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Metagenomic insights into modulation of microbiota community composition, potential metabolic pathways and antimicrobial resistance mechanisms in cage-cultured hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) upon oral immunization with a feed-based inactivated *Vibrio* vaccine

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ABSTRACT

Intensive grouper aquaculture plays a vital role in the economies of Southeast Asia but remains vulnerable to vibriosis outbreaks and the threat of antimicrobial resistance (AMR). This study evaluated the impact of a feed-based inactivated *Vibrio* vaccine on the hindgut microbiota of cage-cultured hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂) reared in an Indonesian fish farm. Fish in the vaccinated group received the feed-based vaccine at 4% body weight daily for five consecutive days during weeks 0, 2, and 6, while control fish were fed trash fish. Hindgut samples were collected for 16S rRNA amplicon sequencing on week 10 to assess microbial diversity and predicted functional profiles. Results revealed significant differences ($p < 0.05$) in both alpha and beta diversity indices, indicating altered microbiota evenness. Notably, *V. harveyi*, an inactivated component of the vaccine, was detected and appeared to significantly suppress the abundance of opportunistic pathogens such as *V. vulnificus* and *Photobacterium damselae*. Additionally, the vaccine enhanced predicted metabolic pathways related to pyrimidine synthesis, L-glutamate metabolism, and vitamin K production. It also reduced the prevalence of genes associated with β-lactam and vancomycin resistance, while preserving potential immune-related microbial functions. Overall, the findings demonstrate that feed-based vaccination can modulate gut microbial ecology in hybrid grouper and contribute to microbiota-mediated mitigation of AMR, even under socially competitive feeding conditions. This approach offers a practical and scalable strategy for enhancing fish health and mitigating AMR risks in mariculture, particularly in low- and middle-income countries where injectable vaccine delivery remains challenging.

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1. Introduction

Marine aquaculture in Southeast Asia has undergone significant development over the past two decades, largely influenced by rising global demand for high-value seafood and capital investment (Akber et al., 2020). Among the marine fish species cultured, the hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) has gained considerable demand, owing to its fast growth rate, resilience compared to other grouper species, and favorable market price, which have been a preferred choice for many fish farmers, especially in coastal areas (Dennis et al., 2020). In Indonesia, grouper farming activity began to increase around 2001, following initiatives led by the Ministry of Marine Affairs and Fisheries (Palm et al., 2015). However, the intensive cultivation of farmed grouper, coupled with poor water quality of stocking system caused massive vibriosis infection, which lead to the decline of grouper cultivation and production in Indonesia (Yanuhar et al., 2022). Bacterial disease, particularly vibriosis, has emerged as a persistent threat, with pathogenic *Vibrio* species such as *V. alginolyticus*, *V. harveyi*, and *V. vulnificus* frequently found in disease outbreaks (Yuan et al., 2024).

The response to these disease problems has varied across farming communities. Especially among small-scale farmers, antibiotics have been used extensively against vibriosis, often without veterinary oversight or adherence to antimicrobial stewardship guidelines (Siahaan et al., 2022). This practice has led to the emergence of antimicrobial-resistant strains, a phenomenon that is increasingly documented in both farmed and wild fish population (Azzam-Sayuti et al., 2021). It was reported that multiple antibiotic resistant *Vibrio* spp. were isolated from intestine of farmed and wild fish species in Indonesian marine waters (Isnansetyo et al., 2022), raising concerns about long-term implications for aquatic health and food safety.

Fortunately, vaccination is one of the most effective antibiotic alternatives to prevent and control fish bacterial diseases such as protecting high economically valued aquaculture fish species, including Atlantic salmon and rainbow trout (Du et al., 2022). On the other hand, recent studies have begun to explore the gut microbiome as a potential modulator of immune response in fish, with growing interest in how microbial composition shifts following vaccination (Sutra et al., 2025). The gut, being a primary interface between host and environment, harbors a diverse microbial community that can influence nutrient absorption, pathogen resistance, and overall health (Luan et al., 2023). In our previous study, we have developed a feed-based vaccine containing formalin-killed *Vibrio* spp., that successfully improved immune response and protective efficacy in orally vaccinated Asian seabass (*Lates calcarifer*) (Mohamad et al., 2022). Moreover, oral administration of fish vaccine is practical, feasible and appear affordable vaccination practice to encourage farmers for adopting vaccination in their fish farming practices (Lan et al., 2023). In a separate study, our feed-based vaccine also enhanced the Asian seabass gastrointestinal microbial diversity and richness in a commercial farm at Pulau Ketam, Malaysia (Sutra et al., 2025). Despite that, in hybrid grouper, the microbiota profile and its response to immunological stimuli from the oral formalin-killed *V. harveyi* vaccine remains poorly understood.

Therefore, this study was designed to address that knowledge gap. Moreover, this study focused also on the field trial for evaluating the effectiveness of the vaccine-incorporated feed under real-world conditions that replicate the complex conditions encountered in commercial aquaculture settings, where fish are exposed to multiple stressors and pathogens simultaneously (Razali et al., 2023; Lan et al., 2025). Using high throughput 16S rRNA amplicon sequencing on hindgut sample, we aimed to characterize the intestinal microbiota of vaccinated hybrid grouper from a farm in Tanjung Pinang, Indonesia, focusing on bacterial taxa associated with immune modulation and pathogen suppression. In addition, the analyses of predicted functional genes and pathways in the hindgut microbiota were conducted using MetaCyc and KEGG databases for investigating potential modulation of microbial metabolism pathways and control of antimicrobial resistance genes (ARGs). Rather than focusing solely on vaccine efficacy in terms of survival or antibody titers, this investigation sought to understand how microbial communities respond to immunization and whether such shifts could inform future strategies for disease prevention. Findings from this study may offer preliminary insights into the interplay between vaccination and gut ecology in farmed marine fish.

Table 1
The sequencing depth and coverage analysis of the treatment groups.

	Sequencing depth (reads)	Good's coverage (%)
Vaccinated group	99956.33 ± 7207.92	100 ± 0
Control group	83425 ± 15077.69	100 ± 0

2. Material and methods

2.1. Confirmation of V. harveyi strain VH1

The *V. harveyi* strain VH1 was cultured and inactivated using formalin following the protocol described by Mohamad et al. (2021). Genomic DNA was extracted using a commercial purification kit (Promega, Wisconsin, USA), with procedures carried out as per the manufacturer's guidelines. Extracted DNA was stored at −20 °C until further use. PCR amplification was performed using REDiant II PCR Master Mix (Apical Scientific, Seri Kembangan, Malaysia), using primers targeting both a specific virulence gene (serine endoprotease, *vhs*) and the universal 16S rRNA gene (Table 1). Thermocycling conditions included 35 cycles of denaturation at 94 °C for 3 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, performed using an Eppendorf Mastercycler Nexus Thermal Cycler (Eppendorf, Hamburg, Germany). Amplified PCR products were visualized and subjected to Sanger sequencing (Apical Scientific, Seri Kembangan, Malaysia). Confirmation of *V. harveyi* strain VH1 identity was based on positive amplification of the *vhs* gene.

2.2. Preparation of feed-based vaccine

The formalin-inactivated cells of *V. harveyi* strain VH1 (10^8 CFU/mL) were incorporated into feed pellets, combined with 10% palm oil adjuvant and commercial marine fish feed (Star Feed, Klang, Malaysia), following Mohamad et al. (2022). All feed preparation was conducted at the Institute of Biosciences, Universiti Putra Malaysia, Selangor. Pellets were standardized to dimensions of 1 cm × 0.5 cm, packed in sterile transparent bags, and stored at room temperature prior to transport to Tanjung Pinang farm, for the field trial.

2.3. Experimental field trial

A field-scale trial was conducted at a private commercial farm in Tanjung Pinang, Riau Islands, Indonesia, using floating cages (10 m × 10 m frame, 3 m net depth). A total of 3000 hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂), averaging 39.7 ± 4.47 g, were sourced from a local hatchery in Bali and distributed into six cages (500 fish/cage). During the 14-day acclimatization period, fish were fed *ad libitum* with locally sourced trash fish. Prior to the start of the study, external appearance of all fish and the intestines of 15 fish were also randomly sampled to ensure no clinical signs of vibriosis. Three cages were randomly assigned to the vaccinated group, and the remaining three served as controls. The vaccinated fish were fed with the feed-based vaccine for five consecutive days at 4% bodyweight during week 0 (day 0 – 5), week 2 (day 14 – 19), and week 6 (day 42 – 47), corresponding to initial vaccination and two boosters. On non-vaccination days, vaccinated fish were fed trash fish, similar to control fish. The feed-based vaccine was finished for the vaccinated group's fish feeding within 5 days upon vaccine-incorporated feed was produced and the new batch of feed-based vaccine was transported to farm within 3 days before the consequent booster vaccination. On week 10 (day 70), 24 live fish were randomly sampled from each group, anesthetized with 50 mg/L MS-222 (Tricaine methanesulfonate; Syndel, Ferndale, USA), and dissected for hindgut collection. Samples were preserved in RNAlater (ThermoFisher Scientific, Waltham, USA) and transported on dry ice to Universiti Putra Malaysia for DNA extraction.

2.4. DNA extraction of fish hindgut

The 12 sampled fish hindguts from the 24 samples of each group were selected randomly as 3 pooled replicates (4 sampled fish hindguts/replicate) for each group. Prior to DNA extraction, the fish hindguts were retrieved from RNAlater solution according to the manufacturer’s manual. The fish hindguts were ground into a fine powder using liquid nitrogen, allowing for efficient DNA extraction. Genomic DNA was extracted from the powdered samples using the PrimeWay Genomic II DNA Extraction Kit (1st BASE, Central Region, Singapore) following the manufacturer's instructions. The quality of the purified DNA was initially assessed by electrophoresis on a 1.7% TAE agarose gel. The DNA concentration was also determined through spectrophotometry using the NanoPhotometer® N60/N50 (Implen, Schatzbogen, Germany) and fluorometric quantification, employing the iQuant™ Broad Range dsDNA Quantification Kit (GeneCopoeia, Rockville, USA). The extracted DNA was subjected to PCR amplification and high throughput 16S rRNA amplicon sequencing.

2.5. High throughput amplicon sequencing

2.5.1. Amplicon library preparation and 16S ribosomal RNA amplicon sequencing

The amplicon library preparation of the samples was conducted according to the “16S-metagenomic sequencing library preparation guide” (Illumina, San Diego, USA). The gene-specific primers' sequences of 16S rRNA fraction containing the V3-V4 region used in this protocol were taken from Klindworth et al. (2012). The gene-specific sequence primer pair included Forward Primer (5′-CCTACGGGNGGCWGCAG-3′) and Reverse Primer (5′-GACTACHVGGGTATCTAATCC-3′). Illumina overhang adapter nucleotide sequences were added to the gene-specific primer sequences respectively. The overhang adapter pair were Forward overhang adapter (5′- TCGTCGGCAGCGTCAGATGTG TATAAGAGACAG-3′) and Reverse overhang adapter (5′-GTCT CGTGGGCTCGGAGATGTGTATAAGAGACAG-3′). The DNA extracted from the shrimp samples underwent the first amplification step. These steps were carried out in 50 μL reactions, employing KOD -Multi & Epi-® (Toyobo, Osaka, Japan). The resulting amplicons were subjected to a cleanup procedure using magnetic AMPure XP beads (Beckman Coulter, Brea, USA) to remove free primers and primer dimer species following the manufacturer's specifications. Subsequently, a second amplification step was performed to introduce dual indices and sequencing adapters, employing the Nextera XT Index Kit v2 (Illumina, San Diego, USA) following the manufacturer's protocols. A second round of cleanup using magnetic AMPure XP beads (Beckman Coulter, Brea, USA) was conducted for library purification. To assess the quality of the libraries, measurements were conducted using the Agilent Bioanalyzer 2100 System with the Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, USA). Additionally, quantification of the libraries was performed fluorometrically using the Helixyte Green™ Quantifying Reagent (AAT Bioquest, Pleasanton, USA). For 16S ribosomal RNA amplicon sequencing, each library was adjusted to a concentration of 4 nM, utilizing 10 mM Tris (pH 8.5) as the diluent. Then, the prepared libraries were loaded with MiSeq Reagent Kit v3 (Illumina, San Diego, USA) into a MiSeq platform (Illumina, San Diego, USA) and the sequencing results were obtained after 300 cycles. The sequences were deposited with Bioproject number: PRJNA1336979 in the NCBI database.

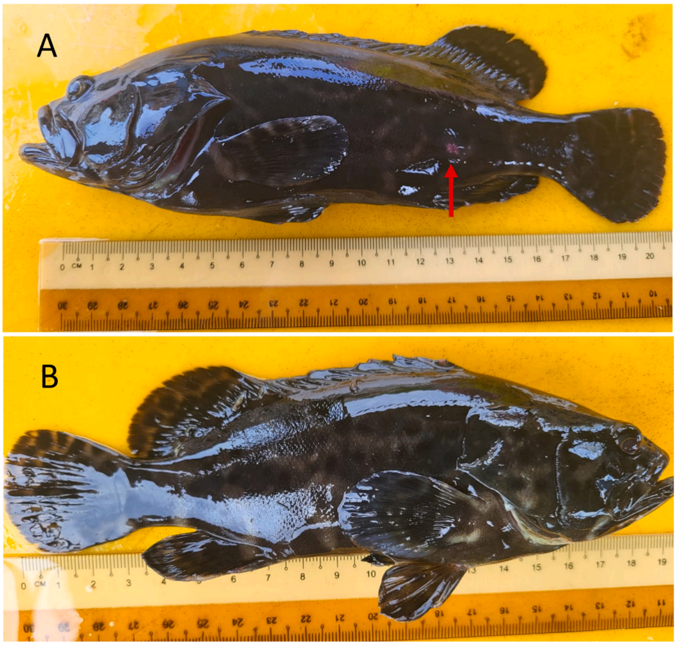

Fig. 1. Clinical symptos of live cultured hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂) on day 70 of field trial. Fig. 1A is representative fish from non-vaccinated group while Fig. 1B is representative fish from vaccinated group. Black arrow indicates patch of ulcer was observed on the non-vaccinated representative fish.

2.5.2. Microbial taxonomy, diversity, and functional prediction analysis

Raw reads were quality-checked using FastQC (Andrews, 2010), and primers/adapters were trimmed with Cutadapt 3.5 (Martin, 2011). Paired-end reads were merged and processed using DADA2 V1.18 (Callahan et al., 2016), with chimera removal and taxonomy assignment based on SILVA database v138.1 (Quast et al., 2013). Amplicon sequence variants (ASVs) and taxonomic tables were exported for downstream analysis. The 16S rRNA sequence of *V. harveyi* VH1 (from Sanger sequencing) was aligned with ASV-assigned sequences using ClustalW in MEGA 11.0. Conserved regions were retained after trimming, and a phylogenetic tree was constructed using the Maximum Likelihood method with Kimura-2 model and 1000 bootstrap replicates. Venn diagrams of ASVs were generated using R software (v3.6.1) with ggVennDiagram (Gao et al., 2021). MicrobiomeAnalyst 2.0 was used for statistical and functional profiling, including taxonomic bar plots, dendrograms, alpha and beta diversity, and network correlation analysis (Lü et al., 2023). The Rarefaction curve analysis was conducted for determining the species richness and sequencing depth. Then, the Good’s coverage with T-test was calculated. Alpha diversity metrics (ACE, Chao1, Shannon, Simpson) and beta diversity (PERMANOVA, PCoA using Bray-Curtis index) were calculated. LEfSe was used for differential abundance analysis, and Pearson correlation assessed microbial network relationships (Weiss et al., 2016). Functional prediction was performed using PICRUSt2, referencing MetaCyc for metabolic pathways and KEGG for antimicrobial resistance genes (Douglas et al., 2020). The differential abundance of the predicted metabolic pathway was analyzed using the LEfSe method. The fold change of predicted *Vibrio*-related pathogenic mechanism and predicted antimicrobial resistance gene was analyzed using the DEseq2 method.

2.6. Statistical analysis

The calculated values were considered significantly different if $p < 0.05$ for all statistical tests that were used in this study.

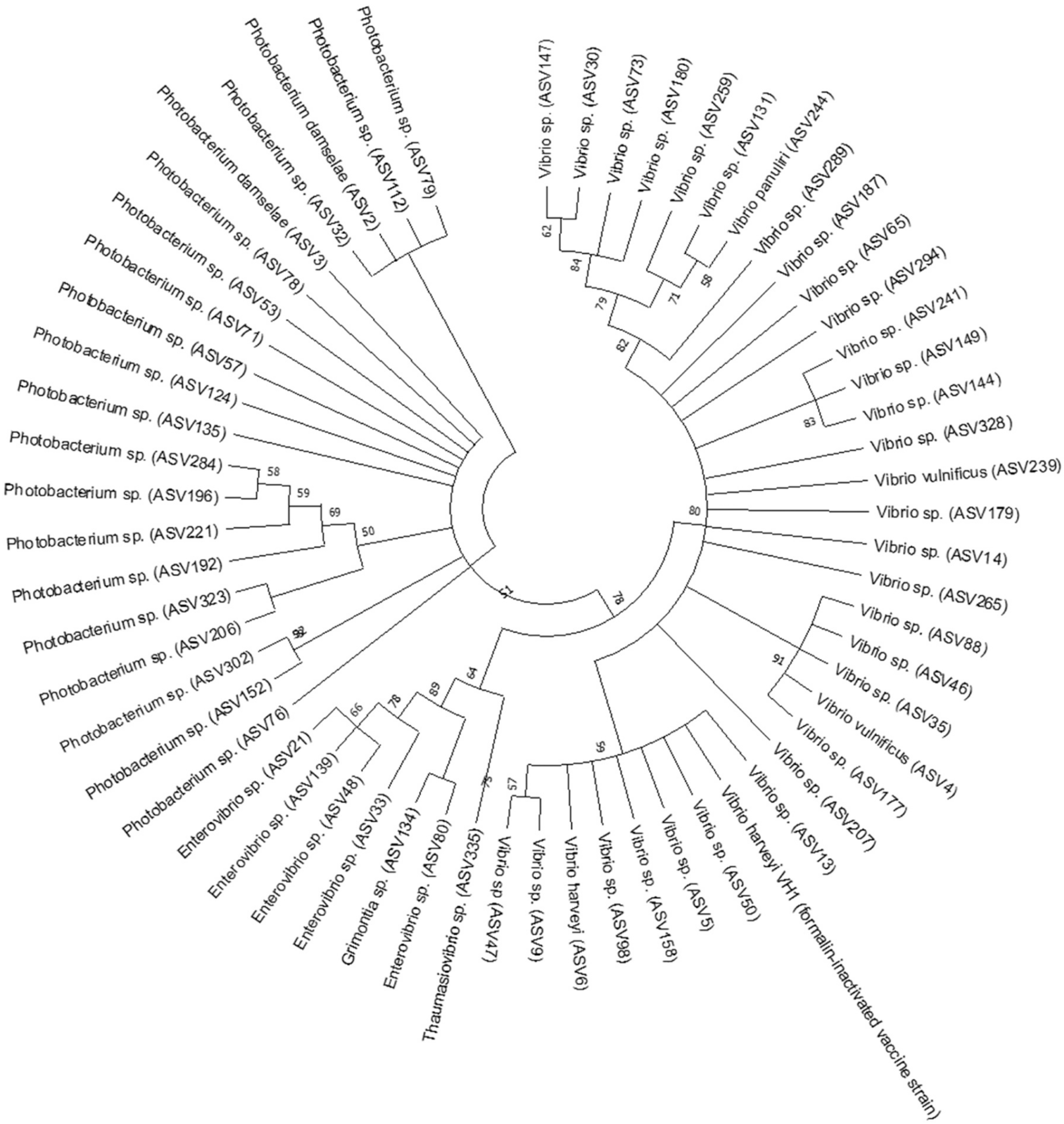

Fig. 2. Maximum Likelihood phylogenetic tree of Vibrionaceae in hindgut microbiota community of vaccinated group and control group of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). The aligned sequence of *V. harveyi* VH1 was the inactivated strain used in the feed-based vaccine.

3. Results

3.1. Clinical observation

By day 70 of the field trial (week 10), there was no statistically significant difference in body weight between the vaccinated group (169.4 ± 21.62 g) and the unvaccinated controls (179 ± 31.13 g) ($p < 0.05$). However, several fish from the control group exhibited ulcerative lesions on their skin (Fig. 1A), whereas none of the vaccinated individuals showed visible signs of clinical abnormality. The absence of external pathology in the vaccinated fish was consistent across all replicates.

3.2. Phylogenetic analysis of vibrionaceae

Out of the 342 total ASVs identified across both groups, 60 were classified under the family Vibrionaceae. Phylogenetic reconstruction using the Maximum Likelihood method revealed that these ASVs clustered into five distinct genera. The genus *Photobacterium* accounted for 33.33% of Vibrionaceae ASVs, comprising *Photobacterium* spp. (n = 18) and *P. damselae* (n = 2). The genus *Enterovibrio* represented 8.33% (n = 5), while *Grimontias* and *Thaumasiovibrio* each contributed a single ASV (1.67%). The genus *Vibrio* was the most dominant, encompassing 55% of Vibrionaceae ASVs, including *Vibrio* spp. (n = 29), *V. harveyi* (n = 1), *V. vulnificus* (n = 2), and *V. panuliri* (n = 1) (Fig. 2). Interestingly, the aligned sequence of *V. harveyi* strain VH1 clustered within the same clade as ASV6, which was annotated as *V. harveyi*. This suggests a high likelihood that the *V. harveyi* strain VH1, originally incorporated into the feed-based vaccine, was detectable within the hindgut microbiota of vaccinated fish.

3.3. Rarefaction curve analysis of reads

The sequencing depth, ranging from 90758 reads to 108360 reads in vaccinated fish samples, whereas 62385 reads to 96944 reads in control samples (Fig. 3). In this circumference, the sequencing depth of vaccinated group was not significantly different ($p > 0.05$) with control

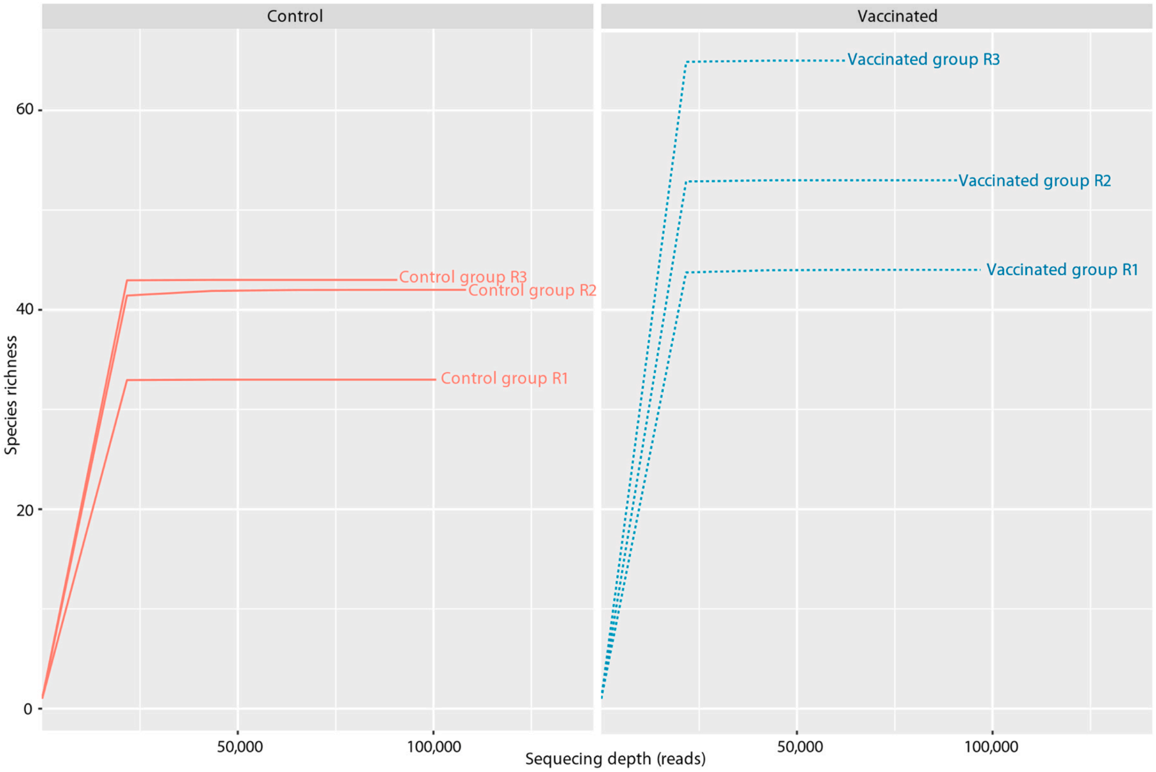

Fig. 3. Rarefaction curve analysis of reads from the hindgut samples of microbiota community in hindgut of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂).

group (Table 1). All treatment group samples demonstrated 100% coverage and not significantly difference ($p > 0.05$) between the vaccinated group with control group (Table 1), which indicates that the samples are well-representative of the population's diversity as the coverages were more than 90%.

3.4. *Microbiota community composition of hindgut*

3.4.1. *Alpha diversity*

Comparative analysis of alpha diversity revealed a significant difference in the Simpson index ($p < 0.05$) between vaccinated and control groups, indicating altered evenness in microbial distribution. However, other indices, such as ACE, Chao1, and Shannon, did not show significant variation ($p > 0.05$), suggesting that overall richness remained relatively stable (Fig. 4). This pattern was further supported by Venn diagram analysis, which showed a Jaccard similarity index of 16.08%, with 55 shared ASVs between the 146 ASVs in the vaccinated group and 141 ASVs in the control group (Fig. 5). While the overlap was limited, it reflected a degree of compositional divergence without dramatic shifts in richness.

3.4.2. *Beta diversity*

PERMANOVA and ANOSIM analysis did not detect significant differences ($p > 0.05$) between groups despite significant level of ANOSIM analysis was $p < 0.2$ in beta diversity (Table 2). The dendrogram clustering showed that Replicate 1 from the vaccinated group grouped alongside control replicates, and vice versa (Fig. 6B). However, ANOSIM yielded borderline significance ($0.05 < p < 0.2$), which loosely aligned with PCoA results based on Bray-Curtis dissimilarity. In those plots, the vaccinated group formed a cluster that was spatially adjacent, but not overlapping with the control group (Fig. 6A), indicating subtle shifts in community structure.

3.4.3. *Composition of microbiota community*

Taxonomic profiling at the genus level revealed considerable variability in bacterial abundance across samples. *Vibrio* was the most dominant genus in both groups, ranging from 2.69% to 81.70% in vaccinated fish and 2.49–48.24% in controls. *Photobacterium* followed, with 5.80–10.14% in vaccinated fish and a broader range of 15.04–74.17% in controls. *Cetobacterium* was also prominent, spanning 1.61–40.35% in vaccinated fish and 1.45–43.08% in controls. *Clostridium* and *Epulopiscium* were present in both groups, though their relative abundances varied (Fig. 7).

Linear discriminant analysis (LDA) identified *Cetobacterium* spp. (LDA score: 5.03) and *V. harveyi* (LDA score: 4.71) as significantly enriched in the vaccinated group ($p < 0.05$). Conversely, *P. damselae* (LDA score: 5.11) and *V. vulnificus* (LDA score: 4.92) were more abundant in controls (Fig. 8). Network correlation analysis further revealed that *V. harveyi* was negatively correlated with both *P. damselae* ($r = -0.880$) and *V. vulnificus* ($r = -0.871$), with statistical significance ($p < 0.05$). These inverse relationships may reflect competitive exclusion or niche displacement following vaccination (Fig. 9).

3.4.4. *Predictive functional metabolism pathway of microbiota community*

Analysis of predicted metabolic pathways revealed distinct profiles between the vaccinated and control groups. In the vaccinated fish, several pathways were significantly enriched ($p < 0.05$), including L-histidine degradation I (MetaCyc: HISDEG-PWY; LDA score: 2.93), pyrimidine deoxyribonucleosides salvage (PWY-7199; 2.58), lipid A biosynthesis IV (NAGLIPASYN-PWY; 2.54), and purine nucleobases degradation (P164-PWY; 2.11) (Fig. 10A). Additional pathways such as glycogen degradation II (PWY-5941; 1.59), pyrimidine biosynthesis from CTP (PWY-7210; 1.41), and *de novo* pyrimidine biosynthesis IV (PWY-7198; 1.27) were also expressed at notable levels. Minor but significant enrichments were observed for menaquinol-8 biosynthesis II (PWY-6263; 1.16) and 1,4-dihydroxy-6-naphthoate biosynthesis II (PWY-7371; 0.72) (Fig. 10A). The highest LDA score of predictive

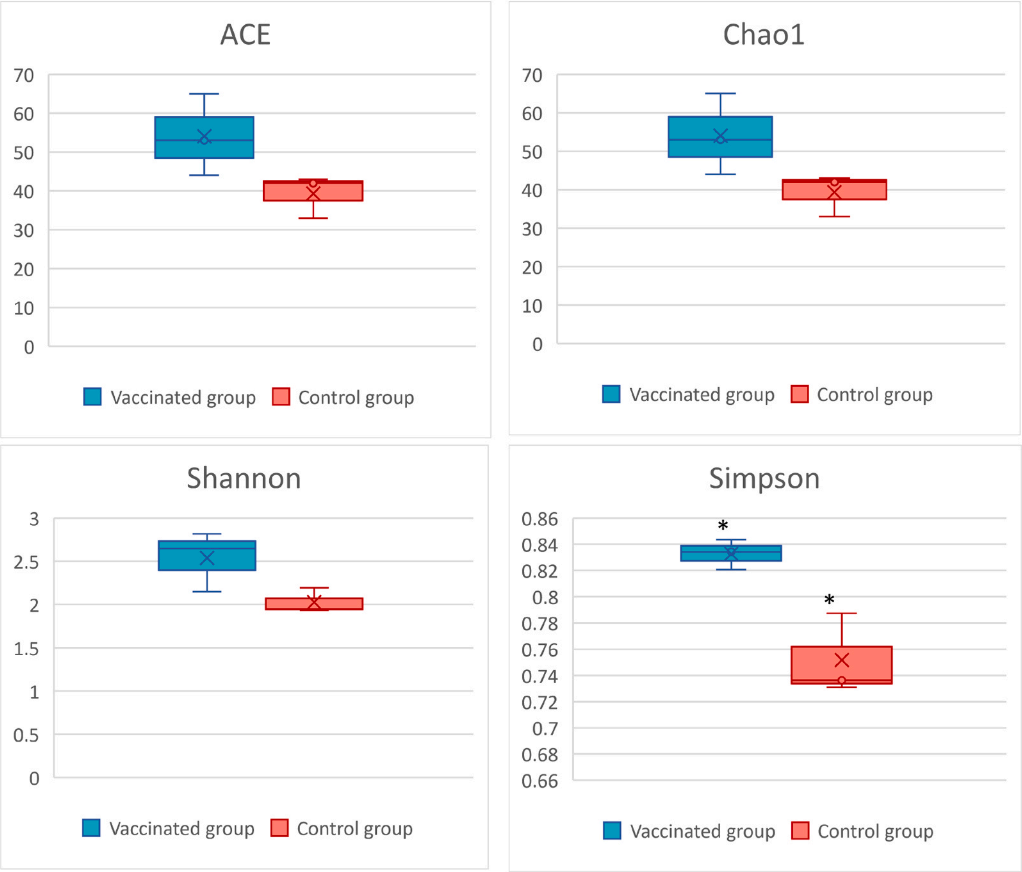

Fig. 4. Alpha diversity metrics of microbiota community in hindgut of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). The significant difference of the Alpha diversity indices was calculated with Student T-test. Figs. 4A, 4B, 4C and 4D indicates ACE, Chao1, Shannon and Simpson indices respectively. Asterisk (*) above the boxplot indicates significant difference ($p < 0.05$) between the two groups.

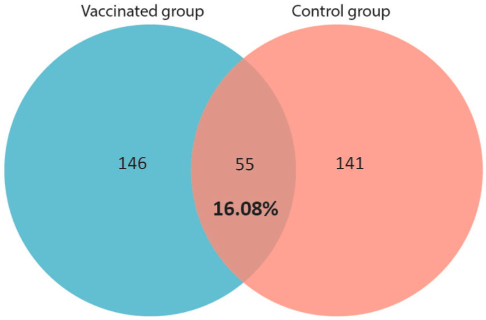

Fig. 5. Venn diagram of shared and unique ASVs of microbiota community in hindgut of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). The Jaccard similarity index percentage was shown between the vaccinated and control group.

Table 2

PERMANOVA and ANOSIM analyses with Bray-Curtis index of microbiota community in hindgut of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂).

Comparison of group		PERMANOVA		ANOSIM	
		Statistical values	Significant difference	Statistical values	Significant difference
Vaccinated group	Control group	F: 1.334 R^2: 0.25	*p*-value: 0.2 ($p > 0.05$)	R: 0.370	*p*-value: $x < 0.2$

pathway of L-histidine degradation I (MetaCyc: HISDEG-PWY) in vaccinated group was shown in Fig. 10B.

Conversely, the control group exhibited higher predicted relative abundance of pathways such as ectoine biosynthesis (P101-PWY; 2.93), peptidoglycan maturation (PWY0–1586; 2.84), and L-arginine degradation II (AST-PWY; 2.75) (Fig. 10A). Core energy metabolism pathways—glycolysis (GLYCOLYSIS; 2.41) and gluconeogenesis I (GLUCONEO-PWY; 2.31)—were also significantly enriched in the control group (Fig. 10A). The highest LDA score of predictive pathway of ectoine biosynthesis (MetaCyc: P101-PWY) in control group was shown in Fig. 10C. These differences indicate a shift in microbial functional capacity following vaccination.

3.4.5. *Predictive Vibrio-related pathogenic mechanism of microbiota community*

Comparative analysis of predicted virulence-associated genes revealed notable shifts in quorum sensing and biofilm formation pathways (Fig. 11). In the vaccinated group, the alkanal monooxygenase alpha (LuxA) and beta chains (LuxB) were enriched functional potential approximately nine-fold ($p < 0.05$), while the LuxR family transcriptional regulator was depleted functional potential by a similar magnitude ($p < 0.05$), indicating a possible disruption in canonical quorum sensing signaling (Fig. 11A). For biofilm-related mechanisms, the accessory colonization factor (AcfC) was significantly enriched functional potential (~9-fold), whereas the outer membrane protein (OmpT) was depleted functional potential (~3-fold) in the vaccinated group (Fig. 11B). These patterns may reflect altered colonization dynamics or competitive interactions within the hindgut microbiota. Other virulence-related genes did not show significant differences between

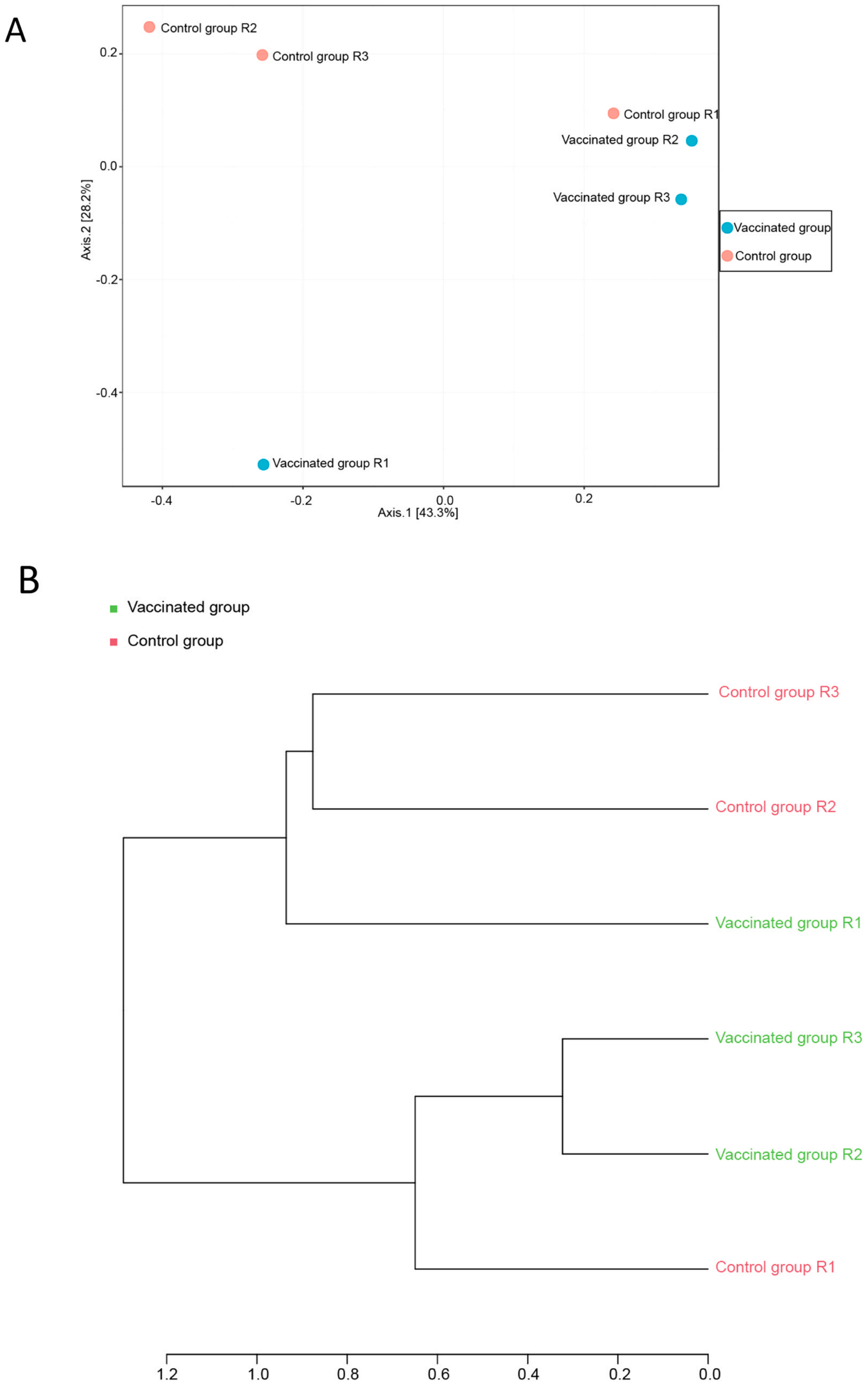

Fig. 6. Beta diversity of microbiota community in hindgut of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). Fig. 6A shows Principal coordination analysis (PCoA) with Bray-Curtis Index. Fig. 6B shows dendrogram (hierarchical tree) analysis with Jaccard Index.

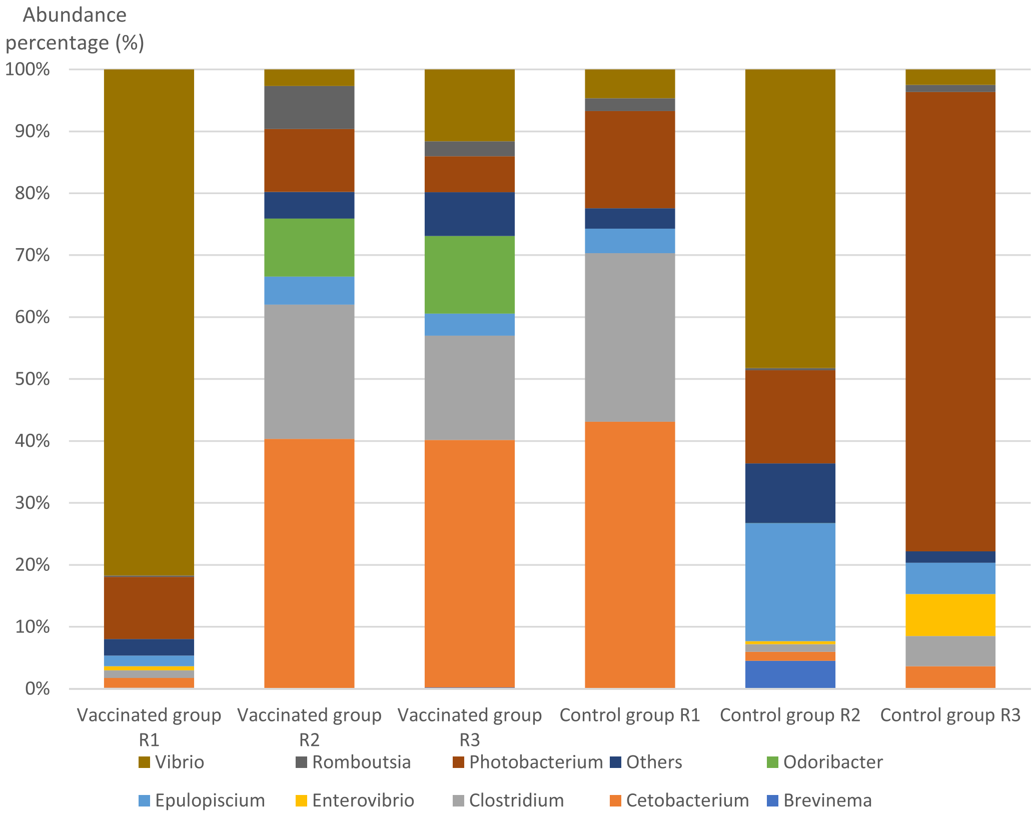

Fig. 7. Relative abundances of bacterial genera in hindgut microbiota community composition of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂).

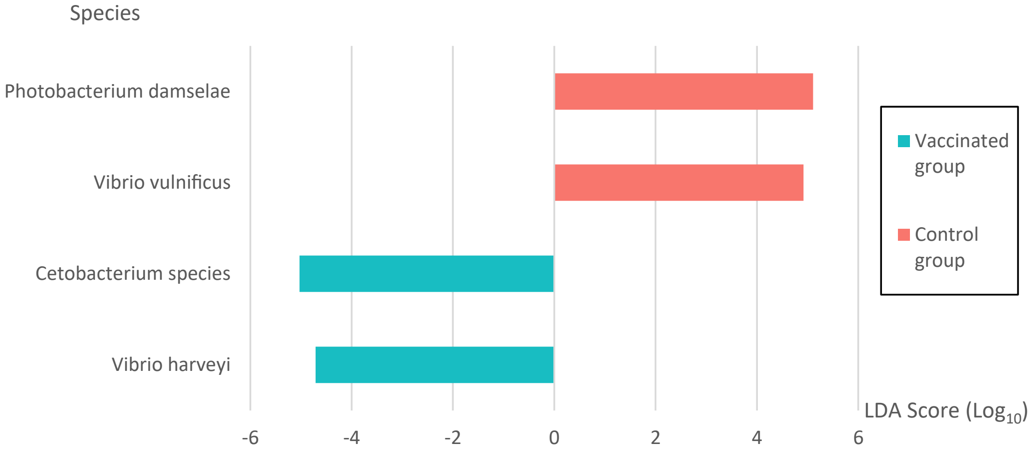

Fig. 8. Linear discriminate analysis of bacterial species in hindgut microbiota community composition of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). The LDA score shown in the figure was significantly different ($p < 0.05$) between the two groups.

groups ($p > 0.05$), indicating that only a subset of *Vibrio*-associated functions were modulated by vaccination.

3.4.6. Predictive antimicrobial resistance mechanism of microbiota community

In terms of antimicrobial resistance (AMR), the vaccinated group exhibited a significant enriched functional potential (~4-fold) of multidrug efflux systems, including genes such as oprM, emhC, ttgC, cusC, adeK, smeF, mtrE, cmeC, and gesC ($p < 0.05$) (Fig. 12A). However, genes associated with beta-lactam resistance, beta-lactamase class A (BlaZ) and penicillin-binding protein (MecA), were depleted functional potential by approximately eight-fold ($p < 0.05$) in the vaccinated fish (Fig. 12A). Additionally, components of vancomycin resistance pathways showed reduced predicted relative abundance: zinc D-Ala-D-Ala dipeptidase (VanX) and sensor histidine kinase (VanS) were depleted functional potential by 2.5-fold and 1.5-fold, respectively ($p < 0.05$) (Fig. 12B). Other AMR-related genes did not differ significantly between groups ($p > 0.05$), indicating that while some resistance mechanisms were suppressed post-vaccination, others remained unaffected (Fig. 12B).

4. Discussion

In the present study, no clinical signs of vibriosis were observed in the vaccinated group, which aligns with earlier findings that vaccines derived from *V. harveyi* can reduce external symptoms such as ulcerative

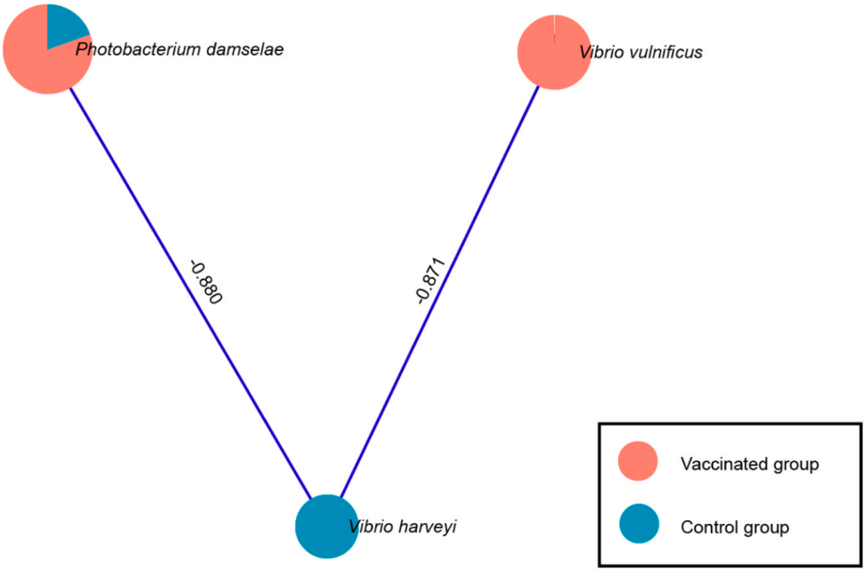

Fig. 9. Network correlation analysis with Spearman rank correlation coefficient algorithm (p < 0.05) for hindgut microbiota community composition of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). The blue colour line indicates negative correlation.

lesions and hemorrhages in injured marine fish (Chin et al., 2019). While the feed-based vaccine did not enhance growth performance in hybrid grouper, it also did not induce any adverse effects, a result that contrasts with previous evaluations in vaccinated *Lates calcarifer*, where growth improvements were reported (Amir-Danial et al., 2022). We postulate that the absence of growth enhancement in this study may be due to the lack of a serious vibriosis outbreak during the field trial period, which would otherwise trigger energy reallocation toward immune defense. Without such a challenge, the control group may have maintained normal growth trajectories (Franke et al., 2024). The species-level assignment from metagenomic analysis suggested the possible presence of DNA fragments contained in the feed-based vaccine, particularly the detection of *V. harveyi* ASV6, which clustered with the *V. harveyi* VH1 strain used in the vaccine. This observation is supported by previous studies showing highly accurate alignment between ASVs and 16S rRNA genes of *V. vulnificus* isolated from the Baltic Sea (Riedinger et al., 2024). We agree with the notion that formalin inactivation, while lethal to gram-negative bacteria, preserves cellular integrity and membrane-associated proteins, thereby retaining intracellular DNA within the bacterial cytoplasm (Taddese et al., 2021).

The feed-based vaccine have high proportion of protein and carbohydrate which similar with typical commercial feed, as well as similar ingredient and formulation of previous studies (Mohamad et al., 2022). The feed-based vaccine appeared to modulate the diversity of hindgut microbiota, particularly in terms of evenness, which is consistent with previous findings in grass carp (*Ctenopharyngodon idella*) where oral administration of a *V. mimicus* DNA vaccine altered microbial distribution (Cao et al., 2021). However, the overall microbial community structure between vaccinated and control groups showed only slight differences. This might be attributed to shared environmental conditions, fish species, or the use of trash fish as the main diet (Luan et al., 2023). Additionally, behavioral factors such as social dominance hierarchy may have influenced feed access and contributed to dispersion in microbiota profiles within the same treatment group (Gao et al., 2023). We assume that the protogynous hermaphroditism of grouper, where larger individuals undergo sex reversal from female to male, may have further impacted social dynamics and feeding behavior, thereby influencing microbial variation (Murata et al., 2021).

Among the dominant genera identified in the hindgut microbiota, *Photobacterium*, *Clostridium*, and *Cetobacterium* were consistently present across both groups. These genera are commonly found in marine piscivores and are likely influenced by the carnivorous diet provided during the trial (Huang et al., 2020). Notably, *Vibrio* and *Photobacterium* are recognized as opportunistic pathogens that exist naturally in the intestinal microbiota of fish hosts. Under unfavorable conditions, such as poor water quality, low nutritional status, or external abrasions, these bacteria may cause direct infections, co-infections, or secondary infections (Deng et al., 2020; Manchanayake et al., 2022). Despite the mild infection signs observed in the control group, the presence of *P. damselae* and *V. vulnificus* is concerning, as both are known to cause pasteurellosis and vibriosis, respectively. These diseases are associated with skin discoloration, hemorrhagic lesions, and hepatic tissue necrosis in grouper (*Epinephelus* spp.) (Hoihuan et al., 2021; Zhuo et al., 2021). Remarkably, the high abundance of *Cetobacterium* spp. in the vaccinated group may confer health benefits, as this bacterium is known to synthesize vitamin B12, which supports metabolic function and immune regulation in fish (Zhang et al., 2023). The elevated presence of *V. harveyi* in the vaccinated group further supports the hypothesis that VH1 DNA was retained and detectable in the hindgut. This aligns with previous findings showing that the VH1 strain can induce strong antigenic responses against its homologous outer membrane proteins (OMPs), and cross-react with heterologous OMPs from pathogenic *Vibrio* spp. and *P. damselae* strains (Mohamad et al., 2021).

Oral vaccination also appeared to influence predicted metabolic functions of the hindgut microbiota of the hybrid grouper. Enrichment of predictive pyrimidine metabolic pathways may contribute to the synthesis of pyrimidine molecules, which are essential for energy generation, cell homeostasis, and immune function in fish (Song et al., 2025). We postulate pyrimidine regulate neuronal function and cell metabolism which crucial for involvement of fish immune response activation (Baharum et al., 2022). The predictive L-histidine degradation might produce bioactive derivative of histidine, with important for marine fish health, including energy production, digestive physiology, immune regulation and neuroendocrine communication (Macías-Reyes et al., 2025). Moreover, the predictive L-histidine degradation pathway may be involved in L-glutamate production, which enhances digestive enzyme activity and strengthens the intestinal barrier (Ramos-Pinto et al., 2021). We assume that preserved antigen may have conferred the lipid A biosynthesis pathway, which is linked to lipopolysaccharide formation, a key antigenic component that can trigger specific antibody responses (Zhao et al., 2018). The glycogen degradation pathway suggests that hindgut microbiota may assist in breaking down glycogen into

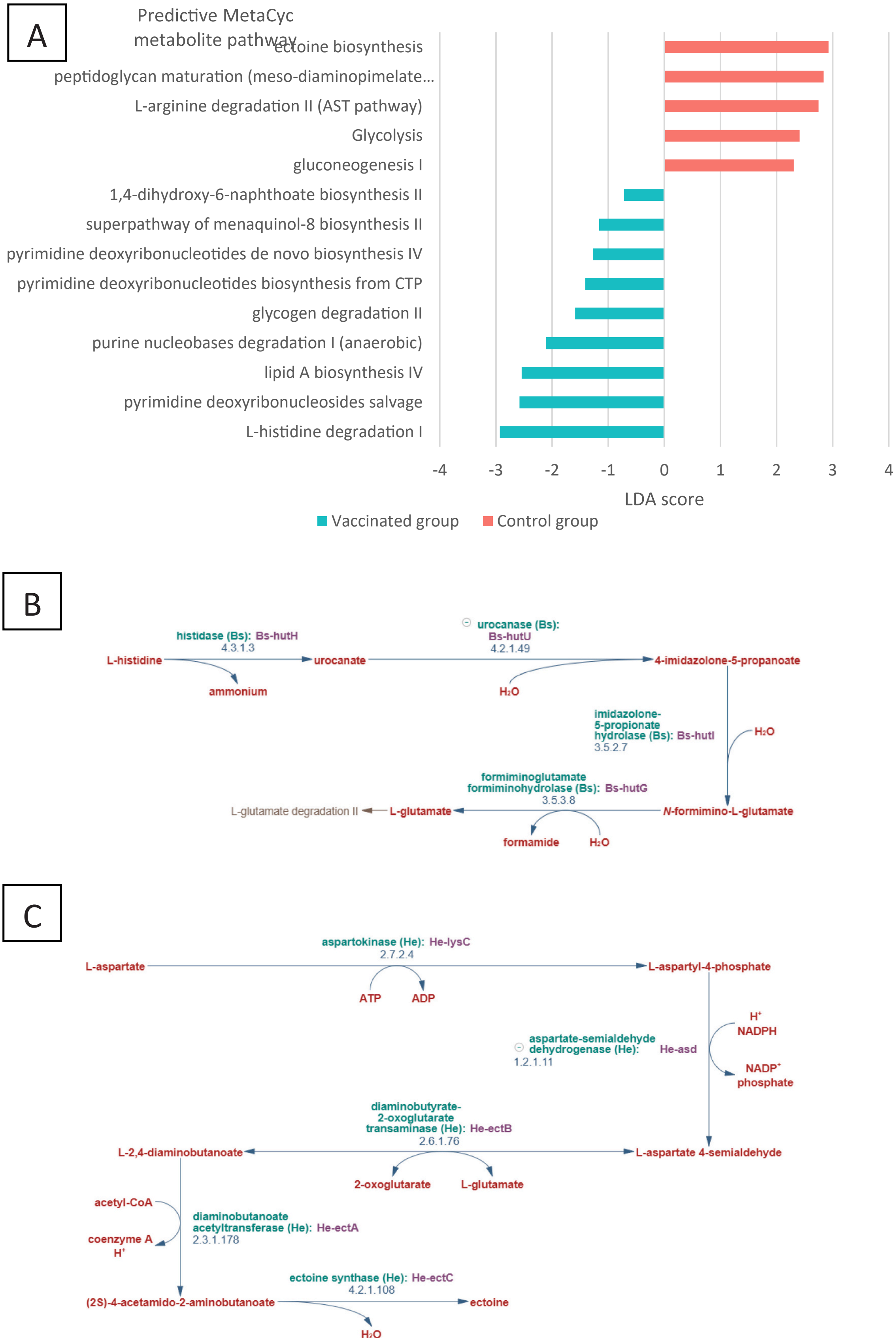

Fig. 10. Predictive MetaCyc metabolism pathway for hindgut microbiota community composition of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). Fig. 10A shows Linear discriminate analysis which the LDA score was significantly different ($p < 0.05$) between the two groups. Fig. 10B shows highest LDA score of predictive pathway, L-histidine degradation I (MetaCyc: HISDEG-PWY) in vaccinated group. Fig. 10C shows highest LDA score of predictive pathway, ectoine biosynthesis (MetaCyc: P101-PWY) in control group.

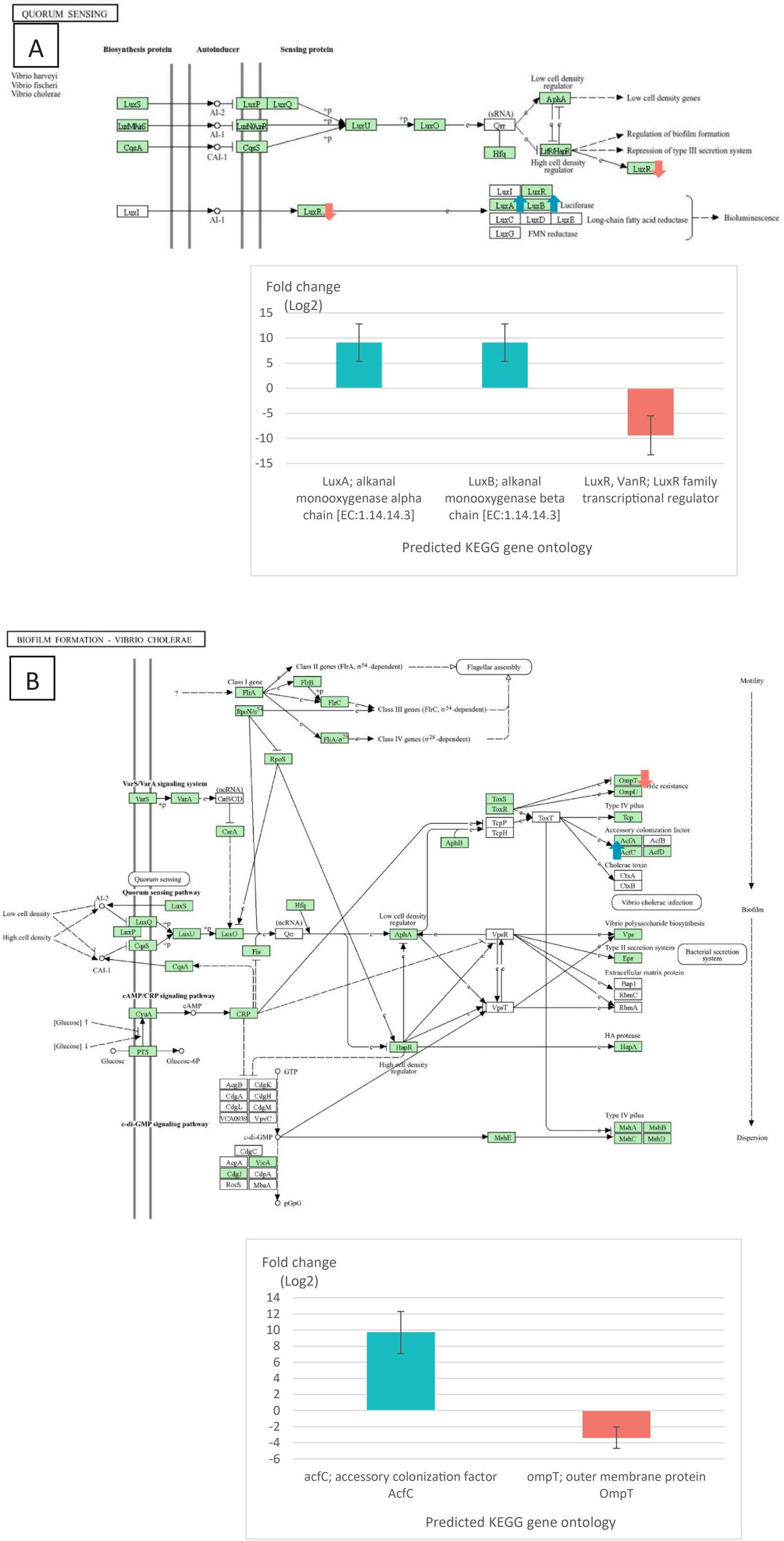

Fig. 11. KEGG Predictive Vibrio-related pathogenic genes and related mechanism of hindgut bacterial community composition of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). Fig. 11A is predictive quorum sensing mechanism while Fig. 11B is predictive *Vibrio* spp. related biofilm formation. The green colour indicates presence of predictive gene. The blue arrow and positive fold change indicates significantly ($p < 0.05$) enriched functional potential, whereas the red

arrow and negative fold change indicates significantly ($p < 0.05$) depleted functional potential for the respected gene of vaccinated group compared with control group.

simpler glucose forms, facilitating absorption and energy availability for the host (Anjaly et al., 2025). Moreover, predictive pathways related to 1,4-dihydroxy-6-naphthoate and menaquinol-8 biosynthesis indicate potential synthesis of vitamin K, a nutrient crucial for blood clotting and tissue recovery, especially in marine fish exposed to physical stress or injury (Wei et al., 2023). On the other hand, vaccination may suppress certain microbial functions that are less desirable in the hybrid grouper. For instance, depletion of ectoine biosynthesis, typically associated with adaptation to extreme salinity, may reflect a shift toward L-aspartate recovery, which could benefit host metabolism (Liu et al., 2021). Reduction in peptidoglycan maturation and L-arginine succinyltransferase (AST) pathways may indicate a decline in gram-negative bacterial populations (Adams et al., 2020; Hernández et al., 2021). Similarly, lower predicted relative abundance of glycolysis and gluconeogenesis pathways may suggest a microbiota composition more suited to carnivorous hosts (Liu et al., 2016).

Vaccination also appeared to modulate predicted virulence mechanisms within the hindgut microbiota. Depleted functional potential of the LuxR gene, a key regulator in quorum sensing, may reduce virulence predicted relative abundance, a trend previously observed in *L. calcarifer* following oral vaccine administration (Sutra et al., 2025). In contrast, LuxA and LuxB genes, which are associated with luminescence but not pathogenicity, were enriched functional potential (Kaku et al., 2021). The OmpT gene, known to produce pathogenic porins that disrupt host cell membranes, was suppressed in the vaccinated group. Meanwhile, the accessory colonization factor (AcfC), which facilitates mucus adhesion during biofilm formation, was elevated. We assume that AcfC may possess immunogenic properties, potentially serving as an antigen capable of stimulating adaptive immune responses (Cai et al., 2018; Liu et al., 2024). The increase in multidrug efflux system genes, including oprM, emhC, ttgC, cusC, adeK, smeF, mtrE, cmeC, or gesC, may reflect enhanced microbial diversity, as these transporters help bacteria expel endogenous and exogenous compounds (Huang et al., 2022). Interestingly, the feed-based vaccine appeared to reduce predicted relative abundance of genes involved in beta-lactam and vancomycin resistance, including antibiotic-binding proteins and degrading enzymes. This suggests a potential role in limiting colonization by antimicrobial-resistant pathogens, which is a promising direction for disease prevention in aquaculture (Stogios and Savchenko, 2020; Martin et al., 2022; Tadesse et al., 2023).

In addition, some limitations of this study were discussed. Establishing baseline information on farmed fish's microbial composition is important to examine microbiome variations systematically for aquaculture management (Jose et al., 2025). However, this study was similar with some previous researches that included gut microbiota data didn't apply first day or week as baseline data, in the gut microbiota studies of tilapia (*Oreochromis* spp.) (Shi et al., 2020; Wu et al., 2022), grouper (*Epinephelus* spp.) (Hou et al., 2025) and large yellow croaker (*Larimichthys crocea*) (Liu et al., 2024; Huang et al., 2023) upon oral vaccination and immune-stimulation treatments. In the further study, the baseline data was recommended to overcome this limitation and validate research directionality. The fish gut colonization with microorganisms from the diet and environmental factors to gain insights into the effects on overall gut microbiome structure and fish host cellular responses (Talwar et al., 2018). However, the fish water environmental microbiome, including microbiota water body and habitat are only slightly affect the fish foregut microbiota and not significantly affect fish hindgut, as the microbial colonization of the microbial communities might be driven by the potential functions of different gut parts (Sun et al., 2022). Therefore, the water environment microbiome was not analysed in this study. Since the 10% palm oil as adjuvant and inactivated *V. harveyi* as vaccine were incorporated with the fish feed as vaccine vector, we assume that the high abundance of *V. harveyi* and its family Vibrionaceae in the feed as high DNA concentration of *V. harveyi* as genetic material, which detectable through PCR and high-throughput sequencing analysis (Hayat et al., 2021). The lipid and other nutrients of the feed-based vaccine were not degraded instantly by natural microbiota proliferation that utilized feed nutrient sources in feed under dry condition before fish feeding (Filipe et al., 2023). Therefore, the feed microbiome was not analysed. The microbiota of feed and environment can be analysed to address possible diet confounding and environmental noise respectively in the further study.

5. Conclusion

This study demonstrates that feed-based vaccine can modulate the diversity of hindgut microbiota in farmed hybrid grouper raised in Indonesia, even under conditions where feeding behavior may be influenced by social hierarchy among fish. The detection of inactivated *V. harveyi* DNA within the intestinal microbial community suggests that the vaccine was successfully delivered and retained through the gastrointestinal tract, supporting its stability and uptake via feed-based administration. Furthermore, the feed-based vaccine appeared to reduce the abundance of opportunistic pathogens such as *V. vulnificus* and *P. damselae*, while promoting beneficial taxa like *Cetobacterium* spp., which are known to contribute positively to fish health. These microbial shifts were accompanied by changes in predicted metabolic functions, including pathways linked to nutrient synthesis, immune modulation, and pathogen suppression. Notably, the feed-based vaccine also influenced the predicted relative abundance of genes associated with virulence and antimicrobial resistance, suggesting a broader ecological impact on the gut microbiota. Taken together, these findings support feed-based vaccination as a promising candidate for vaccination strategies aimed at controlling vibriosis in mariculture. Its potential to reduce AMR-related risks and mitigate production losses offers relevance for aquaculture systems, especially in low- and middle-income countries, where access to cold-chain infrastructure and injectable vaccines remains limited.

CRediT authorship contribution statement

Alim Isnansetyo: Validation, Resources, Project administration. **Indah Istiqomah:** Validation, Resources, Project administration. **Abdulwakil Olawale Saba:** Writing – review & editing. **Dwi Sulistiyono:** Formal analysis, Data curation. **Mohammad Noor Azmai Amal:** Supervision, Conceptualization. **Yong Kit Chin:** Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Annas Salleh:** Supervision, Investigation, Data curation. **Md Yasin Ina-Salwany:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Mohd Zamri-Saad:** Investigation. **Aslah Mohamad:** Project administration, Investigation. **Tharangani K Herath:** Writing – review & editing. **Zahaludin Amir-Danial:** Validation, Data curation. **Norhariani Mohd Nor:** Validation, Software, Conceptualization. **Mohamad Azzam-Sayuti:** Writing – review & editing, Validation, Software, Investigation, Funding acquisition, Conceptualization. **Syazana-Faridon Batrisyia:** Investigation, Formal analysis. **Muhammad Farhan Nazarudin:** Resources, Project administration, Investigation, Conceptualization.

Ethical statement

Vaccination experiments conducted on the hybrid grouper followed the guidelines set forth by the Research Ethics Committee of Universitas Gadjah Mada, Yogyakarta (no: 00080/II/UN1/LPPT/EC/2025), which

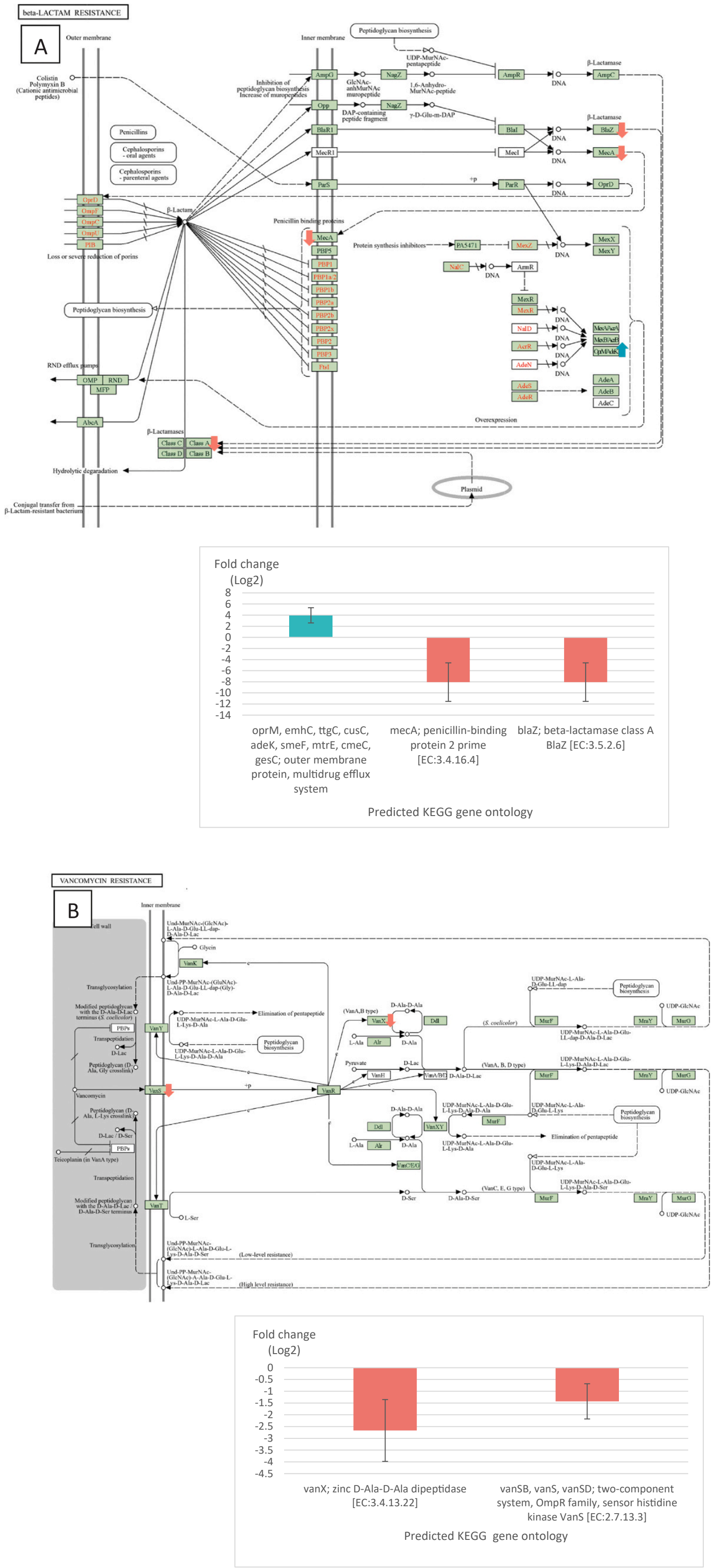

Fig. 12. KEGG Predictive antibiotic resistance genes with related mechanisms of hindgut microbiota community composition of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂). Fig. 12A is beta-lactam resistance mechanism while Fig. 12B is vancomycin resistance mechanism. The green colour indicates presence of predictive gene. The blue arrow and positive fold change indicates significantly ($p < 0.05$) enriched functional potential, whereas the red arrow and negative fold change indicates significantly ($p < 0.05$) depleted functional potential for the respected gene of vaccinated group compared with control group.

is responsible for the control and supervision of animal experiments, including the care and handling of experimental animals.

Declaration of Competing Interest

The authors declare that that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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