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ORIGINAL RESEARCH

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Investigating the effect of bacterial coinfections on juvenile and adult green-lipped mussels (*Perna canaliculus*)

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Abstract

The New Zealand's Greenshell™ mussel (*Perna canaliculus*) aquaculture industry is being affected by summer mortality events associated with increasing seawater temperatures and pathogens. In this study, challenge experiments were conducted to investigate, for the first time, the effects of pathogen coinfection on the survivability and haemolymph immune responses of juvenile and adult mussels. Animals were injected with marine broth (control), *Vibrio mediterranei*, *Photobacterium swingsii*, or a mixture of *V. mediterranei* and *P. swingsii*. Then, mussel survival was monitored for 72 h, and haemolymph was sampled for bacterial quantification and metabolomics analyses at 24- and 48-h post challenge. Coinfected adults and juveniles showed 100% mortality. Bacterial colony counts in haemolymph decreased as infection time continued, especially in juveniles. The haemolymph metabolome of mussels exposed to single bacterial species and coinfection showed response changes largely within energy metabolism. Mussels infected with *V. mediterranei* exhibited increased metabolites linked to the glutathione pathway, branched-chain amino acids, and

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others over time, supporting structural functions. Conversely, mussels infected with *P. swingsii* showed no metabolic differences over time. The coinfection group exhibited large decreases in important metabolites, such as fatty acids as an alternative energy source and amino acids to support immune functions and protein synthesis.

KEYWORDS
bacterial coinfection, green-lipped mussels, metabolomics, *Photobacterium* sp., *Vibrio* sp.

1 | INTRODUCTION

Aquaculture is currently the fastest growing food production sector in the world (Garlock et al., 2020). In New Zealand, the Greenshell™ mussel (*Perna canaliculus*) is the cornerstone of the country's aquaculture industry, with revenues estimated to be NZ$381 million in 2019 (Stenton-Dozey et al., 2021). However, over the past few years, mass mortality events have occurred at mussel farms throughout New Zealand, threatening the industry. During the summer months, increased seawater temperatures act as a stressor on mussels, impacting essential biological processes, such as metabolism, reproduction, growth, behavior, immune response, and survival (Dunphy et al., 2018). Higher temperatures also augment the prevalence of bacteria in marine environments, including *Vibrio* species (Vezzulli et al., 2013). Although the exact reason for these mortalities is unknown, the combination of a weakened host immune system and increased bacterial loading suggests that pathogens may be the cause of these mortality events (De Burgh-Day et al., 2022).

Pathogens associated with mussel summer mortality include species such as *Vibrio mediterranei* and *Photobacterium swingsii*. *V. mediterranei* is a well-researched pathogen, which has been isolated and identified as a dominant bacterium in ailing and dying giant fan mussels (*Pinna nobilis*) during mass mortality events (Prado et al., 2020). *P. swingsii* is known to be pathogenic in oysters (Fichi et al., 2015), abalone (Shi et al., 2017), and octopus (Gomez-Gil et al., 2011). *P. swingsii* and *V. meditteranei* have both been isolated and deemed as highly pathogenic strains in a previous study on Greenshell™ mussels (Azizan et al., 2022). As an opportunistic pathogen, *V. mediterranei* appears to lay dormant in its host, but it can cause disease when protective barriers are breached or when immunosuppression occurs (Prado et al., 2020). Currently, there is limited information available specifically on the dormancy and coexistence patterns of *P. swingsii*. However, within the *Photobacterium* genus, some species, for instance, *Photobacterium damselae* subsp. *piscicida*, have been found to exhibit dormancy or enter resting states under unfavorable conditions (Magarinos et al., 1994). During favorable conditions, *P. swingsii* has exhibited the expression of virulence genes (*hsp60*, *zm*, *vcpA*, *toxR*, *ompU*, *mshA*, *chi*, *lip*, and *plp*), attributing to mussel mortalities in a challenge experiment, demonstrating the pathogenic qualities of this bacterium (Azizan et al., 2022). Likewise, *V. mediterranei* has been reported to possess virulence mechanisms, including adhesion, superoxide dismutase production, and toxin production (e.g., zona occludins toxin (*zot*)) (Reshef et al., 2008). Ultimately, mussels host large quantities of opportunistic bacteria that can become problematic (related to quorum-sensing mechanisms/enhanced virulence factor production) when their host's defenses are weakened (Eggermont, 2017), as seen during marine heatwave events (Ericson et al., 2023). The mussel response, in light of the combined stressors and multiple bacteria, remains unknown and warrants further research.

Many previous studies have investigated the interactions between individual pathogens and their hosts. However, in the natural environment, it is more likely that mussels would be harbored by multiple pathogens simultaneously (Karvonen et al., 2019). Most cases of bacterial infections are caused by mixed infections, rather than one

single species. Coinfections can alter disease dynamics by affecting pathogen transmission and virulence, host immune responses, and the effectiveness of disease control measures (Carella et al., 2020). Coinfections can also result in various outcomes, such as one or both pathogens being amplified, one or both pathogens being suppressed, or one pathogen being amplified and the other suppressed (Kotob et al., 2017b). Currently, there is limited information on how bivalves respond to coinfections. Coinfections have been found to be the driver of mass mortality events in various species (Gay et al., 2004). Previous studies have found that one pathogen may promote the proliferation of another pathogen and alter the progression and severity of disease (Arzul et al., 2012), pathogenesis, and clinical outcomes, and influence the spread of infections at a population level (Lassalle et al., 2007), creating disease outbreaks as seen in corals (Ushijima et al., 2020), fish (Kotob et al., 2017b), and other bivalves (Tracy et al., 2018). Although most research studies investigating bacterial infections in mussels have been conducted on adult animals (Eggermont et al., 2014, Benabdelmouna et al., 2018, Rey-Campos et al., 2019), it is believed that juvenile mussels are likely more susceptible to pathogenic infections (Pruzzo et al., 2005). This susceptibility can be attributed to the fact that smaller mussels have a lower tolerance to pathogens, since juveniles may not have fully developed immune systems (Coen & Bishop, 2015). Therefore, it is important that pathogen–mussel studies incorporate the hosts' different sizes and life stages.

Molecular, biochemical, and physiological biomarkers that respond to stressful conditions provide detailed insights into an organism's health (Delorme et al., 2021). Metabolomics is an approach used to detect changes in relevant metabolites within an organism and can be used to describe complex biological systems at the metabolic level (Young & Alfaro, 2018) while also capturing a snapshot of the organism's physiological state at a given time (Alfaro & Young, 2018). Changes in metabolic pathways can inform how pathogen infections induce stress-related mechanisms and affect molecular regulatory processes (Li et al., 2020). To date, experiments studying *Vibrio* spp.-related infections in *P. canaliculus* using metabolomics have only focused on singular pathogenic infections (Ericson et al., 2022; Nguyen & Alfaro, 2020; Nguyen, Alfaro, Young, et al., 2018), highlighting that more research is needed to understand the responses of mussels during coinfections.

The aim of this study was to use a gas chromatography–mass spectrometry (GC–MS) based metabolomics approach to investigate physiological changes in the haemolymph of juvenile and adult *P. canaliculus* infected with *V. mediterranei*, *P. swingsii*, and a combination of both strains over a 48 h timeframe. This study is the first to explore the mechanisms of pathogen coinfection in Greenshell™ mussels and support efforts to strengthen biosecurity management in New Zealand.

2 | METHODS

2.1 | Animal husbandry and bacterial culture

Mussels ($n = 200$) were obtained from Kaiaua Marine Farms Ltd. (Firth of Thames, New Zealand) and transported to the aquaculture facility of the Auckland University of Technology (AUT). Upon arrival at the laboratory, the mussels were divided into juveniles and adults based on size (juveniles, 50–60 mm; adults, 70–80 mm). The two groups were placed into separate seawater recirculating systems (temperature of 14°C; salinity of 35 ppt; and pH of 8.2) and acclimated for two weeks prior to the start of the infection challenge.

Prior to the infection challenge experiment, isolates of *V. mediterranei* and *P. swingsii* (obtained from AUT's Aquaculture Biotechnology Research Group frozen culture library) were revived and suspended in sterile fresh marine broth to obtain a suspension with an OD600 of 1.0. The cultures were then quantified, and two single-species (*V. mediterranei* or *P. swingsii*) and a mixture (*V. mediterranei* and *P. swingsii*) solution were prepared with a concentration target of 10^7 CFU/mL to be used in the mussel challenge experiment. This bacterial concentration was selected as per previous mussel bacterial infection studies conducted in the same laboratory (Azizan et al., 2022).

2.2 | Mussel bacterial challenge and sampling

Juvenile and adult mussels were divided into four experimental groups based on treatment (Figure 1). The mussels were injected with (1) marine broth (MB), (2) *Vibrio mediterranei* (VM), (3) *Photobacterium swingsii* (PS), and (4) *Vibrio mediterranei* and *Photobacterium swingsii* (VM + PS). Additionally, the mussels from each of the four groups were divided into two cohorts: (1) monitoring cohort—used to track survival overtime (10 juveniles and 10 adults per treatment = 40 juveniles and 40 adults in total), and (2) sampling cohort—used to destructively sample mussels overtime (20 juveniles and 20 adults per treatment = 80 juveniles and 80 adults in total).

To inject mussels, they were slightly opened, and a pipette tip was placed between the valves. Then, a needle attached to a syringe was inserted into the posterior adductor muscle. Injections were performed in the following treatment sequence: MB with 100 μL of marine broth, VM with 100 μL marine broth containing 10^7 CFU/mL of *V. mediterranei*, PS with 100 μL of marine broth containing 10^7 CFU/mL of *P. swingsii*, and VM + PS with 100 μL of marine broth containing a mixture of both *V. mediterranei* and *P. swingsii* (10^7 CFU/mL). Juveniles and adults in the monitoring and sampling cohorts were injected at about the same time during their treatments. After being injected, the mussels were placed into individual 2 L tanks and aerated using air stones, and 50% of the seawater was replaced daily. The water temperature was monitored and maintained between 16 and 18°C. At 24- and 48-h post challenge (hpc), animals displaying uncontrolled gaping were assessed using the British Standard Squeeze method (Dunphy et al., 2015)—if the mussels did not adduct after 10 squeezes, they were classified as dead. Mussels were sexed during the removal of the dead animals via visual observation of the color of the gonads (white in males and orange in females). Observations on spawning occurrences and water quality parameters (nitrite, nitrate, ammonia, and pH) were recorded before daily water exchanges.

At 24 and 48 hpc, haemolymph samples were aseptically obtained from each mussel ($n = 5$ per treatment). A pipette tip was used to maintain the shell opening, and a prechilled needle (25 gauge) attached to a 3 mL syringe was inserted through the opening and into the posterior adductor muscle. Approximately 0.5–1.0 mL of haemolymph was sampled, of which 50 μL was transferred into a microcentrifuge tube for thiosulfate–citrate–bile salts–sugar (TCBS) agar plating, and 400 μL was transferred into a cryo vial and then flash frozen in liquid nitrogen for later metabolomics analyses (Ericson et al., 2022).

2.3 | Bacterial clearance measurements

Aliquots of 10 μL of undiluted haemolymph were spread in duplicate onto TCBS agar media. Agar was used for heterotrophic plate counting because this selective medium provides better support for stressed cells and produces discrete (larger) colonies overnight. Dark milky green colonies were counted as total *V. mediterranei* colonies, light green colonies were counted as total *P. swingsii* colonies, and bacterial loads were estimated as colony-forming units (CFU) formed after 24 h incubation at 22°C. Data obtained from two plates were expressed as the mean ± standard error (SE) for each time point.

2.4 | Metabolite profiling

Frozen haemolymph (400 μL), together with 10 μL of internal standard (10 mM L-alanine-2,3,3,3-d_4 concentration on column, prepared in water), were dried under vacuum for 4 h at 0°C. A two-step sequential extraction method was used for metabolite extraction by adding 500 μL of a cold methanol–water solution (50% MeOH:50% H_2O) to the dried samples, vortexed for 1 min, and centrifuged for 10 min at 208,003 *g* at −9°C. The supernatants were collected and transferred to a new tube. The sample pellets were re-extracted by adding a 500 μL cold methanol–water solution (80% MeOH:20% H_2O), followed by vortexing and centrifugation (as earlier). After combining and freezing the supernatants, the samples were dried in a SpeedVac concentrator before derivatization (Venter et al., 2021).

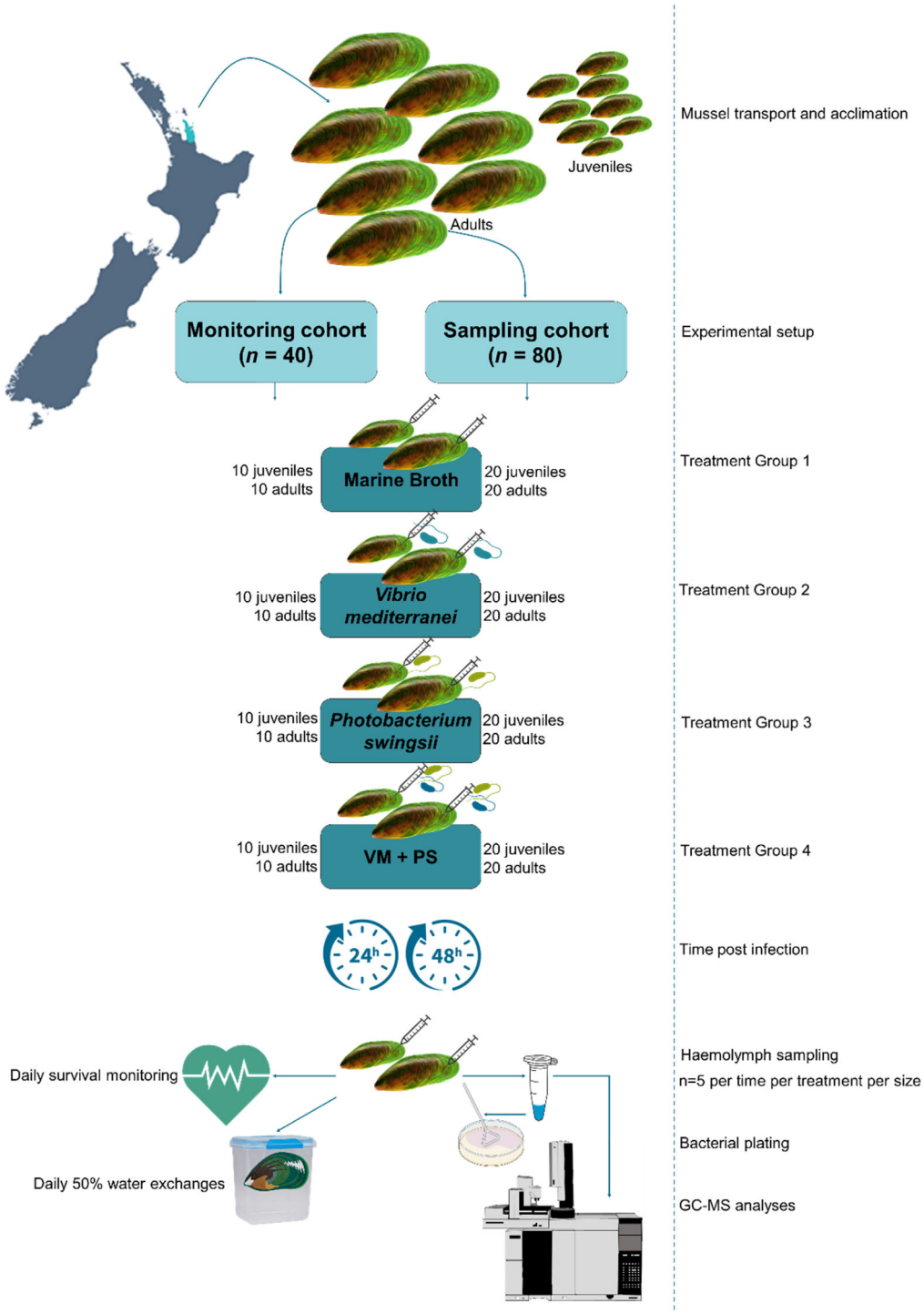

FIGURE 1 Schematic overview of the experimental and sampling design. Following mussel transportation and acclimation, juvenile and adult mussels were divided into a sampling and monitoring cohort, which were injected with either marine broth, *V. mediterranei*, *P. swingsii*, or a combination of both bacteria. Mussels were sampled at 24 and 48 hpc. Then, haemolymph was snap frozen for metabolomics analyses and used to quantify bacterial growth. Additionally, mussel survival was recorded from the monitoring cohort until 96 hpc.

Extracted metabolites were derivatized by methyl chloroformate (MCF) alkylation using an established protocol (Smart et al., 2010). Dried extracts were resuspended in 400 μL of 1M sodium hydroxide (40 g NaOH/1000 mL H_2O) and transferred to salinized borosilicate glass tubes containing 334 μL methanol and 68 μL pyridine. While keeping the samples on a vortex, a volume of 40 μL MCF reagent (Sigma-Aldrich, M35304) was added to the samples and vortexed for 30 s, followed by a second volume of 40 μL MCF reagent for 30 s. Then, 400 μL of chloroform was added and vortexed for 10 s, followed by the addition of 800 μL of 50 mM sodium bicarbonate (4 g $NaHCO_3$/1000 mL H_2O) and vortexed for a further 10 s. The mixture was centrifuged for 5 min at 11,743 *g* at 6°C. The upper aqueous layer was discarded, and approximately 30 mg of anhydrous sodium sulfate was added to remove residual H_2O. The chloroform phase containing the MCF derivatives was transferred to 2 mL amber gas chromatography glass vials fitted with inserts. Quality control (QC) samples were included in every batch by preparing a pooled mixture of the haemolymph or tissue samples. These QC samples were included within the biological sample batches and treated no differently than the samples of interest. The QC samples were injected at regular intervals throughout the analytical run of the analyzed batch to measure repeatability and identify any potential batch effects in the data. A separate standard amino acid mix (100 μL, 20 mM Sigma-Aldrich, 79248) and a sample blank containing 10 μL of the internal standard were similarly derivatized for QC purposes (Young et al., 2019).

Derivatized samples were analyzed on an Agilent 7890A gas chromatograph (GC) coupled to an Agilent MSD5975C mass spectrometer detector (Agilent Technologies, CA, USA), with an electron ionization (EI) source operated at 70 eV. The system was equipped with a DB-1701 GC capillary column (30 m × 250 μm internal diameter × 0.25 μm film thickness) (Agilent, Santa Clara, CA, USA). Helium was used as the carrier gas and was held at a constant flow of 1 mL/minute. Samples (1 μL) were injected under splitless mode with the injector temperature set at 290°C. The GC oven temperature was initially held at 45°C for 2 min and then raised with a gradient of 9°C/min to 180°C; after 5 min, the temperature was further increased from 40°C/min to 220°C. Following another 5 min, the temperature was increased from 40°C/min to 240°C and held for 11.5 min. Finally, the temperature was increased at 40°C/min until it reached 280°C, where it was held for a further 16 min. The interface temperature was set to 250°C, and the quadrupole temperature was set to 250°C. The mass spectrometer was operated in scan mode, starting after 5.6 min, with a mass range of 40–600 atomic mass unit (amu) and a scan time of 0.1 s. Identification of compounds was carried out using mass spectra acquired in scan mode from 40 to 600 amu, with a detection threshold of 80 ion counts (Smart et al., 2010). A derivatized sample blank containing the internal standard, a standard amino acid mix, and a sample of pure chloroform solvent were also injected and analyzed for QC purposes. MCF samples were derivatized and injected in four subbatches over four consecutive days. Samples were completely randomized, and QCs were injected five times at regular intervals to account for potential within-batch signal drift (Young et al., 2019).

Deconvolution of chromatographic data and metabolite identification were performed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS v. 2.66) software based on an in-house mass spectral library of MCF-derivatized commercial standards. Compound identifications were based on matches (>70%) to both the MS spectrum of the derivatized metabolite and its respective chromatographic retention times. As such, the identified compounds can be assigned a Level-1 and -2 identification, whereas unknown features are assigned a Level-3 confidence interval (Schymanski et al., 2014). MassOmics, version 2.5 (Guo et al., 2021), a Windows-based data extraction application, was used to generate a composite list of all metabolites detected in the dataset, containing metabolite identifications, mass spectral identification scores, the most abundant ion for each library match, the number of times each metabolite was detected in the whole dataset, and the retention time drift for each metabolite. A Microsoft Excel file containing peak height data for each metabolite was generated and manually checked for the presence of contaminants (e.g., MCF derivative artifacts). Data were blank corrected, and aberrant records were removed. The data matrices of peak intensities were preprocessed for QC purposes and to meet the distributional requirements before statistical analyses using the web-based tool MetaboAnalyst 5.0 (Chong et al., 2018). Data were normalized against the internal standard (Venter et al., 2021).

2.5 | Data analysis

Mussel weight, length, and number of bacterial colonies were analyzed with the two-way analysis of variance (ANOVA). The different treatment groups (marine broth control, *Vibrio mediterranei*, *Photobacterium swingsii*, and coinfection), time points (24 and 48 hpc), and mussel life stage (juvenile and adult) were selected as within-subject factors, with dependent variables of weight (g), length (mm), and number of bacterial colonies. Tukey pairwise comparisons were used for all analyses to examine the significant differences among the factor levels. When interactions were nonsignificant, these terms were removed from the model to test for single-order effects alone. Survival data were analyzed by a Kaplan–Meier survival plot. All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) with an alpha level of 0.05. For metabolomic data, univariate and multivariate analyses were conducted using MetaboAnalyst 5.0 (Chong et al., 2018). The data were generalized log (glog) transformed to alleviate the dependency of the variance on the compound concentrations and subjected to two-way analysis of variance (ANOVA) to determine the influence of animal life stage and sampling time (between subjects, $p < 0.05$), as summarized in the Venn diagrams.

3 | RESULTS

The average (± SE) mussel lengths were 57 ± 7 mm for juveniles ($n = 80$) and 72 ± 5 mm for adults ($n = 80$). There were no significant differences ($p = 0.1374$) in shell length between the different treatment groups (MB, VM, PS, and VM + PS) within juvenile or adult mussels. However, as expected, juvenile mussels were statistically shorter than adult mussels (two-way ANOVA, $p < 0.001$). There was no interactive effect between treatment groups and mussel stages on length ($p = 0.1416$). Average (± SE) mussel weights were 15 ± 6 g for juveniles and 32 ± 5 g for adults. There were no significant differences amongst the treatment groups (two-way ANOVA; $p = 0.2596$) within mussel stages. However, the weight of juvenile mussels was statistically lighter than that of adult mussels (two-way ANOVA, $p < 0.001$). There was no interactive effect between treatment groups and mussel stages on weight ($p = 0.8455$). Altogether, within the juvenile cohort, 11 males, six females, and 23 underdeveloped mussels were identified. In the adult cohort, the sex was as follows: 21 males, 7 females, and 12 undetermined.

No mortalities were observed in the juvenile mussels injected with marine broth (control animals). Juvenile mussels infected with *V. mediterranei* (VM) showed 90% survival within 24 h of injection and 60% survival by 72 hpc. Juvenile mussels injected with *P. swingsii* (PS) showed 60% survival within 24 h, with 0% survival by 72 hpc. Juvenile mussels injected with both *V. mediterranei* and *P. swingsii* died at the fastest rate in this experiment, with 0% survival at 48 hpc (Figure 2a, Log-rank test, $p < 0.001$). Adult mussels injected with marine broth showed the same response as juvenile mussels, with 100% survival throughout the experiment. Interestingly, adult mussels injected with *V. mediterranei* also showed 100% survival at 72 hpc. When injected with *P. swingsii*, adult mussels showed 50% survival after 24 h and remained at 50% for the remainder of the experiment. Injecting adult mussels with *V. mediterranei* and *P. swingsii* resulted in 40% survival after 24 h, with 0% survival obtained at 72 hpc (Figure 2b, Log-rank test, $p < 0.001$).

The number of bacterial colony-forming units (CFU) detected at 24 and 48 hpc was higher in juvenile mussels compared to adult mussels (mussel stage, $p < 0.0001$) (Figure 3). However, pair-wise comparisons indicated that juvenile mussels injected with *V. mediterranei* ($p = 0.0021$) and the coinfection group *V. mediterranei* + *P. swingsii* ($p = 0.03$) were statistically different when compared to mussels injected with marine broth at 24 hpc. Bacterial counts were also significantly higher in juvenile mussels injected with *P. swingsii* at 24 hpc compared to *V. mediterranei*. No significant differences were found in CFU counts across treatment groups at 48 hpc. For juveniles, no CFU were detected in mussels injected with marine broth at 24 and 48 hpc (Figure 3). On the first day (24 hpc), the highest number of CFU was detected in haemolymph collected from mussels injected with *V. mediterranei* (± 280 CFU) compared to all experimental groups (significant difference from the MB control group, $p = 0.0012$). On

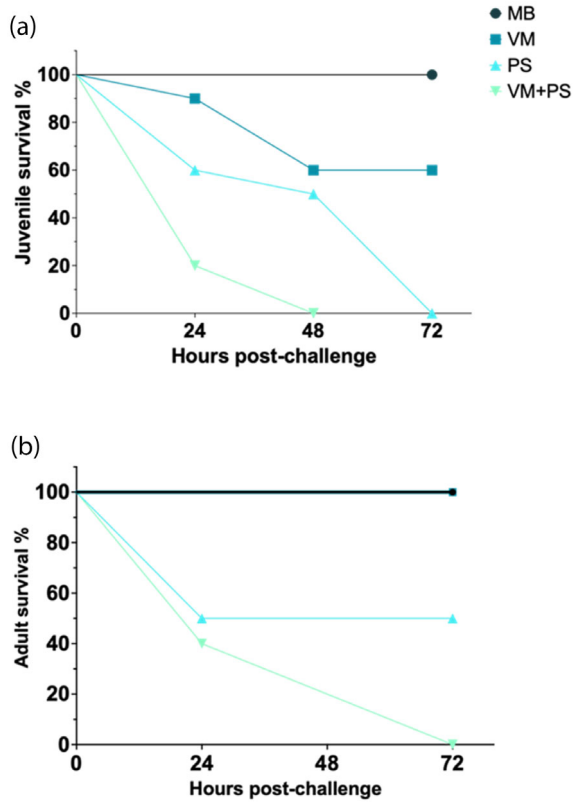

FIGURE 2 Survival plots of juvenile (a) and adult (b) mussels after a 72 h post challenge to marine broth (MB), *V. mediterranei* (VM), *P. swingsii* (PS), and coinfection of both VM and PS (VM + PS).

day two (48 hpc), 73 CFU were detected in mussel haemolymph injected with *V. mediterranei* (no significant difference, $p = 0.7007$). Injection with *P. swingsii* in mussels showed 28 CFU at 24 hpc and 9 CFU at 48 hpc. This was the lowest number of CFU in all groups where bacteria were injected (no significant difference from the MB control group at 24 and 48 hpc, $p = 0.9758$ and $p = 0.9991$). Coinfection of both VM and PS resulted in 197 CFU at 24 hpc (significant difference from the MB control group, $p = 0.0012$) and 158 CFU at 48 hpc (no significant difference from the MB control group, $p = 0.1079$). There was no interaction effect between experimental groups and time points (Treatment*Timepoint, $p = 0.6849$, two-way ANOVA measure).

For adult mussels, injection with marine broth resulted in haemolymph samples with no CFU at both 24 and 48 hpc (Figure 3). At 24 hpc, the largest number of CFU were detected in haemolymph collected from adult mussels injected with VM (± 98 CFU) compared to all experimental groups (no significant difference from the MB control group, $p = 0.3507$). At 48 hpc, 21 CFU were detected in mussel haemolymph injected with VM (no significant difference from the MB control group, $p = 0.9826$). Injection with PS in adults showed 13 CFU at 24 hpc and 5 CFU at 48 hpc. This was the lowest number of CFU in all groups where bacteria were injected (no significant difference from the MB control group at 24 and 48 hpc, $p = 0.9957$ and $p = 0.9998$). Coinfection of both VM and PS resulted in 43 CFU at 24 hpc (no significant difference from the MB control group, $p = 0.4108$) and 43 CFU at 48 hpc (no significant difference from the MB control group, $p = 0.8806$). There was no interaction effect between experimental groups and time points (Treatment*Timepoint, $p = 0.6849$, two-way ANOVA).

The metabolomic analyses resulted in a total of 71 features, which were detected within all sampling groups and sampling times, of which 48 metabolites were successfully identified. Significantly different metabolites ($p < 0.05$) as

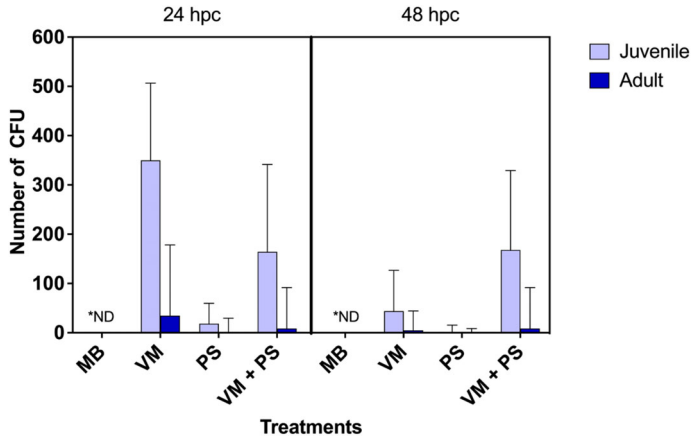

FIGURE 3 Bacterial colony counts are shown between juvenile and adult mussels across treatment groups overtime. The hatching bracket between the two bars represents a significant difference between treatment groups at the same time point. The single asterisk (*) on each bar represents a significant difference at $p < 0.05$, and double asterisks (**) indicate a difference at $p < 0.001$. Different sets of letters (a, b, and c for VM; a', b', and c' for PS; and a'', b'', and c'' for VM + PS at two time points (e.g., MB: 24 hpc vs MB: 48 hpc), respectively) indicate a significant difference between timepoints within a treatment group. *ND = not detectable.

determined by two-way ANOVA can be viewed in the Tables S1–S3. Following injection with marine broth, two-way ANOVA identified nine significantly different metabolites relating to sampling time (Figure 4a). The metabolites asparagine, cysteine, dodecanoic acid, glutamic acid, glutamine, glutathione, margaric acid, trans-vaccenic, and tyrosine were higher in mussels sampled at 48 hpc compared to the mussels sampled at 24 hpc, regardless of life stage when injected with marine broth (Figure S1). Juvenile and adult mussels injected with *V. mediterranei* showed two metabolites (benzoic acid and citric acid), which had higher concentrations in the juveniles (compared to adults) (Figure S2), whereas 21 metabolites showed significant differences due to experimental time (Figure 4b). The metabolites alanine, asparagine, aspartic acid, benzoic acid, cis-4-hydroxyproline, creatinine, cystathionine, cysteine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine displayed an increase in metabolite abundance within the 48-h sampling group (compared to the 24 hpc group) following injection with *V. mediterranei* (Figure S3). Injection with *P. swingsii* resulted in no metabolite differences between adult and juvenile mussels sampled at 24 and 48 hpc (Figure 4c). When injecting both *V. mediterranei* and *P. swingsii* into adult and juvenile mussels, 22 metabolites showed significant differences due to sampling time (Figure 4d). The metabolites arachidonic acid, asparagine, creatinine, cystathionine, docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), glutamine, gondoic acid, hexadecanoic acid, isoleucine, leucine, lysine, margaric acid, octadecanal, ornithine, pentadecanoic acid, phenylalanine, threonine, tryptophan, tyrosine, and valine were lower in the 48 hpc sampling group (compared to the 24 hpc sampling group) (Figure S4).

4 | DISCUSSION

The emergence of *Vibrio* sp. and *Photobacterium* sp. infections in bivalves depends on various factors, including the life stages of the host, the diversification of pathogens and parasites in the bacterial communities, and the weakening of the host's immune system due to prolonged exposure to stressors (Destoumieux-Garzón et al., 2020). The genus *Vibrio* has been described as a disease-causing agent in bivalve molluscs of various life stages (Beaz-Hidalgo et al., 2010). Typically, larvae are more susceptible to vibriosis than adults since the resistance to bacterial infection

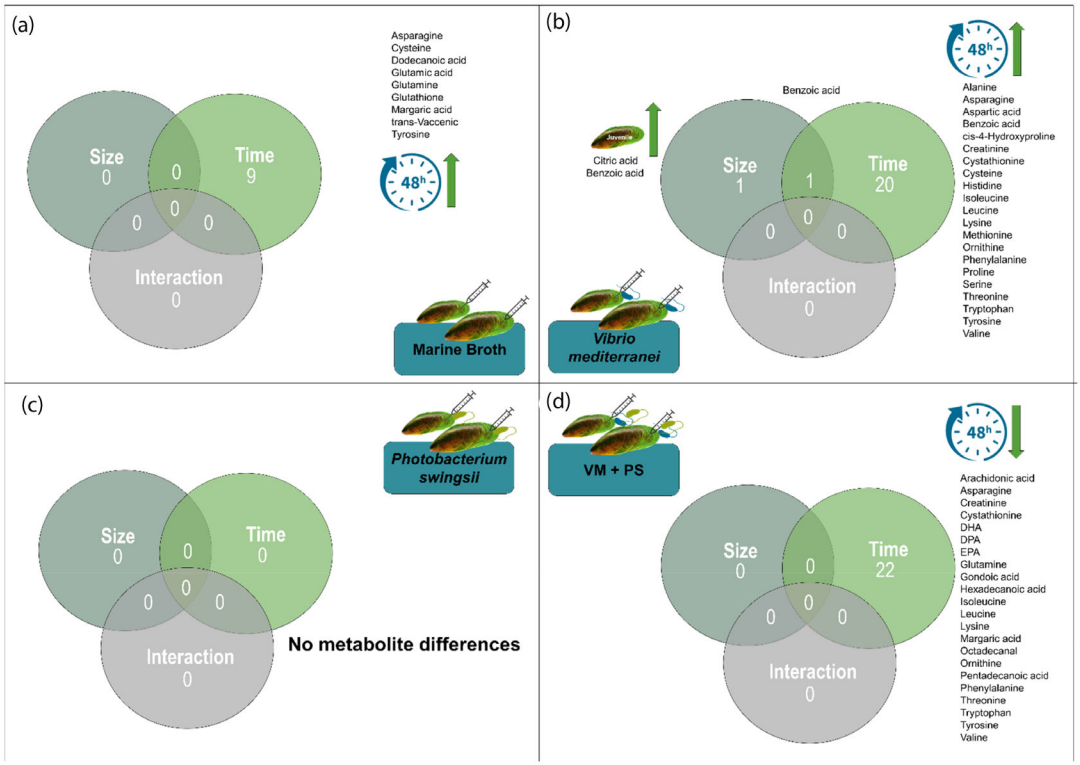

FIGURE 4 Overview of the metabolomics results represented as a Venn diagram indicating the metabolite differences between adult and juvenile mussels sampled at 24 and 48 hpi following injection with marine broth (a), *Vibrio mediterranei* (b), *Photobacterium swingsii*, (c) and VM + PS (d). Metabolite differences are indicated as an increase (↑) or decrease (↓) in metabolite abundance due to affected experimental factors.

increases with age (Dubert et al., 2017). Using juvenile and adult Greenshell™ mussels as hosts, we demonstrated in the current laboratory-based study that mussels succumb more easily to coinfections compared to single infections. This was evident by survival, bacterial colony count, and haemolymph metabolomics data. This study is also the first to report differences between juvenile and adult *P. canaliculus* in infection susceptibility. Indeed, the results revealed that juveniles had lower survival rates than adults when injected with *V. mediterranei*, *P. swingsii*, and both pathogens. This is not surprising, as previous studies on bivalves have shown that juveniles are more vulnerable to pathogens than adults (Albuixech-Martí et al., 2021; Green et al., 2016). In juvenile mussels, the immune systems and defense mechanisms are often not fully developed compared to the adults, leaving them more susceptible to pathogens (Pruzzo et al., 2005). The juvenile response can be attributed to factors such as lower hemocyte production and quantity (Canesi & Pruzzo, 2016), incomplete organ development (Coen & Bishop, 2015), or a reduced capacity for immune response (Lane & Birkbeck, 2000).

4.1 | Marine broth response

The use of marine broth injection as the control group in bivalve pathogen experiments is common practice to assess the putative effect of experimental handling and/or bacterial growth media (Cellura et al., 2007), as demonstrated in the current study. Here, the results showed that no bacterial colonies were detected in haemolymph samples at 24 and 48 hpc from juvenile and adult mussels injected with marine broth. Additionally, no mortalities were recorded

within these groups either. In a previous study on *P. canaliculus*, mortalities were seen within the marine broth injection (control) group within 2–5 days of experimental time, attributed to the act of injection causing disruption to the tissue (Azizan et al., 2022). Typically, marine broth contains all the nutrients (such as minerals, peptone—a source of nitrogen, vitamins and amino acids, yeast extracts—the source of B-vitamins, and inorganic substances) necessary for the growth of marine bacteria (ZoBell, 1941). Yet, marine broth can also affect the metabolism of the host, as seen in the current study, where several amino and fatty acids showed increased levels in the haemolymph after 48 h of injection. It has been noted that seawater has greater salinity than traditional culture media (Iffland-Stettner et al., 2022), resulting in a medium with high osmolarity when using something like marine broth. In the marine broth injected mussels, the increased levels of amino acids asparagine, cysteine, glutamic acid, glutamine, and tyrosine may be a physiological response to manage osmolyte levels and other regulatory processes, such as ammonia detoxification, cellular redox status, and antioxidant functions (Wu, 2009). The choice of media contributes to the metabolite profile (Daskalaki et al., 2018), making it a necessity to analyze an unspent culture medium as a control within a metabolomics experiment to determine the nonbiological changes that occur (Pinu & Villas-Boas, 2017). Comprehensive metabolite profiling of mussel metabolism in response to marine broth contact has not been done to date and remains a topic of interest for future studies.

4.2 | *Vibrio mediterranei* response

Interestingly, from the metabolomics findings of the current study, only injection *with V. mediterranei* resulted in differences between juvenile and adult mussels, with an increase in the levels of benzoic acid and citric acid detected in juvenile mussels. Benzoic acid and citric acid can be used as nonspecific inhibitors of phenoloxidase (PO) activity to investigate defense mechanisms of innate immunity, as shown in a study on limpets (Quinn et al., 2020). Increases in PO activity in the haemolymph of bivalves are a response to bacterial infection, as seen with the use of *Vibrio coralliilyticus* (Van Hung et al., 2019). Although PO activity was not measured in the current study, increases in PO-inhibiting metabolites were seen, suggesting an increased response to counter PO activity in the haemocytes of *P. canaliculus* in response to *V. mediterranei* infection. Strong correlations have been found between the decrease in PO activity and the occurrence of diseases in bivalve invertebrates, leading to mortalities in the host (Luna-Acosta et al., 2017), potentially as seen in the juveniles infected with *V. mediterranei* in the current investigation.

Significantly higher CFU counts were found when infecting both juveniles and adults with this species of *Vibrio*. Mussels may have had more difficulty clearing *V. mediterranei* out of their systems in the first 24 h of infection. However, a reduction in CFU counts was seen at 48 hpc. *V. mediterranei* is known to have virulence mechanisms related to adhesion, superoxide dismutase production, and toxin production, all of which inhibit phagocytosis (Andree et al., 2021; Reshef et al., 2008) and makes it harder for the host to eliminate. Additional virulence genes, including pili and zona occludins toxin (*zot*), identified in *V. mediterranei*, contribute to disease development by promoting immune evasion, attachment, and colonization, enabling the bacterium to counteract the host's defense mechanisms (Reshef et al., 2008). The effect of time on bacterial infection was also seen when considering the metabolite response, where all significantly different metabolites were increased at 48 hpc.

The time-related metabolite response of the *V. mediterranei* injection group significantly differed from the other injection groups. We observed multiple increased metabolites, most of which were amino acids. When undergoing stress, such as a pathogenic infection, mussels can also oxidize amino acids to provide energy for cellular production (Wu et al., 2015) or immune response, causing a large increase in amino acid metabolites (Liu et al., 2013). Resultantly, the increased amino acids observed in the current study can be due to protein catabolism (Muznebin et al., 2022) in response to *V. mediterranei* infection taking place over a 48 h timeframe. Additionally, the findings support the use of amino acids in cellular energy, as reported in clams infected with *V. harveyi*, where elevated levels of amino acids were ascribed as a sign of energy depletion (Liu et al., 2013).

Typically, pathogens trigger an innate immune response, which leads to the release of reactive oxygen species (ROS); this response can be countered by antioxidant metabolites related to the glutathione metabolic pathway (Young et al., 2017). Our study revealed increases in the metabolites cystathionine, cysteine, methionine, ornithine, and serine, which support an active glutathione pathway to partake in oxidative stress mechanisms following *V. mediterranei* infection, as previously reported in heat-stressed *Perna canaliculus* (Delorme et al., 2021). Branched-chain amino acids (BCAA) also support innate immunity (Nguyen, Alfaro, Merien, et al., 2018). The increased levels of leucine, isoleucine, and valine additionally support the immune system by providing energy for the biosynthesis of protective molecules, as seen after 24 h of *V. harveyi* infection in clams (Liu et al., 2013).

Changes in the levels of proline and hydroxyproline can be associated with collagen production and stability (Inglis et al., 2016). Thus, the increased proline metabolites from *V. mediterranei* infection can indicate the degradation of muscle tissue. This is consistent with previous studies that recorded other species of *Vibrio* sp. causing collagen degradation and tissue damage in Greenshell™ mussels (Nguyen, Alfaro, Merien, et al., 2018). An increase in the level of creatinine, a valuable source of carbon and nitrogen (Azizan et al., 2021), was also seen in mussels infected with *V. mediterranei*. Often, a decrease in the levels of amino acids and creatine due to the high energy demands of the mussel responding to infection is reported (Nguyen et al., 2019). However, the increase in the levels of amino acids and creatinine over time from the current study suggests recovery in mussels after being exposed to a pathogenic infection (Nguyen, Alfaro, Merien, et al., 2018). This is further supported by the significant decrease in CFU in the group injected with *V. mediterranei* after 48 h, which suggests that the mussels were able to successfully clear the majority of the *V. mediterranei* after 48 h.

It is also worth noting that the survival of juvenile mussels injected with *V. mediterranei* was higher than that of juveniles injected with *P. swingsii*. In the adults injected with *V. mediterranei*, no mortalities occurred. Some potential justifications for the outcomes of infections of *V. mediterranei* could include host specificity (i.e., a narrower range of host species it can infect and colonize, which could limit its overall virulence), immune evasion mechanism (i.e., the ability to modulate host immune response, allowing it to establish a persistent but less virulent infection), competition with other microorganisms (i.e. competition with other bacterial species in the environment or within the host), and environmental influences (e.g. temperature, nutrient availability, or salinity) (Dubert et al., 2016; Künili et al., 2021; Lattos et al., 2021; Prado et al., 2020). The metabolite response of the group injected with *V. mediterranei* largely differed from the other injected groups of mussel, with the metabolites increasing over time. As stated above, the increase in metabolites and decrease in CFU suggest that the mussels had fought off most of the infection within the first 24 h and were recovering after 48 h. The higher survival rate further supports this, as it indicates that the mussels were able to fight off the infection successfully. This evidence suggests that the mussels' immune system was able to respond and overwhelm the *V. mediterranei* pathogenic infection after 48 h, unlike in the other infection groups.

4.3 | *Photobacterium swingsii* response

Injection of *P. swingsii* in both juvenile and adult mussels showed low CFU counts at 24 hpc and even lower counts by 48 hpc. Adult mussels showed a higher survival rate to *P. swingsii* injection than juveniles. However, there were no significant metabolic differences between the age groups affected by this injection. There were also no significant changes in metabolites over time. This is interesting as *P. swingsii* caused 100% mortality in adults and 50% in juvenile mussels. A similar outcome of adult mussel mortalities due to *P. swingsii* has been previously reported by Azizan et al. (2022), attributed to virulence factors produced by the bacterium. Mortality often occurs in bivalves not as a direct effect of pathogenic assault and only restricted to the host's immune system, but as a consequence of changes in other traits that might have an impact on pathogens' infection processes (Labaude et al., 2017). For instance, factors such as temperature, hypoxia, and filtration rates influence food consumption in mussels, which in turn can

increase the likelihood of pathogen consumption/uptake and subsequent infection (Lopez-Joven et al., 2011; Parisi et al., 2017; Skår & Mortensen, 2007).

4.4 | Coinfection response

Pathogen coinfection concerning *Vibrio* sp. that affect *P. canaliculus* health and their relationship with environmental stress is poorly understood and has been raised as an important knowledge gap. The physiological consequences of the first pathogen infecting the host are likely to affect the host's immune response, by either hindering or enhancing proliferation of the subsequent infections. Hence, these co-occurring pathogens can either act synergistically or antagonistically with each other (Kotob et al., 2017a).

The coinfection group had the highest mortality rates, with 100% of juvenile and adult mussels dying, and this group also showed the highest CFU counts among the treatment groups. This implies that the mussel's immune system struggled to cope with the coinfection of pathogens, and we suspect that the bacteria worked in conjunction to breach the mussel's immune system. This trend has been observed in other coinfection studies in bivalves, such as oysters and fan mussels (Gay et al., 2004; Künili et al., 2021; Tall et al., 1999). In these studies, the pathogens work together potentially in a synergistic manner, where the primary pathogen suppresses the host immune system and avoids phagocytosis, thereby causing the secondary pathogen to worsen disease severity and mussel mortality, causing the infection to spread faster (Tall et al., 1999). As *P. swingsii* and *V. mediterranei* belong to different families, they likely have different pathogenic strategies (Pruzzo et al., 2005). This would result in the pathogens attacking the mussel's immune system using two different methods, making it harder for the mussel's immune system to respond. Potentially, rapid pathogen growth could occur, causing the pathogens to overwhelm the host, followed by death. As our understanding of the immune mechanism of mussels within the context of coinfection is limited, additional research is needed to determine the immunological mechanism underlying these pathogen interactions. Additionally, our observations on disease outcomes (i.e., mortality) showed that this parameter was sensitive to the temporal separation between pathogen exposures. This suggests, along with these data, that future research should investigate the order and timing of pathogen exposure to determine pathogen community structure in hosts and disease outcomes.

The metabolism of the coinfection group also showed a distinctly different pattern from the other infection groups, with significantly different metabolites (amino and fatty acids) decreasing over time, as previously reported in *P. canaliculus* when infected with a single pathogen (Nguyen et al., 2019). The decrease in the levels of amino and fatty acids is believed to be caused by the high energetic demands of the mussel's immune response to the coinfection. Immune responses are known to require high energy expenditure (Ellis et al., 2014), resulting in the decreased metabolite levels observed in the current study. The coinfection with multiple pathogens likely resulted in greater stress on the mussels, creating energetic demands that depleted energy reserves and ultimately resulted in death.

Hosts and bacterial pathogens may share similar nutritional substrates and produce common metabolic products at the infection site with crosstalk between their metabolic pathways potentially influencing infection pathogenesis (Meegha & Prasad, 2021). Considering that the metabolic response is taking place in both the host (in response to the pathogen) and the pathogen (as it adapts and proliferates in the host environment), the metabolite results should also be considered a consequence of the bacteria and not only the mussel, as previously demonstrated in *Vibrio* sp. infected *P. canaliculus* (Ericson et al., 2022). Metabolite profiling of *V. parahaemolyticus* fatty acids reported hexadecenoic (16:1), hexadecanoic (16:0), and octadecenoic (18:1) acids as the major fatty acids of *Vibrio* strains (Jia et al., 2014). The C16 and C18 fatty acids detected in the coinfected group of mussels in the current study can thus be attributed to a bacterial response, in addition to the mussel response.

Pathogenic bacteria are known to utilize compounds other than glucose as carbon sources for growth, such as fatty acids (Ericson et al., 2022). With the reduction in the levels of fatty acids (arachidonic acid, gondoic acid, hexadecanoic acid, margaric acid, octadecanal, and pentadecanoic acid) in the current study, it can be suggested that

Vibrio sp. injected *P. canaliculus* utilize lipid metabolites as an energy source. The importance of fatty acids (lipids) as a metabolic energy source and membrane component is prominent in the literature, with *Vibrio* sp. infections shown to affect the balance of lipid metabolism in clams (Yu et al., 2019). The current study confirms that *Vibrio* sp. affects *P. canaliculus* lipid metabolism; however, the utilization by host or pathogen is unclear, making this an important aspect for future research.

The levels of branched-chain amino acids (leucine, isoleucine, and valine) were decreased in the coinfected group of mussels, suggesting a high demand of BCAAs for host energy production in response to *Vibrio* sp. infection, as reported previously in *P. canaliculus* in response to *Vibrio* sp. DO1 infection (Ericson et al., 2022; Nguyen, Alfaro, Merien, et al., 2018). The effect of *V. parahaemolyticus* infection on the aromatic amino acids tyrosine, phenylalanine, and tryptophan in molluscs (Lu et al., 2017) aligns with our current findings, where decreases in the levels of these amino acids suggest alteration of protein formation and synthesis of neurotransmitter derivatives.

We hypothesize that *Vibrio mediterranei* causes more destruction to mussels than *Photobacterium swingsii*. Yet, when these bacterial species are combined, the mussel response is even more profound, with mortalities occurring faster and bacterial counts remaining high after 48 h. Also, the metabolite response suggests support for immune functions and energy production in the dual-infected cohort. What remains unclear is the order in which events happen and the interplay between bacteria once inside the host. Research investigating the metabolite profiles of *Vibrio* sp. (without a host) will be beneficial in determining which aspects of the metabolite response can be related to the mussel, the bacteria, or a combination of both. Also, the effect of external factors on coinfection mechanisms will benefit from further work. For example, water temperature is an important factor for bacterial growth rates (Hoppe et al., 2002), and both *Vibrio* and *Photobacterium* have shown increased growth in warmer temperatures (Roquigny et al., 2021). However, *V. mediterranei* can grow at lower temperatures and has been known to cause mortalities in mussels from 17°C upward (Prado et al., 2020). Thus, water temperature plays an important role in the experimental design for coinfection experiments, as one temperature might favor the growth of one species and not the other. Future studies are needed to elucidate the potential effects of seawater temperature on the growth of *V. mediterranei* and *P. swingsii* used in the current experiment. Regarding *P. swingsii* specifically, further characterization of this bacterium is needed to determine its dormant capabilities, interactions with other bacterial species, and, especially, its virulence factor production in vivo during bacterial infection. This will provide a complete picture of bacterial pathogenesis.

This study demonstrates that coinfections by the pathogens *P. swingsii* and *V. mediterranei* cause high mortality in Greenshell™ mussels. The results also show that juvenile mussels are more susceptible to coinfections than adult mussels and highlight complex changes in the host metabolome of Greenshell™ mussels when exposed to bacterial coinfection. These findings provide valuable insights into the immune system of mussels, their susceptibility/resilience to pathogenic infections, and potential mechanisms of infection progression for this valuable mussel species. This study forms part of a larger research program focused on the investigation of mussel summer mortality in New Zealand.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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