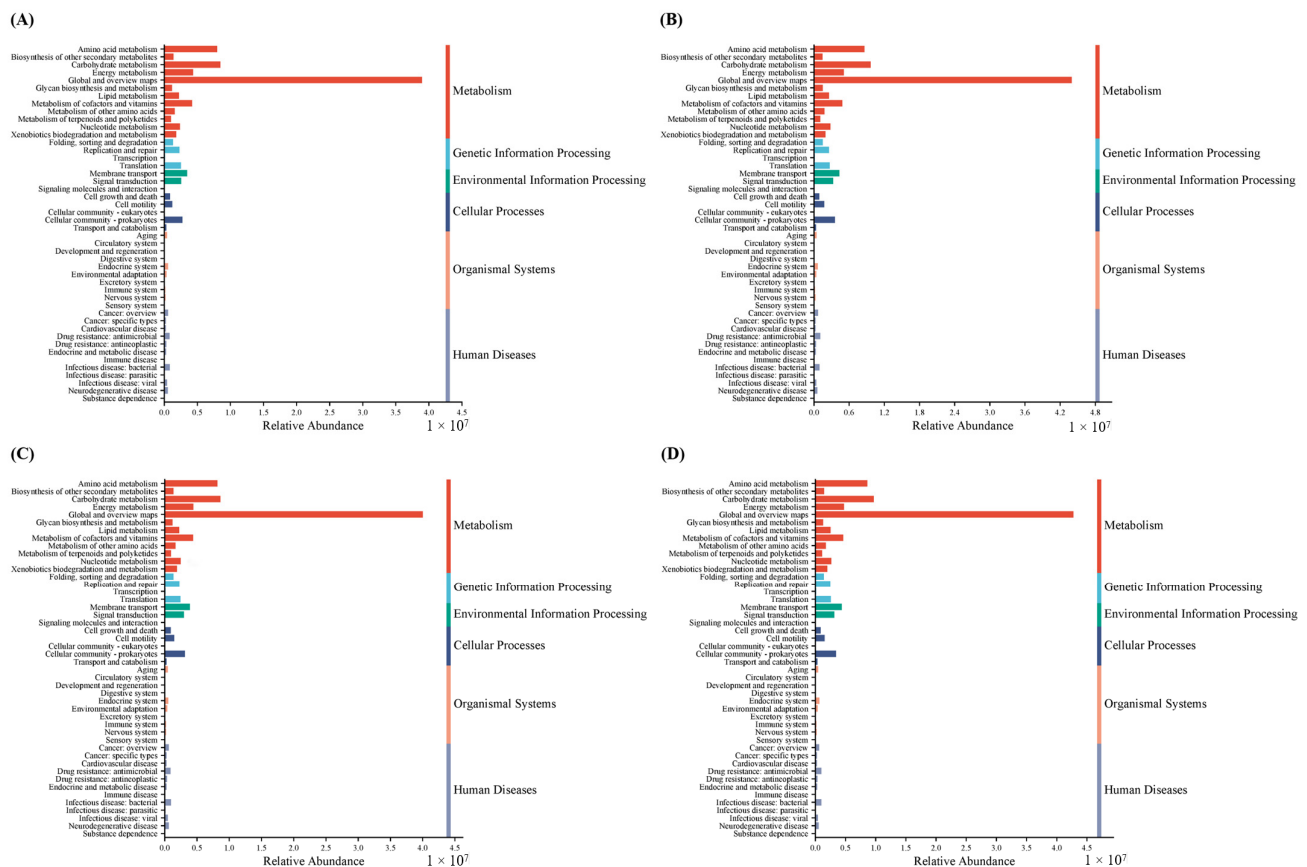


Figure 5. KEGG functional prediction of intestinal microbiota in *M. japonicus* under sand thickness treatments: (A) control group, (B) LG group, (C) MG group, (D) TG group.

4. Discussion

4.1. Effects of Different Sand Laying Thicknesses on the Shrimp Growth Performances

The effects of sediment on crustacean growth were evaluated over a 120-day culture period, during which clear differences in growth performance were observed among experimental groups. Shrimp in the control group exhibited the lowest growth at day 60, while growth performance in the LG group declined noticeably by day 90, particularly with respect to weight gain rate. In contrast, shrimp in the MG and TG groups maintained relatively stable and higher growth rates throughout the culture period. *M. japonicus* undergoes frequent molting during metamorphosis, juvenile growth, and reproduction [15], and molting frequency is strongly influenced by environmental conditions, with increased molting generally associated with enhanced growth [17]. At day 90, molting activity was lowest in the control group, whereas shrimp in the MG and TG groups exhibited higher molting frequencies, which likely contributed to their superior growth performance. Poor growth in the control and LG groups may therefore be attributed to the absence of sand or insufficient sand thickness, indicating that a 5 cm sand layer is inadequate to support optimal growth. By day 120, shrimp in the MG and TG groups clearly outperformed those in the other groups. Previous studies have suggested that a 10 cm sand layer reduces unnecessary movement, conserves energy, and promotes growth in *M. japonicus* [18]. The lack of significant growth differences between the MG and TG groups further suggests that the growth benefits of increasing sand thickness reach a plateau at approximately 10 cm.

Survival data further supported the importance of appropriate sand substrate thickness. The control group exhibited the lowest survival rate (20.5%), whereas survival rates in the LG, MG, and TG groups were markedly higher, at 78.5%, 85.7%, and 87.3%, respectively,

with no significant difference between the MG and TG groups. Daily observations indicated that substantial mortality occurred predominantly in the control group, particularly after day 90, when shrimp appeared severely worn, consistent with previous findings [19]. In sand-free conditions, limited burrowing behavior forced shrimp to interact more frequently with hard surfaces, resulting in increased mechanical damage to appendages. In addition, sand-free culture conditions likely elevated physiological stress and compromised immune function, predisposing shrimp to appendage infections and mortality. The low survival rate in the control group was also associated with intensified aggressive interactions. Under high-density culture conditions, competition for space and food was more pronounced in the absence of sand, leading to increased aggression. Consistent with previous reports, the addition of sediment has been shown to significantly improve growth performance in penaeid shrimp compared with bottomless culture systems [10]. Given the inherently slow growth of *M. japonicus* and its lower growth and survival rates relative to other shrimp species, even under pond culture conditions [20], optimizing sand thickness is particularly important for industrial production. In the present study, both the MG and TG groups exhibited favorable growth and survival outcomes; however, from a practical perspective, a 10 cm sand layer represents a more cost-effective and labor-efficient option for industrial *M. japonicus* culture.

4.2. Effects of Different Sand Thicknesses on the Antioxidant Defense System

ROS are inevitable byproducts of aerobic metabolism, and environmental stress can elevate ROS levels in crustaceans, leading to oxidative stress when antioxidant defenses are insufficient [20]. Antioxidant enzymes and nonenzymatic antioxidants play critical roles in scavenging free radicals and protecting cellular integrity [21]. In the present study, T-AOC activity declined over time in all groups; however, values in the sand substrate groups remained significantly higher than those in the control group during the mid to late culture stages, indicating improved overall antioxidant capacity. In contrast, SOD activity was consistently higher in the control group and peaked at day 90, suggesting increased oxidative challenge under sand-free conditions. CAT activity in the sand substrate groups exhibited a pattern similar to that of SOD, with the TG group showing the highest activity at the end of the culture period. Notably, GSH levels in the TG group were higher than those in the LG and MG groups at day 120, implying greater oxidative stress under excessive sand thickness. GSH-PX is a key antioxidant enzyme involved in protecting cells and DNA from oxidative damage [22]. Although GSH-PX activity declined from day 60 to day 120 in all groups, the MG group exhibited the lowest activity among the sand substrate treatments, suggesting that oxidative stress was relatively alleviated under a 10 cm sand substrate. Consistently, MDA levels, a widely used indicator of lipid peroxidation and oxidative stress in aquatic organisms [23], were markedly lower in the MG group than in the other groups during long-term culture, indicating reduced intestinal lipid peroxidation [24].

Sand thickness also influenced the expression of intestinal immune- and antioxidant-related genes in *M. japonicus*. Among the sand substrate groups, the TG group showed higher expression levels of *SOD*, *CAT*, and *Hsp70* during the mid to late culture stages, suggesting increased oxidative stress under excessive sand thickness. *PO*, a key component of the crustacean immune system involved in host recognition, defense, and melanization [25], exhibited low expression in the control group, further indicating compromised immune function under sand-free conditions. *Lzm*, an important component of the nonspecific immune system [26], and caspase-3, a key regulator of oxidative-stress-induced apoptosis [27], displayed U-shaped expression patterns in the sand substrate groups, decreasing initially and increasing by day 120. Notably, caspase-3 expression was lowest in the MG group, indicating reduced intestinal cell apoptosis under a 10 cm sand substrate during long-term

culture. These results suggest that an intermediate sand thickness provides a more favorable oxidative and immune environment for *M. japonicus* by balancing antioxidant defense and minimizing intestinal cellular damage.

4.3. Effects of Different Sand Thicknesses on Apoptosis

Environmental stress can disrupt intracellular ROS homeostasis, leading to alterations in cellular components and biosynthetic processes and ultimately triggering apoptosis [28]. TUNEL analysis showed that, among the sand substrate groups, the LG group exhibited the highest number of apoptotic cells, followed by the MG and TG groups. These findings indicate that increasing sand substrate thickness effectively reduced oxidative damage in the shrimp intestine and, consequently, decreased apoptotic cell numbers. Unexpectedly, the control group displayed significantly fewer apoptotic cells than the sand substrate groups. This observation may reflect a selection effect, whereby only individuals with stronger stress tolerance survived under sand-free conditions, resulting in an apparently lower level of apoptosis among the remaining shrimp.

Excessive ROS not only induces apoptosis but also compromises intestinal tissue integrity [29]. Environmental stress that damages tissues, cells, and organelles can disrupt normal metabolic processes [30]. Histological examination of intestinal tissues at day 120 revealed swelling and congestion of the intestinal wall in both the control and LG groups, with more severe congestion and wall thickening observed in the LG group. Such inflammatory responses were associated with pronounced thickening of the intestinal wall. In contrast, no obvious congestion or inflammation was detected in the MG or TG groups, and intestinal wall thickness in these groups was markedly reduced compared with that in the control and LG groups. The observed edema and wall thickening were likely attributable to inflammation-induced vascular dilation, cellular swelling, and widening of intercellular spaces. Examination of the intestinal basal lamina further showed that it remained smooth and intact in the control, MG, and TG groups but appeared swollen and disrupted in the LG group. Given that normal intestinal morphology is essential for efficient digestion and nutrient absorption [31], these structural alterations suggest that long-term culture under insufficient sand thickness can impair intestinal integrity and cellular function in *M. japonicus*.

4.4. Effects of Different Sand Thickness on the Intestinal Bacteria

The intestinal microbiota plays a crucial role in crustacean health and homeostasis by influencing community diversity and function, host immunity, metabolism, and growth [32,33]. Previous studies have shown that substrate type affects hindgut microbial diversity in *Urechis unicinctus* larvae and alters crustacean gut microbiota, thereby modulating host metabolism and disease resistance [34,35]. Consistent with these findings, sand substrate thickness in the present study influenced the taxonomic composition, core microbial groups, and interspecific interactions of the intestinal microbiota of *M. japonicus* [36,37]. The absence of sand reduced microbial diversity and altered community structure, which may compromise functional stability and increase disease risk [38]. Notably, a 10 cm sand substrate was associated with improved intestinal microbial diversity, as reflected by a higher Simpson index in the MG group, indicating a more favorable intestinal environment.

Across all experimental groups, *Proteobacteria* dominated the intestinal microbiota, suggesting that this phylum represents a core component of the *M. japonicus* gut microbiome [39]. *Proteobacteria* are widespread in aquatic ecosystems and contribute to nutrient cycling, organic matter mineralization, nitrogen and phosphorus removal, and organic degradation [40]. Given their prevalence in pond sediments, sediment-associated *Proteobacteria* may influence the intestinal microbiota through direct shrimp–sediment interac-

tions [41,42], while increased abundance during later culture stages may also reflect the use of formulated feeds [43]. High *Proteobacteria* abundance has been associated with healthy shrimp intestines [44], and the elevated proportion observed in the MG group suggests that a 10 cm sand substrate may promote intestinal health. *Bacteroidota*, which contribute to intestinal mucosal immune homeostasis and host energy metabolism, were also widely distributed across groups, whereas *Actinobacteriota* and *Chloroflexi* were detected at lower but notable abundances. *Actinobacteriota* are involved in the degradation of polysaccharides and proteins, attenuation of inflammation, and enhancement of host resistance [45], and their abundance has been positively associated with normal shrimp growth [46]. The significantly lower abundance of *Actinobacteriota* in the control group corresponded with its poorer growth performance, supporting this relationship. The presence of anaerobic *Chloroflexi*, some of which possess photosynthetic capacity [47], further suggests that aquaculture water microbiota may contribute to shaping the intestinal microbial community under certain conditions.

Distinct dominant taxa were observed among experimental groups. *Thiotrichaceae* predominated in the control group, *Rhodobacteraceae* in the LG group, *Photobacterium* in the MG group, and *Vibrio* in the TG group. Notably, the relative abundances of *Photobacterium* in the MG group and *Vibrio* in the TG group each reached approximately 20%, exceeding those in the other groups. *Photobacterium* is commonly detected in mariculture systems, where it participates in organic matter degradation [48], produces antibacterial compounds [49], and contributes to host defense by inhibiting pathogenic *Vibrio* species in the shrimp intestine [50]. The enrichment of *Photobacterium* in the MG group may therefore be associated with enhanced resistance to pathogen invasion. In contrast, *Vibrio* is a ubiquitous genus in shrimp aquaculture, and elevated intestinal abundance is often considered an early warning indicator of disease outbreaks [51]. The higher abundance of *Vibrio* in the TG group suggests that excessive sand thickness may increase disease risk in cultured *M. japonicus*. Previous studies have demonstrated that shrimp intestinal microbiota shares greater similarity with pond sediment communities than with rearing water [52], supporting the notion that sediment-derived microorganisms contributed to the increased *Vibrio* abundance observed in the TG group. In practical aquaculture settings, excessive sand thickness has been associated with pond bottom odor and water quality deterioration. It is therefore plausible that a 20 cm sand substrate allows shrimp to burrow more deeply, facilitating the transfer of sediment-associated opportunistic pathogens into the intestine. Despite these compositional differences, functional prediction analyses indicated that intestinal microbiota across all groups were primarily associated with metabolic pathways, particularly carbohydrate and amino acid metabolism. Consistent with findings in *Procambarus clarkii* [53], no significant differences in predicted microbial functions were observed among groups, suggesting that the intestinal microbiota of *M. japonicus* maintains functional stability across different sand thicknesses.

5. Conclusions

In this study, four sand substrate thicknesses were evaluated to determine their effects on the growth performance and intestinal health of *M. japonicus*. The results demonstrated that shrimp reared in a sand-free environment exhibited significantly reduced growth performance, whereas survival and growth rates were highest at a sand thickness of 10 cm. Analyses of intestinal immune responses and antioxidant capacity indicated that decreasing sand thickness increased oxidative stress in shrimp, while the 10 cm sand substrate imposed the least environmental stress by the end of the culture period. Intestinal histological observations and TUNEL assays further showed that insufficient sand thickness increased susceptibility to intestinal tissue damage and apoptosis. In addition, gut microbiota com-

position was clearly influenced by sand substrate thickness. Although the presence of sand enhanced intestinal microbial abundance and diversity, excessive sand thickness was associated with an increased abundance of *Vibrio*. Taken together, these findings indicate that an intermediate sand thickness provides the most favorable balance between growth performance, intestinal health, and microbial stability. Therefore, considering both biological outcomes and practical production efficiency, a sand substrate thickness of 10 cm is recommended for industrial culture of *M. japonicus*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ani16040586/s1>, Figure S1: Top 10 bacterial phyla in *M. japonicus* under sand thickness gradients. (A) Relative abundance, (B) control vs LG (Welch's *t*-test), (C) control vs MG (Welch's *t*-test), (D) NG vs TG (Welch's *t*-test); Figure S2: Dominant bacterial genera (top 10) in *M. japonicus* across sand treatments. (A) Relative abundance, (B–D) Pairwise comparisons: control vs LG/MG/TG (Welch's *t*-test); Figure S3: Core microbiota analysis in *M. japonicus* intestine. (A) Venn diagram, (B) Species distribution; Figure S4: Phylum-level taxa differential analysis (Kruskal–Wallis) in *M. japonicus* intestine; Figure S5: Abundance distribution of key phyla: Proteobacteria, Actinobacteriota, Chloroflexi, Patescibacteria (box plots); Figure S6: Linear discriminant analysis (LDA) effect size (LEfSe) bar plot.

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