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Original Research Article

Host gut-derived *Bacillus* probiotics supplementation improves growth performance, serum and liver immunity, gut health, and resistive capacity against *Vibrio harveyi* infection in hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*)

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ABSTRACT

Several reports have revealed the vital role that probiotics play in fish growth and health. However, few works are available for host gut-derived probiotics on the growth, immunity, and gut microbiota of fish, especially in hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) due to their isolation difficulty and functional verification. This study aimed at assessing 3 host gut-derived *Bacillus* species' effects on the growth, immune and antioxidant-biochemical responses, haematological parameters, intestinal morphology, immune-related gene expression, gut microbiota, and disease resistance against *Vibrio harveyi* in hybrid grouper. A total of 480 hybrid grouper (initial weight = 9.03 ± 0.02 g) were randomly allotted into 4 groups, namely, the group fed a basal diet without probiotic inclusion (control, B0), the group fed the basal diet with *Bacillus velezensis* GPSAK4 (BV), the group fed the basal diet with *Bacillus subtilis* GPSAK9 (BS), and the group fed the basal diet with *Bacillus tequilensis* GPSAK2 (BT) strains at 1.0×10^9 CFU/g. After a 6-week feeding trial, the results revealed significant improvements ($P < 0.05$) in the growth performance, whole fish-body proximate composition, blood haematological parameters, serum, liver, and intestinal biochemical indexes, intestinal morphology, and protection against *V. harveyi* pathogen in the probiotic-treated groups compared with the untreated. Additionally, the expressions of intestinal tight junction genes (occludin and *ZO1*), pro- and anti-inflammatory genes, including *IL1β*, *IL6*, *IL8*, *TNFα*, *MyD88*, *IL10*, and *TGFβ*, were upregulated ($P < 0.05$) after *Bacillus* species administration. Host gut-derived *Bacillus* supplementation shaped the gut microbiota by significantly increasing ($P < 0.05$) the relative abundance of Proteobacteria, Bacteroidetes, Actinobacteria (except the BS group), Acidobacteria (except the BT group), Cyanobacteria (except the BV and BT groups), and Verrucomicrobia phyla, as well as known beneficial genera (*Romboutsia*, *Turicibacter*, *Epulopiscium*, *Clostridium_sensu_stricto* 1 and 13, *Lactobacillus*, and *Bacillus*), but significantly decreased ($P < 0.05$) the abundance of Firmicutes, Chloroflexi, and Fusobacteria phyla, and purported pathogenic genera (*Staphylococcus* and *Photobacterium*) compared with the control group. Collectively, the results suggest that *B. velezensis* GPSAK4, *B. subtilis* GPSAK9 (especially this strain), *B. tequilensis* GPSAK2 dietary supplementation at 1.0×10^9 CFU/g has positive effects on the intestinal health of hybrid grouper via microbial composition modulation, thus

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enhancing the assimilation and absorption of nutrients to boost fish growth, immunity, and disease resistance.

1. Introduction

The increasing global population growth and struggle for land and water resources disclose a parallel trend in the global demand for food. There is a call for more efficient food production systems to deal with the threat of global food insecurity since it can lead to a greater risk of malnutrition and other health problems (FAO et al., 2019). In the last decade, aquaculture has intensified its production to provide cheap protein foods to meet the high global fish demand. For example, aquaculture production increased from 73.8 million tonnes in 2014 to 110.2 million tonnes in 2016 (FAO, 2018).

Hybrid grouper, a new fish species produced by the Universiti Malaysia Sabah in 2007 after the crossing of the female tiger grouper (*Epinephelus fuscoguttatus*♀) with giant male grouper (*Epinephelus lanceolatus*♂) (Ch'ng and Senoo, 2008), is one of the most valuable fish species cultured in most Asian countries. The fish occupies an important position in the aquaculture industry of China as a result of the several advantages it has over its parent specie, such as faster growth, high feed utilization, higher market and nutritional value, higher resistive capacity against diseases, and their ability to withstand higher salinity (Arrokhman et al., 2017; Bunlipatanon and U-taynapun, 2017; Faudzi et al., 2018; Jiang et al., 2015; Sun et al., 2016). In pursuing high production and economic efficiency, farmers have moved into the intensive and super-intensive culturing of this particular specie and others, leading to several challenges emanating from the high stocking densities causing the recurrent occurrences of various challenges such as poor growth and disease infestation. The grouper aquaculture industry has been battling the impacts of disease outbreaks over the years, causing a reduction in their harvest size to about 50% to 70% (Rimmer and Glamuzina, 2019). For instance, iridovirus infection in greasy grouper (*Epinephelus tauvina*) (Qin et al., 2003) and skin ulcer disease infection (caused by *Vibrio harveyi*) in hybrid grouper (*E. fuscoguttatus*♀ × *E. lanceolatus*♂) (Shen et al., 2017) among others have been reported as causing higher economic losses and severe damages to grouper farms and hatcheries. Also, with the aquafeed industry making significant development in sustainable fish feed with the inclusion of plant-based protein diets rather than the costly fishmeal, serious concerns have been raised about mycotoxin contamination. Mycotoxins are toxic, naturally occurring compounds (including aflatoxins, trichothecenes, ochratoxin A, patulin, fumonisins, zearalenone, and nivalenol/deoxynivalenol) produced by several types of moulds which grow on feedstuffs and multiply during adverse weather conditions. Upon ingestion, these mycotoxin-contaminated feedstuffs and the anti-nutritional factors present in the plant-based feedstuffs cause detrimental effects on the growth, immunity, and increase the susceptibility of aquatic animals to diseases (Gonçalves et al., 2020; Amoah et al., 2022a). The use of antibiotics, vaccines, chemotherapies and other prophylactic control mechanisms have been espoused in improving the growth and controlling such diseases. Nevertheless, their excessive usage have caused detrimental effects on humans and animals (Robertsen et al., 1990; Zorriehzahra et al., 2016) with reports of the emergence of antibiotic-resistance genes and bacteria, antimicrobial residues and the suppression of host immune systems. According to the United States Center for Disease Control report (US CDC, 2019), about 2.8 million people suffer serious antibiotic-resistant bacteria infections resulting in about 35,000 yearly deaths of people. Thus, the use of antibiotics has been criticized, and most of them have been banned (Cabello, 2006; European Commission, 2006). There is a need for swift response in the search for a more beneficial alternative to antibiotics in tackling fish disease problems.

Probiotics, which are beneficial microbes, are presently regarded as an effective and environmentally friendly alternative to the various chemicals and antibiotics in dealing with fish diseases (Verschuere et al., 2000). Probiotics have the ability to enhance the growth and protective ability of fish against diseases due to their antagonistic ability as a result of the secretion of bacteriocins and other compounds, ability to enhance the expression of immune-related genes, ability to assuage symptoms of allergy and inflammations, as well as their ability to keep a positive balance of the gut microbial composition (Abarike et al., 2018; Aly et al., 2008a; Amoah et al., 2021a, 2019; Esteban et al., 2014; Kuebutornye et al., 2020; Liu et al., 2009; O'Hara and Shanahan, 2007). Over the years, different probiotic bacteria genera reported to confer health benefits to aquatic animals, include *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Streptococcus*, *Bacillus*, *Enterococcus, Lactococcus*, *Arthrobacter*, *Micrococcus*, *Pediococcus*, *Burkholderia*, *Aeromonas, Pseudomonas*, *Pseudoalteromonas*, *Roseobacter Leuconostoc*, *Pediococcus*, and *Enterobacter* (Gatesoupe, 1999; Nayak, 2010). Nevertheless, the sporulation ability, which helps bacteria in staying viable in harsh conditions for an extended period, and also the ability to produce enzymes of great importance by *Bacillus* species, make them outstanding; thus, has been used in several probiotic-related studies in fish (Soltani et al., 2019).

The gut microbiota, an assortment of microbes dwelling in an animal's gut, plays ardent roles in intestinal homeostasis and development, and immunological fortification resulting in the enhancement of the growth and health of animals (Claus et al., 2016). Recently, there has been extraordinary interest in the studies of the fish gut microbiota as a result of the diversity and defining physiognomies of pathogenic and beneficial bacteria in the gut in relation to host health and diseases. Hence, for a healthy host, it is imperative to keep a balance vis-à-vis the key bacteria species known to affect specific host responses (Sekirov et al., 2010). The gut microbial composition, aside from being shaped by several factors, including host genotype, the stages of host development, host habitat, and physiological condition (Miyake et al., 2015; Mouchet et al., 2012; Rungrassamee et al., 2013), are also affected by dietary supplements such as probiotics (Cotozzolo et al., 2020; Fan et al., 2017; Foysal et al., 2019; Kuebutornye et al., 2020; Poolsawat et al., 2020; Saxelin et al., 2005). Advancing in the scrutiny and modeling of gut microbiota studies has the propensity of broadening our understanding of the health and disease role of gut microbes, which can help in tailoring existing and future prophylactic and therapeutic modalities (Das et al., 2014; Egerton et al., 2018; Ray and Ringø, 2014; Sekirov et al., 2010). Although a plethora of evidence is available on gut microbial studies in fish, little is known about the gut microbial changes after the administration of host-associated probiotics in hybrid grouper.

Probiotic bacteria isolated originally from the intestine of fish have been reported to provide better probiotic effects than others from terrestrial sources (Van Doan et al., 2018). Ramesh et al., (2015) asserted that *Bacillus* spp. isolated from the gut of healthy fish are regarded as one of the best to help control fish diseases and also help in the improvement of the gut microbial composition of host organisms than those from other sources. Recent probiotic-related works conducted in aquaculture have concentrated on using host-associated probiotics, especially *Bacillus* sp., with many noting enhancements of growth and immune parameter boosting. For instance, *Bacillus velezensis* TPS3N and *Bacillus subtilis* TPS4 isolated from Nile tilapia (*Oreochromis niloticus*) gut were reported to enhance the growth, mucosal immunity, intestinal health (morphology, digestive enzyme activities, and gut microbial composition), and resistive capacity of *O. niloticus* against *Aeromonas hydrophila* infection (Kuebutornye et al., 2020). Also, the administration of *B. subtilis* RZ001 revealed an increase in goblet cells and mucus cells which translated into alleviating colitis, thus, improving intestinal integrity (Li et al., 2020). An improvement of the growth, nutrient utilization, and haemato–immunological parameters either through diets or as water additive was achieved in *Labeo rohita* after supplementing *B. tequilensis* KF623287 in diets isolated from the gut of the same fish (Dutta and Ghosh, 2021). Yet, the underlying molecular mechanism for host-associated probiotics in exerting probiotic effects has not been well clarified, especially in *Bacillus*-grouper-related studies. A recent work by Liao et al., (2021) has revealed that very limited works are available on the effects of host gut-derived *Bacillus* species from hybrid grouper on their growth, non-specific immune response, and disease resistance. To the best of our knowledge, there is even no available research conducted on the effects of host gut-derived *Bacillus* species on the gut microbiota of hybrid grouper.

In considering the background given above, the effects of dietary supplementation of 3 host gut-derived *Bacillus* spp. on hybrid grouper's growth, immunity, and disease resistance were evaluated. The 3 isolated strains, namely, *B. velezensis* GPSAK4, *B. subtilis* GPSAK9, and *B. tequilensis* GPSAK2 whose nucleotide sequences obtained, were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers MW548635, MW548634, and MW548630, respectively, were effective at antagonizing *Streptococcus iniae*, *S. agalactiae*, *V. alginolyticus*, and *V. harveyi* in vitro (Amoah et al., 2021b). The 3 strains also displayed properties of utilizing a wide range of carbon sources such as lactose, rhamnose, starch, inositol, citrate, adonitol, and even amino acid arginine, suggesting that they could be helpful in the digestion and hydrolysis of carbohydrates and amino acids in vitro. Furthermore, the strains showed high resistance to low pH (as low as 1), higher bile salt concentration (0.5%) tolerance, high-temperature exposure tolerance (80, 90, 100 °C) (a putative probiotic ability for feed application), high sporulation capacity, and also showed high auto-aggregation and cell surface hydrophobicity capacity indicating their ability to attach to the mucosal surface and epithelial cells (Amoah et al., 2021b). However, it is not clear whether they have a probiotic effect in vivo.

Thus, this current study aimed at assessing the influence of the 3 host gut-derived *Bacillus* species (*B. velezensis* GPSAK4, *B. subtilis* GPSAK9, and *B. tequilensis* GPSAK2) on the growth, immunity, antioxidant and digestive enzyme activity, expression of immune-related genes, gut morphology and microbiota of hybrid grouper as well as its protective effect against *V. harveyi*. A comprehensive assessment of the effects on growth, survival, immunity, disease resistance, and intestinal microbiota offers a concrete theoretical basis for succeeding commercialization and application of the potential probiotic strains.

2. Materials and methods

2.1. Animal ethics statement

The experimental animal's collection and handling were in acquiescence with the ARRIVE guidelines (The ARRIVE guidelines 2.0), and were approved by the Institutional Animal Care and Use Committee board of Guangdong Ocean University (Zhanjiang, China). All methods were carried out in accordance with relevant guidelines and regulations.

2.2. Probiotic spore preparation

The probiotic *Bacillus* species used in the current study were earlier isolated from the intestine of hybrid grouper (♀*E. fuscoguttatus* × ♂*E. lanceolatus*) (Amoah et al., 2021b). Following the previously described methods of Ran et al. (2012) with slight modifications, the spores of the *Bacillus* isolates were prepared by firstly preparing spore preparation agar (peptone, 3.3 g/L; NaCl, 5.0 g/L; beef extract powder, 1.0 g/L; $MgSO_4.7H_2O$, 0.25 g/L; K_2HPO_4, 2.0 g/L; KCl, 1.0 g/L; $MnSO_4$, 0.01 g/L; lactose, 5 g/L; and agar 15 g/L). The bacteria cell suspension was subsequently activated in Luria–Bertani broth media at 37 °C for 7 h, spread onto the spore preparation agar plates, and consequently incubated at 28 °C for 6 days. In collecting the spore suspension, sterilized distilled water (5 mL) was added per individual agar plate. The spores were suspended with an inoculation loop and transferred into a 15-mL tube. In killing the vegetative cells, spores were incubated at 85 °C for 15 min. Following this was a 10-fold serial dilution in 1× phosphate-buffered saline (PBS) to determine the spore suspensions' concentration. The final spore concentration was adjusted with sterile PBS to 1.0×10^9 colony-forming unit (CFU) per mL. Thus, in preparing the spore-amended diets, 90 mL of the spore suspension was added to 1000 g of the formulated diets using bleach and a sterilized pump sprayer to achieve about 9% (vol/wt) spore suspension. It must be noted that the final concentration used in this study was settled upon after completing a preliminary dietary experiment with varying concentrations (10^7, 10^8, and 10^9 CFU/g feed) of the isolates. The results, after revealing 10^9 CFU/g feed as the best concentration (unpublished), made us choose this particular concentration which was as well in agreement with previous studies on fish (Gupta et al., 2014; Amoah et al., 2021a; Panigrahi et al., 2007).

2.3. Diet preparation and viability of bacteria in the diet

The composition of ingredients formulated and the proximate chemical composition analysis of the experimental diets are shown in Table 1. In preparing the diets, while brown fish meal, wheat gluten meal, soy protein concentrate, and castor meal served as the protein sources, soybean oil, fish oil, and soy lecithin also served as the lipid sources. The basal diet was thus formulated to contain 50.92% crude protein, 7.21% crude lipid, 10.56% ash, and 10.06% moisture content after proximate composition analysis (see section 2.6.1). The experimental diets were prepared by adding the bacteria suspension (1.0×10^9 CFU/mL) of individual *Bacillus* isolates to the basal diet as previously described (Amoah et al., 2021a). As a result, 4 experimental diets were made: the basal diet without probiotic supplementation but with an equal volume of PBS serving as the control (B0 group), the basal diet with *B. velezensis* GPSAK4 (BV group), the basal diet with *B. subtilis* GPSAK9 (BS group), and the basal diet with *B. tequilensis* GPSAK2 (BT group) (Fig. 1). After air-drying till reaching a moisture content of approximately 10%, the pellets (2.0 mm and 2.5 mm particle size) were placed in sealed Ziploc bags and stored at −20 °C until

Table 1
Ingredients and nutritional composition of the diets (dry matter basis).

Item	Content, %
Ingredients	
Brown fish meal[1]	46.00
Wheat gluten meal[2]	7.00
Soy protein concentrate[3]	8.00
Wheat flour[2]	22.00
Castor meal[4]	4.00
Fish oil[2]	1.50
Soybean oil[2]	1.00
Soy lecithin[2]	1.00
Vitamin premix[5]	0.50
Mineral premix[6]	0.50
Choline chloride[7]	0.50
Vitamin C[2]	0.05
$Ca(H_2PO_4)_2$[7]	1.50
Attractant[2]	0.10
Ethoxyquin[2]	0.05
Carboxymethyl cellulose[8]	1.00
Microcrystalline cellulose[8]	5.30
Total	100.00
Proximate nutritional composition	
Crude protein	50.92
Crude lipid	7.21
Ash	10.56
Moisture, wet matter basis	10.06

[1] Brown fish meal: crude protein, 70.03%; and crude lipid, 8.24% (supplied by China National Township Enterprises Corporation).
[2] Wheat gluten meal: crude protein, 81.22%; and crude lipid, 0.11%; wheat flour: crude protein, 10.52%; and crude lipid, 0.36%; fish oil; soybean oil; soy lecithin; vitamin C; attractant; ethoxyquin (Zhanjiang Haibao Feed Co. Ltd., Guangdong, China).
[3] Soy protein concentrate: crude protein, 67.87%; and crude lipid, 0.46% (Shandong Changrun Biology Co. Ltd).
[4] Castor meal: crude protein, 58.87%; and crude lipid, 0.42% (Shandong Weifang Supply and Marketing Industrial Co. Ltd., Shandong, China).
[5] Vitamin premix (g/kg mixture): vitamin B_1, 17.00; vitamin B_2, 16.67; vitamin B_6, 33.33; vitamin B_{12}, 0.07; vitamin E, 66.00; vitamin K, 3.33; vitamin D, 33.33, retinyl acetate, 6.67; D-calcium pantothenate, 40.67; nicotinic acid, 67.33; folic acid, 4.17; biotin, 16.67; inositol, 592.72; and cellulose, 102.04 g (Zhanjiang Yuehua Feed Co. Ltd., Zhanjiang, China).
[6] Mineral premix (g/kg premix): $ZnSO_4.H_2O$, 32.0991; $FeSO_4 \cdot 7H_2O$, 18.785; $MgSO_4.H_2O$, 65.19927; $CoCl_2.6H_2O$ (10%), 5.5555; $CuSO_5.5H_2O$, 11.0721; KIO_3, 0.0213; Na_2SeO_3 (10%), 0.5555; KCl, 22.7411; zeolite powder, 843.9777 (Zhanjiang Yuehua Feed Co. Ltd., Zhanjiang, China).
[7] Purchased from Shanghai Macklin Biochemical Co. Ltd., Shanghai, China.
[8] Purchased from Shantou Xilong Chemical Factory, Guangdong, China.

used. The viability of bacteria in diets was analysed in a pre-experiment after storing the prepared diets at 4 °C for 8 weeks following our previously described methods (Amoah et al., 2019). As such, diets were prepared weekly to ensure the viability of bacteria in diets to exert the actual effects.

2.4. Fish rearing management and experimental process

The hybrid grouper fish used in the current study were purchased from a commercial farm in Dong Hai Island (Zhanjiang, Guangdong Province, China). The fish were later cultured temporarily in cement pools with continuous aeration for 2-weeks acclimatization period, during which they were hand-fed twice daily (08:00 and 16:30) with commercial feed (Zhanjiang Aohua Feed Co. Ltd., Guangdong Province, China). A total of 480 juvenile hybrid grouper fish of uniform size after 24 h starvation were weighed (9.03 ± 0.02 g) and randomly divided into 4 groups (B0, BV, BS, and BT). Each treatment group had 4 replicates of 30 fish density per tank, i.e., distributed into 16 cylindrical fiberglass tanks (0.5 m^3). Fish were hand-fed twice daily (08:00 and 16:30) to apparent satiation. The parameters of water quality were maintained daily by renewing 35% of the filtered seawater in the first 2 weeks, and later by renewing 50% of the filtered seawater in the remaining weeks to keep the temperature, pH, dissolved oxygen (kept stable via continuous aeration with air-stones), and salinity ranging from 28 to 30 °C, 7.6 to 8.2, ≥6.5 mg/L, and 27.5‰ to 32‰, respectively (YSI 556 multiprobe system, YSI Inc., US).

2.5. Sample collection and measurements

2.5.1. Survival, growth performance, and morphometric indices

The current study lasted for 6 weeks, and prior to sampling (after the 24 h starvation) period, fish were anesthetized with ethyl-3-aminobenzoate methane-sulfonate (MS-222; Sigma, US) by immersion at 150 mg/L. Subsequently, the total remaining fish were counted and weighed. Three fish were randomly sampled per individual replicate group to measure and record their body weight, body length, liver weight, viscera weight, and intestinal weight and length. Based on the recordings, the growth performance and morphometric indices were calculated as follows:

$$\text{Survival rate (SR, \%)} = 100 \times (\text{Final fish number}/\text{Initial fish number})$$

$$\text{Weight gain rate (WGR, \%)} = 100 \times [(\text{Final fish body weight, g}) - (\text{Initial fish body weight, g})]/(\text{Initial fish body weight, g})$$

$$\text{Specific growth rate (SGR, \%/day)} = 100 \times [\text{Ln (Final fish body weight, g)} - \text{Ln (Initial fish body weight, g)}]/\text{Days of the experiment}$$

$$\text{Feed conversion ratio (FCR)} = (\text{Total dry feed intake, g})/[(\text{Final fish body weight, g}) - (\text{Initial fish body weight, g})]$$

$$\text{Feed intake (FI, \%/day)} = 100 \times (\text{Total diet consumed, g})/\{\text{Days of experiment} \times [(\text{Initial fish body weight, g}) + (\text{Final fish body weight, g})]/2\}$$

$$\text{Condition factor (CF, \%)} = 100 \times [(\text{Fish body weight, g})/(\text{Fish body length, cm})^3]$$

$$\text{Hepatosomatic index (HSI, \%)} = 100 \times [(\text{Fish liver weight, g})/(\text{Fish body weight, g})]$$

$$\text{Viscerosomatic index (VSI, \%)} = 100 \times [(\text{Fish viscera weight, g})/(\text{Fish body weight, g})]$$

$$\text{Intestinal somatic index (ISI, \%)} = 100 \times [(\text{Final fish intestine weight, g})/(\text{Final fish body weight, g})]$$

$$\text{Intestinal length index (ILI, \%)} = 100 \times [(\text{Final fish intestine length, cm})/(\text{Final fish body length, cm})]$$

2.5.2. Blood and tissue sample collection

The fish blood samples were collected with 1-mL sterile syringes from the caudal vein of 6 fish randomly sampled from each replicate tank. A small amount of the blood (0.2 mL) from 2 fish/replicate group of the 6 sampled fish were placed in anti-coagulated ethylenediaminetetraacetic acid (EDTA) tubes for haematological indices testing. The remaining blood samples collected from 4 of the 6 sampled fish were placed into 1.5-mL Eppendorf tubes and stored at 4 °C overnight. Subsequently, the stored overnight blood samples were centrifuged (1252 × *g* for 10 min at 4 °C), and the serums collected were stored at −80 °C for subsequent biochemical

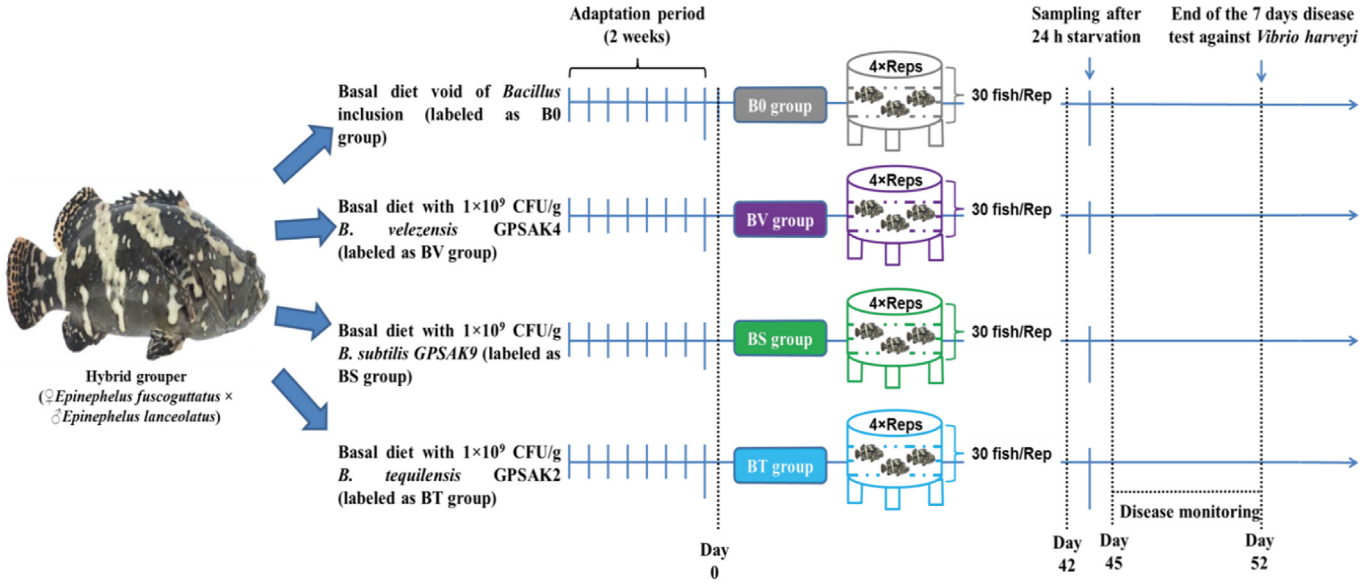

Fig. 1. Experimental design and scheme of the study. Treatment groups, B0, BV, BS, and BT refer to the fish groups fed the basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.

analysis. Three fish were randomly selected per tank to determine the whole body composition. However, the liver and intestinal samples were obtained after dissecting 3 fish from the same fish that blood was drawn under sterile conditions. The liver and intestinal samples (without stool samples) were washed with PBS to clear adipose and mesentery tissues. The samples were then kept in Eppendorf tubes, frozen immediately in liquid nitrogen, and later stored at −80 °C for subsequent liver and intestinal biochemical index analysis. Two fish were randomly selected per individual tank to aseptically dissect the gut to remove the distal intestinal (DI) tissue samples (with stool samples) and kept in Eppendorf tubes. They were placed immediately in liquid nitrogen and later kept at −80 °C for the analyses of the 16S rDNA of gut microbiota. For histological evaluation, the remaining 3 fish from which the blood samples were drawn and an additional randomly sampled fish were dissected to collect the DI tissue samples. The histological DI samples were divided into 2 parts: (1) one part (from 2 of the 4 sampled fish per replicate group) was placed in 4% paraformaldehyde (Wuhan Servieobio Technology Co., Ltd., Wuhan, China) for 24 h until the Alcian Blue-Periodic Acid-Shiff (AB-PAS) staining was conducted, and (2) the other part (from the remaining 2 fish per replicate group) was preserved with electron microscope fixation solution (2.5% glutaraldehyde, Wuhan Servicebio Technology Co. Ltd., Wuhan, China) which was later used for scanning electron microscopy (SEM) analysis. Lastly, 2 fish were randomly selected and aseptically dissected to remove the DI tissue, of which they were cut and placed in 1.5-L test tubes containing RNAlater. The collected samples were placed at 4 °C overnight and then transferred to −80 °C for subsequent gene expression determination.

2.6. Sample analysis

2.6.1. Experimental diet and whole fish body proximate composition analysis

The experimental diet and whole fish body proximate composition analysis, including the crude protein, crude lipid, ash, and moisture contents, were analysed following previously described methods (AOAC, 2002). Briefly, while the crude protein (N × 6.25) contents were determined by the Kjeldahl method, involving Auto Kjeldahl System usage (8400-Autoanalyzer, FOSS, Hoganas, Sweden), crude lipid was determined by the Soxhlet method (using ether extraction). The ash content was rather determined by muffle furnace combustion involving oven incineration at 550 °C (5 h), whereas that of the moisture content was determined by drying samples (feed and fish samples, at 105 °C) in an oven until the attainment of constant weight.

2.6.2. Histological examination

The preparation and analysis of samples for the AB-PAS staining and SEM were conducted following our recently published work (Amoah et al., 2022b). Briefly, for the AB-PAS histological examinations, the tissues after removal from the 4% paraformaldehyde solution were paraffin-embedded (JB-P5, Wuhan Junjie Electronics Co., Ltd.), cut into 4 μm sections using a microtome (Leica Instrument RM 2016, Shanghai, China). The images were thus captured with Olympus model BX51 (Serial number: 9K18395, Tokyo, Japan), and the villi height (VH) (viewing from the villus tip to the submucosa outer edge), villi width (VW) (midpoint measurement of each villus), crypt depth (CD) (from crypt mouth to the base), and intestinal epithelial muscle thickness (MT) (from muscularis mucosae's inner edge to the outer edge of serosa) measured using the Image-Pro Plus 6.3 software (Media Cybernetics, Inc., Rockville, US). The type II mucous cell number on each villus was measured using the cellSens Standard 1.8 software.

2.6.3. Analysis of haematological and biochemical indexes

The Mindray BC-30s automatic haematology analyzer machine, as previously used in detecting haematological parameters (Sakyi et al., 2020), was used to detect parameters such as red blood cells (RBC), white blood cells (WBC), haemoglobin (HGB), mean cell volume (MCV), and haematocrit (HCT).

The stored frozen liver and intestinal samples were weighed, homogenized in a sterile 0.9% saline solution separately at a ratio of 1:9 (wt:vol) by bead homogenizer in ice, later centrifuged (liver, 959 × *g;* intestines, 489 × *g*; all at 4 °C for 10 min), and supernatant

aliquots obtained were used in the quantification of liver and intestinal enzyme activity analysis. The immunoglobulin-M (IgM), lysozyme (LYZ), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), complement 3 (C3), and complement 4 (C4) levels in the serum, liver, and intestine were measured following the described methods (Amoah et al., 2022b). The lipid peroxidation product malondialdehyde (MDA), catalase (CAT), and total antioxidant capacity (T-AOC) levels were measured following the methods of Cai et al. (2017), Lin et al. (2015), and Sun et al. (2010), respectively. The lactate dehydrogenase (LDH) activity was measured using commercial kits (Shanghai Jianglai Biotechnology Co. Ltd., Shanghai, China), where the company's guidelines were strictly followed. On the other hand, the liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities were determined based on the calorimetric method of Reitman and Frankel (1957). Following the company's protocol, the liver and the intestine's biochemical parameters were determined using fish ELISA detection kits (Shanghai Jianglai Biotechnology Co. Ltd., Shanghai, China). The results were later expressed per mg protein concentration (bicinchoninic acid, BCA) (Rider et al., 2009).

2.6.4. RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis

Total RNA was extracted from the fish DI using Trizol reagent (Beijing TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The RNA quality and quantity were assessed by agarose gel (1%) electrophoresis and spectrophotometer (NanoDrop 2000, Thermo scientific) analysis, respectively, where the absorbance ratio of all the samples showed the best quality (absorbance 260:280 nm ratio >1.80 and 260:230 nm ratio > 1.8). PrimeScript RT-PCR Kit (TaKaRa, Kusatsu, Japan) was used to achieve the first-strand cDNA synthesis in RT according to the manufacturer's instructions. The cDNA was kept at −20 °C for subsequent analysis. The primer sequences, including interleukin 1β (*IL1β*), interleukin 6 (*IL6*), interleukin 8 (*IL8*), interleukin 10 (*IL10*), tumor necrosis factor-alpha (*TNFα*), transforming growth factor-beta (*TGFβ*), myeloid differentiation primary response protein 88 (*MyD88*), occludin, and zonula occludens 1 (*ZO1*), used for the RT-qPCR are shown in Table S1. According to our preliminary experimental results concerning the assessment of the internal control genes, β-actin was used as the housekeeping gene to normalize cDNA loading. Consequently, all the real-time PCR reactions were executed on Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, US) using SYBR Premix Ex Taq Kit (TaKaRa). Three replicate qPCR analyses were performed per sample. The target genes' relative gene expression results were analyzed using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001).

2.6.5. Library preparation, sequencing, and analysis of the gut microbiota

2.6.5.1. Intestinal microbiota community discovery and analysis. Following our previously described procedure, the DI microbial composition detection and analysis were performed (Amoah et al., 2019). Briefly, the total genomic DNA was extracted from the stool samples of the fish intestine using E.Z.N.A. stool DNA Kit (Omega Bio-tek Inc., US), following the company's protocol strictly. The DNA purity and concentrations were evaluated by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, US). The variable regions (V3–V4) of the 16S rDNA were amplified (PCR reactions were run with an initial denaturation at 95 °C for 2 min, followed by 27 cycles of denaturation at 98 °C for 10 s, 62 °C for 30 s, 68 °C for 30 s, followed by a final extension of 68 °C for 10 min) using primers 341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT. The PCR reactions were executed in triplicates of 50 μL mixture containing 5 μL of 10 × KOD Buffer, 5 μL of 2.5 mmol/L dNTPs, 1.5 μL of each primer (5 μmol/L), 1 μL of KOD Polymerase, and 100 ng of template DNA.

High-throughput sequencing of the purified PCR products was carried out using the Illumina Hiseq2500 sequencing system. Amplicons after extraction from 2% agarose gels were purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, US) following the company's strict instructions. Samples were quantified using QuantiFluor-ST (Promega, US), pooled in equimolar, and paired-end sequenced (2 × 250) following the standard protocols on an Illumina platform. The library sequencing was conducted by Biomarker Biotechnology Co., Ltd., Guangzhou, China. High-quality reads were acquired by further filtering the raw reads to remove reads containing more than 10% of unknown nucleotides and those with less than 80% of bases with quality (Q-value) > 20.

The paired-end clean reads were compounded as raw tags using FLASH (v 1.2.11). Sequences were analyzed with the help of a Quantitative Insights Into Microbial Ecology (QIIME, v.1.9.1) pipeline. Clean tags were searched against the reference database (http://drive5.com/uchime/uchime_download.html) to perform a reference-based chimera checking UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html). The chimeric tags were finally removed to acquire the effective tags, which were then clustered into operational taxonomic units (OTU; ≥97% similarity) with the help of the UPARSE pipeline, leading to the tag sequence with the highest abundance being selected as a reprehensive cluster within each cluster. The representative sequences were classified into organisms by a naive Bayesian model using the RDP classifier based on the SILVA database (https://www.arb-silva.de/). Taxonomic richness estimators and community diversity metrics were determined for each library in Mothur (version 1.39.1, http://www.mothur.org/). The selection of sequences with the highest abundance at the taxonomic levels as representative sequences were conducted using QIIME software; thus, the alignment of multiple sequences were executed. Alpha diversity was selected to identify Community richness (Chao 1 and ACE (abundance-based coverage)) and Community diversity (Shannon and Simpson), whereas the Good's coverage was also selected to characterize the sequencing depth. The results were generally presented at the phylum and genus taxonomic levels. Beta diversity analysis, such as the Principal Component Analysis (PCA) and Principal Co-ordinates Analysis (PCoA), were used for comparative analysis between groups. While a Venn diagram was generated to illustrate the number of unique and shared OTU, a heatmap was also used to show the normalized abundance.

2.6.5.2. Nucleotide sequence accession number. The DI microbiota data were exported as individual FastQ files and deposited in the Sequence Read Archive (SRA) of the NCBI (SRA, NCBI) under BioProject accession number PRJNA841573.

2.7. Challenge test

Pathogenic *V. harveyi* bacterium previously used in our works (Amoah et al., 2021a, 2021b) was used to evaluate the probiotics' protective ability. The protective ability against *V. harveyi* was assessed according to our previously described methods (Amoah et al., 2019). Briefly, 10 fish per tank were injected intraperitoneally with 0.2 mL of the suspended *V. harveyi* bacterium at 1.6×10^9 CFU/mL concentration for the disease test. The cumulative mortality (CM) per replicate group was observed and recorded daily till the 7th day, and the relative percentage survival was calculated. The formulae used were the following: CM (%) = 100 × (Total mortality per treatment/Total number of fish challenged), whereas that of the relative percentage survival

(%) = 100 × [1 − (Percent mortality in treatment groups/Percent mortality in the control group)].

2.8. Statistical analysis

The statistical analyses of the experiment were conducted using the Statistical Package for Social Sciences (SPSS) for Windows software (IBM SPSS version 20.0, Inc., 2010, Chicago, US). The normality and homogeneity of the variance were tested, followed by a one-way analysis of variance (ANOVA) of all collected datasets (excluding the disease challenge data). The results for the parameters measured were expressed as the mean ± standard error (SE). Differences were statistically significant at $P < 0.05$ among treatment groups using Tukey's Honest Significant Difference (HSD) test. The cumulative survival of the challenge test was identified by the Kaplan–Meier plot Log-Rank (Mantel–Cox) test. Windows GraphPad Prism (version 8, GraphPad Software, La Jolla, California, US) generated the bar charts.

3. Results

3.1. Growth performance, feed utilization, and survival rate

As shown in Table 2, after the 6-week feeding trial, the W_f, WGR, SGR, CF, HSI, and VSI were significantly increased ($P < 0.05$) in fish fed the *Bacillus* species treated diet in comparison to those fed the basal diet. On the other hand, all groups showed no significant differences ($P > 0.05$) in the PER, SR, FI, ISI, and ILI, although higher elevations were witnessed in the probiotic-treated groups. Also, although the B0 group showed higher elevation in the FCR index than the other groups, no significant differences ($P > 0.05$) were observed between the groups.

3.2. Proximate whole fish body composition

The supplementation effects of dietary probiotics on the proximate whole fish-body composition are shown in Table 3. It was observed that the supplementation of host gut-derived *Bacillus* species in hybrid grouper diets significantly increased ($P < 0.05$) the levels of whole fish-body crude protein and crude ash content and decreased significantly ($P < 0.05$) the levels of moisture content in juvenile hybrid grouper as compared to the control. However, the crude lipid content revealed no significant differences ($P > 0.05$).

3.3. Distal intestinal morphological examination based on AB-PAS and SEM

Fig. 2 demonstrates the photomicrographs and measurements of the DI morphological examinations after AB-PAS staining analysis. Clear effects were observed on the target organ after host gut-derived probiotics supplementation. The intestine of the *Bacillus*-treated groups showed taller and wider villi, wider CD, and broader MT than the control group (Fig. 2(A)). Correspondingly, the measurements of the VH, VW, MT, and CD were observed to be significantly higher ($P < 0.05$) in the probiotic-treated groups than in the untreated. However, there were significantly lower ($P < 0.05$) type II mucus cell counts witnessed in the BT and control groups, in contrast to as observed in the BV and BS groups (Fig. 2(B)).

The SEM results are presented in Fig. 3. The B0 group at the end of the study revealed fewer and weaker mucosal surfaces or villi density which contained some orifices. Again, there were noticeable villi detachments from the epithelial layer and villi atrophy, causing some villi to disappear in the B0 group as compared to the other treated groups. On the other hand, the probiotic-treated groups showed more closely packed mucosal surface or villi density, with the BS group revealing the best. It must be stated that the BV group witnessed very few orifices.

3.4. Haematological parameters analysis

Fig. 4 illustrates the blood haematological parameter differences after dietary supplementation of different host gut-derived *Bacillus* species to hybrid grouper. The results exhibited a significantly higher ($P < 0.05$) number of RBC, HGB, HCT, and MCV counts in juvenile hybrid grouper-fed host-associated probiotics diet than in the untreated. Nonetheless, concerning the number of WBC, the BV and BS groups witnessed a significantly higher ($P < 0.05$) number than the BT and B0 groups. No significant differences ($P > 0.05$) were detected between the BT and B0 groups regarding the WBC counts, although higher levels were witnessed in the BT.

3.5. Serum, liver, and intestinal immune, anti-oxidant, and digestive enzyme activities

3.5.1. Serum immune and anti-oxidant enzyme activities

The effects of dietary host gut-derived probiotics on serum immune and anti-oxidant parameters such as IgM, LYZ, SOD, CAT,

Table 2
Growth performance and feed utilization of juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species.

Parameters	B0	BV	BS	BT
W_i, g	9.04 ± 0.01	9.03 ± 0.02	9.03 ± 0.01	9.04 ± 0.01
W_f, g	43.66 ± 1.14^{a}	48.79 ± 0.82^{b}	49.03 ± 0.79^{b}	45.61 ± 1.22^{ab}
WGR, %	383.18 ± 12.13^{a}	440.51 ± 10.04^{b}	442.78 ± 9.24^{b}	404.30 ± 14.05^{ab}
SGR, %/day	2.81 ± 0.05^{a}	3.01 ± 0.03^{b}	3.02 ± 0.03^{b}	2.89 ± 0.05^{ab}
FCR	0.86 ± 0.04	0.73 ± 0.02	0.73 ± 0.03	0.76 ± 0.03
CF, %	2.21 ± 0.11^{a}	2.69 ± 0.09^{ab}	3.12 ± 0.17^{b}	2.76 ± 0.08^{b}
FI, %/day	2.02 ± 0.07	1.89 ± 0.03	1.96 ± 0.03	1.82 ± 0.07
PER, %	2.93 ± 0.11	3.13 ± 0.05	3.01 ± 0.06	3.31 ± 0.12
SR, %	97.77 ± 2.23	95.57 ± 1.13	91.10 ± 4.85	98.90 ± 1.10
HSI, %	4.36 ± 0.30^{a}	5.64 ± 0.07^{b}	6.00 ± 0.28^{b}	5.77 ± 0.37^{b}
VSI, %	10.75 ± 0.35^{a}	12.99 ± 0.52^{b}	12.68 ± 0.27^{b}	12.09 ± 0.23^{ab}
ISI, %	0.95 ± 0.02	1.22 ± 0.04	1.16 ± 0.10	1.39 ± 0.16
ILI, %	136.35 ± 3.61	147.44 ± 6.12	154.76 ± 5.19	161.16 ± 13.24

W_i = initial body weight; W_f = final body weight; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion ratio; CF = condition factor; FI = feed intake; PER = protein efficiency ratio; SR = survival rate; HSI = hepatosomatic index; VSI = viscerosomatic index; ISI = intestinal somatic index; ILI = intestinal length index.
Treatment groups, B0, BV, BS, and BT, refer to the fish groups fed the basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.
The values in the same row with different superscript letters differ significantly among groups ($P < 0.05$) based on Tukey's HSD test. Data are the mean ± SE of 4 replicates (3 fish/replicate group).

Table 3
The proximate whole-body composition of juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species.

Parameters, % dry matter	B0	BV	BS	BT
Crude protein	59.84 ± 0.42[a]	61.57 ± 0.22[b]	61.08 ± 0.26[ab]	63.31 ± 0.21[c]
Crude lipid	15.95 ± 0.21	19.44 ± 0.55	17.75 ± 0.57	19.65 ± 1.80
Crude ash	17.42 ± 0.05[a]	19.17 ± 0.19[c]	18.60 ± 0.19[bc]	18.08 ± 0.21[ab]
Moisture, % wet matter	74.37 ± 0.37[b]	72.01 ± 0.20[a]	71.98 ± 0.69[a]	72.64 ± 0.22[ab]

Treatment groups B0, BV, BS, and BT refer to the fish groups fed the basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.
The values in the same row with different superscript letters differ significantly among groups ($P < 0.05$) based on Tukey's HSD test. Data are the mean ± SE of 4 replicates (3 fish/replicate group).

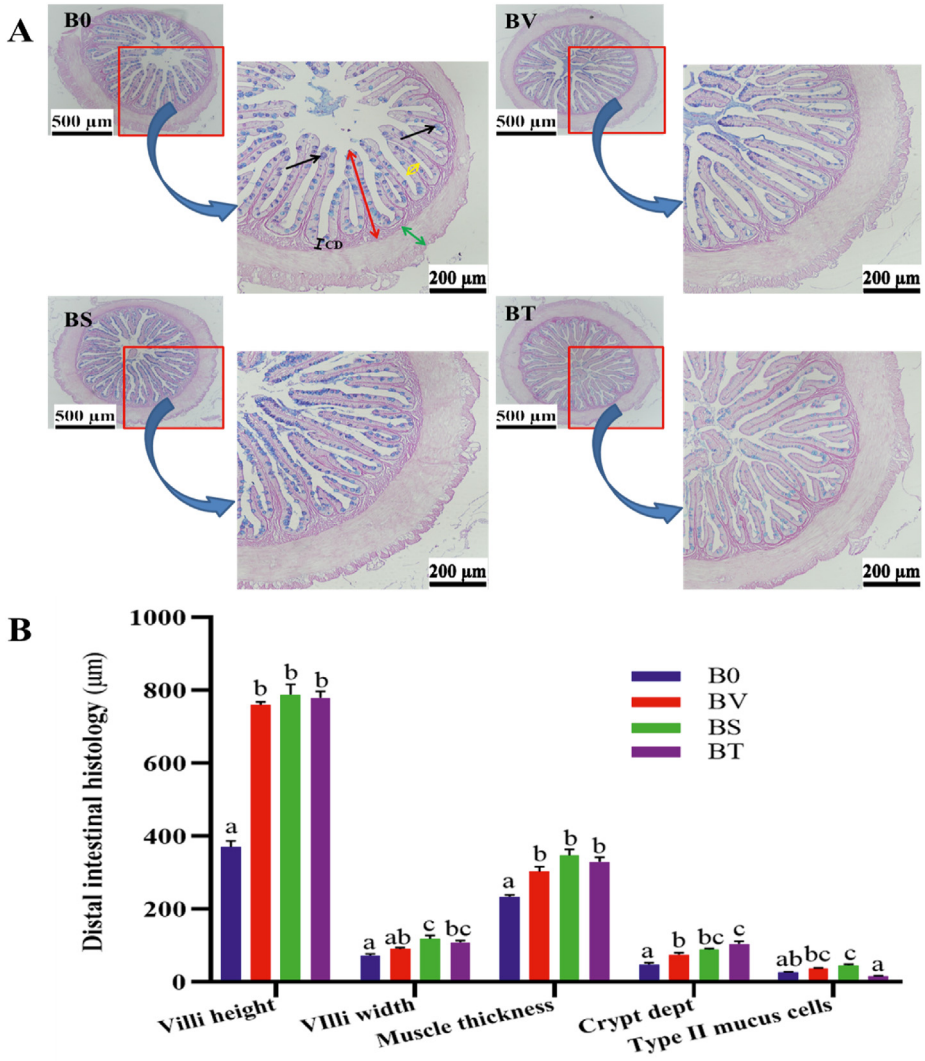

Fig. 2. Photomicrographs (A) and histological measurements (B) of the distal intestinal Alcian Blue-Periodic Acid-Schiff (AB-PAS) staining section of juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species. Red arrow = villi height; yellow arrow = villi width; green arrow = muscle thickness; black arrow = type II mucus cells; and CD = crypt depth. Treatment groups B0, BV, BS, and BT refer to the fish groups fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively. Vertical bars represented the mean ± SE of 4 replicates (2 fish/replicate group). Bars marked with different letters differ significantly ($P < 0.05$) among groups (Tukey's HSD).

LDH, and MDA are shown in Table 4. Compared to the control, dietary *Bacillus* species significantly increased ($P < 0.05$) the serum IgM, SOD, and CAT levels. Serum LYZ was significantly enhanced ($P < 0.05$) in fish fed the BV and BT diets compared to those fed the BS and basal diet (B0), although higher elevations were witnessed in those fed the BS diets. Contrarily, significantly lower ($P < 0.05$) LDH and MDA levels were observed in fish-fed host gut-derived *Bacillus* diets in comparison to those fed the basal diet, with the BS-treated group revealing the least significant levels.

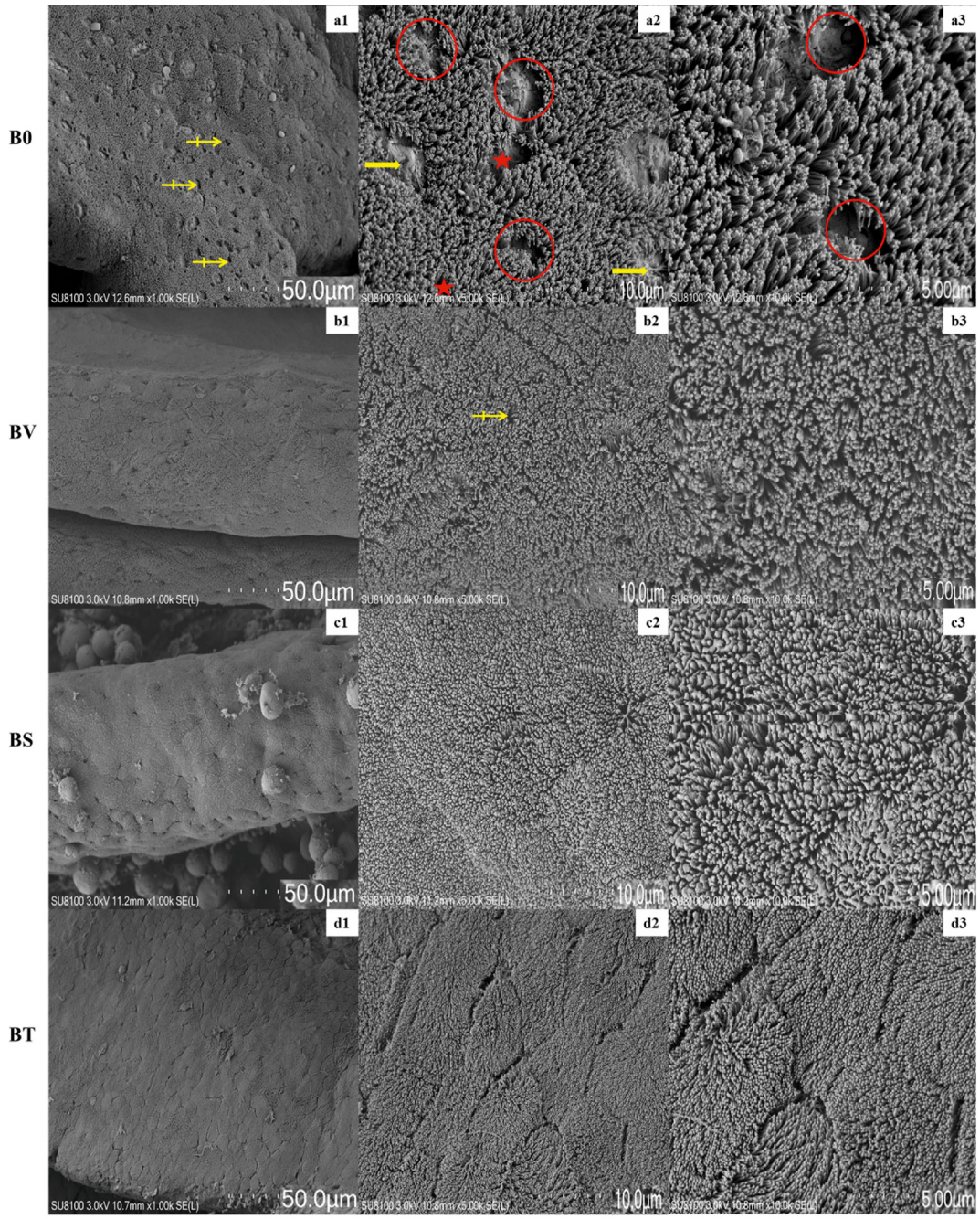

Fig. 3. Scanning electron microscopy (SEM) of the distal intestinal mucosal surface of juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species. Bar markers represent 50 μm (a1, b1, c1, and d1), 10 μm (a2, b2, c2, and d2), and 5 μm (a3, b3, c3, and d3). B0 (a1, a2, a3) shows weaker mucosal surface density comprising of some orifices (crossed arrow), some villi damages (circle), some visible villi detachments from the epithelial layer (stars), and villi atrophy (arrows) causing the disappearance of some villi; BV (b1, b2, b3) shows closely packed mucosal surface density but comprises of very few orifices (crossed arrow); BS (c1, c2, c3) shows the most closely packed mucosal surface density; and BT (d1, d2, d3) shows also more closely packed mucosal surface density. Treatment groups B0, BV, BS, and BT refer to the fish groups fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.

3.5.2. Liver immune and anti-oxidant enzyme activities

Table 4 again displays the results of the liver immune and anti-oxidant activities. At the end of the 6-week feeding trial, all the probiotic-treated groups demonstrated a significant increase ($P < 0.05$) in the IgM, LYZ, SOD, CAT, GSH-Px, and T-AOC, and a significant decrease ($P < 0.05$) in the AST, ALT, and MDA enzyme activities in the liver compared to the B0 group. It must be noted that while the enzyme activity levels of LYZ and CAT were highest in the BV group, that of the SOD and GSH-Px were highest in the BT group. The highest T-AOC activity was achieved in both the BV and BT groups. The group with the lowest liver AST and ALT enzyme activity levels were observed in the BT and BV groups, respectively.

3.5.3. Intestinal immune, anti-oxidant, and digestive enzyme activities

The intestinal immune and anti-oxidant enzyme activities are also illustrated in Table 4. The intestinal IgM activity was

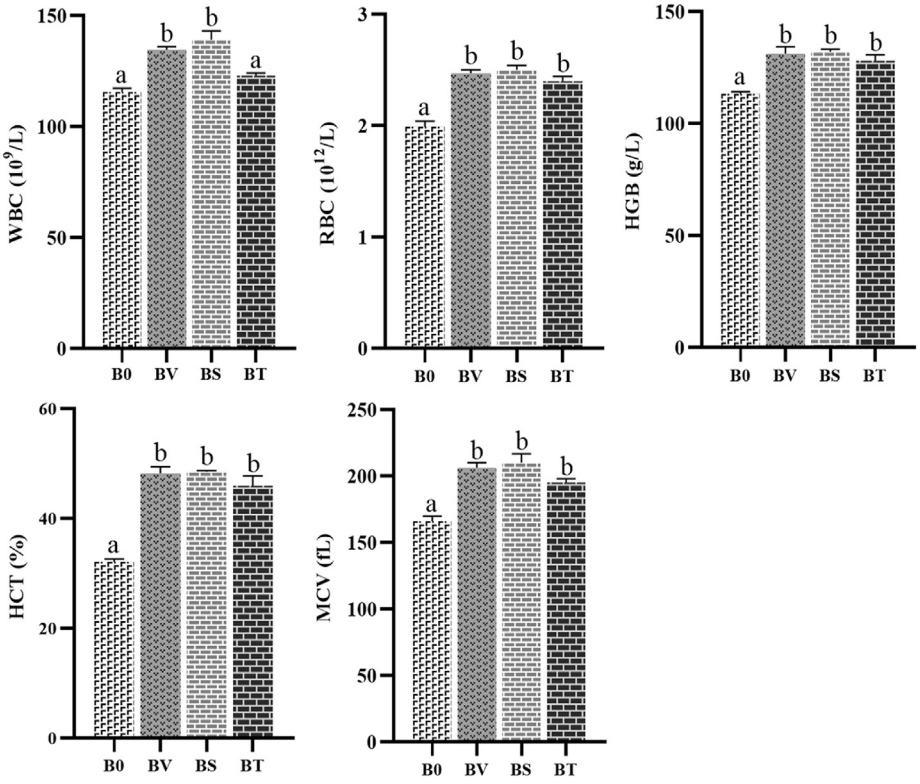

Fig. 4. Blood haematological parameters of juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species. Vertical bars represented the mean ± SE of 4 replicates (2 fish/replicate group). Data marked with different letters differ significantly ($P < 0.05$) among groups (Tukey's HSD). Where: WBC = white blood cell counts; RBC = red blood cell counts; HGB = haemoglobin; HCT = haematocrit; MCV = mean cell volume. Treatment groups B0, BV, BS, and BT refer to the fish group fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.

Table 4
The serum, liver, and intestinal immune response and anti-oxidant enzyme activities in juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species.

Parameters	B0	BV	BS	BT
Serum				
IgM, μg/mL	21.80 ± 0.58^{a}	28.77 ± 0.31^{b}	26.01 ± 1.08^{b}	31.97 ± 0.71^{c}
LYZ, U/L	2.67 ± 0.12^{a}	4.00 ± 0.21^{b}	3.17 ± 0.20^{a}	5.06 ± 0.22^{c}
SOD, ng/mL	7.57 ± 0.22^{a}	15.39 ± 0.14^{c}	13.13 ± 0.29^{b}	13.31 ± 0.28^{b}
CAT, ng/mL	13.67 ± 0.17^{a}	17.65 ± 0.09^{c}	17.83 ± 0.27^{c}	14.87 ± 0.24^{b}
LDH, IU/L	12.26 ± 0.37^{c}	9.63 ± 0.28^{ab}	8.38 ± 0.83^{a}	10.53 ± 0.13^{bc}
MDA, nmol/L	8.60 ± 0.20^{c}	5.96 ± 0.54^{ab}	5.39 ± 0.25^{a}	6.73 ± 0.07^{b}
Liver				
IgM, μg/mg prot	32.52 ± 2.75^{a}	46.04 ± 0.45^{b}	42.16 ± 2.47^{b}	45.86 ± 1.63^{b}
LYZ, mU/mg prot	2.84 ± 0.14^{a}	7.96 ± 0.40^{c}	6.10 ± 0.34^{b}	6.38 ± 0.14^{b}
SOD, ng/mg prot	8.68 ± 0.27^{a}	18.98 ± 0.16^{c}	16.08 ± 0.43^{b}	25.54 ± 0.22^{d}
CAT, ng/mg prot	15.30 ± 0.32^{a}	27.90 ± 1.64^{c}	16.93 ± 0.93^{a}	22.69 ± 0.95^{b}
GSH-Px, ng/mg prot	57.21 ± 0.95^{a}	69.85 ± 1.61^{b}	83.11 ± 4.32^{c}	88.49 ± 0.66^{c}
T-AOC, U/mg prot	14.60 ± 0.29^{a}	26.03 ± 1.73^{c}	20.78 ± 0.39^{b}	27.01 ± 0.79^{c}
AST, mU/mg prot	26.43 ± 0.73^{b}	22.47 ± 0.37^{ab}	23.29 ± 1.73^{ab}	20.35 ± 1.57^{a}
ALT, mU/mg prot	13.59 ± 0.40^{b}	9.38 ± 0.21^{a}	10.58 ± 0.77^{ab}	12.69 ± 1.13^{b}
MDA, nmol/mg prot	14.67 ± 0.20^{b}	8.55 ± 0.13^{a}	10.09 ± 0.67^{a}	9.73 ± 0.20^{a}
Intestine				
IgM, μg/mg prot	30.81 ± 1.24^{a}	32.82 ± 2.38^{a}	41.38 ± 2.43^{b}	32.82 ± 0.60^{a}
LYZ, mU/mg prot	7.10 ± 0.32^{a}	10.96 ± 0.07^{c}	8.06 ± 0.24^{b}	8.83 ± 0.21^{b}
C4, μg/mg prot	173.35 ± 1.86^{a}	256.10 ± 11.03^{b}	243.94 ± 9.58^{b}	232.82 ± 9.98^{b}
C3, μg/mg prot	84.83 ± 2.47^{a}	171.65 ± 15.21^{b}	198.91 ± 4.52^{b}	111.01 ± 5.89^{a}

IgM = Immunoglobulin M; LYZ = lysozyme; SOD = superoxide dismutase; CAT = catalase; LDH = lactate dehydrogenase; MDA = malondialdehyde; GSH-Px = glutathione peroxidase; T-AOC = total anti-oxidant capacity; AST = aspartate aminotransferase; ALT = alanine aminotransferase; C4 and C3 = complements 4 and 3.
Treatment groups B0, BV, BS, and BT refer to the fish groups fed the basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.
The values in the same row with different superscript letters differ significantly among groups ($P < 0.05$) based on Tukey's HSD test. Data are the mean ± SE of 4 replicates (4 fish/replicate group for serum samples and 2 fish/replicate group for tissue samples).

significantly enhanced ($P < 0.05$) in the BS group than in the other groups, including the control. Fish fed the host gut-derived probiotic diets showed significantly higher ($P < 0.05$) LYZ enzyme activities and C4 contents in the intestine than fish fed the basal diet. It was revealed that the intestinal C3 contents were enhanced statistically ($P < 0.05$) in the BV and BS groups than as observed in the BT and B0 groups, although higher elevations of the results were seen in the BT group.

The results of the intestinal digestive enzyme activities are shown in Table 5. It was discovered that the *Bacillus*-treated groups showed significantly higher ($P < 0.05$) LPS activity than the control group. Statistically, a significantly higher ($P < 0.05$) level of intestinal TRP enzyme activities (except the BV and the BS groups) and AMS (except the BS group) was observed in the host gut-derived *Bacillus*-treated groups than in the B0 group.

3.6. Gene expression

The expression of immune-related genes in the DI of hybrid grouper was distinctly affected after dietary supplementation of different host gut-derived *Bacillus* species, as shown in Fig. 5. Compared to the B0 group, a significant up-regulation ($P < 0.05$) of *IL1β*, *IL6*, *IL8*, *TNFα*, and *MyD88* was observed in the isolated *Bacillus*-treated groups, but with significant higher expression of *IL1β*, *IL8*, *TNFα*, and *MyD88* in the BS group (Fig. 5(A)). The *IL1β* and *MyD88* gene expression revealed no significant difference ($P > 0.05$) between the BV and BT groups. The expression of anti-inflammatory (*IL10* and *TGFβ*) and tight junction (occludin and *ZO1*) genes are shown in Fig. 5(B). Similarly, there were significantly higher ($P < 0.05$) expressions of *IL10*, *TGFβ*, occludin, and *ZO1* genes in groups fed host gut-derived *Bacillus* supplemented diets compared to the control. The BS group showed the highest levels in the expression of the *TGFβ*. Nevertheless, no significant differences ($P > 0.05$) were observed in the expression of the *IL10* and occludin genes among the probiotic-treated groups.

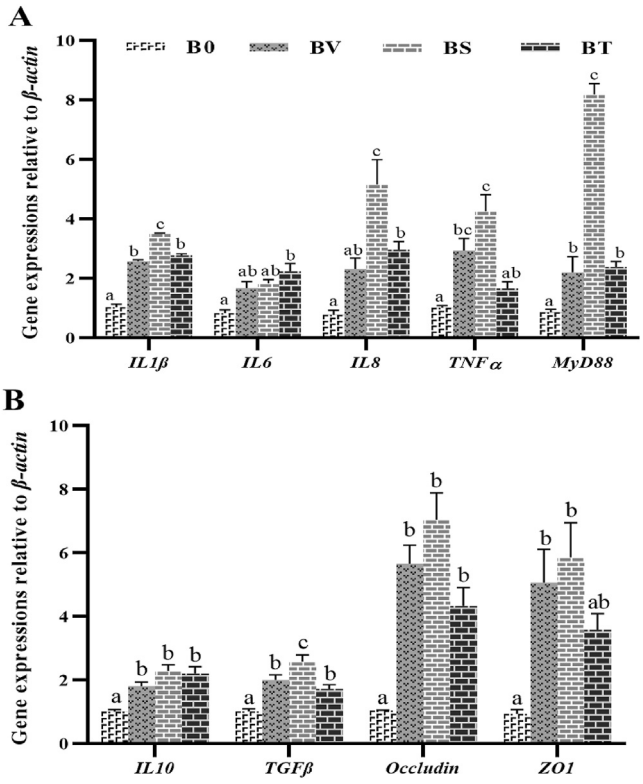

Fig. 5. Effects of different host gut-derived *Bacillus* species supplementation on the distal intestinal (A) pro-inflammatory and (B) anti-inflammatory and tight junction gene expression of juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*). Vertical bars represented the mean ± SE of 4 replicates (2 fish/replicate group). Data marked with different letters differ significantly ($P < 0.05$) among groups (Tukey's HSD). *IL1β* = interleukin 1 beta; *IL6* = interleukin 6; *IL8* = interleukin 8; *TNFα* = tumor necrosis factor alpha; *MyD88* = myeloid differentiation primary response protein 88; *IL10* = interleukin 10; *TGFβ* = transforming growth factor beta; *ZO1* = zonula occludens 1. Treatment groups B0, BV, BS, and BT refer to the fish groups fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.

3.7. High-throughput sequencing analysis of 16S rRNA gene amplicons

3.7.1. Obtained sequence information of the distal intestinal microbiota

A total of 960,059 raw reads were obtained from 12 fish gut microbiota samples (triplicates of the B0 group (control), BV group, BS group, and BT group), with an average of 80,005 sequences per sample (ranging from 79,622 to 80,430 sequences). After data quality filtering, 955,631 clean reads were acquired, averaging 79,636 sequences per sample (ranging from 79,266 to 80,075 sequences). Again, after filtering the chimeras, subsequent analysis of fish's gut clean reads revealed 912,319 effective tags, with an average of 76,027 sequences per sample (ranging from 74,898 to 77,723 sequences). No significant difference ($P > 0.05$) was witnessed among the treatment groups with regards to the reads (Table 6).

3.7.2. Microbiota of the distal intestinal α- and β-diversity analysis

The OTU and alpha diversity metrics, including the community richness (Chao1 and ACE) and diversity (Shannon and Simpson) indexes of the DI microbiota in hybrid grouper after the supplementation of dietary *Bacillus* species, are as well displayed in

Table 5
The intestinal digestive enzyme activities in juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species.

Parameters	B0	BV	BS	BT
TRP, U/mg prot	1638.17 ± 36.37^a	1783.73 ± 15.35^a	1651.26 ± 87.71^a	2222.38 ± 102.74^b
LPS, U/mg prot	574.82 ± 29.49^a	804.14 ± 30.45^b	936.81 ± 8.86^c	1006.39 ± 30.52^c
AMS, U/mg prot	271.33 ± 9.39^a	431.19 ± 8.39^b	314.89 ± 27.31^a	587.04 ± 22.00^c

TRP = trypsin; LPS = lipase; AMS = amylase.
Treatment groups B0, BV, BS, and BT refer to the fish groups fed the basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.
The values in the same row with different superscript letters differ significantly among groups ($P < 0.05$) based on Tukey's HSD test. Data are the mean ± SE of 4 replicates (2 fish/replicate group).

Table 6
Statistical analysis of the operational taxonomic units (OTU), alpha diversity and richness indexes of the distal intestinal microbiota of juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species.

Parameters	B0	BV	BS	BT
OTU	378.50 ± 15.50	376.00 ± 37.00	379.00 ± 19.67	376.00 ± 21.00
Raw reads	80,086.67 ± 47.62	79,908.00 ± 72.75	79,905.00 ± 145.48	80,120.00 ± 178.12
Clean reads	79,731.00 ± 40.93	79,509.33 ± 60.78	79,542.00 ± 141.35	79,761.33 ± 179.30
Effective reads	76,131.33 ± 745.01	76,328.67 ± 732.37	75,957.67 ± 886.74	75,688.67 ± 274.99
Chao 1	515.79 ± 33.96	558.97 ± 59.70	519.31 ± 25.72	450.64 ± 14.00
ACE	479.47 ± 11.12	483.12 ± 9.15	502.52 ± 3.16	445.22 ± 25.75
Shannon	1.88 ± 0.58	1.95 ± 0.34	2.28 ± 0.54	2.16 ± 0.12
Simpson	0.65 ± 0.02	0.50 ± 0.07	0.75 ± 0.05	0.60 ± 0.05

Treatment groups B0, BV, BS, and BT refer to the fish groups fed the basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.
No significant differences ($P > 0.05$) were witnessed among treatment groups. Data are the mean ± SE of 3 replicates (2 fish/replicate group).

Table 6. Generally, all the alpha diversity indexes, including Chao 1, ACE, Shannon, and Simpson, showed no significant differences ($P > 0.05$) among all groups. The goods coverage values obtained for the B0, BV, BS, and BT groups were 0.9984 ± 0.00, 0.9984 ± 0.00, 0.9983 ± 0.00, and 0.9986 ± 0.00, respectively, which meant that most of the microbial diversity within the treatment samples were sufficiently captured.

The unique OTU analysis in the 4 groups was shown by a Venn diagram (Fig. 6). It was revealed that a total of 374 OTU were uniquely shared among treatment groups. On the other hand, the core OTU numbers in the B0, BV, BS, and BT were 48, 74, 28, and 19, respectively, with the BV group obtaining the highest. To evaluate the overall difference in the β-diversity of bacteria between groups, principal component analysis (PCA) and principal coordinate method (PCoA) were used based on unweighted-Unifrac distance. The PCA two-dimensional plot is displayed in Fig. 7(A), whereas that of the unweighted-Unifrac PCoA is shown in Fig. 7(B).

3.7.3. Microbiota composition, relative abundance analysis, and comparison

In obtaining the microbial community composition, the sequencing reads' classification similarity was ≥97%. Judging from the classifications, the sequences obtained were distributed at 21 bacterial phyla, 56 bacterial classes, 116 bacterial orders, 193 bacterial families, 413 bacterial genera, and 471 bacterial species. Fig. 8 demonstrates the relative abundance comparison of the gut microbiota in juvenile hybrid grouper after dietary supplementation of different host gut-derived *Bacillus* species. At the phylum taxonomic level, the 10 most predominant phyla were Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, Cyanobacteria, Verrucomicrobia, Chloroflexi, Fusobacteria, and Planctomycetes, with Proteobacteria bing the most abundant (Fig. 8(A)). The results obtained displayed a significant increase ($P < 0.05$) in the relative abundance of Proteobacteria (BV group obtained the highest), Bacteroidetes (BS group obtained the

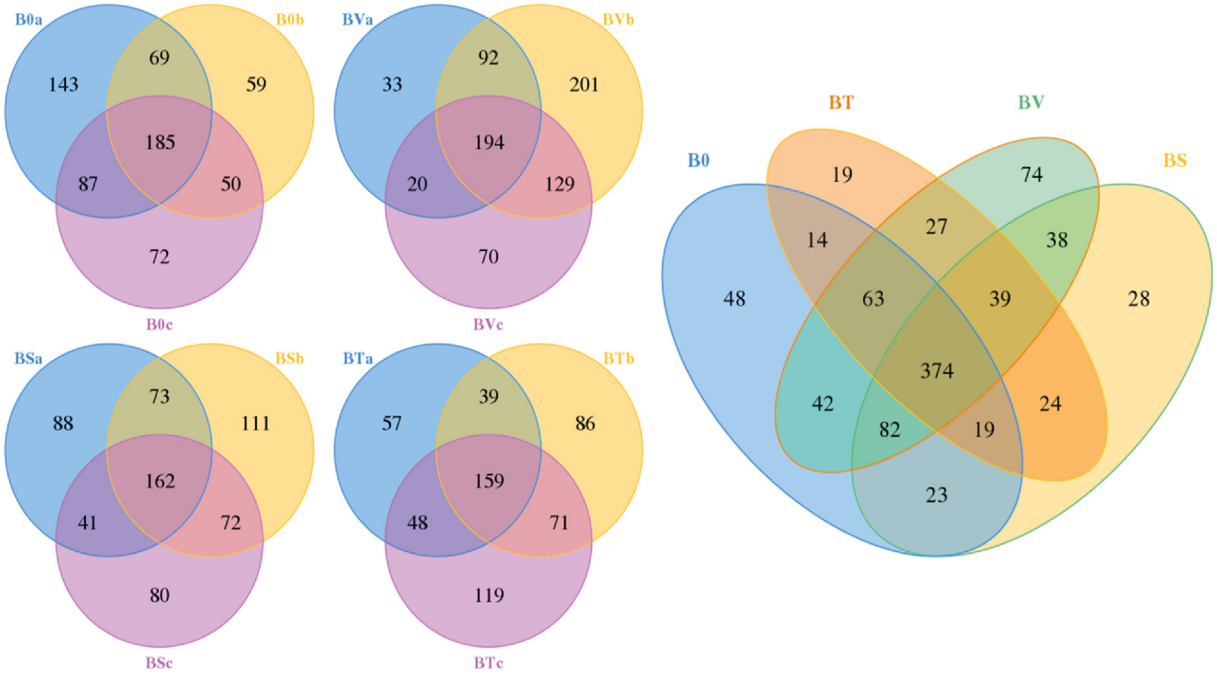

Fig. 6. The Venn diagram showing the distribution of the unique and shared operational taxonomic units (OTU) of intestinal microbiota after dietary supplementation of host gut-derived *Bacillus* species in hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*). Treatment groups B0, BV, BS, and BT refer to the fish groups fed the basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively. B0a, B0b and B0c are treatment replications 1, 2 and 3 of the B0 group; BVa, BVb, BVc are treatment replications 1, 2 and 3 of the BV group; BSa, BSb, and BSc are treatment replications 1, 2 and 3 of the BS group; and BTa, BTb, and BTc are treatment replications 1, 2 and 3 of the BT group.

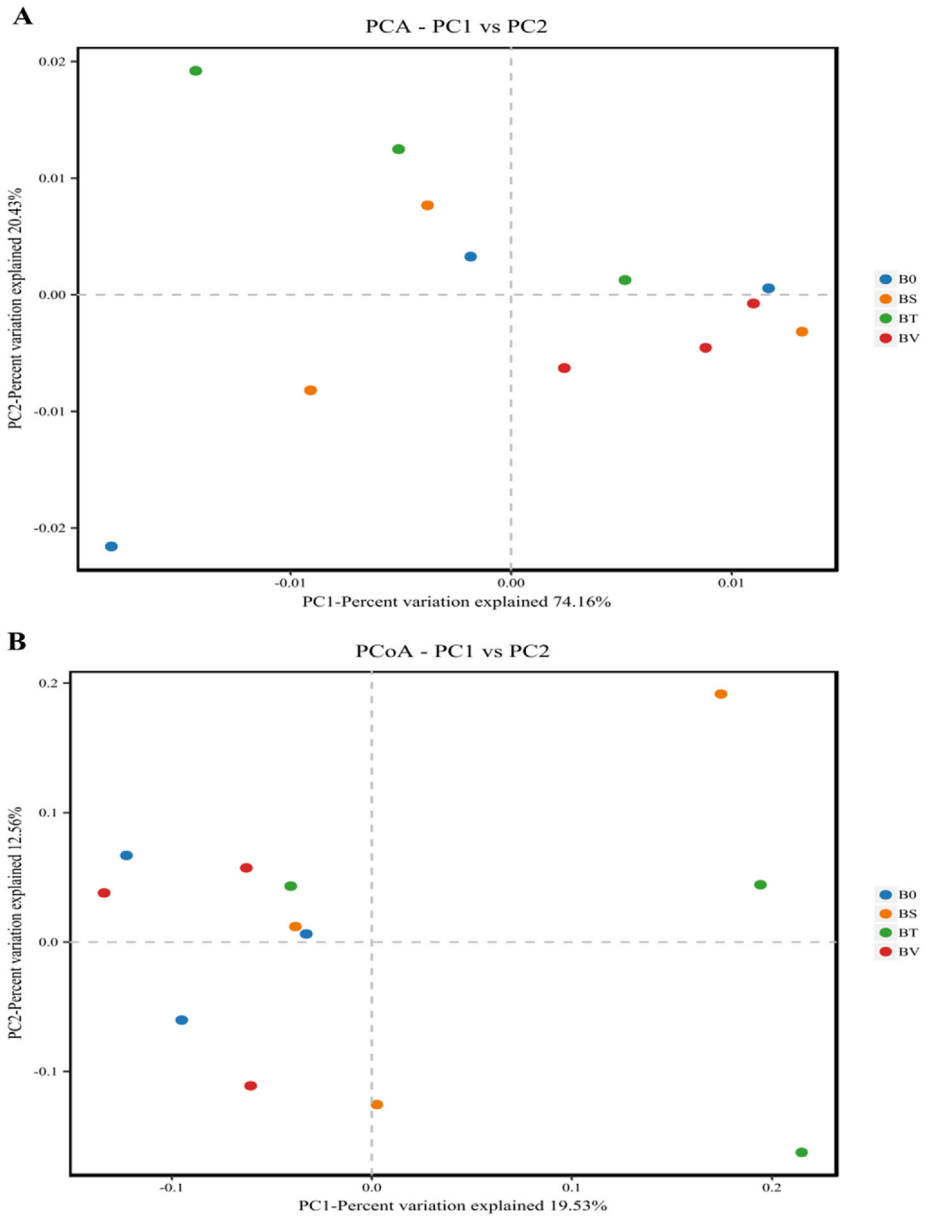

Fig. 7. Beta diversity of gut microbiota based on unweighted UniFrac distance of intestinal microbiota after dietary supplementation of host gut-derived *Bacillus* species in hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*). (A) Principal component analysis (PCA) plot and (B) principal coordinate analysis (PCoA) plot. Treatment groups B0, BV, BS, and BT refer to the fish groups fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.

highest), Actinobacteria (except the BS group, with the BV group obtaining the highest), Acidobacteria (except the BT group), Cyanobacteria (except the BV and BT groups), and Verrucomicrobia (BV obtained the highest), and a significant decrease ($P < 0.05$) in the relative abundance of Firmicutes (BV group obtained the least), Chloroflexi (BS and BT groups obtained the least), and Fusobacteria in the *Bacillus* treated groups in comparison to the control group (Fig. 8(B)).

Fig. 8(C) illustrates the 10 most predominant bacteria observed at the genus taxonomic level after feeding host gut-derived *Bacillus* species to juvenile hybrid grouper. A significantly higher ($P < 0.05$) relative abundance of *Romboutsia* (except the BV group with the BT group witnessing the highest), *Turicibacter*, *Epulopiscium*, *Clostridium sensu stricto* 1, *C. sensu stricto* 13, *Lactobacillus*, *Bacillus* (BS group witnessed the highest), and a significantly lower ($P < 0.05$) relative abundance of *Staphylococcus*, and *Photobacterium* (except the BV group) were observed in the groups fed the *Bacillus*-treated diets in comparison to those fed the basal diets (Fig. 8(D)). Fig. 8(E) displays the phylogenetic tree with genus taxonomic level features. The top 100 bacteria genera observed after feeding fish with host gut-derived *Bacillus* species are shown by a heatmap in Fig. 9. The genus heatmap analysis showed a higher abundance of *Blautia*, *Bifidobacterium*, *Bacillus*, and *Lactobacillus*, and a lower abundance of *Vibrio* genera in the *Bacillus*-treated groups than as observed in the untreated group.

3.8. Challenge test

After the challenge with *V. harveyi* for 7 days, host gut-derived *Bacillus* species' inclusion in diets significantly increased

($P < 0.05$) the survival of hybrid grouper, and the supplementation with BS could effectively improve the survival of fish (Fig. 10). The results of the cumulative mortality obtained were in the order 63%, 40%, 23%, and 27% for fish fed with B0, BV, BS, and BT diets, respectively. The relative percent survival (%) was highest in the BS (63.2%), BT (57.9%), and then lastly in the BV (36.8%) group.

4. Discussion

Managing diseases efficiently in aquaculture is crucial for the fruitful production of aquatic animals and the aquaculture industry's sustainability (Aly et al., 2008b; Buruiană et al., 2014). The wide and wrong usage of antibiotics has resulted in severe biological and ecological concerns, primarily resulting in the emergence of antibiotic-resistant bacteria (Cabello, 2006; Das et al., 2013). Probiotics, recognized as beneficial microbes, are considered an effective and environmentally friendly alternative to antibiotics due to their colossal health benefits to the host and their resistive abilities against pathogens (Lazado and Caipang, 2014). The growth and development of aquaculture are premised primarily on probiotics usage since these microorganisms, besides their numerous benefits, are eco-friendly, unlike antibiotics and other chemicals, which not only pose adverse effects on animals and the environment but are also cost-effective (Aly et al., 2008a; Amoah et al., 2019; Liu et al., 2009; Sankar et al., 2017; Verschuere et al., 2000). Among the several known beneficial bacteria, *Bacillus* spp. has been reported as one of the most outstanding probiotics due to their weighty attributes, such as being able to withstand harsh conditions to stay viable for an extended period. They are also known to enhance the growth performance, immune response, digestive enzyme activity and increase resistive capacity against diseases in aquatic animals (Amoah et al., 2019; Buruiană et al., 2014; Liu et al., 2009; Reda et al., 2018). Research has shown that isolated probiotics from the intestine of host organisms not only enhance the host's growth performance after its supplementation but can also help improve the haematological parameters, intestinal histology, and gut microbial composition (Adorian et al., 2019; Van Doan et al., 2018; Ramesh et al., 2015; Reda et al., 2018; Sahu et al., 2007). Due to the benefits of host-associated probiotics, current studies are advancing in this field, although less is being done. Fewer reports show the effects of isolated *Bacillus* species derived from the gut on the growth, immune response, antioxidant and digestive enzyme activities, and disease resistance in hybrid grouper (Liu et al., 2012; Son et al., 2009; Sun et al., 2010; Zhou et al., 2019). There are no studies conducted on the effects of *B. tequilensis* GPSAK2 (accession number: MW548630), *B. velezensis* GPSAK4 (accession number: MW548635), and *B. subtilis* GPSAK9 (accession number: MW548634) that we previously isolated on hybrid grouper. Therefore, this study evaluated the effects of the above-mentioned host gut-derived *Bacillus* spp. on the growth, immunity, and intestinal health (histology and gut microbiota) in hybrid grouper.

The results obtained in the present study revealed that feeding hybrid grouper with diets supplemented with host-associated *Bacillus* species enhanced the W_f (final weight), WGR, SGR, CF, HSI, and VSI. Again, in contrast to the moisture content results, dietary supplementation of *Bacillus* species significantly increased the whole fish crude protein and ash content than as observed in fish fed the control diet. Although there were no statistical differences in the crude lipid content, higher elevations in the values were witnessed in the *Bacillus*-treated groups. Vijayavel and Balasubramaniam (2006) have shown that the whole-body proximate composition analysis serves as the best indicator of physiological wellness and an improvement in meat quality. The results obtained in the study agree with previously documented reports where an increase in the growth performance and whole-body proximate composition was observed in *Cyprinus carpio* after dietary supplementation of 3 *Bacillus* spp., *Bacillus coagulans* (MTCC 9872), *Bacillus licheniformis* (MTCC 6824), and *Paenibacillus polymyxa* (MTCC 122) (Gupta et al., 2014), *Lates calcarifer* after dietary supplementation of 2 *Bacillus* spp., *B. licheniformis* and *B. subtilis* (Adorian et al., 2019), *L. rohita* after dietary supplementation of *B. subtilis* (Kumar et al., 2006), *Litopenaeus vannamei* after dietary

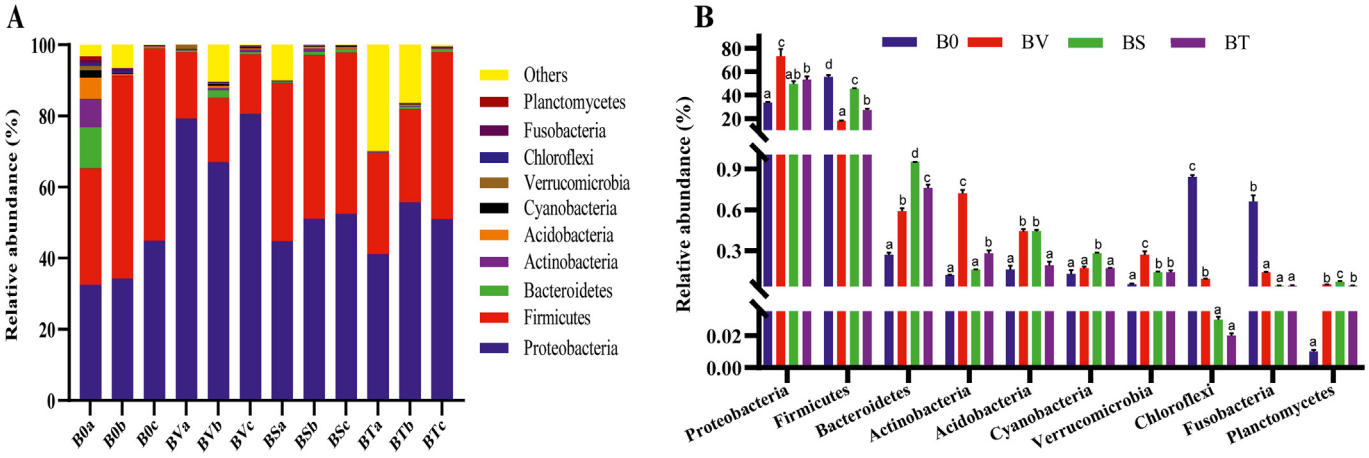

Fig. 8. Comparison of bacterial composition and relative abundance of the intestinal microbiota in juvenile hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) fed different host gut-derived *Bacillus* species. (A) and (B) represent relative abundance comparison at the phylum level; (C) and (D) represent relative abundance comparison at the genus level; and (E) represents the phylogenetic tree with features at the genus taxonomic level (Each branch represents a specie, and the branch length is the evolutionary distance between 2 species, i.e., the degree of species difference. The annular figure shows the phylogenetic tree of species with the same colour of the genus name representing the same phylum). Vertical bars represented the mean ± SE of 3 replicates (2 fish/replicate group). Data marked with letters differ significantly ($P < 0.05$) among groups (Tukey's HSD). Treatment groups B0, BV, BS, and BT refer to the fish groups fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively. B0a, B0b and B0c are treatment replications 1, 2 and 3 of the B0 group; BVa, BVb, BVc are treatment replications 1, 2 and 3 of the BV group; BSa, BSb, and BSc are treatment replications 1, 2 and 3 of the BS group; and BTa, BTb, and BTc are treatment replications 1, 2 and 3 of the BT group.

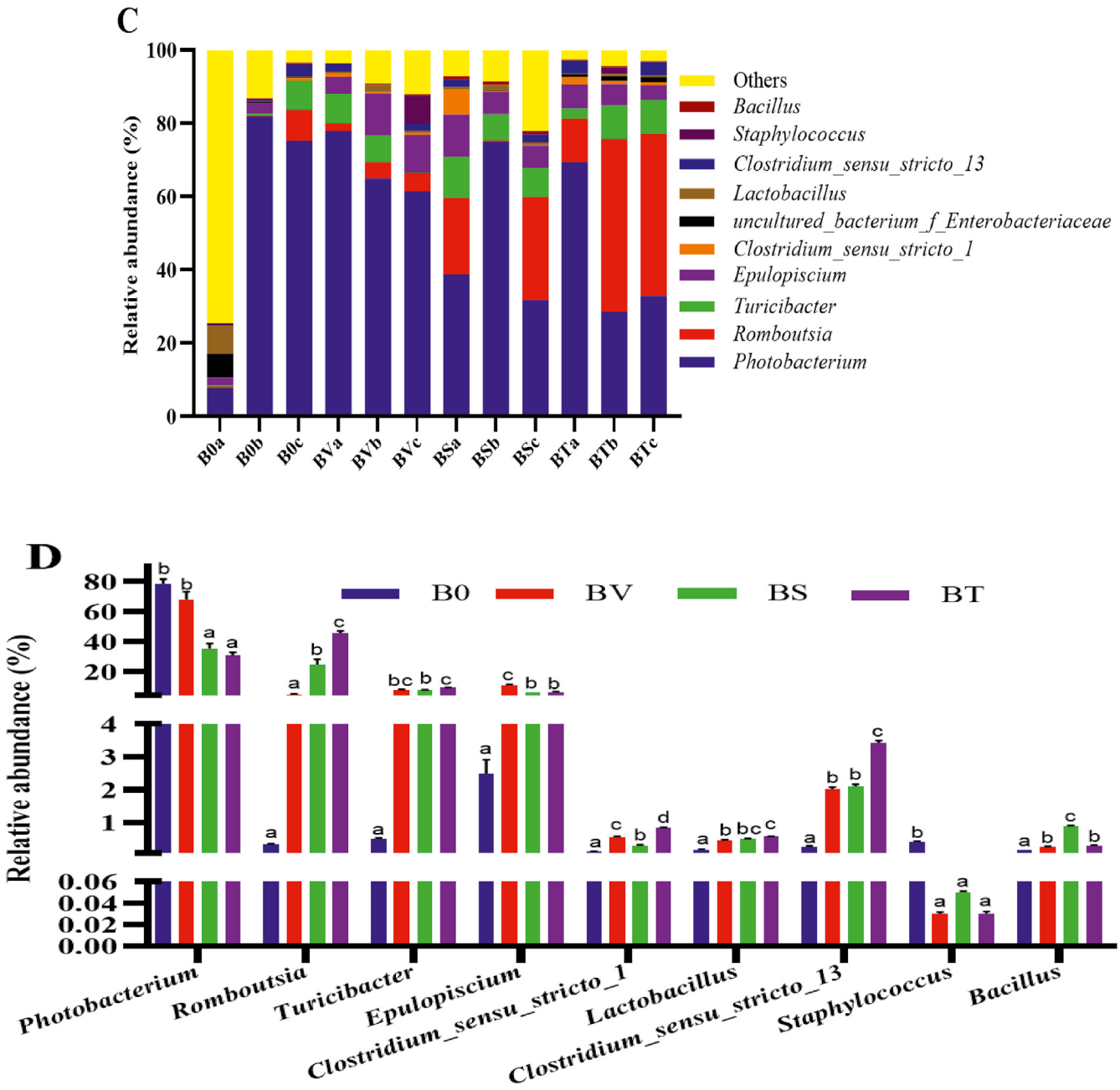

Fig. 8. (*continued*).

supplementation of *B. subtilis* (Zokaeifar et al., 2012) and *B. coagulans* (Amoah et al., 2019), and *L. calbasu* (Hamilton, 1822) after dietary supplementation of 3 isolated *Bacillus* spp., *B. subtilis* (KX756706), *B. cereus* (KX756707), and *B. amyloliquefaciens* (KX775224) (Kavitha et al., 2018). Hong et al. (2005), in their studies, revealed *Bacillus* species as having the ability to influence the production of digestive enzymes, which in turn help in the breaking down of food into smaller particles; thus, there is a higher absorption and distribution of nutrients. The improved growth performance and proximate body composition can be attributed to the enhancement of the digestive enzymes, immune response, the expression of immune-related genes, and the modulation of the gut microbiota of the treated groups that was witnessed in the present study.

Digestive enzymes indicate an organism's ability to metabolize nutrients (Berges and Mulholland, 2008). It was observed in the current study that the supplementation of host gut-derived *Bacillus* species in diets led to a significant increase in the LPS digestive enzymes. Concerning the TRP, only fish that received the BT diets showed the highest significant value compared to the other groups. Also, only the BV and BT groups revealed a significantly higher AMS activity when compared to the other groups. However, it must be stated that higher elevations in the enzymes were observed in the *Bacillus*-treated groups than in the control group. The results in the present study are similar to previous findings where *Bacillus* supplementation increased digestive enzymes (Adorian et al., 2019; Liu et al., 2009; Sankar et al., 2017; Zokaeifar et al., 2012). In addition to the increase in digestive enzyme activities, fish-fed dietary *Bacillus*

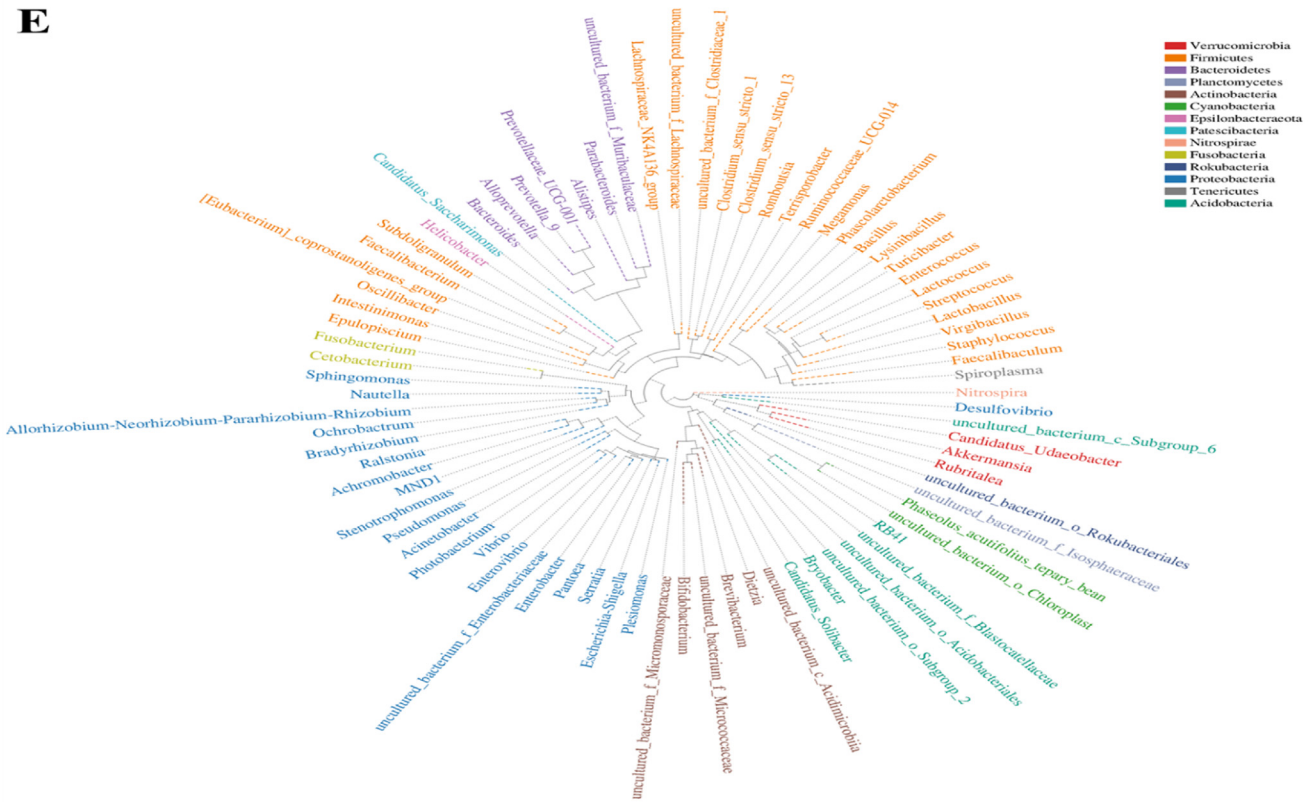

Fig. 8. (*continued*).

diets experienced higher significant VH, VW, MT, CD, and type II mucus cells (except those fed the BT diets) than the control (Cardoso et al., 2015; Fiertak and Kilarski, 2002; Petrinec et al., 2005). The physiology of fish has been conveyed to be affected by the changes in the intestinal structure. Thus, an increase in the intestinal VH, VW, MT, CD, and type II mucus cells translates into better digestion and absorption of nutrients, which go a long way in improving the health and well-being of fish. The current study also showed in the SEM analysis that more closely packed mucosal villi densities with no damage were observed in the probiotic-treated groups. The BS group revealed the best, unlike the fewer and weaker villi density observed in the B0 group. A plethora of data suggests that taller VH, wider VW, broader MT, and CD enhance growth performance, feed utilization, and disease resistance against pathogens. This occurs because the intestinal mucosal tissues provide a broader absorptive surface area for higher amounts of nutrients to be absorbed (Kristiansen et al., 2011). Similarly, highly significant intestinal VH, VW, MT, and CD coupled with more thick villi densities than the control group after *Bacillus* supplementation have been reported in fish (Kuebutornye et al., 2020). The BS group showed the highest type II mucus cells. Similarly, the administration of *B. subtilis* RZ001 revealed an increase in goblet and mucus cells which translated into alleviating colitis and improved intestinal integrity (Li et al., 2020).

The modulation of immune and anti-oxidant activities is one of the key benefits of probiotics (Nayak, 2010). After the 6-week feeding trial, the isolated *Bacillus* species administration in fish diets caused significant enhancement in blood haematological parameters, as well as serum, liver, and intestinal immune and anti-oxidant enzyme activities. As pathophysiological indices, the WBC, RBC, HGB, HCT, and MCV counts are noted as key tools used in detecting fish health and its physiological condition (Harikrishnan et al., 2010; Lim et al., 2000). The study revealed a significant increase in the probiotic-treated groups concerning the WBC (except for the results obtained in the BT group, although higher elevations were observed than the B0 group), RBC, HGB, HCT, and MCV than as observed in control group, corroborating with previously conducted studies (Adorian et al., 2019; Kuebutornye et al., 2020; Kumar et al., 2006; Reda et al., 2018).

Serum, liver, and intestinal immune and anti-oxidant parameters (i.e., IgM, LYZ, SOD, CAT, GSH-Px, T-AOC, C3, C4, LDH, AST, ALT, and MDA), which are key players in the assessment of fish health and also play critical role in fish defense were examined in this study. IgM helps in bacterial opsonization, toxin, and virus neutralization. IgM is liable to phagocyte destruction in the host organism (Beck et al., 2015; Wang et al., 2019). Lysozyme attacks, hydrolyzes, and breaks glycosidic bonds in the peptidoglycan (Magnadóttir, 2006). While SOD supports catalyzing reactive O^{-2} to H_2O_2 partitioning (Shen et al., 2010), the CAT is known to help to hydrolyze H_2O_2 into H_2O and O_2 (Wang et al., 2017). GSH-Px is primarily known to display the detoxification of H_2O_2 and other peroxides, such as lipid hydroperoxides (Wang et al., 2017), whereas the T-AOC serves as an overall indicator of an animal's anti-oxidant status, representing the amount of enzymes and non-enzyme anti-oxidants of the host body (Xiao et al., 2004). Complements are mainly responsible for the annihilation and eradication of toxins. C3 and C4 are mainly produced by hepatocytes which can be activated to participate in immune response (Ekdahl et al., 2019). AST, ALT (Cheng et al., 2018; Kamada et al., 2016), and LDH (Cheng et al., 2017; Shi et al., 2015) serve as reliable indicators for tissue injuries caused by toxicants in the host organism, whereas MDA also illustrates the extent of peroxidation of lipid representing all toxic processes caused due to free radicals (Yang et al., 2017). At the end of the 6 weeks study, the probiotic-treated groups showed

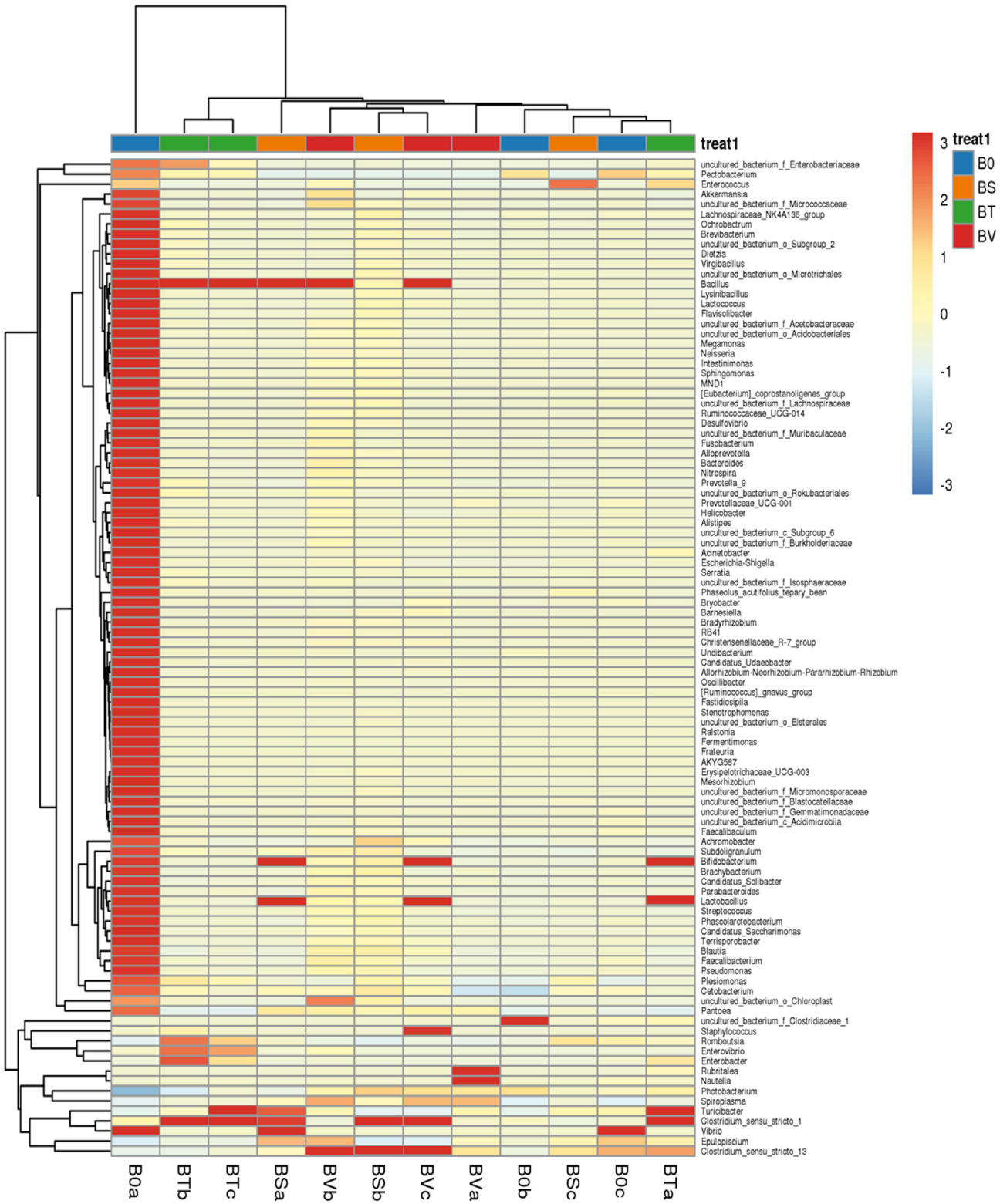

Fig. 9. Heatmap of the top 100 predominant bacteria of hybrid grouper's (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) distal intestinal bacteria composition at the genus taxonomic level after dietary supplementation of different host-gut derived *Bacillus* species. Treatment groups B0, BV, BS, and BT refer to the fish groups fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively. B0a, B0b and B0c are treatment replications 1, 2 and 3 of the B0 group; BVa, BVb, BVc are treatment replications 1, 2 and 3 of the BV group; BSa, BSb, and BSc are treatment replications 1, 2 and 3 of the BS group; and BTa, BTb, and BTc are treatment replications 1, 2 and 3 of the BT group.

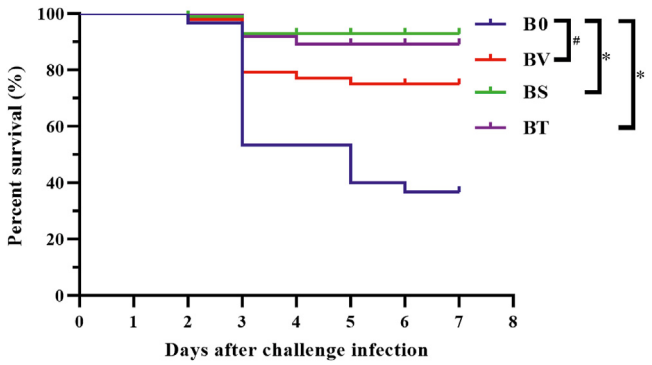

Fig. 10. Cumulative survival of hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) after 7 days post-challenge with *Vibrio harveyi*. Differences in cumulative mortality levels between the B0 and experimental groups were analysed by the Kaplan–Meier plot Log-Rank (Mantel–Cox) test. The number sign (#) indicates $P = 0.001$ and asterisk (*) indicates $P < 0.0001$ (4 replicates with 10 fish/replicate group). The Chi-square values of each comparison object are 10.86 for B0 versus BV groups, 44.05 for B0 versus BS groups, 32.30 for B0 versus BT groups, and 54.74 for all four groups. Treatment groups B0, BV, BS, and BT refer to the fish group fed basal diet without probiotic addition, the basal diet with 1×10^9 CFU/g *B. velezensis* GPSAK4 probiotic strain addition, the basal diet with 1×10^9 CFU/g *B. subtilis* GPSAK9 probiotic strain addition, and the basal diet with 1×10^9 CFU/g *B. tequilensis* GPSAK2 probiotic strain addition, respectively.

a significant enhancement in the serum, liver, and intestinal IgM, LYZ (except for the serum results obtained in the BS group, although higher elevations were witnessed); serum and liver SOD; serum and liver CAT (except the serum results obtained in the BS group although higher elevations were witnessed); liver GSH-Px and T-AOC; intestinal C3 and C4; and a significant decrease in serum LDH; serum and liver MDA, AST, and ALT enzyme activities than as observed in the control group. The BS group revealed the best results. The results affirm the probiotic's ability to reduce oxidative stress and enhance the host organism's immunity. It was thus reasonable to deduce and conclude that the isolated probiotics used in the current study improved humoral immune defenses in hybrid grouper. Other previously conducted studies (Van Doan et al., 2018; Harikrishnan et al., 2010; Kavitha et al., 2018; Kuebutornye et al., 2020; Ramesh et al., 2015) have shown a similar result trend after probiotic supplementation.

Probiotic use in aquaculture studies has modulated the expression of relevant genes in fish. *Bacillus* probiotics have been particularly reported to induce the expression of inflammatory genes, anti-oxidant genes, growth-related genes, genes encoding tight junction proteins, genes related to digestion, and genes associated with the transport of proteins (Abarike et al., 2018; Esteban et al., 2014; Kuebutornye et al., 2020; Zokaeifar et al., 2012). Although the intestine is essential for playing a crucial role in digestion, energy and nutrient absorption, and immune response, they also serve as a congenital barrier against the entry of harmful agents such as pathogens, toxins, and foreign antigens to maintain a relatively stable internal environment (Constantinescu and Chou, 2016); thus, it serves as a good indicator for analyzing whether there are changes in fish health. Fish immunity has been reported to be closely connected to inflammation which is initiated and regulated by cytokines (Sun et al., 2018). Inflammation is characterized by a relationship between pro- and anti-inflammatory cytokines. Also, there are instances where fish immune has been linked to tight junction protein expression due to acellular diffusion of intestinal bacteria and other antigens between epithelial cells affected (Zhao et al., 2014). Probiotics activation may cause the production of several pro- and anti-inflammatory mediators. Anti-inflammatory cytokines are dependent on cell communications. In the present study, the analysis of the expression of pro-inflammatory genes (including *IL1β*, *IL6*, *IL8*, *TNFα*, and *MyD88*), anti-inflammatory genes (*IL10* and *TGFβ*), and tight junction protein (occludin and *ZO1*) genes were conducted. *IL1β* is a foremost player in the immune response of fish as in mammals serving as a key arbitrator in response to microbial invasion and tissue injury. They are as well known to cause the stimulation of immune response via the activation of lymphocytes. Another way to make this happen is by inducing other cytokines capable of macrophage triggering (Secombes and Ellis, 2012). *TNFα* is an effective paracrine and endocrine facilitator of inflammatory and immune functions known for controlling the differentiation and growth of a comprehensive multiplicity of cells (Zou et al., 2003). *TGFβ* and *IL10* serve as important anti-inflammatory cytokines to limit inflammatory responses. *TGFβ* is a potent immune-deviating cytokine with essential roles in prompting active immune tolerance in marginal and mucosal tissues. It wields reflective effects on immune cells including macrophages, lymphocytes, and dendritic cells (Singh et al., 2019; Zhang et al., 2021). Tight-junction proteins such as occludin and *ZO1* is the foremost tightly connected membrane protein noted to control the acellular space between epithelial cells, thus preventing the acellular diffusion of intestinal bacteria and other antigens between epithelial cells (Zhao et al., 2014). *IL8* is a chemoattractant cytokine, and its production is initiated by a multiplicity of tissue and blood cells, prompting neutrophils to stimulate chemotaxis, free lysozyme enzyme, to control the angiogenesis and inflammatory process (Das et al., 2011). The present study revealed an up-regulation of *IL1β*, *IL6*, *IL8*, *TNFα*, *MyD88*, *IL10*, *TGFβ*, occludin, and *ZO1* in the probiotic-treated groups than the untreated group, with the BS group showing the highest significant expressions. Similarly, our study supports previous studies where similar up-regulation of such genes were witnessed (Kim and Austin, 2006; Panigrahi et al., 2007; Perez-Sanchez et al., 2011). The up-regulation of the tight junction genes can be attributed to the significant increase in beneficial bacteria such as *C. sensu stricto* and *Turicibacter* in the gut since these bacteria are reported as having the ability to cause an up-regulation of these genes (Fan et al., 2017).

The fish gut is composed of multifarious microbiota where their interaction with the epithelial cells induces countless host functions related to nutrition, immunity, digestion, and disease resistance. The gut is unceasingly opened to foreign substances, such as opportunistic pathogens, which easily cause diseases when the host's susceptibility is weakened (Hooper et al., 2002; Sekirov et al., 2010). Thus, the improvement of the gut microbiota has recently gained much attention due to the immense contributions toward shaping the intestinal structure via the digestion of food, absorption of nutrients, competition, and conquering of other unwanted microbes to improve the survival and health status of organisms (Hooper et al., 2002; Lazado and Caipang, 2014; Li et al., 2008; Romero et al., 2014; Sekirov et al., 2010). A plethora of data is available noting probiotics' ability to change the gut's microbial composition concerning the abundance of opportunistic pathogens and beneficial microbes (O'shea et al., 2012; Romero et al., 2014). There has been considerable interest in the strain specificity of the gut microbiota and immune boosting by probiotics. There are fewer reports on the changes host-associated probiotics exert on fish gut microbiota, warranting more research in such fields. Our results showed that dietary probiotic supplementation in hybrid grouper shaped the diversity of the gut microbiota. It was observed that regardless of the experimental diet, the 10 most relatively abundant bacterial species in the intestine of hybrid grouper fish at the phylum taxonomic level were Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, Cyanobacteria, Verrucomicrobia, Chloroflexi, Fusobacteria, and Planctomycetes, with the most abundant being the Proteobacteria phyla, which also portray

to be the ordinarily predominant phyla in most fish species (Amoah et al., 2021a; Egerton et al., 2018; Ingerslev et al., 2014; Kuebutornye et al., 2020). At the genus taxonomic level, a significantly higher ($P < 0.05$) relative abundance of *Romboutsia* (except the BV group, with the BT group witnessing the highest), *Turicibacter*, *Epulopiscium*, *C. sensu stricto* 1, *C. sensu stricto* 13, *Lactobacillus*, *Bacillus* (BS group witnessed the highest), and a significantly lower ($P < 0.05$) relative abundance of *Staphylococcus* and *Photobacterium* (BS and BT obtained the least) were observed in the groups fed the *Bacillus*-treated diets in comparison to the fish groups fed the basal diets. Also, the analysis of the genus heatmap (illustrating the top 100 predominant genera) showed a higher abundance of *Blautia* and *Bifidobacterium* and a lower abundance of *Vibrio* genera in the *Bacillus*-treated groups than as observed in the untreated group. The *Romboutsia* genus, a member of the family Peptostreptococcaceae, is an obesity-related genus that positively correlates with lipid profiles and lipogenesis in the liver. Bacteria of this group, when high, can reduce the level of obesity due to their several metabolic abilities, which can cause carbohydrate fermentation and aid in the utilization of amino acids (Therdtatha et al., 2021). *Turicibacter*, *Epulopiscium*, *C. sensu stricto* 1, *C. sensu stricto* 13, *Lactobacillus*, *Blautia*, *Bifidobacterium*, and *Bacillus* genera are widely regarded as probiotics, whereas *Staphylococcus*, *Photobacterium*, and *Vibrio* genera bacteria are known opportunistic pathogens. Bacteria belonging to the *Turicibacter* genus help regulate inflammation in the digestive tract. Reports suggest that bacteria species under the *Turicibacter* genera mediate intestinal tumorigenesis, which is associated with high fat-diet-induced obesity (Cotozzolo et al., 2020; Fan et al., 2017). *C. sensu stricto* abundance in animals' gut is known to be closely associated with *Epulopiscium*, and *Turicibacter* genera. *C. sensu stricto* and *Turicibacter* are noted to increase tight junction genes, translating into significant enhancement in intestinal structural ability (Angert and Clements, 2003; Flint et al., 2005; Kuebutornye et al., 2020). Dietary administration of *Clostridium* bacteria species is reported to enhance the growth performance, intestinal histology, immune response, antioxidant enzyme activities, haematological parameters, the expression of immune-related genes, as well as a decrease in the abundance of purported pathogenic bacteria such as *Vibrio* and *Aeromonas* in tilapia fish (*O. niloticus*) (Poolsawat et al., 2020) and freshwater crayfish, marron (*Cherax cainii*) (Foysal et al., 2019). *Lactobacillus* are lactic acid bacteria with almost all their species marketed as probiotics due to their higher health benefits on the host, including improving the intestinal mucosa (Saxelin et al., 2005). *Blautia* genera species such as *Blautia obeum* are noted to play an essential role with other bacteria in the recovery process after *Vibrio cholera* infection (Hsiao et al., 2014). Members of the *Bifidobacterium* genera aid in stimulating and manipulating the gut immune response, inducing intestinal homeostasis (Vieira et al., 2013). *Epulopiscium* bacteria genera have been reported as being high in abundance in herbivores sturgeon fish intestinal tract (Miyake et al., 2016) and also confirmed to have the closest relation with *Clostridium* bacteria species (Angert and Clements, 2003; Flint et al., 2005). Although little is known about *Epulopiscium* bacteria species, Flint et al. (2005) in their work, have asserted them to be one of the series of players that together form consortia in the breaking down of food for easy absorption of nutrients in the gut. Nonetheless, *Staphylococcus*, *Photobacterium* and *Vibrio* bacteria genera are known opportunistic bacteria whose abundance cause detrimental effects on host organisms (Amoah et al., 2021a; Kimmig et al., 2021; Rivas et al., 2013). Thus, the increase in the relative abundance of *Romboutsia*, *Turicibacter*, *Epulopiscium*, *C. sensu stricto* 1, *C. sensu stricto* 13, *Lactobacillus*, *Bacillus*, *Blautia* and *Bifidobacterium* and the decrease in the relative abundance of *Staphylococcus*, *Photobacterium* and *Vibrio* genera in the gut of the *Bacillus*-treated groups than the untreated group in this study might have been the reason for the enhancement of the growth performance, intestinal histology, immune response, antioxidant and digestive enzyme activities, and the immune related genes witnessed in the probiotic treated groups.

The fish's immunity relies on its ability to increase its resistive capacity in fighting against various pathogenic bacteria, which causes severe infection and high mortality. The 7-day challenge against *V. harveyi* showed that the fish fed with probiotic-treated diets demonstrate strong resistance than those fed the control diet. Similar reports of probiotics increasing resistance to disease infections in *O. niloticus* (Kuebutornye et al., 2020; Poolsawat et al., 2020), *C. carpio* (Gupta et al., 2014), *Ictalurus punctatus* (Lim et al., 2000), and *Epinephelus bruneus* (Harikrishnan et al., 2010) have been documented. The disease-resistant enhancement can be attributed to the highly significant relative abundances of the known beneficial bacteria and the significantly lower abundances of opportunistic pathogens in the *Bacillus*-treated groups than in the untreated. The increased immune and antioxidant biochemical parameters could also be a reason for the enhanced disease resistance.

5. Conclusion

This study revealed that the inclusion of host gut-derived *Bacillus* spp. (*B. tequilensis* GPSAK2, *B. velezensis* GPSAK4, and *B. subtilis* GPSAK9) in diets at 1.0×10^9 CFU/g in hybrid grouper enhanced the growth performance, feed utilization, immune response, antioxidant and digestive enzyme activities, haematological parameters, intestinal health (histology, microbiota, and expression of immune-related genes), and also increased the resistive capacity of fish against *V. harveyi* bacteria. In comparing the results obtained, the fish fed the *B. subtilis* GPSAK9 diet revealed the best performance regarding the measured parameters. The use of new omics technologies, such as proteomics and/or metabolomics, would be considered in future studies. A limitation of this study is the small sample size used (although the sample size used was in line with previous work), which will be delt with in future studies.. Host-associated probiotics of many fish species have not been applied due to the difficulty in isolation and functional verification. Studies concerning the role of host gut-derived *Bacillus* species in regulating mucosal immunity and intestinal microbiota are very rare; thus, we recommend more research to be conducted on more strains that are of superior benefit to fish.

Author contributions

Kwaku Amoah: Conceptualization, Data curation, Investigation, Methodology, Interpreted the statistical outcome; Writing - Original draft, Writing - review & editing; **Xiaohui Dong** and **Beiping Tan**: Conceptualization, Funding acquisition, Methodology, Resources, Supervision; **Shuang Zhang**: Formal Analysis, Investigation, Interpreted the statistical outcome, Methodology; **Shuyan Chi, Qihui Yang,** and **Hongyu Liu**: Data curation, Investigation, Project administration, Interpreted the statistical outcome, Formal Analysis; **Yuanzhi Yang**: Writing - review & editing, Formal Analysis, Visualization; **Haitao Zhang**: Software, Writing - review & editing, Visualization.

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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Appendix Supplementary data

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